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RESEARCH ARTICLE

Pre-treatment of whey protein concentrate using calcium sequestering salts and high-pressure homogenisation to modify component partitioning during microfiltration

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Whey protein concentrate (WPC) undergoes microfiltration (MF) to produce whey protein isolate (WPI), generating a lower value MF retentate as a co-product. Higher-than-expected protein retention in the retentate, attributed to protein aggregation, has been shown to limit WPI yield. Strategies to reverse or reduce aggregation would be expected to increase protein transmission during MF. This study investigated the effects of pre-treating WPC with 5 mM trisodium citrate (TSC), a calcium-binding salt and high-pressure homogenisation (HPH) at 650 bar, both individually and in combination, on protein transmission during MF. A WPC solution (2.4% protein) was pre-treated with TSC, HPH, TSC followed by HPH (TSC + HPH), or HPH followed by TSC (HPH + TSC). Microfiltration was performed using a 1000 kDa polyethersulfone membrane. Processing time, component partitioning and chemical composition in feed, retentate and permeate were analysed. Protein profiles were assessed using SDS-PAGE and RP-HPLC, in addition to whey protein denaturation. Data were obtained from three independent trials, with all analyses conducted in triplicate. Treatment significantly reduced processing time and increased protein permeation ($P < 0.05$). Processing time decreased by 6.4–11.0%, with TSC and HPH having the strongest effects. Compared with the control, protein retention in MF retentate from pre-treated samples decreased by 7.5–11.5%, with HPH + TSC showing the greatest effect, while permeate protein content increased by 5.45–9.64% ($P < 0.05$). SDS-PAGE confirmed lower levels of protein aggregation, particularly in HPH + TSC, coinciding with the lowest sedimented protein level (43.4%). Trisodium citrate pre-treated samples showed significantly lower ($P < 0.05$) calcium and magnesium levels, providing evidence that cations are involved in mediating protein aggregation. The results indicate that WPC treatment can modify protein permeation, improving the yield of WPI while also generating an MF retentate further enriched in polar lipids, supporting more sustainable dairy processing.

Keywords Whey, Whey protein concentrate, Microfiltration, Homogenisation.

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INTRODUCTION

Microfiltration (MF) of whey protein concentrate (WPC) is an established technological approach for producing high-purity protein streams (Price 2019; Carter *et al*. 2021). Such MF processes utilise membranes with pore sizes of 0.1–1.0 μm, designed to retain aggregated whey proteins, residual fat and milk fat globule membrane (MFGM) fragments in the retentate. The permeate stream is further processed into whey protein isolate (WPI), while the retentate stream undergoes further processing to create whey protein phospholipid concentrate (WPPC), also known as high-fat WPC (HFWPC) (ADPI 2023). Our previous research (Mestawet *et al*. 2024) has demonstrated high levels of protein retention in such MF retentate, which aligns with other studies reporting similar findings for WPPC (Levin *et al*. 2016; Ozturk *et al*. 2022). The

high protein retention is likely due to the heat-induced whey protein aggregation during WPC production (Dissanayake and Vasiljevic 2009; Ma *et al*. 2015; Munir *et al*. 2019; Zhang *et al*. 2021).

Heat treatment alters whey protein structures, especially for those proteins with free sulfhydryl groups (Wang and Lucey 2003; Barone *et al*. 2020; Laursen *et al*. 2023), causing denaturation and exposure of reactive thiol groups, which can interact with thiol groups on other proteins, resulting in the formation of disulphide bonds (Čurlej *et al*. 2022). This leads to unfolding, cross-linking, aggregation, oxidation and Maillard reactions (Singh and Creamer 1991; Zhang *et al*. 2021; Coşkun *et al*. 2023). In addition, calcium ions (Ca^{2+}) can promote aggregation of β-lactoglobulin (β-lg) through electrostatic interactions (Fox *et al*. 2015; Joyce *et al*. 2017; Laursen *et al*. 2023). Furthermore, native milk fat globules and MFGM fragments, which are present in the WPC retentate, provide both hydrophobic regions and reactive sites that facilitate protein aggregation (Ozturk *et al*. 2022). Specifically, the denatured whey proteins interact with the hydrophobic regions of these fat globules and fragments, while exposed thiol groups form disulfide bonds, resulting in covalent cross-linking. This structural modification increases particle size, thereby enhancing protein retention in the MF retentate (Nuzzo *et al*. 2017; Guralnick *et al*. 2021). In addition, the aggregation of proteins and resulting larger particles contribute to membrane fouling, leading to a decline in permeate flux during MF.

Although nonthermal milk and whey treatment techniques, including high-pressure processing and pulsed electric fields, have advanced in recent years (Bevilacqua *et al*. 2019; Munir *et al*. 2019), thermal processing remains the most used technology. With the current applications of HFWPC/WPPC limited mainly to animal feed (e.g. milk replacer) and confectionery products, the high protein retention in MF retentate presents an opportunity for further value creation in both streams (i.e. WPPC and WPI). Reducing or reversing protein aggregation has the potential to increase protein transmission during MF, allowing for greater enrichment of MFGM and phospholipids (PLs) in the resulting MF retentate, while also increasing the yield of WPI, a high-value ingredient. Despite its potential, little research has been published on approaches to enhance protein transmission by mitigating aggregation. In the light of these considerations, the authors conducted preliminary trials to investigate the chemical interactions within the aggregated protein matrix, following the approach outlined by Laursen *et al*. (2023). These trials involved pre-treating WPC feed with various chemicals, including tris, sodium dodecyl sulphate, dithiothreitol and ethylenediaminetetraacetic acid (EDTA), to explore their potential in disrupting protein aggregates. In addition, physical methods such as homogenisation at different pressures (350, 500 and 650 bar) and high-shear mixing of the WPC feed were tested as treatment approaches to evaluate their impact on protein disaggregation and permeation. After applying both chemical and physical methods, particle size was measured, and confocal laser scanning microscopy was conducted to assess changes in particle size. The chemical treatments indicated that ionic interactions played a significant role in protein aggregation, aligning with our previous findings of high concentrations of divalent ions (Ca^{2+} and magnesium, Mg^{2+}) in the MF retentate stream (Mestawet *et al*. 2024). Preliminary trials using high-pressure homogenisation (HPH) demonstrated a significant reduction in particle size, consistent with other studies reporting that HPH reduces particle size in milk protein solutions/dispersions (D'Incecco *et al*. 2018; Warncke and Kulozik 2020; Hebishy *et al*. 2022), whereas high-shear mixing did not show such effects. Please see Supplementary figure (S1) for further information.

In the current experiment, trisodium citrate (TSC), a food-grade calcium-binding salt, was selected, along with HPH, as an approach to treatment. Trisodium citrate, commonly used as a food additive and buffering agent, sequesters calcium and other divalent cations, thereby preventing cation-mediated protein aggregation and enhancing protein solubility (McCarthy *et al*. 2017; Hebishy *et al*. 2019). Similarly, HPH reduces particle size and enhances product stability by breaking down protein aggregates into smaller particles or individual molecules, facilitating better dispersion (D'Incecco *et al*. 2018). Building on these properties, this study was designed to investigate the effects of pre-treating WPC with TSC and HPH, both individually and in combination, on improving protein transmission during MF. The results of this study will be important for creating added value and identifying better utilisation opportunities for the underutilised and undervalued MF retentate stream.

MATERIALS AND METHODS

Materials, chemical reagents and treatments

Whey protein concentrate powder was kindly provided by a local Irish dairy company (Carbery Group, Ballineen, Co. Cork, Ireland). All chemicals, reagents and standards for high-performance liquid chromatography (HPLC), unless otherwise stated, were obtained from Sigma-Aldrich (Wicklow, Ireland) and were of analytical grade.

Sample preparation and treatments

The experimental design included a control and four treatments, designed to evaluate the impact of individual and combined treatment approaches, in different sequences, on protein disaggregation and subsequent permeation during MF. Reconstituted WPC feed was prepared by mixing the powder with ultrapure water at 50°C and stirring for 3 h using a three-blade paddle fitted to an overhead stirrer (IKA® EUROSTAR 60 digital, Germany) at 300 rpm to

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achieve a 2.4% (w/v) protein solution. The reconstituted WPC solution (pH adjusted to 6.4 at 22°C) was then maintained at 4°C overnight, with continuous stirring at 200 rpm to ensure complete rehydration, which represented the control (CTRL). For Treatment 1 (TSC), after reconstituting the WPC feed for 3 h as per the CTRL, 5 mM TSC was added, and the pH was readjusted to 6.4 at 22°C. The sample was subsequently stirred for an additional 2 h and then maintained at 4°C overnight with continuous stirring at 200 rpm. This treatment aimed to evaluate the effect of TSC addition on protein disaggregation and subsequent permeation during MF. For HPH treatment, the control sample was passed through a two-stage valve homogeniser (APV 1000, SPX Flow, Charlotte, NC, USA) in a single pass at a total pressure of 650 bar, with 500 bar applied at the first stage and 150 bar at the second stage; this treatment was designed to assess the effects of physical disruption on protein aggregates without any chemical treatment. Treatment 3 (TSC + HPH) involved preparing the sample as for the TSC treatment, followed by HPH at 650 bar the following day. The HPH + TSC treatment involved following the same preparation steps as for the CTRL, followed by the application of HPH at 650 bar, and then adding 5 mM TSC at 22°C; the sample was subsequently maintained at 4°C overnight with continuous stirring at 200 rpm. The sequential treatments in TSC + HPH and HPH + TSC were designed to assess the combined impacts of TSC and HPH on dissociation of protein aggregates, and ultimately, protein permeation. Homogenisation disrupts protein aggregates through mechanical forces, potentially exposing reactive sites buried within the aggregates. The subsequent addition of TSC in HPH + TSC has the potential to enhance its ability to access and chelate these exposed sites, promoting further disaggregation. This aimed to determine whether the sequence of addition of TSC, either after or before homogenisation, would more effectively enhance protein disaggregation and improve permeation during MF.

Microfiltration setup

A laboratory-scale, pressure-driven tangential-flow filtration device (Pellicon 2 mini-holder; Merck Millipore, Tullagreen, Carrigtwohill, Ireland) was utilised following the procedure outlined by France *et al*. (2021a) to conduct three independent MF experiments, each comprising a control and four treatments. A polyethersulfone (PES) membrane cartridge, V-screen, 1000 kDa molecular weight cut-off (Biomax, Merck Millipore) with a membrane area of 0.1 m^2 was used. A peristaltic pump (Watson-Marlow 520S, Watson-Marlow Pumps Group, Falmouth, Cornwall TR11 4RU, UK), operating at 90 rpm, was utilised to circulate WPC feed through the filtration system.

After assembling the filtration unit, 5 L of deionised water at 50°C was passed through the system to remove any residual storage solution from the membrane. Next, 3 L of WPC feed was pumped through the filtration unit, discarding the initial permeate and retentate material until all residual water was removed. The feed was then circulated through the filtration unit under total recirculation mode, with both retentate and permeate lines returned to the feed vessel, until stable temperature and pressure were attained. During MF, the retentate stream was continuously cycled back to the feed vessel, while the permeate was collected until a volume concentration factor (VCF) of 6 was reached. MF was performed at a pump speed of 90 rpm and an initial transmembrane pressure (TMP) of 0.55 bar. The temperature throughout MF was maintained at 12.8 ± 1°C using a water bath (Grant LT ecocool 100, UK) connected to a plate heat exchanger (Complete Stainless Engineering Ltd, Limerick, Ireland) linked to the retentate line. From each treatment, three types of samples were obtained: WPC feed, MF retentate and MF permeate. After each MF trial for each treatment, the normalised water permeability (NWP) of the membrane was measured (see In-process analysis section). To ensure experimental consistency and avoid cross-contamination, the filtration system was thoroughly cleaned after each trial following the protocol outlined by Crowley *et al*. (2015). This cleaning step was particularly essential as fouling on the membrane, caused by protein aggregates and other particulates during MF, reduced permeate flux over time. The removal of fouling material and restoration of the membrane's performance were critical to obtaining accurate and reproducible results for each treatment.

In-process analysis

Permeate flux ($L/m^2/h$), TMP, pH and conductivity were measured according to the methods outlined by Mestawet *et al*. (2024). NWP and membrane fouling were assessed following the approaches described by France *et al*. (2021a).

Gross chemical composition

Total solids (TS), ash, protein and fat contents in samples were determined following standard methods (AOAC International 2000; IDF 2001, 2008, 2010). TS, protein and fat partitioning between retentate and permeate during MF were calculated as a percentage of the contents in the WPC feed.

Mineral profile analysis

The mineral profile analysis of samples was conducted using inductively coupled plasma-mass spectrometry (ICP-MS) following microwave-assisted digestion with a Mars Express Digester (MARS 6®, CEM Microwave Technology Ltd.) as outlined in International Organization for Standardization (2013) guidelines, as previously described by Mestawet *et al*. (2024). The minerals analysed included calcium (Ca), phosphorus (P), magnesium (Mg), sodium (Na), potassium (K) and chloride (Cl).

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Protein profile analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
The protein profile of the samples was analysed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) following the method of Laemmli (1970), with minor adjustments as described by Mestawet *et al*. (2024). The feed gel, consisting of feed samples from all treatments, was loaded with 10 μL of sample at a protein concentration of 1 mg/mL and with 5 μL of SDS-PAGE broad-range protein standard (Bio-Rad, Hercules, CA) serving as a molecular weight marker. To assess the effect of WPC feed treatments on protein partitioning between the MF retentate and permeate streams, the control sample was loaded with 10 μL of sample at a protein concentration of 1 mg/mL. However, pre-treated samples were loaded using the same volume (10 μL) as the control, without standardising their protein concentration to 1 mg/mL. This approach was specifically chosen to emphasise differences in band intensity on SDS-PAGE gels, providing a visual representation of how treatments influenced protein partitioning, independent of standardised protein concentration adjustments.

Reverse-phase high-performance liquid chromatography
The protein profile of all samples was further analysed using reversed-phase high-performance liquid chromatography (RP-HPLC) with an Agilent 1220 Infinity II LC system following the protocol previously described by Mestawet *et al*. (2024).

Whey protein denaturation
The extent of whey protein denaturation was assessed by calculating the difference between the nitrogen content of the soluble protein fraction at pH 4.6 and that of the respective original sample, following the methodology outlined by O'Kennedy and Mounsey (2006) and Schmidmeier *et al*. (2019). The nitrogen content of the pH 4.6-soluble fraction was determined following a standard method of IDF (2008).

Particle size distribution analysis
The particle size distribution in all samples was analysed using dynamic light scattering (DLS) with a Zetasizer Nano ZS instrument (Malvern Instruments Ltd, Malvern, Worcestershire, UK), equipped with a temperature-controlled cell holder, according to the protocol previously described by Mestawet *et al*. (2024). The particle size data were reported as intensity-weighted (Z-average) values.

Statistical analysis

All samples were derived from three independent trials, and all analyses were conducted in triplicate. The experimental data obtained were analysed using a completely randomised design by employing the GLM one-way ANOVA procedure through the Statistical Analysis System (SAS Institute Inc 2008), version 9.1.3 computer software program. Statistically significant differences between means were determined using a Tukey's HSD post hoc test, with a confidence level of 95%.

RESULTS AND DISCUSSION

Microfiltration process performance

During MF, in-line measurements were taken to assess MF process performance. Across the three independent trials, the temperature was maintained at 12.8 ± 1°C. Treatment of WPC feed significantly reduced ($P < 0.05$) membrane fouling, as evident by the smaller reduction in NWP for all treatments compared to the CTRL (which exhibited a 47.3% reduction in NWP after MF); in addition, the CTRL required a significantly longer ($P < 0.05$) processing time to reach the desired VCF of 6 compared to the pre-treated samples (Table 1). This effect was reflected in the permeate flux for the CTRL, which decreased from 32.0 to 18.1 L/m^2/h (Figure 1), with a corresponding increase in TMP from an initial 0.55 bar to a final 1.1 bar. In comparison, the initial and final flux values for TSC, HPH, TSC + HPH and HPH + TSC were 31.6–22.2, 33.6–20.0, 34.8–20.4 and 30.8–19.6 L/m^2/h, respectively, with comparable final TMPs of approximately 0.8 bar. The results indicate that treatment of WPC improves permeate flux and reduces fouling. A review on protein-induced fouling in ultrafiltration and MF highlights that protein aggregation promotes fouling by adhering to the membrane surface and blocking pores, leading to reduced membrane performance (Tanudjaja *et al*. 2022).

Table 1 Reduction in normalised water permeability and processing time to generate 2.5 L of permeate following microfiltration of whey protein concentrate feed with different treatments at 12.8 ± 1°C.

Treatment	Reduction in NWP (%)	Processing time (min)
CTRL	47.3 ± 1.73^a	70.3 ± 0.14^a
TSC	37.4 ± 1.09^b	62.5 ± 1.44^c
HPH	37.7 ± 2.24^b	63.0 ± 1.73^c
TSC + HPH	34.7 ± 3.25^b	65.8 ± 1.17^b
HPH + TSC	39.1 ± 3.77^b	65.7 ± 1.45^b

CTRL, control whey protein concentrate (WPC) feed; HPH + TSC, WPC feed pre-treated with high-pressure homogenisation at 650 bar followed by trisodium citrate; HPH, WPC feed homogenised at 650 bar; NWP, Normalised water permeability; TSC + HPH, WPC feed pre-treated with trisodium citrate followed by high pressure homogenisation at 650 bar; TSC, WPC feed pre-treated with trisodium citrate.
$^{a–c}$Means with different superscript letters within the same column are significantly different ($P < 0.05$).

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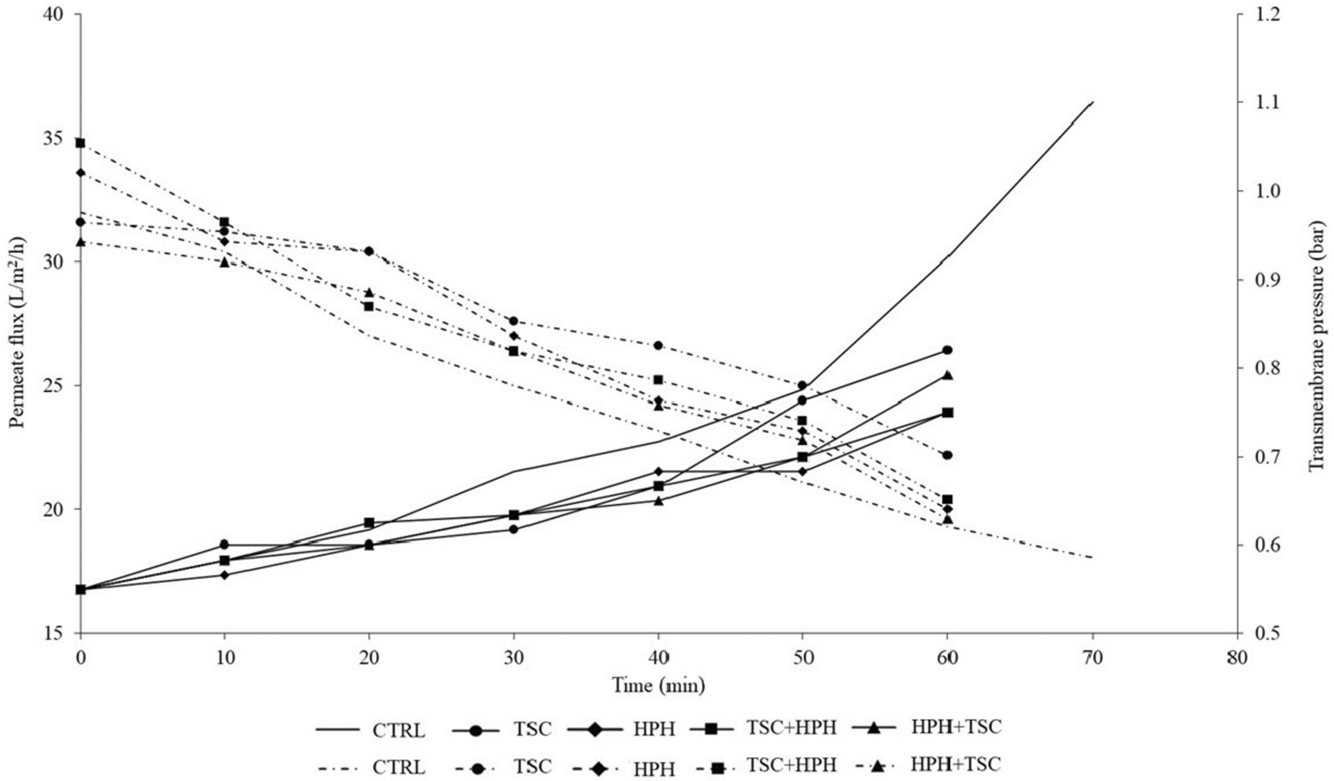


Figure 1 Permeate flux and transmembrane pressure during microfiltration of different whey protein concentrate (WPC) pre-treated feed samples at 12.8 ± 1°C as a function of processing time. CTRL, Control WPC feed; HPH + TSC, WPC feed pre-treated with high-pressure homogenization at 650 bar followed by trisodium citrate; HPH, WPC feed homogenised at 650 bar; TSC + HPH, WPC feed pre-treated with trisodium citrate followed by high pressure homogenization at 650 bar; TSC, WPC feed pre-treated with trisodium citrate.

The pH in both the retentate and permeate samples was lower in the TSC-treated samples (TSC: 6.47 and 6.54; TSC + HPH: 6.52 and 6.58; HPH + TSC: 6.47 and 6.53, in retentate and permeate samples, respectively) compared to the CTRL- and HPH-treated samples, which had pH values of 6.55 in the retentate and 6.62 in the permeate. Initially, the addition of TSC increased the pH slightly in the pretreated samples, consistent with findings in previous studies with similar protein solutions (Hebishy *et al*. 2019). However, following TSC addition, the pH was manually adjusted to 6.4 at 22°C in all pre-treated samples, using HCl, to standardise conditions before MF (which was ultimately performed at a lower temperature). The citrate ions in TSC, being weak acids, act as buffering agents, contributing to the observed lower pH in the TSC-treated streams after MF. These ions chelate divalent cations such as Ca^{2+} and Mg^{2+}, disrupting cation-mediated protein aggregates, which could further influence the pH balance (Ozcan-Yilsay *et al*. 2007).

In contrast, conductivity was higher in the TSC-treated samples (TSC: 2.45 mS/cm in the retentate and permeate; TSC + HPH: 2.45 and 2.43 mS/cm; HPH + TSC: 2.47 and 2.46 mS/cm) compared to the CTRL and HPH treatments (1.75 mS/cm in the retentate and 1.61 mS/cm in the permeate). This higher conductivity is attributed to the higher ionic strength from TSC addition. Trisodium citrate dissociates into Na^+ and citrate ($C_6H_5O_7^{3-}$) ions, increasing the concentration of free ions in the solution and thereby increasing conductivity (Fidaleo and Moresi 2013). High-pressure homogenisation, being a mechanical process, did not affect pH or ionic balance and thus had no influence on conductivity. Therefore, the observed changes in pH and conductivity in the TSC-treated samples can be attributed solely to TSC addition and subsequent pH adjustment.

Gross composition

The WPC feed samples were reconstituted to contain 2.4% protein and 4% TS. The fat content of the WPC powder was approximately 5% by weight on a dry basis, typical for commercial WPC products (Svanborg *et al*. 2015; Mehra *et al*. 2021). Trisodium citrate pre-treated feed samples (TSC, TSC + HPH and HPH + TSC) showed slightly higher, but not significantly different ($P > 0.05$), ash content compared to the CTRL and HPH samples. This minor increase is likely attributable to the addition of TSC and pH adjustment during treatment, which introduces Na^+ and

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$C_6H_5O_7^{3-}$, and the pH adjustment process, which can contribute additional minerals to the solution.

WPC treatment significantly influenced component partitioning, as demonstrated by the gross chemical composition and mineral profile in the retentate and permeate streams (Table 2). Total solids were significantly higher ($P < 0.05$) in the CTRL retentate compared with the pre-treated retentate samples, with reductions of 3.48, 6.09, 6.96 and 11.3% in TSC, HPH, TSC + HPH and HPH + TSC, respectively, relative to the CTRL retentate. These lower levels suggest that treatments improved transmission of components through the membrane, with the greatest effect observed for HPH + TSC. In this HPH + TSC treatment, the combination of HPH followed by TSC appeared to enhance permeation more effectively than other treatment strategies.

Protein content in the retentate of the CTRL was significantly higher ($P < 0.05$) than that in the retentates of the pre-treated samples. Protein retention in the pre-treated samples was reduced by 9.0%, 7.5%, 8.2% and 11.5% for the TSC, HPH, TSC + HPH and HPH + TSC treatments, respectively, relative to the CTRL retentate. Native whey proteins, which have molecular weights ranging from 14 to 150 kDa, are expected to permeate through the 1000 kDa membrane used in the study. However, the high protein retention observed in the CTRL is likely attributed to heat-induced denaturation and aggregation of whey proteins during WPC production, leading to increased protein aggregate size that hinders their passage through the membrane (Levin *et al*. 2016; Mulcahy *et al*. 2017; Ozturk *et al*. 2022; Tanudjaja *et al*. 2022). Treatments, particularly those involving TSC, disrupted these aggregates, thereby enhancing protein permeation, particularly in HPH + TSC. Treatment using HPH also showed increased protein permeation. The role of HPH in reducing protein aggregate size is further supported by the findings of Hebishy *et al*. (2022), who demonstrated that application of homogenisation at pressures between 5 and 10 MPa during the rehydration process significantly reduced the particle size of milk protein concentrate dispersions, leading to enhanced solubility and heat stability. This aligns with the observed improvement in protein permeation in HPH, where HPH disrupted protein aggregates, increasing their solubility and enabling higher transmission through the membrane.

The difference in protein permeation between TSC + HPH and HPH + TSC can be attributed to the sequence of treatments. In TSC + HPH, TSC is added first, allowing for overnight chelation of Ca^{2+} and Mg^{2+} ions, which disrupts cation-mediated protein aggregates. However, the subsequent homogenisation might not have fully disrupted the remaining aggregates, as cation-mediated interactions are only one consideration in the stabilisation of protein aggregates. Other forces, such as hydrophobic interactions, hydrogen bonding and disulfide bridges, may have further stabilised the aggregates, making complete disaggregation challenging. By contrast, in HPH + TSC, homogenisation is applied before TSC treatment, which likely disrupted larger protein aggregates, exposing previously buried regions. The subsequent addition of TSC may then act on these newly exposed sites, leading to more effective chelation of Ca^{2+} and Mg^{2+} and further disaggregation of aggregated proteins, ultimately resulting in the greater protein permeation observed for this treatment. Thus, the order of application of TSC and homogenisation treatments in HPH + TSC facilitated a more efficient disruption of protein aggregates, increasing protein permeation.

In terms of fat retention, the CTRL had significantly lower ($P < 0.05$) fat content (0.78%) than TSC (0.92%), HPH (1.00%), TSC + HPH (0.90%) and HPH + TSC (0.94%). Our previous research utilising the same WPC powder showed that 25.1% of the fat in this WPC is phospholipid (Mestawet *et al*. 2024). Based on this, we assume that treatment also contributed to an increased concentration of PLs in the retentate. The fat concentration, expressed as a percentage of total solids, increased by 15%, 22%, 13% and 17% in TSC, HPH, TSC + HPH and HPH + TSC treatments, respectively, compared to the CTRL retentate. This apparent increase in fat content in the retentate is likely due to enhanced protein permeation in the pre-treated samples, which reduced the total amount of nonfat solids, thereby increasing the relative proportion of fat as a percentage of the remaining total solids. The absolute fat content remained consistent across treatments, as no fat was expected to permeate the membrane. Since fat particles are too large to permeate the membrane, their relative concentration in the retentate increased as more permeable components passed through. In the permeate streams of pre-treated samples, TS and protein content were significantly higher ($P < 0.05$) than in the CTRL permeate. The CTRL permeate had the lowest TS (2.34%) and protein (1.04%), while TSC, TSC + HPH and HPH + TSC showed higher TS (2.44–2.49%) and protein (1.13–1.15%) contents, indicating more extensive whey component transmission.

In summary, the compositional differences between the retentate and permeate streams demonstrate the impact of treatment on the MF performance of WPC (Ozcan-Yilsay *et al*. 2007; Hebishy *et al*. 2019). The addition of TSC, particularly in combination with HPH, played a crucial role in disrupting cation-mediated protein aggregates, thereby improving protein and mineral permeation (Ozcan-Yilsay *et al*. 2007; Li *et al*. 2018; Hebishy *et al*. 2019; Jin *et al*. 2022). The order of treatments in HPH + TSC, with homogenisation followed by TSC treatment, led to the most extensive protein permeation, highlighting the importance of the sequence of treatment.

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Table 2 Gross chemical composition and mineral profile of feed, retentate and permeate samples generated following microfiltration of whey protein concentrate feed with different treatments at 12.8 ± 1°C.

		Gross composition (%)				Mineral composition (mg/100 g)					
Stream	Sample	Total solids	Crude protein	Fat	Ash	Ca	P	K	Na	Mg	Cl
Feed	CTRL	3.96 ± 0.01^{a}	2.40 ± 0.02^{a}	0.19 ± 0.01^{a}	0.16 ± 0.00^{a}	24.3 ± 0.33^{a}	18.7 ± 0.33^{a}	38.0 ± 1.15^{a}	9.30 ± 0.33^{b}	3.67 ± 0.33^{a}	14.7 ± 0.67^{b}
	TSC	4.06 ± 0.03^{a}	2.41 ± 0.05^{a}	0.20 ± 0.01^{a}	0.18 ± 0.02^{a}	23.7 ± 0.33^{a}	18.0 ± 1.00^{a}	38.3 ± 1.76^{a}	41.7 ± 2.03^{a}	3.30 ± 0.33^{a}	18.0 ± 0.58^{a}
	HPH	3.99 ± 0.01^{a}	2.40 ± 0.03^{a}	0.20 ± 0.01^{a}	0.15 ± 0.02^{a}	22.7 ± 0.67^{a}	18.3 ± 0.67^{a}	38.3 ± 1.45^{a}	9.30 ± 0.33^{b}	3.30 ± 0.33^{a}	14.0 ± 0.58^{b}
	TSC + HPH	4.09 ± 0.01^{a}	2.41 ± 0.62^{a}	0.19 ± 0.01^{a}	0.17 ± 0.01^{a}	24.3 ± 0.33^{a}	19.0 ± 0.58^{a}	39.0 ± 0.00^{a}	44.0 ± 0.58^{a}	3.70 ± 0.33^{a}	17.0 ± 1.15^{a}
	HPH + TSC	4.09 ± 0.11^{a}	2.40 ± 0.01^{a}	0.20 ± 0.01^{a}	0.18 ± 0.01^{a}	23.7 ± 0.33^{a}	19.0 ± 0.00^{a}	40.0 ± 0.58^{a}	43.0 ± 0.58^{a}	3.30 ± 0.33^{a}	18.0 ± 0.58^{a}
Retentate	CTRL	11.5 ± 0.12^{a}	8.14 ± 0.01^{a}	0.78 ± 0.05^{c}	0.28 ± 0.01^{b}	53.3 ± 0.33^{a}	37.7 ± 0.33^{a}	53.7 ± 1.45^{a}	13.0 ± 0.58^{b}	7.00 ± 0.00^{a}	11.0 ± 0.58^{c}
	TSC	11.1 ± 0.08^{b}	7.44 ± 0.53^{b}	0.92 ± 0.00^{b}	0.35 ± 0.00^{a}	29.3 ± 0.88^{b}	38.0 ± 1.73^{a}	54.7 ± 2.60^{a}	58.0 ± 3.05^{a}	4.00 ± 0.00^{b}	13.3 ± 0.89^{a}
	HPH	10.8 ± 0.01^{c}	7.53 ± 0.01^{b}	1.00 ± 0.01^{a}	0.28 ± 0.01^{b}	54.0 ± 0.58^{a}	37.0 ± 0.58^{a}	53.7 ± 1.20^{a}	13.0 ± 0.58^{b}	6.70 ± 0.33^{a}	11.0 ± 0.58^{c}
	TSC + HPH	10.7 ± 0.09^{c}	7.47 ± 0.11^{b}	0.90 ± 0.01^{b}	0.31 ± 0.01^{a}	31.0 ± 0.00^{b}	36.7 ± 0.89^{a}	55.7 ± 0.33^{a}	60.5 ± 0.29^{a}	4.00 ± 0.00^{b}	13.7 ± 0.89^{a}
	HPH + TSC	10.2 ± 0.13^{d}	7.20 ± 0.04^{c}	0.94 ± 0.02^{ab}	0.32 ± 0.01^{a}	31.7 ± 0.89^{b}	36.7 ± 0.89^{a}	55.0 ± 0.00^{a}	58.3 ± 0.33^{a}	4.30 ± 0.33^{b}	13.3 ± 0.89^{a}
Permeate	CTRL	2.34 ± 0.00^{c}	1.04 ± 0.02^{c}	0.01 ± 0.00^{a}	0.11 ± 0.00^{b}	15.3 ± 0.33^{b}	14.7 ± 0.33^{c}	27.3 ± 1.86^{d}	8.00 ± 0.00^{b}	2.30 ± 0.33^{b}	14.7 ± 0.67^{b}
	TSC	2.49 ± 0.00^{a}	1.13 ± 0.01^{ab}	0.01 ± 0.00^{a}	0.17 ± 0.00^{a}	20.7 ± 0.88^{a}	14.7 ± 0.33^{c}	32.3 ± 0.89^{c}	36.0 ± 0.58^{a}	3.00 ± 0.00^{a}	19.0 ± 0.00^{a}
	HPH	2.44 ± 0.00^{b}	1.10 ± 0.01^{b}	0.01 ± 0.00^{a}	0.12 ± 0.00^{b}	15.7 ± 0.89^{b}	13.3 ± 0.33^{c}	27.3 ± 2.84^{d}	7.70 ± 0.33^{b}	2.30 ± 0.33^{b}	13.7 ± 0.89^{b}
	TSC + HPH	2.49 ± 0.50^{a}	1.13 ± 0.01^{ab}	0.02 ± 0.00^{a}	0.19 ± 0.00^{a}	20.3 ± 0.89^{a}	14.3 ± 0.33^{c}	31.7 ± 0.89^{cd}	36.7 ± 0.89^{a}	3.00 ± 0.00^{a}	18.0 ± 1.15^{a}
	HPH + TSC	2.49 ± 0.03^{a}	1.15 ± 0.01^{a}	0.01 ± 0.02^{a}	0.20 ± 0.01^{a}	20.0 ± 1.15^{a}	14.0 ± 0.00^{c}	33.0 ± 0.58^{c}	37.0 ± 0.58^{a}	3.00 ± 0.00^{a}	18.3 ± 0.33^{a}

Ca, calcium; Cl, chloride; CTRL, control whey protein concentrate (WPC) feed; HPH + TSC, WPC feed pre-treated with high pressure homogenization at 650 bar followed by trisodium citrate; HPH, WPC feed homogenised at 650 bar; K, potassium; Mg, magnesium; Na, sodium; P, phosphorous; TSC + HPH, WPC feed pre-treated with trisodium citrate followed by high pressure homogenization at 650 bar; TSC, WPC feed pre-treated with trisodium citrate.

[a–d]Means with different superscript letters within the same column and stream are significantly different ($P < 0.05$).

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Mineral profile of the different samples

In the WPC feed samples, similar levels of Ca, K, P and Mg were observed across treatments (Table 2). However, treatments involving TSC (TSC, TSC + HPH and HPH + TSC) showed higher concentrations of Na and Cl than that of the CTRL and HPH feeds. This increase is attributable to the addition of TSC during treatment, which introduces Na, and the subsequent pH adjustment to 6.4 with HCl, which adds Cl. Specifically, these treatments exhibited a 4.6-fold higher Na content and a Cl content that was approximately 23% higher. The Ca, P, Mg and K contents in the WPC feed were consistent with those reported in the literature (Smithers 2008; Joyce *et al*. 2017; Barone *et al*. 2020).

In the retentate streams for the TSC, TSC + HPH and HPH + TSC treatments, the Ca content was significantly lower ($P < 0.05$) than that of the CTRL, with levels of 45%, 42% and 41%, respectively, expressed as a % of the CTRL; the corresponding values for Mg were 43%, 43% and 39%, respectively. This reduction in Ca and Mg contents of the retentate streams following TSC treatment supports the hypothesis that TSC chelated these divalent cations, disrupting cation-mediated protein aggregates and thereby facilitating higher protein permeation. This is further supported by the corresponding higher Ca and Mg contents in the permeate streams of these treatments. In the permeate streams from TSC pre-treated samples, the Ca and Mg contents were approximately 35% and 32% higher than that of the CTRL and HPH samples, respectively. The Na content in the permeate from TSC pre-treated samples was more than 3.6 times higher than that of the CTRL and HPH samples, while the Cl content was 29%, 22% and 25% higher in the TSC, TSC + HPH and HPH + TSC samples, respectively, than in the CTRL. These results suggest that the ability of TSC to chelate divalent ions disrupted protein aggregates and enhanced MF performance by reducing aggregate size and improving component transmission (Ozcan-Yilsay *et al*. 2007; Hebishy *et al*. 2019; Laursen *et al*. 2023). However, the higher contents of Na and Cl in the permeate streams of TSC-treated samples, attributed to TSC addition and pH adjustment using HCl, may necessitate further processing (e.g. nanofiltration) to reduce these concentrations for certain applications.

The concentrations of K and P in the retentate streams of various treatments were approximately 55 and 37 mg/100 g, respectively, with no significant differences observed between treatments. This is consistent with previously reported values for WPPC (Swaminathan *et al*. 2021; Viswanathan *et al*. 2021). These values also align closely with those reported by other studies on WPC products (Levin *et al*. 2016; Carter *et al*. 2021; Ozturk *et al*. 2022). This consistency in K and P contents across treatments reflects the selective ability of TSC to chelate divalent cations such as Ca^{2+} and Mg^{2+}, while HPH physically disrupts protein aggregates without altering the mineral distribution during subsequent MF.

Component partitioning between different process streams

Component partitioning between retentate and permeate during MF is influenced by membrane characteristics, concentration factor, processing parameters and the physical state of feed components (Price 2019; Carter *et al*. 2021; Salunke *et al*. 2021). Treatment of the WPC feed had a significant effect ($P < 0.05$) on component partitioning, as evidenced by the differences in TS, protein and mineral distribution between the retentate and permeate streams (Tables 2 and 3). The measured differences are likely attributable to the ability of TSC to sequester minerals, which disrupted cation-mediated protein aggregates, and the size reduction in aggregates achieved using homogenisation (Ozcan-Yilsay *et al*. 2007; McCarthy *et al*. 2017; Li *et al*. 2018). All pre-treated samples showed increased permeation of TS and protein into the permeate, compared to the CTRL, which showed the highest retention. Among the treatments, HPH + TSC was the most impactful, achieving the highest permeation of TS and protein into the permeate. HPH + TSC showed the highest permeation of TS and protein into the permeate, resulting in the lowest retention of TS (42.3%) and protein (50.1%) in the retentate, compared to 47.4% TS and 56.5% protein retention in the CTRL. This outcome is likely due to the sequence of treatments in HPH + TSC, where homogenisation was performed prior to TSC addition. Homogenisation effectively disrupted protein aggregates, exposing reactive sites that facilitated subsequent chelation by TSC, thereby enhancing protein disaggregation and increasing permeation during MF.

Component partitioning between the retentate and permeate was calculated through mass balance, which also accounts for material associated with the membrane. It is important to note that the sum of components partitioning between retentate and permeate did not equal 100%, as some components adhered to the membrane surface or contributed to fouling layers on/in the membrane during MF. Previous studies have shown that smaller aggregates, formed because of treatment, are more likely to accumulate on the membrane surface, which may contribute to fouling and slightly reduce component transfer efficiency (Baldasso *et al*. 2011; France *et al*. 2021b; Swaminathan *et al*. 2021; Tanudjaja *et al*. 2022). Nonetheless, the increased permeation observed in pre-treated samples indicates that, despite the potential for fouling, the treatments enhanced transmission across the membrane by improving protein disaggregation and reducing aggregate size.

Although there was a significant difference ($P < 0.05$) in the extent of fat partitioning among all retentates, fat

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Table 3 Component partitioning between retentate and permeate streams following microfiltration of whey protein concentrate feed with different treatments at 12.8 ± 1°C.

		Partitioning (%)		
Stream	Sample	Total solids	Protein	Fat
Retentate	CTRL	47.4 ± 0.45[a]	56.5 ± 0.09[a]	68.4 ± 0.14[e]
	TSC	46.6 ± 0.27[b]	51.9 ± 0.09[b]	80.7 ± 0.14[c]
	HPH	45.2 ± 0.35[b]	52.1 ± 0.18[b]	89.1 ± 0.38[a]
	TSC + HPH	43.8 ± 0.14[c]	51.9 ± 0.11[b]	81.8 ± 0.18[b]
	HPH + TSC	42.3 ± 0.14[d]	50.1 ± 0.17[c]	76.9 ± 0.14[d]
Permeate	CTRL	49.7 ± 0.26[b]	38.2 ± 0.11[c]	4.87 ± 0.61[a]
	TSC	50.9 ± 0.24[a]	39.2 ± 0.18[b]	4.90 ± 0.01[a]
	HPH	50.9 ± 0.14[a]	39.2 ± 0.06[b]	5.01 ± 0.18[a]
	TSC + HPH	50.8 ± 0.14[a]	38.9 ± 0.09[b]	4.84 ± 0.01[a]
	HPH + TSC	50.9 ± 0.12[a]	39.9 ± 0.11[a]	4.86 ± 0.02[a]
Membrane associated	CTRL	2.89 ± 0.58[c]	5.34 ± 0.20[c]	27.7 ± 1.02[a]
	TSC	2.47 ± 0.49[d]	8.83 ± 0.12[b]	14.4 ± 0.14[c]
	HPH	3.83 ± 0.22[c]	8.73 ± 0.24[b]	5.85 ± 0.38[d]
	TSC + HPH	5.33 ± 0.29[b]	9.17 ± 0.03[b]	13.4 ± 0.18[c]
	HPH + TSC	6.77 ± 0.09[a]	10.0 ± 0.29[a]	18.2 ± 0.13[b]

CTRL, control whey protein concentrate (WPC) feed; HPH + TSC, WPC feed pre-treated with high pressure homogenisation at 650 bar followed by trisodium citrate; HPH, WPC feed homogenised at 650 bar; TSC + HPH, WPC feed pre-treated with trisodium citrate followed by high pressure homogenisation at 650 bar; TSC, WPC feed pre-treated with trisodium citrate.

[a–e]Means with different superscript letters within the same column and stream are significantly different ($P < 0.05$).

transmission into the permeate did not differ significantly ($P > 0.05$) between treatments, indicating the membrane's effectiveness in retaining fat. The high protein content in the retentate stream suggests that, despite the treatments, some aggregated material persisted. This observation highlights that aggregation is not solely driven by cation interactions but also involves other stabilising forces, including hydrophobic interactions, hydrogen bonding and disulfide bridge formations, which chelation or HPH alone cannot fully dissociate (Fox *et al*. 2015). Consequently, although TSC and HPH treatments effectively disrupted cation-mediated aggregates and decreased aggregate size, they were insufficient in disrupting all aggregates due to the presence of these additional stabilising interactions.

Protein profile of the process samples

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Visual assessment of proteins *via* SDS-PAGE revealed complex protein profiles across all samples, primarily characterised by a high abundance of the low-molecular-weight proteins, such as α-lactalbumin (α-la) and β-lg, within the range 10–20 kDa (Figure 2). Under nonreducing conditions, the intensity of unresolved proteins was low in the TSC pre-treated samples (TSC, TSC + HPH and HPH + TSC) of WPC feed and MF retentate, demonstrating the role of the chelating agent in disaggregating proteins. A study that analysed this unresolved high-molecular-weight aggregated material with liquid chromatography–tandem mass spectrometry (LC–MS/MS) based proteomics identified 36 different types of proteins, including lactoferrin (LF), immunoglobulins (Ig) and bovine serum albumin (BSA; Xiong *et al*. 2021).

Under nonreducing conditions, in the WPC feed gel, the lanes with TSC pre-treated samples (TSC, TSC + HPH and HPH + TSC) showed reduced aggregation, particularly with HPH + TSC lacking bands or other signs of aggregated protein between ~20 and 50 kDa. On the retentate gel under nonreducing conditions, unresolved proteins with molecular weights >250 kDa were more intense in CTRL and HPH compared with the other treatments. These proteins are primary components of complexed/aggregated proteins (Xiong *et al*. 2021; Ozturk *et al*. 2022). Under nonreducing conditions, lanes loaded with retentate samples from pre-treated feed (TSC, HPH, TSC + HPH and HPH + TSC) showed reduced aggregation, with the most noticeable effect in HPH + TSC compared to the CTRL retentate. Lower (α-la and β-lg) and higher molecular weight proteins (Ig and xanthine oxidase) were significantly less abundant in pre-treated retentate samples compared to the CTRL. This reduction in band intensities indicates that treatment, particularly with TSC, enhances the dissociation of aggregated proteins and reduces their retention in the retentate. Trisodium citrate acts as a chelating agent,

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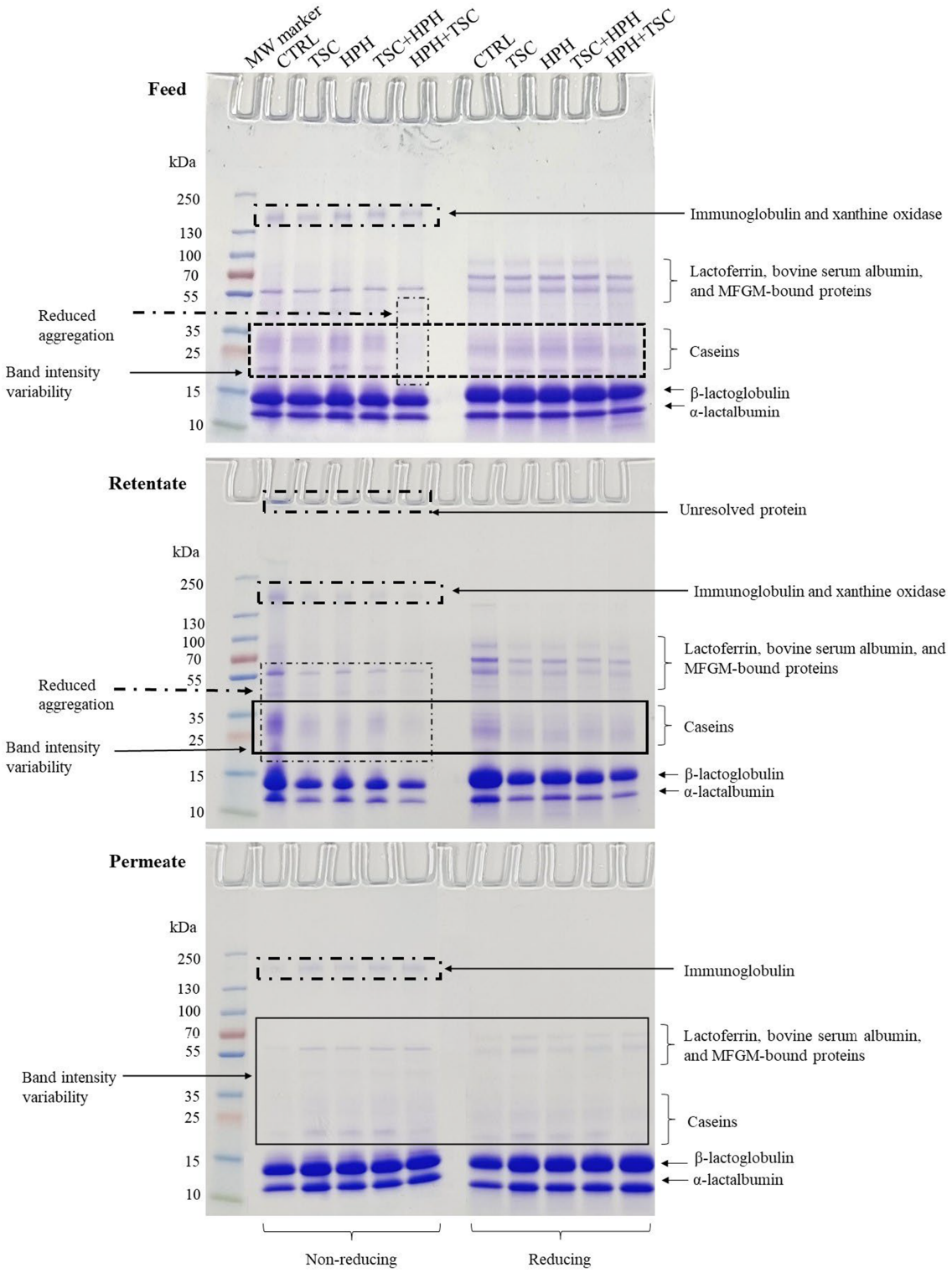


Figure 2 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis protein profiles of feed, retentate and permeate samples generated following microfiltration of different whey protein concentrate (WPC) pre-treated feed samples at 12.8 ± 1°C under nonreducing and reducing conditions. All samples on feed gel are loaded at 1 mg/mL protein concentration. On the retentate and permeate gels, the control sample was loaded at 1 mg/mL concentration, while the pre-treated samples were loaded on a volume basis. CTRL, Control WPC feed; HPH + TSC, WPC feed pre-treated with high-pressure homogenization at 650 bar followed by trisodium citrate; HPH, WPC feed homogenised at 650 bar; TSC + HPH; WPC feed pre-treated with trisodium citrate followed by high-pressure homogenization at 650 bar; TSC, WPC feed pre-treated with trisodium citrate.

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Table 4 Protein profile of feed, retentate and permeate samples generated following microfiltration of whey protein concentrate feed with different treatments at 12.8 ± 1°C.

		Gross composition (mg mL^{-1})			
Stream	Sample	α-la	β-lg	GMP	α-la:β-lg
Feed	CTRL	2.77 ± 0.03[a]	10.5 ± 0.50[a]	4.61 ± 0.33[a]	0.26 ± 0.01[a]
	TSC	2.72 ± 0.12[a]	10.3 ± 0.26[a]	4.71 ± 0.87[a]	0.27 ± 0.03[a]
	HPH	2.76 ± 0.12[a]	10.5 ± 0.46[a]	4.99 ± 0.28[a]	0.26 ± 0.02[a]
	TSC + HPH	2.74 ± 0.13[a]	10.5 ± 0.44[a]	4.99 ± 0.24[a]	0.26 ± 0.01[a]
	HPH + TSC	2.68 ± 0.01[a]	10.6 ± 0.27[a]	4.59 ± 0.74[a]	0.25 ± 0.04[a]
Retentate	CTRL	7.51 ± 0.19[a]	40.1 ± 0.15[a]	12.9 ± 1.51[a]	0.19 ± 0.03[a]
	TSC	5.52 ± 0.21[b]	37.5 ± 1.46[b]	11.8 ± 0.47[a]	0.15 ± 0.01[b]
	HPH	6.01 ± 0.12[b]	38.6 ± 2.41[b]	13.9 ± 1.85[a]	0.16 ± 0.01[ab]
	TSC + HPH	4.63 ± 0.11[b]	36.5 ± 0.90[b]	12.6 ± 0.91[a]	0.13 ± 0.01[cd]
	HPH + TSC	4.22 ± 0.11[c]	34.8 ± 1.22[b]	12.1 ± 1.02[a]	0.12 ± 0.01[d]
Permeate	CTRL	2.19 ± 0.14[b]	5.19 ± 0.10[c]	2.82 ± 0.18[a]	0.42 ± 0.03[a]
	TSC	2.28 ± 0.03[b]	5.41 ± 0.05[b]	2.78 ± 0.10[a]	0.42 ± 0.01[a]
	HPH	2.23 ± 0.04[b]	5.38 ± 0.07[a]	2.80 ± 0.04[a]	0.41 ± 0.00[a]
	TSC + HPH	2.36 ± 0.09[a]	5.49 ± 0.41[a]	2.81 ± 0.08[a]	0.43 ± 0.02[a]
	HPH + TSC	2.43 ± 0.04[a]	5.58 ± 0.04[a]	2.87 ± 0.05[a]	0.44 ± 0.01[a]

CTRL, control whey protein concentrate (WPC) feed; GMP, glycomacropeptide; HPH + TSC, WPC feed pre-treated with high pressure homogenisation at 650 bar followed by trisodium citrate; HPH, WPC feed homogenised at 650 bar; TSC + HPH, WPC feed pre-treated with trisodium citrate followed by high pressure homogenisation at 650 bar; TSC, WPC feed pre-treated with trisodium citrate; α-la, α-lactalbumin; β-lg, β-lactoglobulin.
[a–d]Means with different superscript letters within the same column and stream are significantly different ($P < 0.05$). The protein profile of all samples was analysed using reversed-phase high-performance liquid chromatography (RP-HPLC) with an Agilent 1220 Infinity II LC system.

binding to divalent cations such as Ca^{2+} and Mg^{2+}, which are involved in cation-mediated protein aggregation (Ozcan-Yilsay *et al*. 2007; McCarthy *et al*. 2017). By chelating these cations, TSC disrupts the ionic bridges stabilising protein aggregates, leading to their dissociation and formation of smaller protein particles. This reduction in aggregate size enhances protein transmission, subsequently reducing retention in the resulting retentate.

The presence of unresolved protein complexes in the wells of TSC pre-treated samples suggests that the aggregation is not solely mediated by cations. In addition, homogenisation improves permeation through size reduction, but does not dissociate chemically associated protein complexes, as evidenced by unresolved proteins >250 kDa. Under reducing conditions, band intensity is higher for all proteins in the CTRL retentate compared to retentates from pre-treated feed. No clear distinction exists in the intensity of protein bands between retentate samples from different pre-treated feeds under reducing conditions.

In the gel loaded with permeate samples, the intensity and number of visible bands were greater in samples generated from the pre-treated samples compared to the CTRL sample. Under nonreducing conditions, in the lane loaded with CTRL, the clearly identifiable bands were α-la and β-lg, with a low-intensity band at 150 kDa most likely representing Ig. In the pre-treated samples, in addition to α-la, β-lg, and Ig, additional bands appear around 20 kDa, likely casein fragments, and 55 kDa, corresponding to either BSA and/or LF (Saxena *et al*. 2009; Svanborg *et al*. 2015; Mehra *et al*. 2021). Previous studies have shown that during MF of whey generated from cheese processing, which has undergone multiple heat treatments, most of the Ig were associated with other proteins and retained in the MF retentate stream (Xiong *et al*. 2021). This could be a reason for the low intensity of the Ig band in the CTRL. In the samples generated following treatment, the intensity of the band representing Ig was high, providing evidence that treatment of WPC feed effectively achieved protein dissociation and ultimately increased permeation of Ig into the permeate stream.

Reverse-phase high-performance liquid chromatography

As expected, the most abundant protein in the samples from different treatments was β-lg (Table 4), which constitutes approximately half of the whey protein in bovine milk (Levin *et al*. 2016; Swaminathan *et al*. 2021; Ozturk *et al*. 2022). Retentate samples from TSC, TSC + HPH and HPH + TSC showed significantly lower ($P < 0.05$) levels of α-la and β-lg compared to the CTRL and HPH. This suggests that TSC, through its chelating and sequestration properties, releases these proteins from the aggregated protein matrix, enabling their permeation and subsequent reduction

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in the retentate stream (McCarthy *et al*. 2017; Li *et al*. 2018; Hebishy *et al*. 2019; Jin *et al*. 2022). Furthermore, in retentates, the ratio of α-la to β-lg was significantly reduced ($P < 0.05$) in TSC, TSC + HPH and HPH + TSC compared to the CTRL and HPH, indicating that α-la is predominantly released from the aggregated protein matrix. In the permeate streams, α-la levels were significantly higher ($P < 0.05$) in TSC + HPH and HPH + TSC than in the CTRL and other treatments, consistent with the lower α-la to β-lg ratio observed in the retentate stream. This suggests increased permeation of α-la into the permeate stream due to treatment of WPC. In addition, β-lg levels were significantly higher ($P < 0.05$) in pre-treated samples than the CTRL, further evidencing the impact of treatment of WPC on component partitioning mechanisms. However, the ratio of α-la to β-lg did not show significant variation ($P > 0.05$) between the different treatments. Our analysis indicated no significant difference ($P > 0.05$) in glycomacropeptide (GMP) content between different treatments within each stream. Ozturk *et al*. (2022) detected small amounts of κ-casein in WPPC. The content determined in this study could possibly include both GMP and residual κ-casein, as they are known to co-elute using the method used (van der Schaaf *et al*. 2024).

The observed differences in the partitioning mechanisms of key whey proteins, with increased permeation into the permeate stream, present a new opportunity for enhancing the yield of WPI. In addition, this process enriches important components such as PLs and MFGM-associated proteins in the retentate stream, consequently increasing the value of the retentate for potential applications in developing specialised ingredients. These ingredients could find utility in infant formula enrichment, as well as in products tailored for elderly and sports nutrition, owing to their significant biological, physiological, therapeutic effects and techno-functional properties (Levin *et al*. 2016; Córdova-Dávalos *et al*. 2019; Barone *et al*. 2020; Ozturk *et al*. 2022).

Whey protein denaturation

The amount of sedimented protein, expressed as a % of total protein, varied significantly ($P < 0.05$) among the treatments and streams (Table 5). HPH + TSC feed exhibited the lowest sedimentation (48.3%), significantly lower ($P < 0.05$) than the CTRL (51.7%) and other treatment feeds. This lower level of sedimentation in HPH + TSC is indicative of reduced protein aggregation and is due to the combination of HPH and TSC treatments. While homogenisation is known to cause physical disruption to whey protein aggregates, the subsequent addition of TSC in the HPH + TSC treatment, combined with extended storage time, would have created additional active sites for TSC, facilitating more extensive disaggregation of cation-mediated aggregates compared to using HPH alone. The proportion of

Table 5 Particle size and sedimented protein at pH 4.6 as a function of total protein, in the feed, retentate and permeate streams generated following microfiltration of whey protein concentrate with different treatments at 12.8 ± 1°C.

Stream	Sample	Z-average (nm)	Sedimented protein as a function of total protein (%)
Feed	CTRL	460 ± 6.08^{a}	51.7 ± 0.45^{a}
	TSC	466 ± 10.8^{a}	50.8 ± 0.43^{a}
	HPH	309 ± 46.2^{b}	49.2 ± 0.47^{b}
	TSC + HPH	339 ± 8.73^{b}	49.6 ± 0.41^{b}
	HPH + TSC	359 ± 2.19^{b}	48.3 ± 0.39^{c}
Retentate	CTRL	450 ± 7.28^{a}	65.0 ± 0.91^{a}
	TSC	453 ± 1.54^{a}	61.6 ± 1.72^{b}
	HPH	365 ± 10.4^{b}	51.4 ± 0.98^{c}
	TSC + HPH	357 ± 10.0^{b}	53.8 ± 2.18^{c}
	HPH + TSC	351 ± 10.3^{b}	43.4 ± 1.60^{d}
Permeate	CTRL	12.2 ± 0.47^{a}	29.8 ± 1.22^{d}
	TSC	11.3 ± 0.46^{a}	39.8 ± 1.17^{a}
	HPH	12.7 ± 1.47^{a}	32.7 ± 0.88^{c}
	TSC + HPH	12.2 ± 0.63^{a}	38.9 ± 0.56^{a}
	HPH + TSC	10.8 ± 0.93^{a}	35.7 ± 0.48^{b}

CTRL, control whey protein concentrate (WPC) feed; HPH + TSC, WPC feed pre-treated with high pressure homogenisation at 650 bar followed by trisodium citrate; HPH, WPC feed homogenised at 650 bar; TSC + HPH, WPC feed pre-treated with trisodium citrate followed by high pressure homogenisation at 650 bar; TSC, WPC feed pre-treated with trisodium citrate.

$^{a–d}$Means with different superscript letters within the same column and within the same stream are significantly different ($P < 0.05$).

denatured protein in the CTRL (51.7%) aligns with the range reported by Sodini *et al*. (2006), who reported 10–53% aggregated protein in WPC, while the results of the current study are higher than those reported by Barone *et al*. (2020), at 31.4%. These differences in the levels of native and denatured whey protein could be attributed to variation in the manufacturing processes in WPC, as highlighted in previous studies (Sodini *et al*. 2006; Walstra *et al*. 2006; Fox *et al*. 2015; Barone *et al*. 2020; Laursen *et al*. 2023). Factors such as heat treatment intensity, pH conditions during processing and the drying methods employed in WPC production can significantly influence protein denaturation and aggregation levels, potentially explaining the slightly higher aggregated protein content measured in our study.

Among the retentate streams, the CTRL showed the highest sedimentation (65%, $P < 0.05$), whereas HPH + TSC showed the lowest (43.4%, $P < 0.05$), following a pattern similar to that observed in the feed stream. Retentate from CTRL, TSC, HPH and TSC + HPH contained higher proportions of denatured material compared to HPH + TSC,

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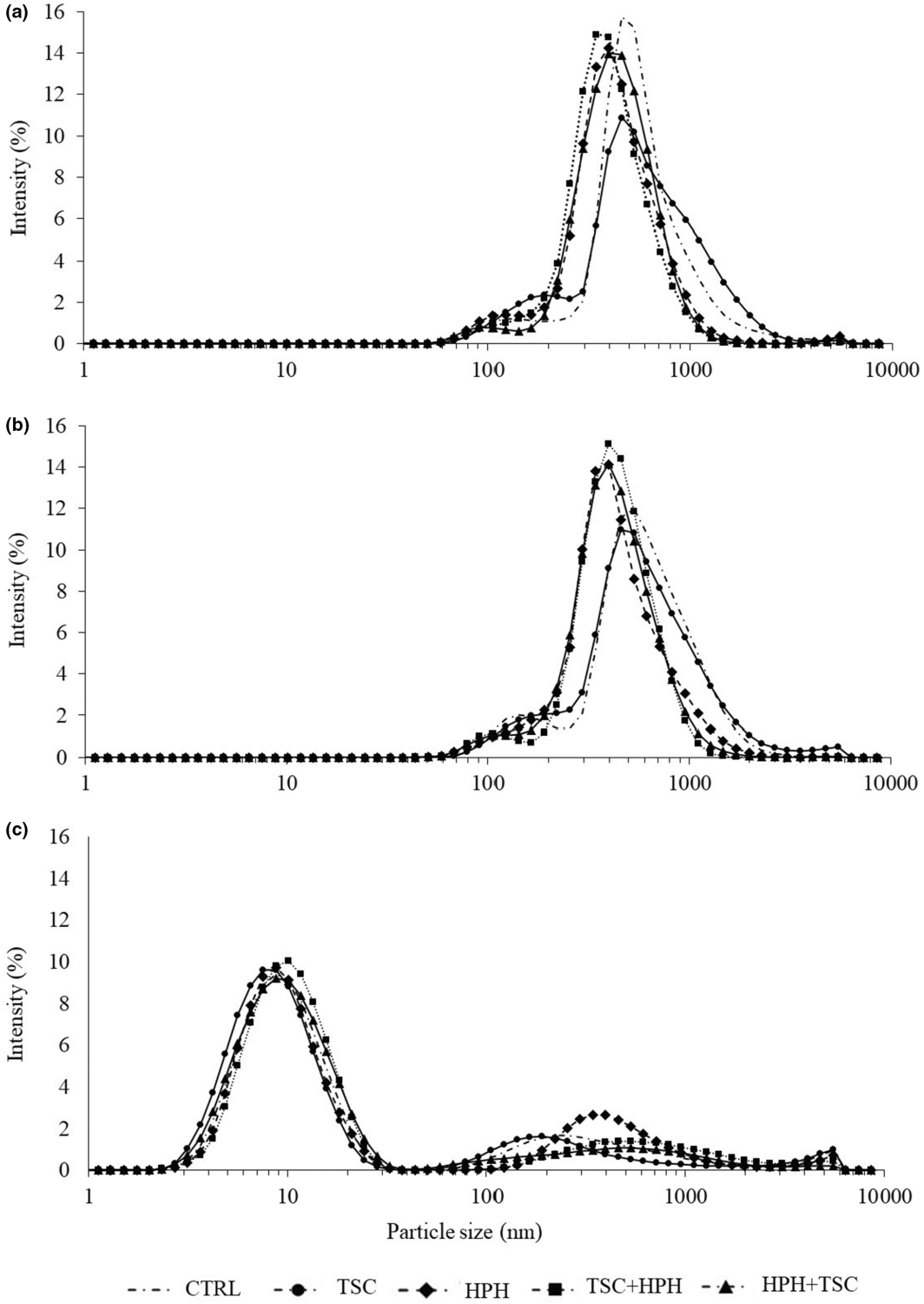


Figure 3 Particle size distribution (Z-average) in whey protein concentrate (WPC) feed (a), retentate (b) and permeate (c) samples generated following microfiltration of different WPC pre-treated feed samples at 12.8 ± 1°C. CTRL, Control WPC feed; TSC, WPC feed pre-treated with trisodium citrate; HPH, WPC feed homogenised at 650 bar; TSC + HPH, WPC feed pre-treated with trisodium citrate followed by high pressure homogenization at 650 bar; HPH + TSC, WPC-feed pre-treated with high-pressure homogenization at 650 bar followed by trisodium citrate.

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consistent with the SDS-PAGE gel analysis (Figure 2). The presence of larger aggregated and denatured particles likely contributed to the higher sedimentation in these samples, leading to the higher measured protein retention during MF. In contrast, smaller aggregates and native proteins were more likely to permeate, consistent with findings from previous studies (McCarthy *et al*. 2017; Price 2019; Reig *et al*. 2021). In the permeate stream, the CTRL exhibited significantly lower ($P < 0.05$) sedimentation than the permeate samples from pre-treated feed, indicating that the increased protein permeation achieved through TSC and HPH treatments predominantly involved smaller aggregates.

Particle size distribution

The particle size of samples from various treatments and streams was determined by dynamic light scattering (DLS) and the data were presented as intensity z-average diameter values. Samples showed a bimodal distribution (Figure 3), but further analysis with the correlogram–correlation function revealed that over 99.9% of the particles had a unimodal distribution, with the second peaks attributable to less than 0.01% of larger particles emitting high light intensity. Therefore, the reported distribution is unimodal, with intensity-based z-average values provided.

In this study, treatment of WPC feed significantly influenced particle size. Samples subjected to HPH, TSC + HPH and HPH + TSC showed significantly smaller ($P < 0.05$) particle size than those of CTRL and TSC, being 100–150 nm smaller (Table 5). Interestingly, TSC pre-treated feed showed slightly larger particle size compared to CTRL, which can be attributed to the sensitivity of DLS measurements to light scattering by small proportions of large aggregates. Despite this, the chelation of divalent ions (e.g. Ca^{2+} and Mg^{2+}) by TSC likely reduced aggregate stability, thereby improving protein solubility and contributing to an overall decrease in particle size across treatments. The observed large particle size in WPC feed may be associated with heat-induced denaturation and aggregation during the manufacture of WPC, a phenomenon well documented in whey protein powder products (Mulcahy *et al*. 2017; Hebishy *et al*. 2019; Yates *et al*. 2022).

The particle size data for the feed and retentate samples across different treatments indicate the presence of larger protein aggregates, consistent with the 670 nm particle size of WPC reported by Barone *et al*. (2020). In contrast, significant differences ($P < 0.05$) in particle size were observed in the permeate samples across treatments. However, the smallest particle size was recorded for HPH + TSC, suggesting that the combined effect of homogenisation followed by TSC treatment facilitated further disaggregation of aggregates, resulting in smaller particle sizes, which ultimately translated into higher protein permeation. The particle sizes measured in the permeate samples for the current study align with those of previous studies, including Mulcahy *et al*. (2017), who reported 20–30 nm, Ryan *et al*. (2012), with 43–66 nm, and Dissanayake and Vasiljevic (2009), who observed particle size ranging from 10 to 100 nm, while characterising the properties of WPI, native and soluble aggregates of WPI, and whey proteins, respectively.

CONCLUSION

This study demonstrated that pre-treating WPC effectively reduced protein aggregation, leading to increased permeate flux and modified component partitioning during MF. The reduction in protein content, particle size and mineral concentrations in the MF retentates from pre-treated samples was evidence of reduced protein aggregation. Among the treatments studied, the combined use of HPH and TSC was the most effective in modifying component transmission during subsequent MF. This effectiveness was attributed to the sequential action of homogenisation, followed by TSC treatments, likely exposing additional active sites for TSC to bind minerals, further enhancing protein disaggregation. Indeed, the significantly lower concentrations of calcium and magnesium in the retentate of TSC-treated samples underscores the role of these cations in mediating protein aggregation. This study is the first to show improved protein permeation during the MF of WPC as a result of targeted treatments designed to enable the enrichment of bioactive compounds, such as PLs, in the retentate stream, contributing to valorisation and fostering sustainable innovation in dairy ingredient development.

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AUTHOR CONTRIBUTIONS

Asfaw T. Mestawet: Conceptualization; investigation; funding acquisition; writing – original draft; methodology; validation; visualization; formal analysis; project administration. **Thomas C. France:** Investigation; writing – review and editing; supervision. **Patrick G. J. Mulcahy:** Conceptualization; writing – review and editing; methodology; supervision. **James A. O'Mahony:** Conceptualization; investigation; funding acquisition; writing – review and editing; visualization; validation; methodology; project administration; supervision; resources.

CONFLICT OF INTEREST STATEMENT

All authors declare that they have no conflict of interest.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

The following supporting information is available for this article:

Figure S1. Pretrial results presented in different figures. (a) particle size distribution (Z-average) in whey protein concentrate (WPC) feed and chemical-treated WPC samples, (b) confocal microscopy images visualising particle size in WPC feed and WPC treated with ethylenediaminetetraacetic acid, (c) particle size distribution (Z-average) in WPC feed samples treated with various concentrations of trisodium citrate, (d) particle size distribution (Z-average) in WPC feed samples pretreated with different levels of high-pressure homogenisation and (e) particle size distribution (Z-average) in WPC feed and samples treated with 5 mM trisodium citrate and high-pressure homogenisation at 350, 500 or 650 bar.

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