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Heat stability of reconstituted casein micelle dispersions: changes induced by salt addition

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Abstract – The aim of the present work was to study the heat stability of reconstituted casein micelle dispersions (RCMD) after addition of various salt solution: NaCl, $CaCl_2$, $MgCl_2$, sodium citrate and sodium phosphate. Casein micelle stability to heat treatments (95 °C – 10, 20 and 30 min) was evaluated. Modifications of the pH, of the casein micelle pellet water content and of the mineral and casein distribution between aqueous and micellar phase were measured. NaCl addition decreased the RCMD pH, increased the amount of diffusible calcium and decreased the amount of supernatant casein. $CaCl_2$ and $MgCl_2$ addition decreased the RCMD pH and the amount of supernatant casein. Sodium citrate addition, with a solution at pH 7.4, increased the RCMD pH, drastically increased the amount of diffusible calcium, phosphorus and of supernatant casein. It caused the casein micelle's destructuration. Sodium phosphate addition, with a solution at pH 7.4, did not modify the RCMD pH and increased the amount of supernatant casein. Acidification, beyond a pH value of 7.0, induced heat aggregation. Calcium and magnesium chloride addition was detrimental to casein micelle heat stability. NaCl, sodium citrate and sodium phosphate addition enhanced reconstituted casein micelle heat stability. The determinant role of the aqueous phase on reconstituted casein micelle physico-chemical properties was emphasized by this study. © Inra/Elsevier, Paris

casein micelle / salt / heat stability

Résumé – Stabilité thermique de dispersions de micelles de caséine reconstituées : modifications produites par l'addition de sels minéraux. L'objectif du présent travail était d'étudier la stabilité thermique de dispersions de micelles de caséine reconstituées après l'addition de différentes solutions salines : NaCl, $CaCl_2$, $MgCl_2$, citrate de sodium et phosphate de sodium. La stabilité au traitement thermique (95 °C, 10–20–30 min) des dispersions des micelles de caséine reconstituées était évaluée. Les modifications du pH, de la teneur en eau des culots des dispersions de micelles de caséine et de la répartition des minéraux entre phase aqueuse et micellaire étaient mesurées. L'addition de NaCl diminuait le pH des dispersions, augmentait la teneur en calcium diffusible et diminuait la teneur en

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caséine des surnageants d'ultracentrifugation. L'addition de $CaCl_2$ et de $MgCl_2$ diminuait le pH des dispersions et la teneur en caséine des surnageants d'ultracentrifugation. L'ajout de citrate de sodium, à l'aide d'une solution à pH 7,4, augmentait le pH des dispersions, augmentait fortement les teneurs en calcium et phosphore diffusibles et de façon drastique la teneur en caséine des surnageants d'ultracentrifugation. Cet ajout entraînait une déstructuration de la micelle. L'addition de phosphate de sodium, à l'aide d'une solution à pH 7,4, ne modifiait pas le pH des dispersions et la teneur en caséine des surnageants d'ultracentrifugation. Une acidification à des valeurs de pH inférieures à 7,0 entraînait une agrégation après traitement thermique. L'addition de chlorure de calcium et de magnésium diminuait la stabilité thermique des micelles reconstituées. L'addition de NaCl, de citrate de sodium et de phosphate de sodium améliorait la stabilité thermique des micelles de caséine reconstituées. Dans cette étude, nous avons souligné le rôle déterminant de la phase aqueuse sur les propriétés physico-chimiques des dispersions de micelles de caséine reconstituées. © Inra/Elsevier, Paris

micelle de caséine / minéraux / stabilité thermique

1. INTRODUCTION

Incidence of various heat treatments on milk has been extensively studied. Depending on the heat intensity, various modifications in phosphocalcic equilibria, in whey protein solubility as well as formation of complexes between κ-casein and β-lactoglobulin, between ε-amino groups of proteins and lactose and degradation of lactose occur [1]. Some of these changes are reversible; others are irreversible and lead to a degradation of the nutritional value and of the technological properties of milk.

Most of the published studies on the effect of heating on casein micellar stability have been carried out with normal or evaporated milks [11, 35, 37, 46], i.e., with casein micelles dispersed in a very complex solution. Some works have used solutions of artificially formed casein micelles as models for studying the effect of individual casein ratio on heat stability [21, 32, 33]. To our knowledge, no complete study used dispersions of micellar casein without interaction with the aqueous phase during heat treatment, i.e., in the absence of lactose and whey proteins.

Recent developments in membrane microfiltration processing of milk have allowed the preparation of micellar phosphocaseinate with no effect on the casein micelle structure [29, 30]. Micellar phosphocaseinate powder with a high bacteriological quality can be produced in large quantities by microfiltration with a 0.1 μm pore size membrane [10], carried out in a system using the uniform transmembrane concept developed by Sandblom [31], of skim milk previously treated by the Bactocatch process and submitted to diafiltration before spray-drying [34]. This product has been shown to exhibit a micellar-like behaviour in terms of rennet coagulation before and after a 100 °C-5 min heat treatment [29], of β-casein dissociation [30] and of acid gelation [9].

Milk and its proteinaceous derivatives are commonly used in formulated foods containing various mineral salts and other ingredients. Physico-chemical properties of milk proteins, as well as their heat stability, are generally not predictable because of the complexity of the possible interactions between the added proteins and the food components. Therefore, it appears necessary to study the behaviour of model protein solutions in progressively more complex systems submitted to heat treatments usually carried out by the food industry.

This paper describes the effect of various environments (pH, NaCl, $CaCl_2$, $MgCl_2$, sodium citrate, sodium phosphate) on physico-chemical properties of reconstituted casein micelle dispersion (RCMD) which

is considered to be an interesting model for proteins susceptible to be used per se in milk protein mixture as an ingredient, before and after three heat treatments at 95 °C (10, 20, and 30 min).

2. MATERIALS AND METHODS

2.1. Preparation of RCMD

The micellar phosphocaseinate powder was prepared as described by Pierre et al. [29] and Schuck et al. [34]. The chemical composition of the micellar phosphocaseinate powder is reported in *table I*. Powder was reconstituted in ultrapure water (milliQ) at 50 °C. In one experiment, powder was reconstituted in milk ultrafiltrate at 30 °C obtained with a 10 000 Da cut-off membrane at 50 °C. The final casein concentration was 25 $g \cdot L^{-1}$ (i.e., 31.1 $g \cdot L^{-1}$ of powder). In all cases, 0.1 $g \cdot L^{-1}$ of thimerosal (Sigma, Saint Louis, Missouri, USA) was added to the dispersion to prevent bacterial and fungal growth.

2.2. Acidification of RCMD in water

The RCMD were acidified with 1 $mol \cdot L^{-1}$ HCl to reach the pH values: 6.99, 6.93 and 6.67. Ultrapure water was added to compensate for volume changes.

2.3. Addition of salts to RCMD in water

The salt solutions used were sodium chloride (NaCl; 4.15 $mol \cdot kg^{-1}$), calcium chloride ($CaCl_2$; 3.77 $mol \cdot kg^{-1}$), magnesium chloride ($MgCl_2$; 2.12 $mol \cdot kg^{-1}$), a mixture of disodium and of trisodium citrate (final pH value: 7.4; 1.25 $mol \cdot kg^{-1}$) and a mixture of sodium dihydrogen orthophosphate (NaH_2PO_4) and of disodium hydrogen orthophosphate (Na_2HPO_4) (final pH value: 7.4; 0.45 $mol \cdot kg^{-1}$). Reagents were of the analytical grade. Salt solutions were added to the RCMD in water, at room temperature. Salt concentrations added in final dispersions are reported in *table II*. During salt additions, the RCMD were stirred vigorously to ensure rapid and complete mixing. Samples were stirred for one hour at room temperature. For some experiments carried out at an adjusted pH, HCl 1 N or NaOH 1 N was added to reach the required pH value. pH adjusted samples were stirred for one hour at room temperature and pH was checked and eventually readjusted during the hour the sample was left standing. Ultrapure water was added to compensate for volume changes induced by salt addition or pH correction.

Table I. Chemical composition of the phosphocaseinate powder ($g \cdot kg^{-1}$ powder).

Tableau I. Composition chimique de la poudre de phosphocaséinate natif ($g \cdot kg^{-1}$ poudre).

Dry matter	914.7
Total Protein (N × 6.38)	832.7
Total Casein (N × 6.38)	794.0
NCN (N × 6.38)	38.7
NPN (N × 6.38)	2.3
Lactose	5.4
Ashes	76.8

NPN: non protein nitrogen. NCN: non casein nitrogen.
NPN : azote non protéique. NCN : azote non caséinique.

Table II. Final concentrations of added salts in reconstituted casein micelle-like dispersions (RCMD) ($mmol \cdot kg^{-1}$).

Tableau II. Concentrations finales des sels ajoutés dans les dispersions de micelles de caséines reconstituées ($mmol \cdot kg^{-1}$).

salt added	1st concentration	2nd concentration	3rd concentration
NaCl	17.4	94.8	177.8
$CaCl_2$	10.5	13.7	19
$MgCl_2$	2.6	9.3	19.3
Sodium citrate	6.0	10.6	17.8
Sodium phosphate	1.74	4.19	7.80

2.4. Heat treatments

Ten g of RCMD in sealed pyrex tubes (external dimensions: 10 × 1.6 cm; volume = 15 mL) were submerged in an oil-bath. The temperature was thermostatically regulated (± 0.1 °C) at the required assay temperature (95.0 °C). A rocking rate of 2 rev·min^{-1} was used (RCMD flows from one end of the tube to the other 4 times·min^{-1}). The heating times were 10, 20 and 30 min. These times included a 5-min heating-up period (experimental determination). After heating, RCMD were immediately cooled to 20 °C and then analysed. RCMD reconstitutions, heat treatments and subsequent analyses were carried out in duplicate.

2.5. Physico-chemical analyses

Physico-chemical analyses were carried out at 20 °C. Analyses were carried out before and after salt additions and heat treatments. Heated and control dispersions were centrifuged at 160*g* for 5 min, in order to remove insoluble aggregates. RCMD samples consisted of these supernatants.

pH values were measured with a Portames 752 Calimatic pHmeter at 20 °C (Bioblock, Illkirch, France). Total protein contents were determined by Kjeldahl method using a 6.38 converting factor. The diffusate phases of RCMD were obtained by ultrafiltration on Centriflo CF 25 (cut-off: 25 000 Da; Amicon, Epernon, France) after centrifugation at 500 *g* for 1 h at 20 °C.

Cation concentrations (Na, Ca and Mg) were determined by atomic absorption spectrometry as described by Brulé et al. [5] on RCMD samples and on ultrafiltrates. Phosphorus concentrations were determined according to the IDF method [17] on RCMD samples and on ultrafiltrates. Mineral concentrations in ultrafiltrates were converted into diffusible mineral concentrations in RCMD by multiplying by a 0.96 correcting factor as described by Pierre and Brulé [27]; this correction takes into account the excluded volume effect.

The RCMD samples were ultracentrifuged at 20 °C for 2 h at 77 000 *g* in a L8-55 ultracentrifuge with a 50.2 Ti rotor (Beckman Instrument, Gagny, France). The casein micelle pellet water contents were determined after a 22-h freeze drying of the RCMD pellet. Each sample was separated in 2 tubes. One was used to estimate micelle pellet water content and the other one to estimate total protein content of the dry pellet. Casein micelle pellet water contents were expressed as g of water per g of total protein.

Supernatant protein contents were defined as casein which did not sediment after a 2-h centrifugation at 77 000 *g* at 20 °C. They were estimated by optical density (OD) measurements at 280 nm (spectrophotometer Beckman, DU 62, Beckman Instrument, Gagny, France) of the supernatants diluted in 10 mmol·L^{-1} EDTA, pH 10 (adapted from Driehuis and Teernstra [8]). OD of supernatants were calculated by multiplying the OD measured by the dilution factor. Reverse-phase high performance liquid chromatography (RP-HPLC) analysis allowed the evaluation of the casein contents in the following samples: the RCMD samples, a heat-induced precipitate, and ultracentrifugal supernatants. Before RP-HPLC analysis, the heat-induced precipitate was redissolved in 7 mol·L^{-1} urea and 15 mmol·L^{-1} EDTA. Analyses were performed after treatment in 10 mmol·L^{-1} DTT (Sigma, Saint-Quentin-en-Yvelines, France) for 1 h at 30 °C. The system was composed of a Waters 600E multisolvent delivery system, a Waters 486 tunable absorbance detector set at 214 nm and a Nelson analytical Data System (Perkin Elmer, Saint-Quentin-en-Yvelines, France). The reverse-phase column was a 15 cm Vydac C4, 214 TP 54 (Touzart et Matignon, Vitry-sur-Seine, France). The chromatographic conditions were those of Jaubert and Martin [18]. The percentages of solubilized α_{s1}- and β-caseins were estimated by ratio of individual casein area in the ultracentrifugable supernatants to individual casein area obtained for the RCMD before ultracentrifugation.

3. RESULTS AND DISCUSSION

3.1. Characterization of RCMD in water

The pH of RCMD was 7.33. The diffusible calcium and phosphorus concentrations were about 0.8 and 1.0 mmol·kg^{-1}, respectively. These results are 10 times less than the diffusible calcium and phosphorus concentrations determined in milk: 10 and 12 mmol·L^{-1}, respectively [15]. As shown by Schuck et al. [34], calcium determination confirms that spray-drying treatment had no effect on Ca salt equilibrium since the content determined in the microfiltration retentate (data not shown) and in recons-

tituted RCMD are identical. Increase of pH resulted from the decrease of ionic strength (the diffusible ash content of RCMD was 0.2 $g \cdot kg^{-1}$) which induces an increase of the apparent pK's of casein carboxyls residues and the displacement of protons from the aqueous to the micellar phase. This also allowed the ionization of groups which possess an apparent pK close to the pH of dispersion, i.e., His and SerP.

After heat treatment of RCMD, no pH variation was detected. Such a result is explained by the lack of milk aqueous phase which induces formation of formic acid during heating of milk with a concomitant release of H^+ [1].

Casein micelle pellet water content of RCMD (2.05 g of water per g of total protein before heat treatment) was reduced to 1.78 by the treatment of 30 min at 95 °C. Some disintegration of casein micelles could have occurred during the heating leading to a preferential release of κ-casein [36], which is followed by a casein micelle reassociation during cooling [2]. An irreversible change of colloidal calcium phosphate (CCP) could also occur during heating [28, 45]. The slight modification of the micelle organization is confirmed by the observed data (control samples in *tables III* and *IV*): the applied heat treatment induced an aggregation of around 4 % expressed in protein content of RCMD. The same slight polymerization of casein was found by Zin el Din and Aoki [47] when whey protein-free milk without lactose was heated at 80 °C for 15 to 75 s. Singh et al. [38] suggested that heat treatment of milk appeared to weaken the interaction forces between casein components within the micelles. In the present study, the micelle pellet water content could have been reduced by this incomplete restoration of the original micellar organization after heat treatment and cooling.

3.2. Acidification of RCMD in the pH range 7.33–6.67

The diffusible calcium concentrations of the RCMD adjusted to pH 6.99, 6.93 and 6.67 were respectively 1.20, 1.44 and 2.23 $mmol \cdot kg^{-1}$. As expected, the pH decrease led to calcium solubilization which is similar to what Famelart et al. [9] observed.

When pH was adjusted to a value lower than 7.0, there was an aggregation caused by the applied heat treatment (*figure 1*). At pH 6.67, about 40 % of total protein and

Table III. Concentrations of total protein ($g \cdot kg^{-1}$) of reconstituted casein micelle-like dispersions (RCMD) as a function of $CaCl_2$ concentrations ($mmol \cdot kg^{-1}$) and after heating at 95 °C, 30 min. Before analysis, a centrifugation (160 *g*, 5 min) was carried out.

Tableau III. Concentrations en matière protéique totale ($g \cdot kg^{-1}$) des échantillons de dispersions de micelles de caséines reconstituées en fonction de la concentration en $CaCl_2$ ($mmol \cdot kg^{-1}$) et après chauffage à 95 °C, 30 min. Les échantillons ont subi une centrifugation (160 *g*, 5 min) avant analyse.

	$CaCl_2$ ($mmol \cdot kg^{-1}$)			
heating time (min)	0	10.5	13.7	19.0
0	25.60	25.17	25.16	25.02
10	ND	0.85	0.91	0.93
20	ND	0.84	0.85	0.89
30	24.40	0.84	1.25	0.84

ND: not determined. Total protein = N × 6.38.
ND : non déterminé. Matière protéique totale: N × 6,38.

Table IV. Concentrations of total proteins ($g \cdot kg^{-1}$) of reconstituted casein micelle-like dispersions (RCMD) as a function of $MgCl_2$ concentrations ($mmol \cdot kg^{-1}$) and after heating at 95 °C, 30 min. Before analysis, a centrifugation (160 *g*, 5 min) was carried out.

Tableau IV. Concentrations en matière protéique totale ($g \cdot kg^{-1}$) des échantillons de dispersions de micelles de caséines reconstituées en fonction de la concentration en $MgCl_2$ ($mmol \cdot kg^{-1}$) et après chauffage à 95 °C, 30 min. Les échantillons ont subi une centrifugation (160 *g*, 5 min) avant analyse.

	$CaCl_2$ ($mmol \cdot kg^{-1}$)			
Heating time (min)	0	2.6	9.3	19.3
0	25.19	25.04	24.92	24.72
10	ND	18.88	1.19	1.3
20	ND	18.25	1.17	1.06
30	23.89	16.61	1.22	1.06

ND: not determined. Total protein = N × 6.38.
ND : non déterminé. Matière protéique totale : N × 6,38.

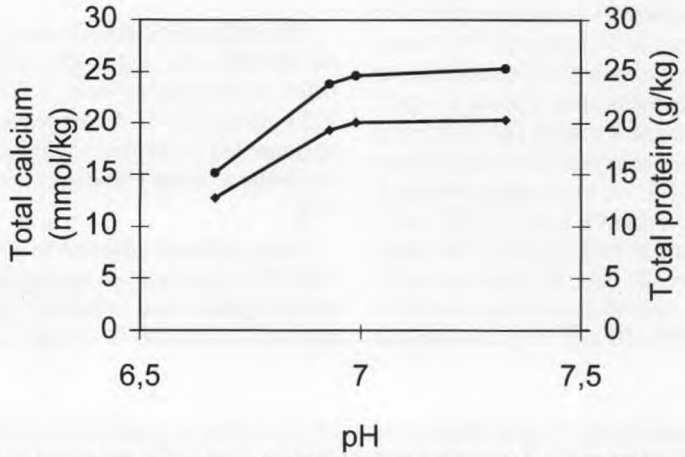

Figure 1. Concentrations of total protein (●) and total calcium (◆) in RCMD supernatants (160 *g*, 5 min) after heat treatment (95 °C, 30 min) as a function of pH.

Figure 1. Concentrations en matière protéique totale (●) et en calcium total (◆) dans les surnageants de centrifugation (160 *g*, 5 min) de phosphocaséinate natif après traitement thermique (95 °C, 30 min) en fonction du pH.

calcium were in the sediment obtained by centrifugation at 160 *g*-5 min. RP-HPLC analysis of both the non-heated RCMD sample and sediment samples showed that there was no selective precipitation of caseins: the proportion of α_{s1}- and β-caseins were similar (results not shown). Application of the same heat treatment to a similar dispersion of casein micelles in milk ultrafiltrate at pH 6.70 did not induce aggregation (results not shown). Such a difference in the behaviour of casein micelles can be attributed to the higher isoelectric pH of precipitation obtained at low ionic strength when compared to the milk ionic strength. Isoionic pH of individual caseins are higher than

isoelectric ones at milk ionic strength ($I = 0.08$ mol·L^{-1}). When micelles are dispersed in a medium, they are subject to charge neutralization at a higher pH when the ionic strength of the medium is less than 0.08 mol·L^{-1}. Micellar casein might also be regarded as a globulin which was considered to be a class of protein insoluble in pure water but soluble in dilute saline solutions [4]. Acidification through gluconodeltalactone addition gave similar results for heat precipitation (results not shown).

3.3. NaCl addition

NaCl addition (0–177.8 mmol·kg^{-1}) to the RCMD led to an average pH decrease

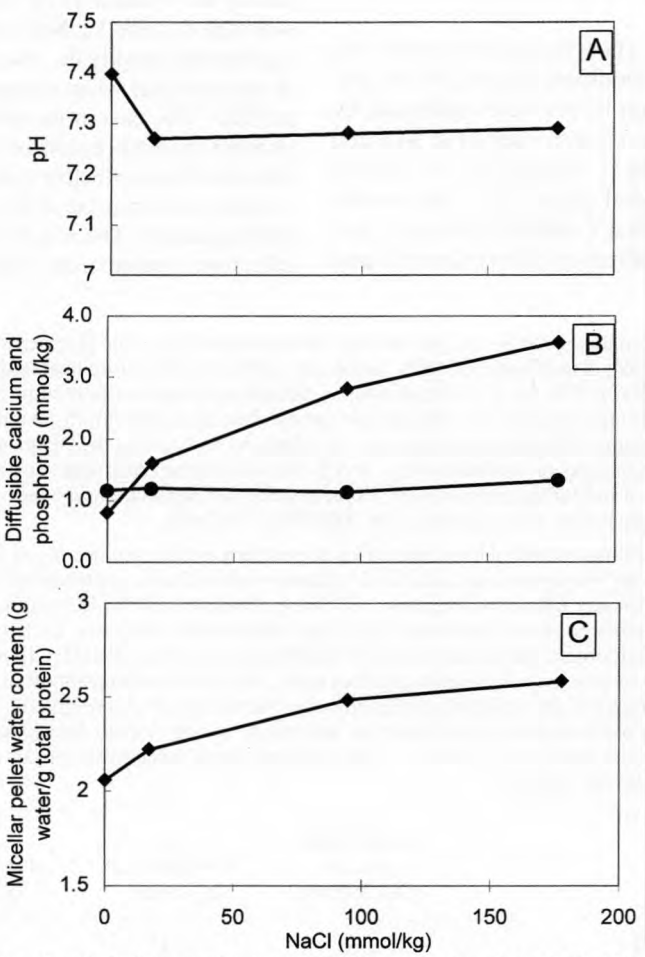

Figure 2. Physico-chemical characterization of RCMD samples after NaCl addition before heat treatment. (**A**) pH; (**B**) diffusible calcium (◆) and phosphorus (●) concentrations; (**C**) micellar pellet water content.

Figure 2. Caractérisation physicochimique des échantillons de dispersions de micelles de caséines reconstituées après ajout de NaCl, avant traitement thermique. (**A**) pH ; (**B**) concentrations en calcium (◆) et phosphore (●) diffusibles ; (**C**) teneur en eau des culots micellaires.

of about 0.1–0.15 unit (*figure 2A*). H^+ release is due to the effect of ionic strength which decreases the activity coefficients of the diffusible ions and consequently, increases dissociation of the ion pairs. H^+ release is also due to the exchange between Na^+ and H^+ as suggested by Grufferty and Fox [14] and Strange et al. [40] because approximately 5 % of the Na^+ added is displaced onto the micellar phase (result not shown).

Diffusible phosphorus levels were not significantly modified (*figure 2B*) but diffusible calcium levels were multiplied by factors of 2.0, 3.5, 4.5 when 17.4, 94.8 and 177.8 mmol·kg^{-1}, respectively, of sodium ions were added (*figure 2B*). Our results confirm that NaCl addition induces a preferential solubilization of the calcium bound to phosphoseryl residues as hypothesized by Grufferty and Fox [14], Le Graet and Brulé [22] and Famelart et al. [9] owing to the increase in ionization of these residues.

The casein micelle pellet water content was increased by 8, 21, 25 %, respectively for the aforementioned sodium ion additions (*figure 2C*) as is expected in milk [6, 9, 14, 35, 43]. The amount of supernatant protein estimated by OD measurement decreased by 25 % when 177.8 mmol·kg^{-1} NaCl was added (*table V*). NaCl addition did not significantly modify the casein solubilization in supernatants when compared to control RCMD. The casein micelle pellet water content increase could be due to a lower ultracentrifugal effective compression and to a subsequent increase of the amount of pelleted micelles. The lower ultracentrifugal effective compression could be explained

Table V. Supernatant protein and percentage of supernatant α_{s1}- and β-caseins of reconstituted casein micelle-like dispersions (RCMD) containing different salts before heat treatment. Ultracentrifugation: 77 000 *g*, 2 h, 20 °C. Control sample: phosphocaseinate suspensed in water without salt addition. Supernatant protein was obtained by optical density at 280 nm in 10 mmol·L^{-1} EDTA of RCMD supernatants; OD of supernatants were calculated by multiplying the OD measured by the dilution factor. Percentages of solubilized α_{s1}- and β-caseins in ultracentrifugal supernatants were estimated by ratio of individual casein area in ultracentrifugal supernatant to the individual casein area in control sample before ultracentrifugation (RP-HPLC analysis).

Tableau V. Caséines non ultracentrifugeables et pourcentage de caséines α_{s1} et β non ultracentrifugeables dans les dispersions de micelles de caséines reconstituées contenant différents sels avant traitement thermique. Ultracentrifugation : 77 000 *g*, 2 heures, 20 °C. Échantillon témoin : phosphocaséinate remis en dispersion dans l'eau, sans addition de minéraux. Le taux de caséine non sédimentable était obtenu par mesure de la DO à 280 nm en présence d'EDTA 10 mmol·L^{-1} dans les surnageants de dispersion de phosphocaséinate natif ; la DO des surnageants était calculée en multipliant la DO mesurée par le facteur de dilution. Les pourcentages de caséines α_{s1} et β solubilisées étaient estimés par le rapport entre la surface obtenue pour une caséine dans le surnageant d'ultracentrifugation et la surface de la même caséine obtenue dans l'échantillon témoin avant ultracentrifugation (analyse RP-HPLC).

Sample	Supernatant protein (OD 280 nm)	% of solubilized α_{s1}-casein	% of solubilized β-casein
Control RCMD	2.63	0.17	9.80
NaCl (177.8 mmol·kg^{-1})	1.97	0.06	7.10
$CaCl_2$ (19.0 mmol·kg^{-1})	1.36	0.05	2.68
$MgCl_2$ (19.3 mmol·kg^{-1})	1.60	0.18	2.78
Sodium citrate (17.8 mmol·kg^{-1})	12.49	43.30	67.30
Sodium phosphate (6.8 mmol·kg^{-1})	3.00	0.17	9.80

by the increase of protein aggregation. Sood et al. [39] found a negative correlation between the voluminosity of casein micelles and their calcium content. Even though we can not conclude directly to the existence of an increase in the hydration of the casein micelle from our results, we can conclude to the existence of an increase in casein micelle voluminosity content after sodium chloride addition because of this correlation.

RCMD supplemented in NaCl showed no variation of pH, of total protein content and of mineral content (results not shown) when the different heat treatments were applied, regardless of the initial pH (whether adjusted to 6.8 or not) whereas control RCMD precipitated (*figure 1*). Horne and Davidson [16] show that NaCl addition to casein micelles in skim milk stabilize them against aggregation by an increase of the hairy layer but with no modification of the casein hydrodynamic radius. NaCl addition stabilized casein micelles in water against heat precipitation probably by enhancement of steric repulsions.

3.4. $CaCl_2$ and $MgCl_2$ additions

$CaCl_2$ and $MgCl_2$ (0–19; 0–19.3 mmol·kg^{-1}, respectively) additions decreased the pH by 0.5 and 0.4 pH unit, respectively, for the highest concentrations added (*figures 3A* and *4A*, respectively). Although the proportion of added calcium ions or sodium ions determined in the diffusible phase was the same (90 %), addition of calcium ions led to a greater pH decrease than addition of sodium ions did (*figures 2A* and *3A*), as could be expected from the relationship existing between the activity coefficient and the ion valence [46]. As also envisaged for NaCl addition, the pH decrease is also due to an H^+ release from the micelles following the fixation of the 10 % calcium added to the micelle.

Diffusible calcium concentrations were increased by factors of 2.3, 4.3, 6.0 when 2.6, 9.3, 19.3 mmol·kg^{-1} of Mg^{2+} were added, respectively (*figure 4B*). Mg^{2+} addition appears to induce a more complex phenomenon than Ca^{2+} addition with only a partial displacement of bound calcium since the total amount of solubilized Ca^{2+} and Mg^{2+} is close to the added Mg^{2+}. $CaCl_2$ and $MgCl_2$ addition decreased the diffusible phosphorus concentrations only by 0.3 mmol·kg^{-1} for both at the highest concentrations (*figure 4B*) thus confirming that the RCMD is almost entirely colloidal.

The amount of supernatant protein decreased by 48 and 39 % when 19 and 19.3 mmol·kg^{-1} of CaCl2 and $MgCl_2$, respectively, were added (*table V*). As for pH, Ca^{2+} addition led to a greater decrease in the amount of supernatant protein than Mg^{2+} addition did. Solubilized β-casein was reduced to 2.68 and 2.78 % when 19 and 19.3 mmol·kg^{-1} of $CaCl_2$ and $MgCl_2$ were added, respectively (*table V*). The decrease in the amount of supernatant protein could be due to the aggregation of the calcium sensitive caseins.

A 19 mmol·kg^{-1} calcium ion addition decreased micellar pellet water content by approximately 8 % whilst a 19.3 mmol·kg^{-1} magnesium ion addition did not significantly modify it at unadjusted pH (*figures 3C* and *4C*, respectively). Calcium ions induced a higher decrease in pH, in the micelle pellet water content and in the amount of casein in supernatants than magnesium ions did. This could be attributed to their different hydrated ionic radius (Ca^{2+} = 0.412 nm, Mg^{2+} = 0.428 nm [26]) and electronegativities (Ca = 1.2; Mg = 1 on the Pauling scale [22]).

After 30 min of heat treatment, less than 5 % of the initial total protein content remained dispersed with an addition of about 19 mmol·kg^{-1} divalent ions (*tables III* and *IV*). Similar experiments carried out at an adjusted pH of 7.4, after divalent ion addition, also induced casein aggregation (results not shown). As observed in milk [37], addition of $CaCl_2$ or $MgCl_2$ to RCMD has a destabilizing effect mainly caused by their

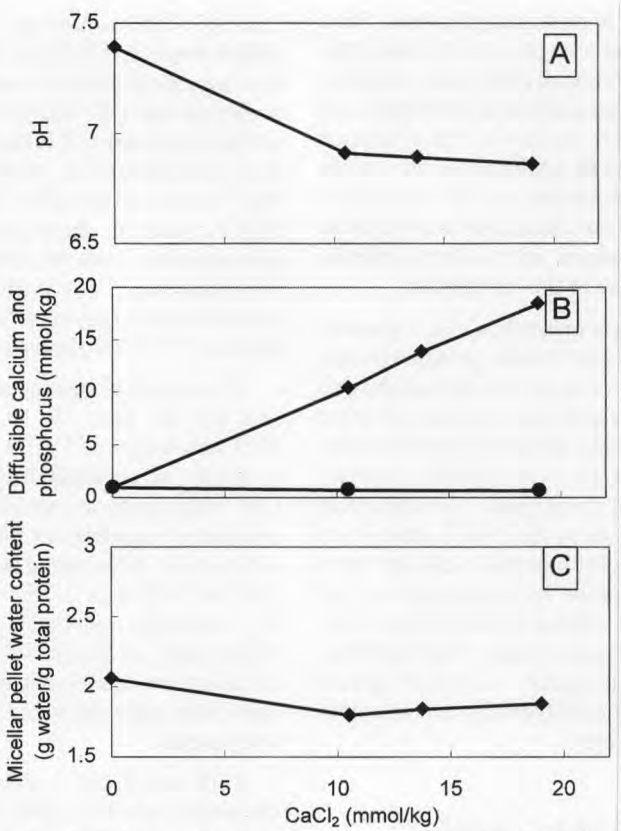

Figure 3. Physico-chemical characterization of RCMD samples after $CaCl_2$ addition before heat treatment. (**A**) pH; (**B**) diffusible calcium (◆) and phosphorus (●) concentrations; (**C**) micellar pellet water content.

Figure 3. Caractérisation physicochimique des échantillons de dispersions de micelles de caséines reconstituées après ajout de $CaCl_2$, avant traitement thermique. (**A**) pH ; (**B**) concentrations en calcium (◆) et phosphore (●) diffusibles ; (**C**) teneur en eau des culots micellaires.

double charge which shields, depending on the added concentration, the net negative charge of the casein micelles. Consequently, the zeta potential is reduced [7] as well as the electrostatic repulsions. Jeurnink and de Kruif [19] point out that calcium chloride addition to milk makes casein micelles shrink and as a consequence of the lower hydration, the van der Waals attraction would be larger. Hence, interactions between the micelles increase. The results presented here are consistent with both analyses. Reduction of steric repulsion of casein micelles and of electrostatic repulsion, and increase of van der Walls attractions by addition of divalent cations (Ca^{2+}, Mg^{2+}) resulted in a lowered stability to heat.

3.5. Sodium citrate addition

Citrate addition (0–17.8 $mmol \cdot kg^{-1}$) to the RCMD increased the pH up to 8.08 (*figure 5A*), despite a citrate solution pH value of 7.4. By chelating the colloidal calcium, citrate increased the second apparent pK of the phosphoseryl residues and allowed

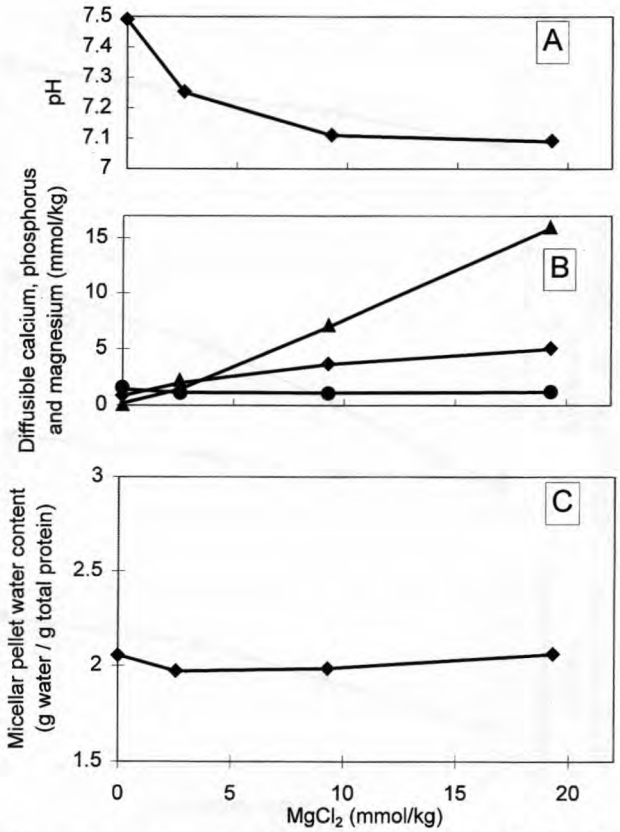

Figure 4. Physico-chemical characterization of RCMD samples after $MgCl_2$ addition before heat treatment. (**A**) pH; (**B**) diffusible calcium (◆), phosphorus (●) and magnesium (▲) concentrations; (**C**) micellar pellet water content.

Figure 4. Caractérisation physicochimique des échantillons de dispersion de micelles de caséines reconstituées après ajout de $MgCl_2$, avant traitement thermique. (**A**) pH ; (**B**) concentrations en calcium (◆), phosphore (●) et magnésium (▲) diffusibles ; (**C**) teneur en eau des culots micellaires.

groups which possess pKs close to the pH of the dispersion to bind protons; both phenomena resulted in the RCMD pH increase. Moreover, because of the low buffering capacity of the dispersing phase, the consequence of this phenomenon on the dispersion pH is higher than in milk serum (data not shown). Calcium and magnesium chloride addition act conversely by inducing fixation of calcium and magnesium onto the micellar phase.

Citrate ions increased the diffusible calcium ion concentrations by 4.1, 6.3 and 8.8 times for addition of 6.0, 10.6 and 17.8 $mmol \cdot kg^{-1}$, respectively (*figure 5B*). Diffusible phosphorus concentrations were correspondingly increased by factors of 1.3, 1.7 and 2.4 (*figure 5B*). The binding of calcium and phosphorus to micelles due to the pH increase was more than compensated by their solubilization caused by citrate addition. The ratio of solubilized calcium to solubilized phosphorus was 2.4 $mol \cdot mol^{-1}$ when 17.8 $mmol \cdot kg^{-1}$ of citrate were added, a value far from the classical one of 1.5 found in casein micelles [1] which could be explai-

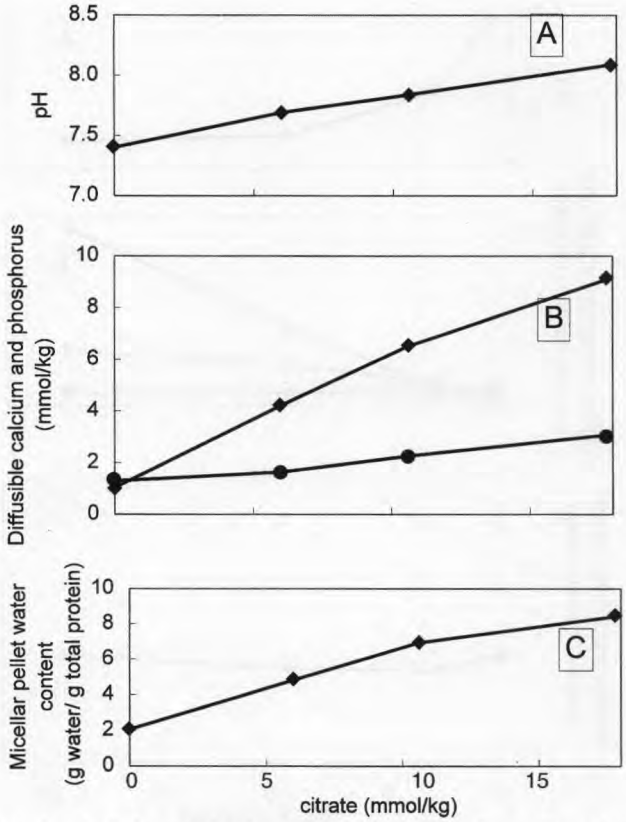

Figure 5. Physico-chemical characterization of RCMD samples after sodium citrate addition before heat treatment. (**A**) pH; (**B**) diffusible calcium (◆) and phosphorus (●) concentrations; (**C**) micellar pellet water content.

Figure 5. Caractérisation physicochimique des échantillons de dispersions de micelles de caséines reconstituées après ajout de citrate de sodium avant traitement thermique. (**A**) pH ; (**B**) concentrations en calcium (◆) et phosphore (●) diffusibles ; (**C**) teneur en eau des culots micellaires.

ned by a sequestrating effect of the added citrate on both, calcium involved in CCP and bound to phosphoseryl residues.

The amount of supernatant protein increased by a factor of 4.7 when 17.8 mmol·kg^{-1} sodium citrate was added to RCMD (*table V*). Solubilized α_{s1}-casein represented 43.30 % of the casein total area when 17.8 mmol·kg^{-1} sodium citrate was added (*table V*). Solubilized β-casein represented 67.30 % of this casein total area (*table V*). Citrate addition induced a release of large amounts of casein from the micellar phase and a solubilization of α_{s1}-casein because of the disorganization of CCP. Lin et al. [23] and Griffin et al. [13] reported the dissociation of casein micelles after EDTA addition to milk through dialysis with a non-discriminate solubilization of the four caseins. The dissociation of reconstituted casein micelles which occurred in the present study, after sodium citrate direct addition, happened with a higher solubilization of the most phosphorylated casein quantified: the α_{s1}-casein. The discrepancy between our results and the aforementioned results can be explained by the

mode of addition: direct addition as opposed to dialysis addition.

Citrate addition also drastically increased the casein micelle pellet water content from 2.05 to 8.41 g of water·g^{-1} of total protein for a 7.8 mmol·kg^{-1} addition (*figure 5C*). This increase in the amount of the casein micelle pellet water content can be explained by the reduction of the micellar size induced by sodium citrate addition (results not shown). As mentioned for sodium chloride, Sood et al. [39] found a negative correlation between the voluminosity of casein micelles and their calcium content. We can indirectly conclude that an increase of casein micelle voluminosity after citrate addition occurs because of this correlation.

Our results are in agreement with those obtained with milk. Citrate ion addition to milk brings about substantial disintegration of the micelles by chelation of calcium ions thereby causing a shift in the distribution from the colloidal to the diffusible phase [25]. As a result, a number of interrelated chemical and physical properties of the milk are altered: a) reduced sedimentation of the micelles by ultracentrifugation [25], b) increased viscosity [45], c) reduced turbidity [20, 25] and lightness decrease [20], and d) increased numbers of small particles and residual open micelle 'skeleton' [45].

No modification of the pH, of the mineral distribution between aqueous and micellar phase and of protein content was observed after heat treatments (results not shown). A heat treatment under the same experimental conditions but at an adjusted pH of 6.7 was carried out and showed no casein aggregation (results not shown). Sodium citrate protected casein micelles against heat aggregation as it improves milk heat stability [24] probably by solubilizing micellar salts.

3.6. Sodium phosphate addition

Phosphate ion addition to the RCMD (0–6.8 mmol·kg^{-1}) induced no modification of the pH values (*figure 6A*). The pH of the sodium phosphate solution was close to the RCMD control pH. The diffusible calcium concentrations were not modified (*figure 6B*). It suggests that the added inorganic phosphate ions did not displace calcium ions from the micellar to the diffusible phase as citrate did. The calcium affinity is higher for phosphoseryl residues and for inorganic phosphate contained in CCP than for the inorganic phosphate added to RCMD. This affinity difference can be explained by the relatively high electronegativity of the phosphoseryl clusters and/or by their local conformation [12]. Solubilized phosphorus represented 56, 63 and 71 % of the phosphorus concentration added (*figure 6B*). So a part of the phosphate added was bound to casein micelles. The potential sites of interaction for the negatively charged inorganic phosphate could be casein micellar calcium and magnesium sites but also basic amino acid residues (arginyl, lysyl residues and free α-amino terminal) [42]. Calcium phosphate solubility in RCMD diffusible phase is lower than in milk diffusible phase. According to Walstra and Jenness [46], the apparent solubility product of $CaHPO_4$ in milk diffusible phase is 6 times higher than in an infinitely diluted solution. In our case, less phosphorus and calcium ions were found in the diffusible phase because of the low ionic strength of the medium. The CCP solubilization is limited by its solubility product in the RCMD aqueous phase.

Casein micelle pellet water content increased by 8, 16 and 34 % for addition of 1.74, 4.19 and 7.8 mmol·kg^{-1}, respectively (*figure 6C*). As explained for sodium chloride and sodium citrate addition, we can conclude that phosphate ion addition increased casein micelle voluminosity. Phosphate addition increased the amount of supernatant protein by 14 % when compared to control RCMD (*table V*). It did not modify the percentage of solubilized α_{s1}- and β-caseins (*table V*). Phosphate addition causes a nondiscriminate solubilization of the casein studied.

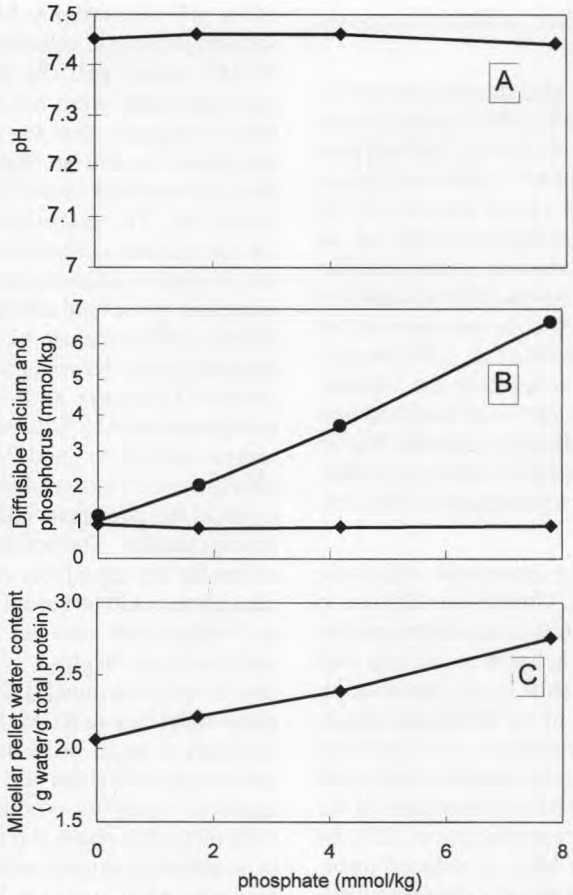

Figure 6. Physico-chemical characterization of RCMD samples after sodium phosphate addition before heat treatment. (**A**) pH; (**B**) diffusible calcium (◆) and phosphorus (●) concentrations; (**C**) micellar pellet water content.

Figure 6. Caractérisation physicochimique des échantillons de dispersions de micelles de caséines reconstituées après ajout de phosphate de sodium, avant traitement thermique. (**A**) pH ; (**B**) concentrations en calcium (◆) et phosphore (●) diffusibles ; (**C**) teneur en eau des culots micellaires.

After heat treatments, no modification of pH, mineral repartition between aqueous and micellar phase and of protein content was measured (same concentrations added, same heat treatment; results not shown). An experiment carried out at an adjusted pH of 6.8 did not induce the heat aggregation of casein micelles. Such results agree with the improvement of the milk heat stability by addition of sodium phosphate which was shown by Sweetsur and Muir [41]. Sodium phosphate addition protect the RCMD against heat instability probably by solubilizing the micellar minerals.

4. CONCLUSION

The dispersing aqueous phase which surrounds casein micelles strongly influences the major physico-chemical properties for the casein micelle dispersions (pH, micelle

pellet water content, mineral and individual casein distribution between micellar and aqueous phase). Sodium chloride, sodium citrate and sodium phosphate addition by reducing the amount of micellar mineral and increasing stabilizing forces (steric repulsions, etc.) protect RCMD against heat instability. Calcium and magnesium chloride by increasing the amount of micellar minerals and the van der Waals attraction and reducing steric and electrostatic repulsions induced heat instability.

Sodium chloride was also found to stabilize faba bean protein (vicilin and legumin) against thermal denaturation whilst calcium and magnesium chloride destabilized the protein isolate [3]. The denaturation temperature of whey protein concentrate determined by differencial scanning calorimetry was reduced by calcium and magnesium chloride addition but increased in the presence of sodium chloride on the alkaline side of the isoelectric zone [44]. Chloride salts seem to have similar effects on casein micelles dispersions, on whey protein concentrate and on faba bean protein isolate.

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