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Effects of depleting ionic strength on ^{31}P nuclear magnetic resonancespectra of micellar casein during membrane separation and diafiltration of skim milk

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ABSTRACT

Membrane separation processes used in the concentration and isolation of micellar casein-based milk proteins from skim milk rely on extensive permeation of its soluble serum constituents, especially lactose and minerals. Whereas extensive literature exists on how these processes influence the gross composition of milk proteins, we have little understanding of the effects of such ionic depletion on the core structural unit of micellar casein [i.e., the casein phosphate nanocluster (CPN)]. The ^{31}P nuclear magnetic resonance (NMR) is an analytical technique that is capable of identifying soluble and organic forms of phosphate in milk. Thus, our objective was to investigate changes to the ^{31}P NMR spectra of skim milk during microfiltration (MF) and diafiltration (DF) by tracking movements in different species of phosphate. In particular, we examined the peak at 1.11 ppm corresponding to inorganic phosphate in the serum, as well as the low-intensity broad signal between 1.5 and 3.0 ppm attributed to casein-associated phosphate in the retentate. The MF concentration and DF using water caused a shift in the relevant ^{31}P NMR peak that could be minimized if orthophosphate was added to the DF water. However, this did not resolve the simultaneous change in retentate pH and increased solubilization of micellar casein protein. The addition of calcium in combination with orthophosphate prevented micellar casein solubilization and simultaneously contributed to preservation of the CPN structure, except for overcorrection of retentate pH in the acidic direction. A more complex DF solution, involving a combination of phosphate, calcium, and citrate, succeeded in both CPN and micellar casein structure preservation while maintaining retentate pH in the region of the original milk pH. The combination of ^{31}P NMR as an analytical technique and experimental probe during MF/DF processes provided useful insights into changes occurring to CPN while retaining the micellar state of casein.

Key words: phosphoprotein, nuclear magnetic resonance, casein, phosphate nanocluster, micellar structure

INTRODUCTION

Since its discovery, nuclear magnetic resonance (**NMR**) spectroscopy (Bloch, 1946; Purcell et al., 1946) has been successfully applied to many branches of science. In food chemistry, ^{1}H NMR has been used to analyze all major categories of food (Mannina et al., 2012), particularly with respect to product quality and structure. In the case of milk proteins, ^{31}P NMR (Spyros and Dais, 2009) lends itself to the study of casein because of the presence of phosphoserine residues. Casein proteins are divided in 2 categories, calcium-sensitive (α_{s1}-, α_{s2}-, and β-CN) and noncalcium-sensitive (κ-CN; Horne, 2006); however, the aforementioned calcium-sensitive caseins are mainly phosphorylated (West, 1986) and, thus, frequently referred to as phosphoproteins. Caseins self-assemble in milk to form colloidal particles known as micelles (**MC**; Fox and Brodkorb, 2008). Phosphoserines in conjunction with other carboxylic residues can chelate Ca and P (Cross et al., 2005) to form casein phosphate nanoclusters (**CPN**; Lenton et al., 2015). The behavior of milk proteins during UF, microfiltration (**MF**), and diafiltration (**DF**) has been investigated in various studies (Hernández and Harte, 2009; Hurt and Barbano, 2010; Ferrer et al., 2011, 2014); however, the influence of these processes on CPN is not totally understood and needs further investigation (Gaucher et al., 2007; Li and Corredig, 2014; Liu et al., 2014). The first ^{31}P NMR spectra of milk was generated in 1985 (Belton et al., 1985), and in subsequent studies the technique was used to identify the small organic phosphorus molecules (Wahlgren et al., 1986) present in the soluble phase of milk and to differentiate between the caseins of different species (Belton and Lyster, 1991). The in-

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tensity of the NMR signal has been used to elucidate the redistribution of milk minerals during temperature treatment (Wahlgren et al., 1990). In addition, solid state NMR (MAS NMR) is capable of distinguishing between soluble and insoluble CPN (Thomsen et al., 1995), as well as detailing the distribution of P in cheese (Gobet et al., 2013). A study on the diffusion of ^{32}P in milk (Kolar et al., 2002) using the ^{31}P NMR relaxation signal with subsequent deconvolution showed how the population of casein nanoclusters is not uniform, but represented by different types of nanoclusters. The ^{31}P NMR spectral changes in response to pH alteration, as well as differentiation between micellar and solubilized casein (in the form of sodium caseinate), have also been studied (Gonzalez-Jordan et al., 2015). The same research group later investigated the mobility of organic and inorganic P in a sodium caseinate suspension in response to the addition of different concentrations of Ca and orthophosphate ions (Thomar et al., 2016). Those studies demonstrated the capability of NMR to generate spectra that define the CPN and, furthermore, revealed that its structure is not uniform, contrary to widely held views by other researchers (Holt, 2004). To date, no relationship has been established between the spectra generated by ^{31}P NMR and the different P species in milk and their interactions with Ca. Furthermore, as P plays a key role in micelle formation via the CPN, it would be valuable to ascertain the usefulness of ^{31}P NMR to monitor effects on CPN during milk processes, such as membrane separation or DF, which disturb the ionic equilibrium of milk through depletion of its soluble salts.

MATERIALS AND METHODS

MF of Skim Milk

A NovaSet-LS membrane (TangenX, Shrewsbury, MA) was used to microfilter (pore size 0.1 μm) pasteurized skimmed bovine milk at 40°C and a constant feed pressure of 100 kPa. Six DF steps were performed, all based on an original starting volume of 1 L of milk. The DF process was initiated after concentrating the retentate to 500 mL and adding 500 mL of DF solution to restore the volume to 1 L. Thus, a total of 3 L of DF solution was used during the course of the successive DF steps. The individual solutions used for DF were (1) MilliQ water (**DF-W**; Millipore, Billerica, MA); (2) 20 m*M* sodium dibasic orthophosphate (**DF-P**); (3) 10 m*M* citric acid (**DF-Cit**); (4) 20 m*M* sodium dibasic orthophosphate + 10 m*M* calcium chloride [**DF-(P+Ca)**]; (5) 10 m*M* citric acid + 10 m*M* calcium chloride [**DF-(Cit+Ca)**]; and (6) 20 m*M* sodium dibasic orthophosphate + 10 m*M* citric acid + 10 m*M* calcium chloride [**DF-(P+Cit+Ca)**]. The individual or collective minerals included in the DF solutions (2–6) were chosen to minimize depletion of those specific minerals from milk that typically occurs during the DF process. Calcium and citrate (**Cit**) were added to the respective DF solutions at a level corresponding to their soluble content in milk (Gaucheron 2005). Phosphorus addition at 20 m*M*, however, corresponded to the typical concentration of inorganic P in milk. All mineral solutions were adjusted to pH 7 using 0.1 *M* sodium hydroxide to control the influence of pH during the DF process. Samples were collected at the beginning of the process (milk sample), at the end of the first concentration (**R1**) and at the end of DF [DF-W, DF-P, DF-Cit, DF-(P+Ca), DF-(Cit+Ca), and DF-(P+Cit+Ca)]. Sodium azide 0.3 mg/mL was added to all samples to control microbial growth. Two different solutions of DF-(P+Ca) and DF-(P+Cit+Ca) were used to avoid precipitation of the insoluble calcium phosphate salt. Each DF step in these 2 cases was executed by first adding of 250 mL of a 40 m*M* P solution, immediately after which was added 250 mL of 20 m*M* calcium or 20 m*M* calcium and Cit. This was performed to avoid Ca-casein aggregation, which is not reversible following the addition of P (Thomar et al., 2016).

Phosphorus and Calcium Analyses

Total Ca and P analyses were conducted by an external laboratory (Eurofins, Eurofins Cork Limited, Glanmire, Ireland), using atomic absorption (FT013) for determination of total Ca content and a colorimetric method (FT014) for total phosphorus content.

pH Measurement

The pH of milk and the final retentate was determined using a Mettler Toledo pH meter (Mettler-Toledo Ltd., Beaumont Leys, Leicester, UK). The pH meter was calibrated with standard pH solutions. The pH of milk and the retentate was measured directly during the MF process at a temperature of 40°C.

NMR Spectroscopy

The ^{31}P NMR spectra were collected using a 500-MHz Bruker spectra (Bruker UK Ltd., Coventry, UK) at 25°C using an external phosphate solution as reference. A volume of 0.9 mL of sample was added to 0.1 mL of deuterium water to perform the signal lock. Data acquisition was obtained using a frequency of 202 MHz in proton decoupling, using an acquisition time of 0.4 s,

and a scan number of 5,024. All spectra were analyzed with Top Spin 3.2 (Bruker UK Ltd.).

Dynamic Light Scattering

Evaluation of micellar casein size was estimated using a Nano–ZS, (Malvern Zetasizer UK, Malvern). Samples were diluted 1:100 using the permeate solution obtained during the DF process to minimize multiple scattering effects. Measurements were performed in triplicate at 25°C. Results were calculated as cumulated mean diameter and referred to as hydrodynamic diameter (nm).

SDS PAGE Electrophoresis

The protein profiles of the samples and its distribution between retentate and serum fractions was assessed using SDS PAGE. The final retentate of the MF process was diluted 15 times using distilled water to obtain a protein content of ~4 μg of protein/μL; in the case of the starting milk sample the dilution used was 7.5 times (the half of the finale retentate), to obtain similar protein content to the retentates. Diluted samples were then mixed with Nu PAGE (BIO-Sciences Ltd., Dun Laoghaire, Ireland) SDS-reducing buffer and diluted to ~1 μg of protein/μL (in 4 dilution). The samples were loaded (10 μL) on to precast gels 12% Bis-TRIS (1.0 mm × 10 well; Novex, Life Technologies, Carlsbad, CA) and run for 50 min at 200 V. The gels were stained with 0.25% (wt/vol) Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories Ltd., Watford, UK) and destained in methanol:acetic acid (15:10%). The soluble protein fractions were obtained by centrifuging the retentates and milk samples at 20,000 × g for 2.5 h at 25°C in a Eppendorf Centrifuge 5417R (Eppendorf UK Limited, Stevenage, UK) using rotor type F45–30–11. The supernatants were then carefully removed to avoid mixing with the pellets, and those were prepared as already explained for SDS PAGE, using a similar dilution to that used for the total suspension.

Scanning Electronic Microscopy

The samples were diluted 1:200 with milliQ water before scanning electron microscopy analysis. The diluted samples were dried onto the of sample holder, attached to scanning electron microscope stubs, and then coated with chromium (K550X, Emitech, Ashford, UK). Scanning electron microscopy images were collected using a Zeiss Supra 40P field emission SEM (Carl Zeiss SMT Ltd., Cambridge, UK) at 3.00 kV. Representative micrographs were taken at 10,000×, 25,000×, and 50,000× magnification to visualize the MC.

Statistical Analysis

Samples obtained from MF and DF processes as well as the initial milk feedstock were analyzed in triplicate for pH, TS, Ca, and P content. An ANOVA was undertaken using Minitab version 17 (Minitab Inc.). The level of significance was established at $P < 0.05$. Fisher's multiple-comparison test was used for paired comparison of treatment means and the level of significance was determined at $P < 0.05$.

RESULTS AND DISCUSSION

Mineral Equilibrium and ^{31}P NMR

The main objective of our study was to investigate changes to the ^{31}P NMR spectra of skim milk during MF and DF by tracking movements of different species of P [e.g., casein-associated phosphate in the retentate and inorganic phosphate ($\mathbf{P}_i$) in the serum]. The ionic composition of milk is typically altered during different processing steps (e.g., membrane filtration and cheesemaking). Changing the concentration of the mineral or ionic species present in milk may influence the equilibrium of the CPN according to the stoichiometric reaction conditions illustrated in Figure 1. To date however, little information exists regarding the effect of ionic depletion on CPN; hence, it was opportune to monitor such changes during membrane filtration of milk using a benchtop membrane system. Furthermore, it was possible to modify the DF conditions by incorporating specific minerals in the DF solutions to partially compensate for the losses due to permeation across the membrane. Irrespective of DF conditions, it was noticeable that this separation process did not completely destroy the MC conformation as supported by scanning electron microscopy of the retentates produced (Figure 2) and also by SDS PAGE (Figure 3A). However, differences were evident in the total concentration of P and Ca present in the final retentates following the different DF processes (Table 1). Both soluble and CPN phosphates were identified in the ^{31}P NMR spectrum of milk (Figure 4), in accordance with that originally characterized by Wahlgren et al. (1986). Of particular interest was the peak occurring at 1.11 ppm associated with the P_i ($H_2PO_4^-$ and HPO_4^{2-}) in the serum. In addition, it was possible to observe a low-intensity broad signal between 1.5 and 3.0 ppm. This was attributed to casein-associated phosphate (Belton and Lyster, 1991), the low intensity of which was related to the micellar status of the casein. However, this signal was more visible following concentration of the micellar casein by microfiltration (Figure 4). In previous research (Li and

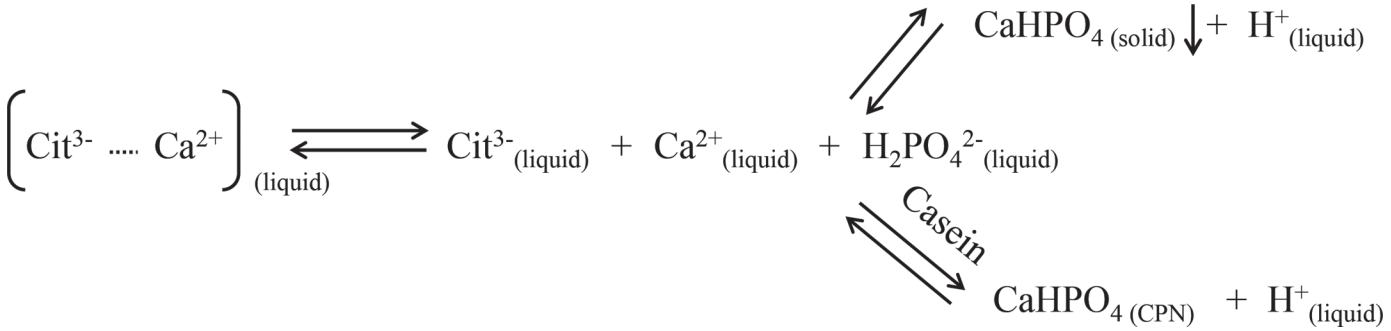

Figure 1. Mineral equilibrium between aqueous and micellar states in milk. The charges shown are the most prevalent species according to Gaucheron (2005). Cit = citrate; CPN = casein phosphate nanocluster.

Corredig, 2014), concentration by UF had no effect on the behavior of CPN.

Water DF

Diafiltration with water reduced all soluble milk minerals, led to a small reduction in casein micelle size, and increased retentate pH (Table 1). The P-to-Ca ratio changed from 1.03 (milk) to 0.81 in the case of the final retentate (DF-W); however, there appeared to be no visible difference between the soluble casein levels (following sample centrifugation) of the final retentate and the initial milk, as seen in the SDS PAGE analyses (Figure 3B, lane 4 and 5). It was possible to observe that the signal from the small organic phosphorus molecules present in both milk and R1 (Figure 4) had completely disappeared at the end of the DF-W (3 different peaks visible from 0.62 ppm to 0.05, 2 peaks at 0.95 and 1.36 ppm) in the ^{31}P NMR spectrum of the final retentate following DF–W (Figure 5). On the other hand, due to its higher concentration, P_i was still present in the retentate at the end of DF, but with a peak shift to 1.4 ppm. The CPN signals, originally positioned at 1.5 to 3.0 ppm (Figure 4), shifted to higher ppm (2.5–3.5 ppm), and with sharper definition (i.e., within a range of 1 ppm). Thus, the 1-ppm distance between the CPN and P_i peaks in the case of DF-W compares with 0.5 ppm in the case of R1 (Figure 4).

Phosphate DF

Diafiltration was executed using DF-P (20 m*M*) or DF-(P+Ca) (at 20 and 10 m*M*, respectively). The objective of these DF steps was to maintain a constant concentration of P and P and Ca, respectively, in solution and to avoid a shift in ionic equilibrium that could cause dissociation of the nanocluster (Figure 1). In the ^{31}P NMR spectra for both of these DF treatments (Figure 6), a more intense signal was found for P_i at 1.4 ppm compared with that in DF-W (Figure 5). During DF-P, the signal of the CPN does not shift to a higher value of ppm, but remains low (2–3 ppm). It was possible to observe how DF-P reduced the mean diameter of the MC but did not influence retentate pH (Table 1), showing a final pH similar to DF-W. At the same time, the amount of nonsedimentable protein increased during this MF process (Figure 3B, lane 6). During DF-(P+Ca), casein micellar size remains similar ($P < 0.05$) to DF-W (Table 1). However, the addition of Ca in the form of DF-(P+Ca) reduced the solubilization of MC as seen in the SDS PAGE gel of the DF-P serum, with no casein bands visible in the serum of DF-P+Ca (Figure 3B, lane 7). At the same time, pH was reduced (Table 1) in line with the likely formation of new CPN or calcium phosphate precipitates (Figure 1). Casein peaks in DF-(P+Ca) (Figure 6) appear to be comparable with those of DF-P (Figure 6), except for a shift of signal to lower ppm due to an associated decrease in pH.

Citrate DF

The stability of MC was reduced during DF-Cit in a manner similar to that described previously when Cit was added to milk (Le Berre and Daufin, 1998; Hernández and Harte, 2009). This destabilization resulted in the appearance of a bimodal particle size distribution. It is possible to distinguish between 2 different apparent particle diameter populations, particularly the appearance of a separate peak of smaller mean particles size (23 nm) alongside that of the regular peak at 174 nm (Table 1) for micellar casein not subjected to the effects of added Cit. The destabilization was evident in the SDS PAGE gel where the soluble fraction (Figure 3B, lane 8) shows high intensity bands, whereas the bands corresponding to its total casein content (Figure 3A) were of similar intensity with respect to those obtained with other DF methods. In addition, the pH increased

to a higher value compared with DF-W (Table 1). Figure 7 shows the ^{31}P NMR spectra obtained following DF-Cit and DF-(Cit+Ca). During DF-Cit, a new multiplet appears at 3.3 to 4.0 ppm, in addition to a peak at 2.96 ppm, and to a change in P_i that now occurs at 2.46 ppm. In DF-(Cit+Ca), the combination of Ca and Cit during DF mitigates the Ca-chelating effect of Cit, thus reducing solubilization of the MC, visible by the intensity of the bands in the electrophoresis gel (Figure 3B, lane 9). Micelle size and pH of the final retentate were similar to those of DF-W ($P < 0.05$; Table 1). The peaks of CPN and P_i remain comparable with the DF-W spectra (except for a peak at 2.2 ppm), where addition of Ca to DF-Cit prevented dissociation of the MC.

Combined Salt Solution (P, Cit, and Ca) Diafiltration

The DF process which included all 3 ions (P, Ca, and Cit) minimized the effect of conventional DF (i.e.,

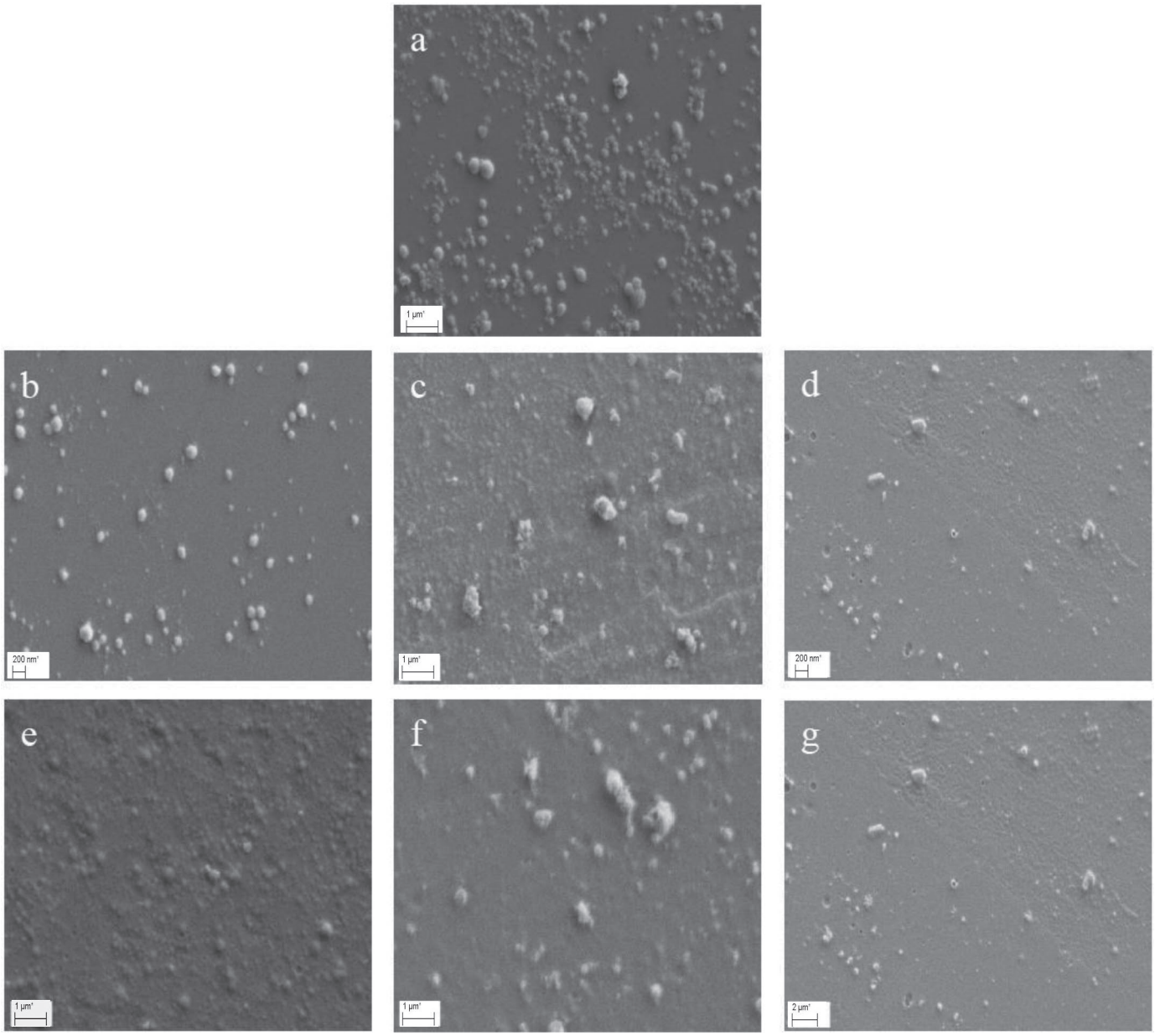

Figure 2. Scanning electron microscopy image of the final retentate following microfiltration and diafiltration of skim milk. Picture letters correspond to a = retentate (R1), and different diafiltration conditions; b = water; c = phosphate (P); d = P + Ca; e = citrate (Cit); f = Cit + Ca; and g = P + Ca + Cit.

Table 1. pH, TS, micelle size (hydrodynamic diameter), and phosphate (P) and calcium (Ca) concentration (m*M*) of the final retentate following skim milk microfiltration/diafiltration (MF/DF)[1]

		Diafiltration solutions					
Measured parameter	Skim milk	DF-W	DF-P	DF-(P+Ca)	DF-Cit	DF-(Cit+Ca)	DF-(P+Cit+Ca)
pH	6.55 ± 0.01^{d}	7.02 ± 0.01^{b}	7.00 ± 0.01^{b}	6.22 ± 0.04^{e}	7.88 ± 0.01^{a}	6.98 ± 0.01^{b}	6.83 ± 0.04^{c}
TS (% wt/wt)	9.11 ± 0.05^{a}	6.93 ± 0.05^{g}	7.71 ± 0.02^{f}	8.88 ± 0.04^{b}	7.97 ± 0.01^{e}	8.31 ± 0.03	8.62 ± 0.04^{c}
Hydrodynamic diameter (nm)	167 ± 2^{b}	157 ± 4^{cd}	147 ± 4^{e}	150.8 ± 0.2^{de}	174 ± 10^{a} 23 ± 4^{f}	157 ± 1^{cd}	161 ± 2^{bc}
P (m*M*)	33 ± 3^{e}	38.5 ± 0.1^{d}	60.6 ± 0.1^{c}	94 ± 2^{a}	22.7 ± 0.2^{f}	40.6 ± 0.1^{d}	67 ± 5^{b}
Ca (m*M*)	32 ± 3^{e}	47.5 ± 0.2^{d}	48.5 ± 0.1^{d}	103 ± 2^{a}	19.3 ± 0.4^{f}	64.9 ± 0.2^{c}	70 ± 5^{b}
P/Ca (m*M*/m*M*)	1.03 ± 0.03^{c}	0.811 ± 0.005^{f}	1.250 ± 0.005^{a}	0.912 ± 0.003^{e}	1.18 ± 0.03^{b}	0.626 ± 0.001^{g}	0.95 ± 0.02^{d}

[a–g]Means on the same row not sharing a common superscript letter are different ($P < 0.05$).

[1]DF-W = diafiltration with water; DF-P = diafiltration with phosphate; DF-(P+Ca) = diafiltration with a combined solution of phosphate and calcium; DF-Cit = diafiltration with citrate; DF-(Cit+Ca) = diafiltration with a combined solution of citrate and calcium; DF-(P+Cit+Ca) = diafiltration with a combined solution of phosphate, citrate, and calcium.

DF-W influence on pH and hydrodynamic diameter of the final retentate). Micelle size and pH were least influenced by DF-(P+Cit+Ca) (Table 1) and remained similar at the end of the process to the starting values of milk. Treatment with DF-(P+Cit+Ca) (Figure 3B, lane 10,) resulted a moderate visible increase in inten-

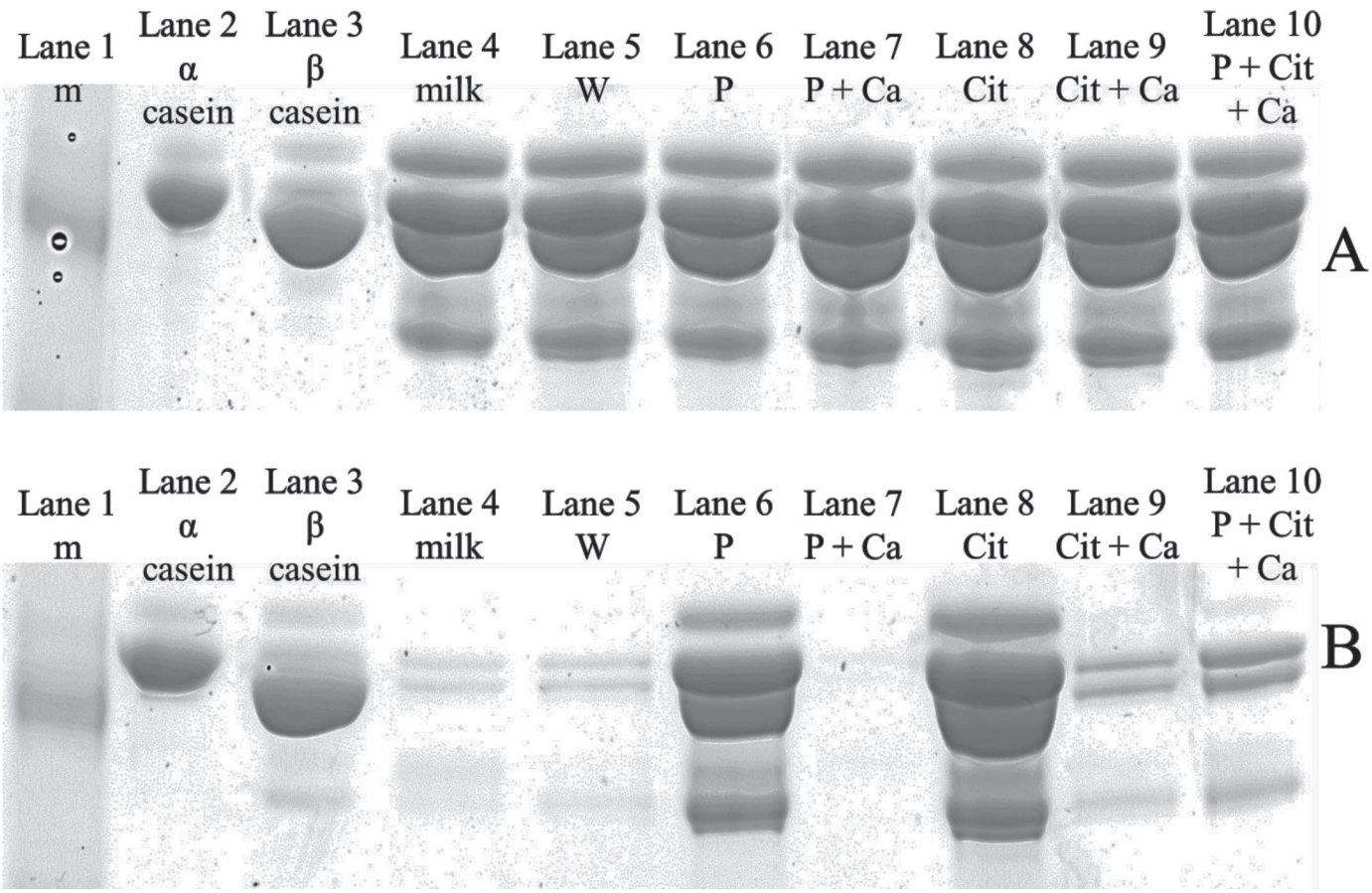

Figure 3. Reducing SDS PAGE of final retentates (gel A) and their corresponding supernatants (soluble fractions; gel B) obtained after centrifugation (20,000 × *g* for 2.5 h at 25°C) following microfiltration and diafiltration of skim milk. Lane 1 = protein markers; lane 2 = α_s-CN; lane 3 = β-CN; lane 4 = milk; lane 5 = diafiltration with water (W); lane 6 = diafiltration with phosphate (P); lane 7 = diafiltration with a combined solution of P and Ca; lane 8 = diafiltration with citrate (Cit); lane 9 = diafiltration with a combined solution of Cit + Ca; lane 10 = diafiltration with a combined solution of P + Ca + Cit.

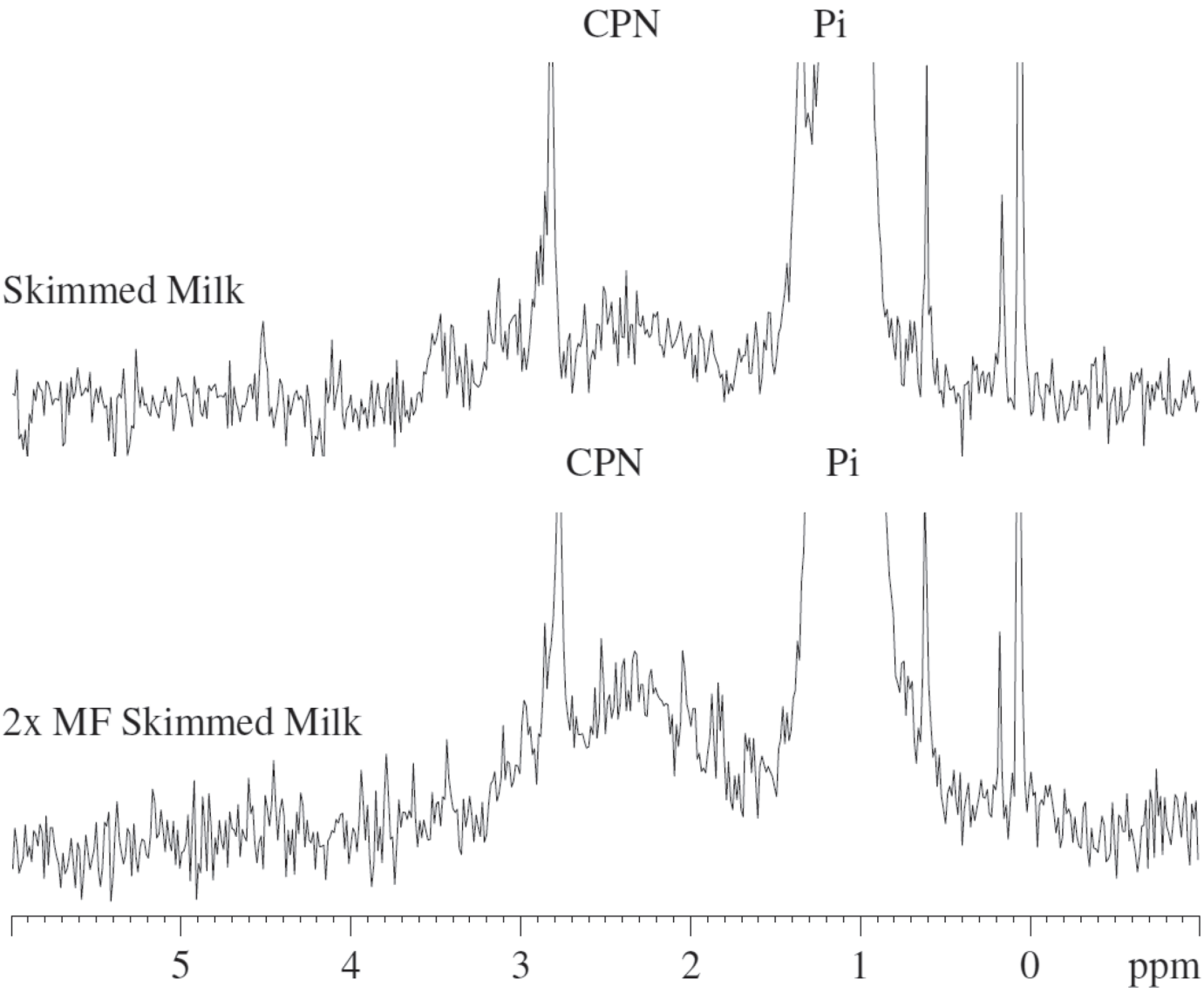

Figure 4. The ^{31}P nuclear magnetic resonance (NMR) of milk (top) and of 2× microfiltration (MF) skim milk retentate (bottom). The spectra show the peaks of the casein phosphate nanocluster (CPN) and inorganic phosphate (Pi), plus different peaks from organic ester phosphate.

sity of the soluble protein fraction, which is considerably lower than that caused by DF-P or DF-Cit, but slightly more intense than the starting milk. Figure 8 shows the ^{31}P NMR spectra of the final retentate obtained following DF-(P+Cit+Ca). The signal from the CPN does not change position during DF and remained between 2.0 to 3.0 ppm for R1 (Figure 4), P, and P+Ca (Figure 6).

Calcium Phosphate Nanocluster in the ^{31}P NMR Spectra of Milk During MF

During the DF process, the retentate pH typically increased except for a decrease in the case of DF-(P+Ca). This effect is clearly visible in the ^{31}P NMR spectra, for which correlations have already been established (Gonzalez-Jordan et al., 2015). However, pH change alone does not explain the ppm shifts observed in the NMR signal of CPN. Such shifting of ^{31}P NMR signals to lower or higher ppm indicates that some structural changes to CPN are likely to have taken place during milk MF or DF. In practice, such events typically occur during the manufacture of both micellar casein and milk protein concentrate-based commercial dairy ingredients. Our study showed for the first time direct evidence of the presence of 2 distinct patterns of ^{31}P NMR peaks for CPN. This is in agreement with Kolar et al. (2002), who relied on NMR relaxation and de-

convolution to prove the presence of different groups of nanoclusters while investigating the diffusion of ^{32}P in milk, and with Lenton et al. (2015), who theorized a nonuniform CPN structure. If we consider that CPN is a nonstoichiometric compound, it is likely to find the presence of different structural populations of CPN corresponding to different ratios of P to Ca. A change in the ratio between P and Ca in the milk serum will favor shifting of the equilibrium visible in Figure 1, as well as 1 of the 2 populations of clusters with different P-to-Ca ratios. For example, a high concentration of Ca ions in solution would favor a higher Ca content on the CPN structure. An increase of Ca^{2+} in such a structure will lead to a reduction in H^+ bound to P and an increase of the negative charge on the P group with consequent increase in the alkalinity of the nanocluster. This change would consequently shift the ^{31}P NMR signal to higher ppm, similar to that induced by an increase in milk pH (Gonzalez-Jordan et al., 2015).

In conclusion, it is possible to speculate that the structure of CPN could change under different ionic conditions in milk, and that the structure of the nanocluster would be largely influenced by the P-to-Ca molar ratio. This change could be followed using the ^{31}P NMR; for example, data presented in the current paper showed that the ^{31}P NMR signal of CPN shifted to higher ppm (2.5–3.5 ppm) only if the total P-to-Ca ratio was <0.9. The opposite is the case for a ratio >0.9, where the signal of ^{31}P NMR remains stable at 2 to 3 ppm. This shift was also shown in the study of Thomar et al.

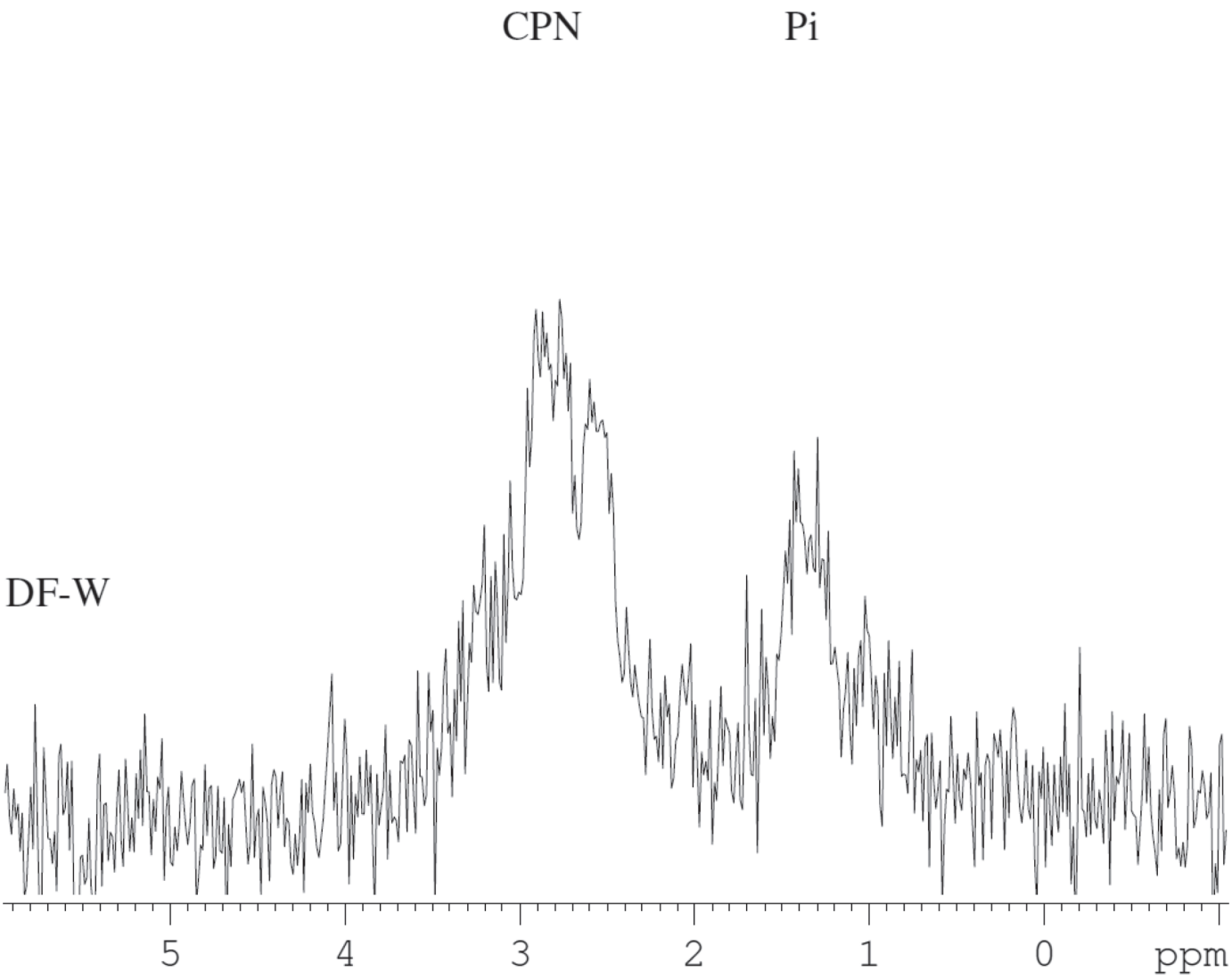

Figure 5. The ^{31}P nuclear magnetic resonance (NMR) spectra of final skim milk retentate following diafiltration with water (DF-W). The spectra show the peaks from the casein phosphate nanocluster (CPN) and inorganic phosphate (Pi).

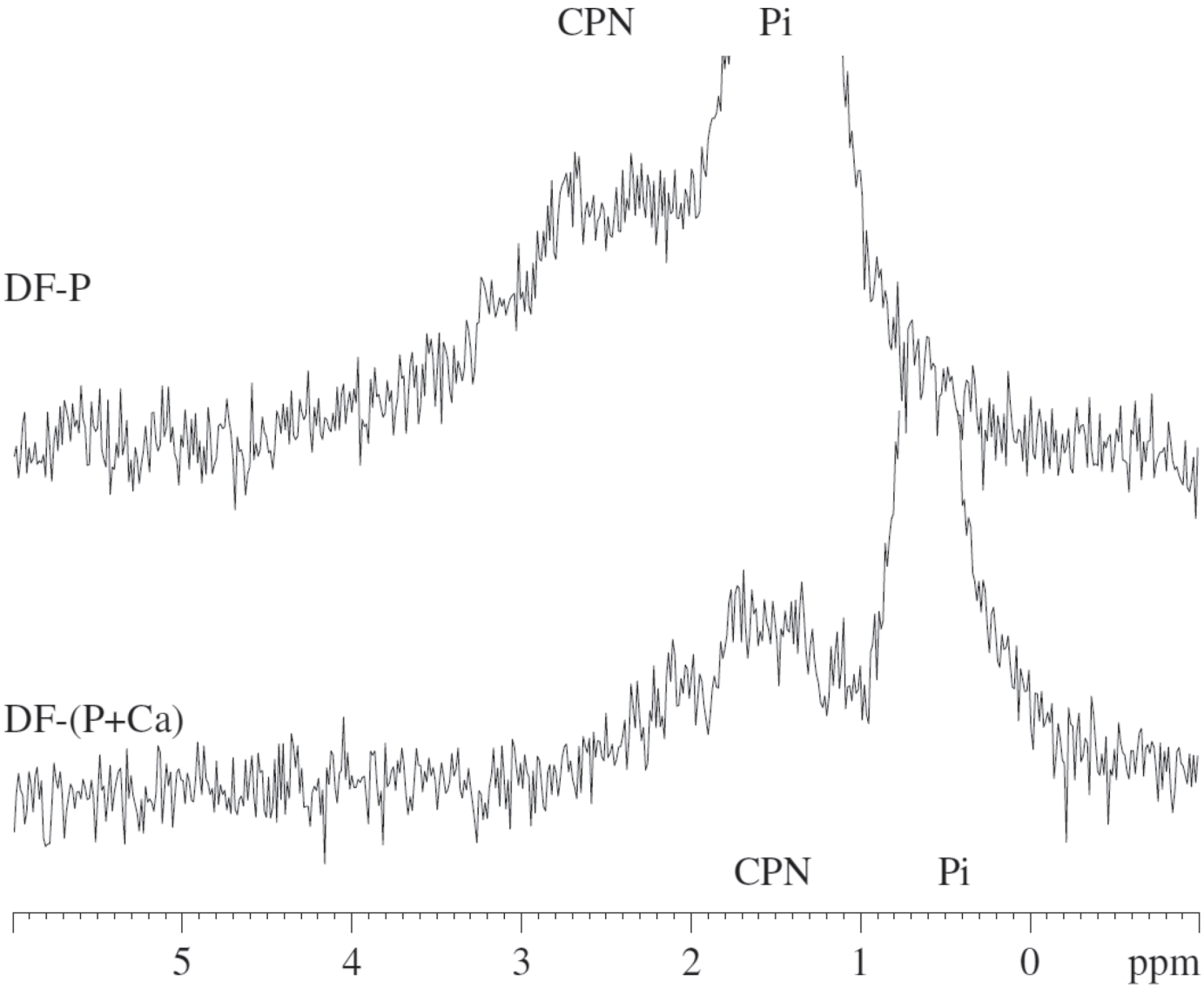

Figure 6. The ^{31}P nuclear magnetic resonance (NMR) spectra of final retentate following microfiltration or diafiltration of skim milk with orthophosphate (DF-P) and orthophosphate + calcium [DF-(P+Ca)]. The spectra show the peaks from the casein phosphate nanocluster (CPN) and inorganic phosphate (Pi).

(2016), when sodium caseinate was suspended at different concentrations of P and Ca. Thomar et al. (2016) concluded that this shift was caused by the different affinities of Ser-P for Ca^{2+}. This explanation, however, could not be applied to the results shown in the current paper, as the signal of casein phosphate derived from Ser-P and inorganic phosphorus are incorporated in the CPN group. Observing the spectra of milk and R1 (Figure 4), it is possible to observe a CPN signal positioned between 3.0 and 1.5 ppm, which overlaps at higher ppm values for both CPN DF-W (3.3 to 2.3) and CPN DF-P (3.3 to 1.9) signals (Figure 5 and 6). These 2 DF systems are at the outer limits of the P-to-Ca ratio generated by our experiment, showing the 2 possible extremes of the CPN structure (e.g., DF-W will have an high Ca content CPN structure, whereas DF-P will have a low Ca content). The overlapping of the milk CPN ^{31}P NMR signal creates a compelling case that the CPN in milk is defined by its dual mineral structural elements (P/Ca <0.9 and P/Ca >0.9 CPN).

^{31}P NMR and DF Interventions During MF

By influencing the ionic strength during different dairy processes, it should be possible to favor one or another structure, as illustrated by the results obtained

during DF in our paper. The influence of ionic interventions on mineral distributions was demonstrated using ^{31}P NMR, whereas their process effect on the distribution of casein proteins within the soluble or the colloidal fractions was demonstrated using SDS PAGE. The DF-W showed no apparent effect on protein distribution, leaving it similar to the starting milk. The addition of Ca-chelating agents (DF-P and DF-Cit) increased the soluble fraction of casein in the final retentate. However, DF interventions incorporating Ca (DF-P+Ca, DF-Cit+Ca, and DF-P+Cit+Ca) were shown to act as protectors against the P and Cit effects by reducing the dissociation of MC. Diafiltration with different solutions altered mineral concentrations in line with that found by Gaucheron (2005). Phosphorus and Ca contents change in accordance with the increase in concentration of micellar casein (2×) and the depletion of soluble phase when comparing skim milk with DF-W. A measure of the distribution of P between the soluble and colloidal phases may be obtained by observing the intensity of the ^{31}P NMR P_i peak. Hence, it was possible to observe that the concentration of P_i is higher in DF-P, DF-P+Ca, and DF-P+Ca+Cit and lower in DF-W and DF-Cit+Ca. Following modification of the mineral contents of DF solutions, the ratio between P and Ca changed from 1.03 in the case of

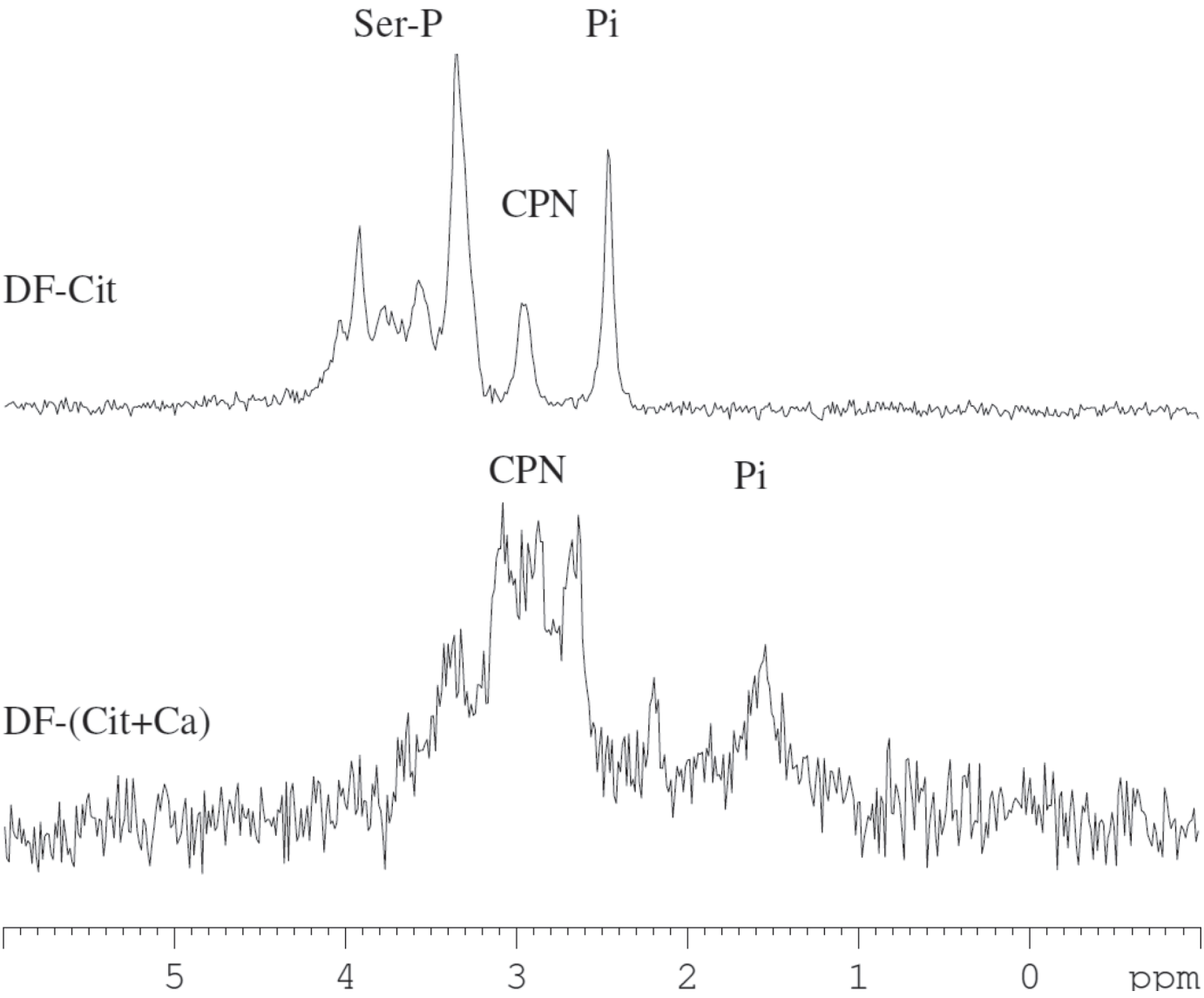

Figure 7. The ^{31}P nuclear magnetic resonance (NMR) spectra of final retentate following microfiltration or diafiltration of skim milk with citrate (DF-Cit) and of citrate + calcium [DF-(Cit+Ca)]. The spectra show the peaks of the casein phosphate nanocluster (CPN), soluble serine phosphate residue (Ser-P), and inorganic phosphate (Pi).

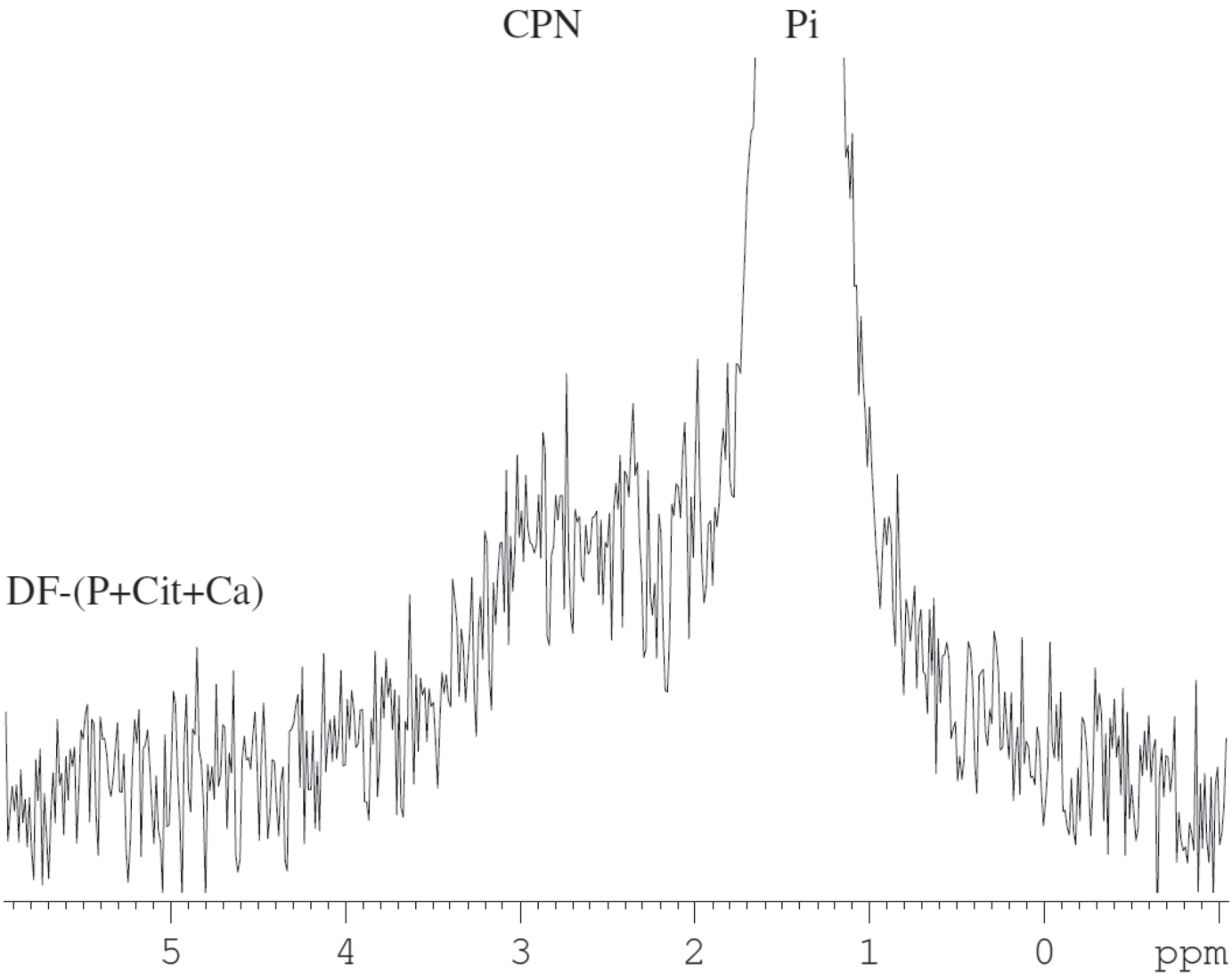

Figure 8. The ^{31}P nuclear magnetic resonance (NMR) spectra of final retentate following microfiltration or diafiltration with phosphate + citrate + calcium [DF-(P+Cit+Ca)]. The spectra show the peaks of the casein phosphate nanocluster (CPN) and inorganic phosphate (Pi).

milk to 1.25 (DF-P) and 0.63 [DF-(Cit+Ca)] with its attendant influences on the structure of CPN. The ^{31}P NMR spectra showed that DF in the presence of P is necessary to maintain the structure of the nanocluster (as defined by the original ^{31}P NMR spectrum) close to that of milk apart from an increase in retentate pH and an increase in soluble casein; Hernández and Harte (2009) obtained a similar result. However, the increase of soluble fraction of casein, visible in Figure 3B (lane 6), was not accompanied by an increase in the signal of soluble Ser-P in the ^{31}P NMR results (Figure 6), but was visible during DF-Cit (Figure 7).

This distinction between DF-P and DF-Cit highlights differences in the behavior between P and Cit chelation of Ca. The DF-P spectrum shows a broad CPN peak without any sharp peak, indicating that the CPN structure is not influenced by DF-P solubilization and that the soluble casein is present as small particles not sedimentable by centrifugation within the CPN (i.e., similar to MC CPN at its core structure). This view of CPN aggregation is also supported by evidence according to the hydrodynamic diameter (Table 1), where DF-Cit showed a bimodal distribution, whereas DF-P was monomodal but with a smaller diameter compared with DF-W. This result is in agreement with Gaucher et al. (2007), who concluded that at low concentrations ($<$50 mM) added P does not displace Ca from CPN because the affinity of Ca is higher for micellar P than for added P_i. Gaucher et al. (2007) also found that P concentration in the diffusible phase was less than added levels, thus leading to the conclusion that precipitation or combination with the MC-calcium-phosphate com-

plexes was occurring. In the present study, the extra P concentration (Table 1) in the DF-P permeate corresponded to the amount of added P (~20 m*M*), whereas the Ca concentration remained the similar ($P < 0.05$) for DF-W and DF-P. Hence, we found no evidence of added P-induced precipitation with soluble Ca, thus suggesting that it associated with CPN increase in the P-to-Ca ratio.

Introducing Ca along with P [DF-(P+Ca)] also preserved CPN and MC structure but overcorrected the pH change in the acidic direction. The pH change and the high concentration of P and Ca in the final retentate of DF-(P+Ca) (Table 1) are indicative of precipitation of calcium phosphate salts, as predicted by Gaucher et al. (2007). Unfortunately, this precipitate was not detectable in the ^{31}P NMR spectrum. The addition of Ca during DF or MF prevented the tendency by Ca-chelation agents to increase the amount of the soluble casein fraction (as evident in Figure 3B, lane 7). However, the optimum solution was to incorporate Cit along with the aforementioned DF-(P+Cit+Ca) to accomplish both objectives (i.e., CPN/MC structural preservation and maintenance of retentate pH in the region of the original milk pH). On the other hand, an excess of Cit addition during DF reduced the stability of micellar casein. In milk, over 90% of the Cit is soluble, except for that proportion associated with the micellar phase in the form of Ca Cit (Gaucheron, 2005); its ability to chelate Ca^{2+} (Singh et al., 1991) reduces the stability of milk during processing (Le Berre and Daufin, 1998; Hernández and Harte, 2009). Excess Ca chelation is antagonistic to the stability of CPN and the overall micellar status of casein. During DF-Cit, this effect is manifested by the appearance of a bimodal particle size distribution, in line with the findings of previous research (Le Berre and Daufin, 1998; Hernández and Harte, 2009) and with the SDS PAGE results (Figure 3). The DF-Cit causes a shift to pH 7.88, and this was reflected in a corresponding shift in ^{31}P NMR spectra to higher ppm (Figure 7); this increase in pH was attributable to solubilization of CPN (Figure 1).

A loosening of the micellar state of casein is noticeable in the ^{31}P NMR spectrum in the region of the typical multiplet (3.3–4 ppm) corresponding to soluble casein (Gonzalez-Jordan et al., 2015). Hernández and Harte (2009) found that MF retentates retained some casein protein following treatment with 50 m*M* Cit 5 volume DF with respect to the starting sample's volume. Figure 3B, lane 8, shows that the greater part of the MC was destabilized by the DF-Cit process, leading to an increase in the intensity of the bands in the soluble protein fraction in the electrophoresis gel. However, the presence of pellets following centrifugation of DF-Cit retentates confirms that a certain proportion of casein remained in the micellar state. In addition, ^{31}P NMR analysis confirmed the presence of retentate micellar casein according to a peak at 2.96 ppm, which was also evident from the scanning electron microscopy micrograph (Figure 2e). This observation (scanning electron microscopy, ^{31}P NMR, and previous work) leads to the conclusion that a small portion of casein in the final retentate obtained with DF-Cit retains its micellar state. The corresponding peak on the ^{31}P NMR spectrum could be compared with a low ppm value signal from micellar casein following DF-P, as its distance from P_i (2.46 ppm) was only of the order of 0.5 ppm, whereas in DF-W its distance was 1 ppm, in agreement with the P and Ca influence already shown.

The DF-(Cit+Ca) showed how the effect of Ca chelation by Cit was the driving force behind micelle dissociation. The soluble protein fraction of this DF process [DF-(Cit+Ca)] showed (Figure 3B, lane 9) a low content of protein, even if slightly higher than for DF-W. The final retentate pH and micellar size of DF-(Cit+Ca) were similar to those of DF-W. The ^{31}P NMR spectra of DF-(Cit+Ca) were similar to DF-W, except for a lower intensity peak at 2.20 ppm, which coincided with the signal obtained with DF-Cit, thus attributing the effect to the presence of excess Cit. These results show that ^{31}P NMR is a useful analytical tool to help understand the effects of industrial processes on submicellar changes at CPN level. Therefore, ^{31}P NMR may be useful when retrospectively analyzing nanocluster status to identify changes induced during the processing of milk at industrial scale (e.g., if minimal shifts in the NMR spectra are required, the use of more complex DF conditions, as outlined in our study, should aim to maintain retentate P/Ca >0.9 so as to ensure that the ^{31}P NMR signal remains stable at 2–3 ppm). In terms of practical application, the successful intervention of DF-P as a means of protecting the CPN structure could be readily used during membrane separation processes to minimize subtle compositional changes to the CPN. These results support the findings of Hernández and Harte (2009) at a molecular level, that DF with P reduced the retention of whey protein during MF, even at the expense of greater casein protein loss. When simultaneous pH control and retention of MC structure is desired, a more complex DF solution involving P, Ca, and Cit may be required. It is reasonable to expect that similar micellar and sub-micellar effects would also be likely to occur with UF during the course of milk protein concentrate manufacture, or indeed where UF milk retentates are produced for other dairy product applications with or without the use of diafiltration. One exception is that the higher concentration of whey protein in the final UF retentate could have an additive influence on CPN equilibrium by acting as a chelator

of Ca. Further studies involving spray-drying of the retentates prepared using different DF conditions are needed to understand the extent to which such proactive measures for both CPN and pH control affect the functionality of dairy powders produced from casein-based high-protein retentates.

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