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# Calcium equilibrium in milky systems between colloidal and soluble phase - study of calcium - protein interactions

Latha-Selvi Canabady-Rochelle

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# INSTITUT NATIONAL POLYTECHNIQUE DE LORRAINE

Ecole Nationale Supérieure d'Agronomie et des Industries Alimentaires (*Nancy, France*)  
Laboratoire des Sciences et Génie Alimentaires

Centre de Recherche et de Développement d'Unilever (*Vlaardingen, Les Pays-Bas*)

## THESE

Présentée devant l'Institut National Polytechnique de Lorraine

Pour obtenir le grade de Docteur de l'INPL

*Spécialité : Procédés Biotechnologiques et Alimentaires*

par

**Latha-Selvi CANABADY-ROCHELLE**

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## Équilibres en Calcium dans les Systèmes Lactés – Étude des Interactions Calcium-Protéines

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## Calcium Equilibrium in Milky Systems between Colloidal and Soluble Phase - Study of Calcium-Protein Interactions

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**SUCCESS IS THE ABILITY TO GO FROM ONE FAILURE  
TO ANOTHER ONE WITH NO LOSS OF ENTHUSIASM.**

**Winston Churchill**

A mes Etoiles...





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**ABSTRACT.** Calcium (Ca) is a mineral essential for life. Hence Ca supplementation of food is a world-wide health stake. Ca equilibrium between soluble and colloidal phases was studied in milky systems (milk, Non Hydrolysed, NH, or Hydrolysed, H, soy milks). Calcium chloride supplementation (CC, 25 mmol.kg<sup>-1</sup>) was followed by pH cycle (pH<sub>min</sub> 5.5 or 3.5). pH, Ca<sup>2+</sup>, turbidity and apparent viscosity were recorded *in situ*. Ca equilibria were related to protein phase variations. Contrarily to milk, Ca<sup>2+</sup> concentration was initially negligible in soy milks. Yet, whatever the milky system, Ca<sup>2+</sup> increased upon CC addition and with acidification, and decreased during alkalization. For reference milk, pH cycle to 5.5 was reversible neither on Ca<sup>2+</sup> variations nor on protein phase contrarily to CC-milk. This could be due to the previous capture of Ca during supplementation, involving casein micelles reinforcement through Ca-protein interactions. For pH cycle to 5.5, acid-induced aggregation was partially and completely reversible upon alkalization for NH and H-soy milks, respectively. Once CC addition, Ca-induced aggregation was irreversible and pH cycle had minor effects. Whatever the system, the irreversibility of phenomena was observed for pH cycle to 3.5. Ca-(milk or soy) protein interactions studied by ITC showed similar endothermic signals, probably due to the water release occurring upon interaction. Ca binding should rather be described as H<sup>+</sup>/Ca<sup>2+</sup> exchange with respect to the electrostatic forces involved. Finally, Ca-binding sites were identified with FTIR spectroscopy. A decrease of the absorption energy in the amide I and II region and in the carboxylate region occurred upon CC-addition, with higher variations in soy milks.

**Key words:** calcium, supplementation, milk, soy milk, pH cycle, protein, interaction, ITC.



**RÉSUMÉ.** Le calcium (Ca) est un minéral essentiel pour la vie et de ce fait, la supplémentation en Ca est un enjeu de santé mondial. Les équilibres en calcium (Ca) entre la phase soluble et la phase colloïdale ont été étudiés dans des systèmes lactés (laits de vache, de soja Hydrolysé ou Non-Hydrolysé). La supplémentation en Ca ( $\text{CaCl}_2$ , CC,  $25 \text{ mmol} \cdot \text{kg}^{-1}$ ) a été suivie d'un cycle de pH ( $\text{pH}_{\text{min}}$  5,5 ou 3,5). Le pH, la concentration en calcium ionisé ( $\text{Ca}^{2+}$ ), la turbidité et la viscosité apparente ont été reliés aux variations de la phase protéique. La concentration en  $\text{Ca}^{2+}$ , initialement négligeable dans le lait de soja, augmente avec l'addition en Ca, ainsi qu'avec l'acidification et diminue lors de l'alcalinisation. Pour le lait de vache non supplémenté, le cycle de pH à 5,5 n'est réversible ni sur les variations en  $\text{Ca}^{2+}$ , ni sur les variations de la phase protéique, contrairement au lait de vache supplémenté en Ca. Ceci pourrait être dû à la capture préalable en Ca, entraînant un renforcement des micelles de caséines. Pour des cycles de pH à 5,5, l'agrégation induite par l'acidification est partiellement ou complètement réversible lors de l'alcalinisation pour les laits de soja NH et H, mais l'agrégation induite par le Ca est irréversible. Quelque soit le système étudié, des phénomènes irréversibles sont observés lors de cycle du pH à 3,5. Les interactions Ca-protéines (de vache ou de soja) étudiées par CTI montrent des signaux endothermiques similaires, probablement dû au relargage de molécules d'eau. La liaison du Ca pourrait être décrite comme un échange  $\text{H}^+/\text{Ca}^{2+}$  étant donné les forces électrostatiques impliquées. Les sites de fixation du Ca ont été identifiés par IR-TF. Une diminution de l'énergie d'absorption dans les régions amides I et II et dans la région carboxylate s'est produite lors de l'addition de Ca, avec de plus grandes variations pour les laits de soja.

**Mots clefs:** calcium, supplémentation, lait de vache, lait de soja, cycle de pH, protéines, interactions, Calorimétrie Isothermale.



## List of Abbreviations

<b><math>\epsilon</math></b> : Dielectric constant	<b>NaCl</b> : Sodium Chloride
<b><math>\lambda</math></b> : Wavelength (cm)	<b>NH</b> : Non Hydrolysed
<b><math>\mu</math></b> : Ionic strength	<b>O.M</b> : Optical Microscopy
<b><math>\mu_E</math></b> : Electrophoretic mobility	<b>pH<sub>i</sub></b> : isoelectrical pH
<b>AA</b> : Amino Acid	<b>pH<sub>min</sub></b> : minimal pH of pH cycle
<b>AAS</b> : Atomic Absorption Spectrometry	<b>pK<sub>R</sub></b> : reactive pK
<b>Ca ISE</b> : Calcium Ion Selective Electrode	<b>RDA</b> : Recommended Daily Allowance
<b>CC</b> : Calcium Chloride	<b>RDI</b> : Recommended Daily Intake
<b>CCP</b> : Colloidal Calcium Phosphate	<b>rpm</b> : round per minute
<b>CMA</b> : Cow Milk Allergy	<b>SDS-PAGE</b> : Sodium Dodecyl Sulphate PolyAcrylamid Gel Electrophoresis
<b>CP</b> : Colloidal Phase	<b>SHMP</b> : Sodium HexaMetaPhosphate
<b>DP</b> : Differential Power ( $\mu\text{cal}\cdot\text{sec}^{-1}$ )	<b>SM</b> : Soy Milk
<b>DSC</b> : Differential Scanning Calorimetry	<b>SP</b> : Soluble Phase
<b>e.m.u.</b> : electrophoretic mobility unit ( $\mu\text{m}\cdot\text{cm}\cdot\text{s}^{-1}\cdot\text{V}^{-1}$ )	<b>SPC</b> : Soy Protein Concentrate
<b>EAA</b> : Essential Amino-Acid	<b>SPF</b> : Soy Protein Formula
<b>EDTA</b> : Ethylen Diamin Tetra Acetate	<b>SPI</b> : Soy Protein Isolate
<b>FTIR</b> : Fourier Transformed Infra-Red	<b>T<sub>1</sub></b> : end of probes stabilization
<b>H</b> : Hydrolysed	<b>T<sub>2</sub></b> : end of calcium equilibrium
<b>HP</b> : High Pressure	<b>T<sub>3</sub></b> : end of acidification
<b>IP</b> : Isoelectrical Point	<b>T<sub>4</sub></b> : end of alkalization
<b>ITC</b> : Isothermal Titration Calorimetry	<b>UT</b> : Ultraturrax
<b>K</b> : Binding constant ( $\text{M}^{-1}$ )	<b>w/v</b> : weight by volume (g/L)
<b>kDa</b> : kiloDalton	<b>w/w</b> : weight by weight (g/g)
<b>MC</b> : Milk Calcium	<b><math>\zeta</math></b> : zeta potential (mV)
<b>MCP</b> : Micellar Calcium Phosphate	<b><math>\Delta H</math></b> : heat change ( $\text{cal}\cdot\text{mole}^{-1}$ )
<b>MW</b> : Molecular Weight	<b><math>\Delta S</math></b> : entropy ( $\text{cal}\cdot\text{mol}^{-1}\cdot\text{deg}^{-1}$ )
<b>N</b> : Number of sites	<b>ATR</b> : Attenuated Total Reflection





## **RESUME EN FRANCAIS**



## **INTRODUCTION**



Le calcium est un minéral essentiel pour l'homme, particulièrement durant certaines phases de la vie, telles que la croissance pour la constitution des os (Bonjour et al., 2005), la lactation et durant la vieillesse pour lutter contre l'ostéoporose (Renner, 1994 ; Nordin, 1997 ; Heaney, 2000, 2001 ; O'Connell et Stamm, 2004). Cependant, certaines populations spécifiques à risque ne satisfont pas les Apports Journaliers Recommandés (AJR) en calcium. Des études récentes sur les habitudes alimentaires rapportent que 50 à 75% des adolescentes, des femmes de plus de 55 ans, des hommes de plus de 65 ans et des personnes âgées ne satisfont pas ces Apports Journaliers Recommandés en calcium (Guégen, 2000). D'après une enquête Individuelle et Nationale sur les Consommations Alimentaires (Volatier et al., 2000), même dans les pays occidentaux ces populations à risque ne satisfont pas ces AJR en calcium. Plusieurs études scientifiques ont conclu qu'un apport approprié en calcium durant l'enfance empêcherait l'apparition de l'ostéoporose à l'âge adulte (Chan, 1991). De plus, en dehors de l'effet du calcium dans la prévention de l'ostéoporose, ce minéral est aussi reconnu dans la prévention du cancer du colon (Wallace et al., 2004), de l'hypertension (Tsuda et al., 2001) et de nombreuses autres maladies (Miller et al., 2001 ; Mc Carron et Heaney, 2004)...

Les autorités ont pris en considération l'importance de satisfaire les apports recommandés en calcium. De ce fait, de nombreux gouvernements et organismes de santé ont mis l'accent sur l'importance de l'enrichissement en calcium des aliments, et de nos jours la fortification en calcium montre une tendance croissante dans l'industrie agro-alimentaire.

Avec une population vieillissante, et plus particulièrement dans les pays occidentaux, les dépenses de santé liées aux fractures ostéoporitiques sont considérables. Par conséquent, la prévention de l'ostéoporose et de ses complications constitue une priorité socio-économique essentielle. Cette déficience nutritionnelle en calcium présente un enjeu de santé mondiale et peut être compensée par la supplémentation alimentaire en calcium.

Les produits laitiers, naturellement riches en calcium, présentent une grande biodisponibilité en calcium, et constituent les aliments appropriés pour la supplémentation en calcium. En tant que source naturelle en calcium, avec les micelles de caséines comme nano-vecteur, le lait de vache constitue un aliment approprié dans la stratégie de supplémentation en calcium. De plus, le lait et les produits laitiers ont une image « santé » et sont des vecteurs idéaux pour délivrer le calcium additionnel. Néanmoins, pour diverses raisons, éthiques, religieuses ou économiques, le lait de vache ne peut être consommé par certaines populations.

De ce fait, le « lait » de soja constitue une alternative au lait de vache et plus particulièrement pour les populations souffrant d'allergies aux protéines de lait de vache (Exl et Fritsché, 2001) et/ou d'intolérance au lactose (Swagerty et al., 2002). Ce lait d'imitation a une valeur nutritive similaire à celle du lait de vache (Desikachar et Subramanyan, 1946 ; INRA, 1995) et est dépourvu de cholestérol et de lactose. Néanmoins, le lait de soja contient seulement un cinquième à un huitième de calcium en comparaison au lait de vache (Souci et al., 1994 ; Ciquel, INRA, 1995). De ce fait, la supplémentation en calcium du lait de soja à un niveau similaire à celui du lait de vache présente un intérêt spécifique pour les personnes qui désirent changer leurs habitudes alimentaires tout en conservant leurs apports en calcium, ou pour des groupes spécifiques de la population tels que les personnes intolérantes au lactose ou allergiques aux protéines de lait de vache.

Jusqu'à maintenant, la fortification du lait de soja à un niveau similaire à celui du lait de vache a pu être réalisée en présence d'un complexant du calcium et/ou d'un stabilisant (Zemel et Shelef, 1986) mais en leur absence, la haute sensibilité des protéines de soja au calcium induit leur agrégation (Saio et al., 1969 ; Lee et Rha, 1977).

Dans ce contexte, il semblerait utile d'imiter le vecteur naturel du calcium qui est présent dans le lait de vache, à savoir les micelles de caséines. En effet, l'élaboration de vecteurs ressemblants aux micelles de caséines avec des protéines de soja et de calcium ajouté pourrait être une alternative aux complexants et/ou aux stabilisants communément utilisés dans le cas de la fortification en calcium du lait de soja. L'utilisation d'un cycle de pH après la supplémentation en calcium du lait de soja pourrait être un moyen intéressant de créer de nouvelles structures de protéines de soja. Dans le cadre d'une approche plus fondamentale, les interactions calcium-protéines devraient être caractérisées. Enfin, les équilibres calciques entre la phase soluble et la phase protéique ainsi que les interactions calcium-protéines devraient être comparées entre le lait de vache et le lait de soja.

Ce mémoire de thèse est constitué de trois chapitres principaux : un chapitre bibliographie, une partie matériels et méthodes, suivie des résultats et discussions.

La bibliographie traite dans un premier temps de l'importance de l'élément calcium dans le corps humain et plus particulièrement de l'enjeu de santé mondiale lié à la supplémentation en calcium des aliments. Les équilibres calciques ont ensuite été étudiés dans

le lait de vache soumis à diverses variations de paramètres physico-chimiques tels que le pH, la température, la force ionique ect...La dernière partie bibliographique aborde le lait de soja et l'influence de la supplémentation en calcium sur les équilibres en calcium dans ce dernier système. L'influence de divers paramètres physico-chimiques tels que le pH, la température et la supplémentation en calcium sur les protéines de soja a été également étudiée.

La partie sur le matériel et les méthodes utilisés pour l'étude des équilibres calciques entre la phase soluble et la phase colloïdale et pour l'étude des interactions calcium-protéines dans le lait de vache et le lait de soja est décrite dans le second chapitre de ce mémoire de thèse.

Dans le troisième chapitre, les résultats obtenus sont présentés et discutés. Les équilibres calciques entre la phase soluble et la phase colloïdale sont étudiés dans des systèmes lactés supplémentés en calcium puis soumis à un cycle de pH (partie 1 pour le lait de vache et partie 2 pour le lait de soja). Les interactions calcium-protéines dans le lait de vache et les laits de soja (partie 3) ont été caractérisées principalement par Titration Calorimétrique Isothermale et par Infra-Rouge à Transformée de Fourier.

Enfin, en conclusion générale, les deux systèmes lactés étudiés, le lait de vache et le lait de soja, sont comparés et des perspectives concernant ce travail de thèse sont proposées.





## **MATÉRIEL ET MÉTHODES**



# **1. Étude des équilibres en calcium entre la phase soluble et la phase colloïdale dans des laits soumis à un cycle de pH**

## **1.1. Préparation des laits**

### **1.1.1. Lait de vache**

La poudre de lait Low heat (Ingrédia, Arras, France) a été reconstituée à 12% (p/p) dans de l'eau distillée et a été soumise à agitation pendant 3 heures afin de permettre la ré-équilibration des minéraux (Anema et Li, 2003). Le lait ainsi reconstitué a été ensuite conservé toute la nuit à 4°C avant d'être utilisé. Deux kg de lait écrémé ont été préparés pour chaque expérience. Dans ces conditions opératoires, la concentration en protéines dans le lait écrémé reconstitué était égale à 4,2% (p/p).

### **1.1.2. Laits de soja**

#### **1.1.2.1. Isolats de Protéines de Soja**

Deux Isolats commerciaux de Protéines de Soja (IPS) ont été utilisés dans la reconstitution des laits de soja : un IPS Non-Hydrolysé (**NH**) SPI (SUPRO 760, IPS Non-GM, The Solae company, Barcelone, Espagne) et un IPS Hydrolysé (**H**) (IPS 219 Non-GM, Dupont Protein technology, St Louis, MO, USA) contenant respectivement 88,2 et 86% de protéines (p/p).

#### **1.1.2.2. Reconstitution du lait de soja et stabilisation**

Les laits de soja hydrolysés et non hydrolysés ont été reconstitués à partir de leur IPS respectifs, dans de l'eau distillée, à une concentration de 4,2% en protéines (p/p), similairement à la concentration en protéines contenue dans le lait écrémé reconstitué.

La présence d'agrégats protéiques dans les isolats de soja a conduit à une grande instabilité des suspensions reconstituées. Pour pallier cette instabilité physique, un traitement de stabilisation approprié a été appliqué : un cisaillement à 13500 rpm pendant 5 min (ultraturrax, Ultralightnin, Bioblock, Advantech, Strasbourg, France) suivi d'un traitement par homogénéisation par hautes pressions à 500 bars durant 5 cycles (EmusiFlex-C3, Sodexim

S.A, Muizon, France). Les laits de soja non stabilisés et stabilisés seront respectivement référés à des solutions d'IPS et à des laits de soja.

## **1.2. Supplémentation en sels de calcium**

### **1.2.1. Supplémentation du lait de vache**

La supplémentation en sel de calcium a été effectuée avec du Chlorure de Calcium (CC,  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , pour analyse, Carl Roth GmbH, Karlsruhe, Allemagne) ou du calcium naturel de lait (Calcium du lait, MC, Fieldgate natural dairy calcium, First district association, Minnesota, USA). Le CC a été choisi pour sa grande solubilité et son utilisation triviale tandis que le MC a été étudié comme source naturelle de calcium. Le calcium naturel du lait, est principalement constitué de phosphate de calcium (28%, p/p) mais aussi d'autres composés tels que du lactose et des minéraux. Ce pourcentage en calcium a été pris en compte lors de la supplémentation en calcium.

Deux kg de lait écrémé reconstitué ont été supplémentés en CC ou en MC. Pour chaque source de calcium, les concentrations en calcium ont été fixées à 10, 15 et 25 mmoles/kg de lait écrémé, d'après les valeurs de concentration communément mentionnées dans la littérature (Guillaume et al., 2002). Le lait écrémé non-supplémenté constituait la référence.

Après une période d'équilibre en calcium (2 h), le lait écrémé supplémenté en calcium a été soumis à un cycle de pH. Dans chacune des expériences menées, le temps d'équilibre après la supplémentation en calcium a été maintenu constant afin de permettre la comparaison des cinétiques, quelque soit la source de calcium étudiée.

### **1.2.2. Supplémentation du lait de soja**

Après homogénéisation, le lait de soja reconstitué a été supplémenté avec 25 mmoles de  $\text{CaCl}_2$  (CC, Carl Roth GmbH, Karlsruhe, Allemagne) par kg de lait de soja afin d'atteindre un niveau de supplémentation en calcium similaire à celui du lait de vache. Après une période d'équilibre de 2 heures, le lait de soja a été soumis à un cycle de pH.

### 1.3. Procédure du cycle de pH

#### 1.3.1. Procédure générale

Le cycle de pH a été mené en utilisant un auto-titrateur (Multiburette 2S, Crison, La Souterraine, France). La cuve thermostatée contenant le lait a été soumise à une agitation constante. L'acidification du lait de vache ou du lait de soja a été effectuée à l'aide de HCl 1N (Riedel-de Haën, Seelze, Sigma Aldrich, Allemagne) jusqu'à atteindre une valeur de pH minimale ( $pH_{\min}$ ). Ensuite, directement après l'acidification, l'étape de neutralisation par alcalinisation a été effectuée par addition du NaOH 1N (Riedel-de Haën, Seelze, Sigma Aldrich, Allemagne). L'acide et la base ont été ajoutés dans le milieu à vitesse constante. Le cycle de pH a été stoppé lorsque le pH initial du système lait a été atteint. Tous les paramètres de la titration sont résumés dans le tableau ci-dessous et sont proportionnels au volume du réacteur.

Tableau 1. Paramètres de titration

	Lait de vache	Lait de soja
Volume de la cuve	2L	500 mL
Agitation	250 rpm	150 rpm
Température	4°C	25°C
pH Initial	6,65	7,1-7,2
$pH_{\min}$ du cycle de pH	5,5, 5,0, ou 3,5	5,5 ou 3,5
Vitesse d'acidification et d'alcalinisation	1 mL/min	0,25 mL/min

A la fin de chacune des étapes de la cinétique ( $T_1$  = stabilisation des sondes,  $T_2$  = période d'équilibre en Ca,  $T_3$  = acidification et  $T_4$  = alcalinisation), les échantillons ont été collectés pour des analyses ultérieures. Chaque expérience a été dupliquée. Le principe et la cinétique de l'expérience sont respectivement présentés dans la figure ci-après.

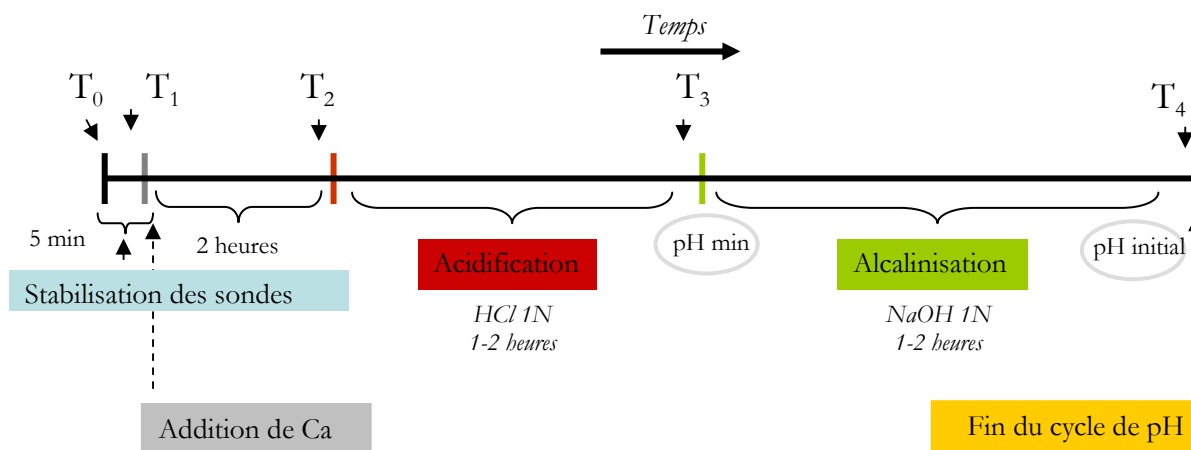


Figure 1. Expérience de cycle de pH sur le lait de vache et le lait de soja en fonction du temps.  $T_1$  = stabilisation des sondes,  $T_2$  = période d'équilibre en Ca,  $T_3$  = acidification et  $T_4$  = alcalinisation.

### 1.3.2. Protocole expérimental

L'ensemble de la cinétique (supplémentation en calcium suivie d'un cycle de pH) a été suivi par plusieurs sondes immergées dans le liquide : le pH-mètre (Radiometer analytical, Remiremont, France), la sonde de conductivité (CDM 210, Radiometer analytical, Remiremont, France) et l'électrode sélective aux ions calcium (**Ca ISE**, Sentek, Estate, Braintree, Royaume-Uni). Le turbidimètre a été placé perpendiculairement à la cuve thermostatée (Analite NEP 160, McVan Instruments, Mulgrave, Australie) afin d'éviter les perturbations liées à l'agitation.

Le contrôle continu (Almemo 8990-8 V5, Ahlborn, Holzkirchen, Allemagne) du pH, de la conductivité, du calcium ionisé et de la turbidité a été effectué en utilisant un collecteur de données couplé à un PC équipé d'un logiciel adapté (AMR WinControl pour Almemo, Akrobit<sup>®</sup>, Gera, Allemagne; figure 2). La calibration des électrodes a été effectuée quotidiennement. Ce protocole expérimental a été décrit précédemment par Gaiani et al., (2005) dans une étude cinétique concernant la réhydratation des poudres laitières.

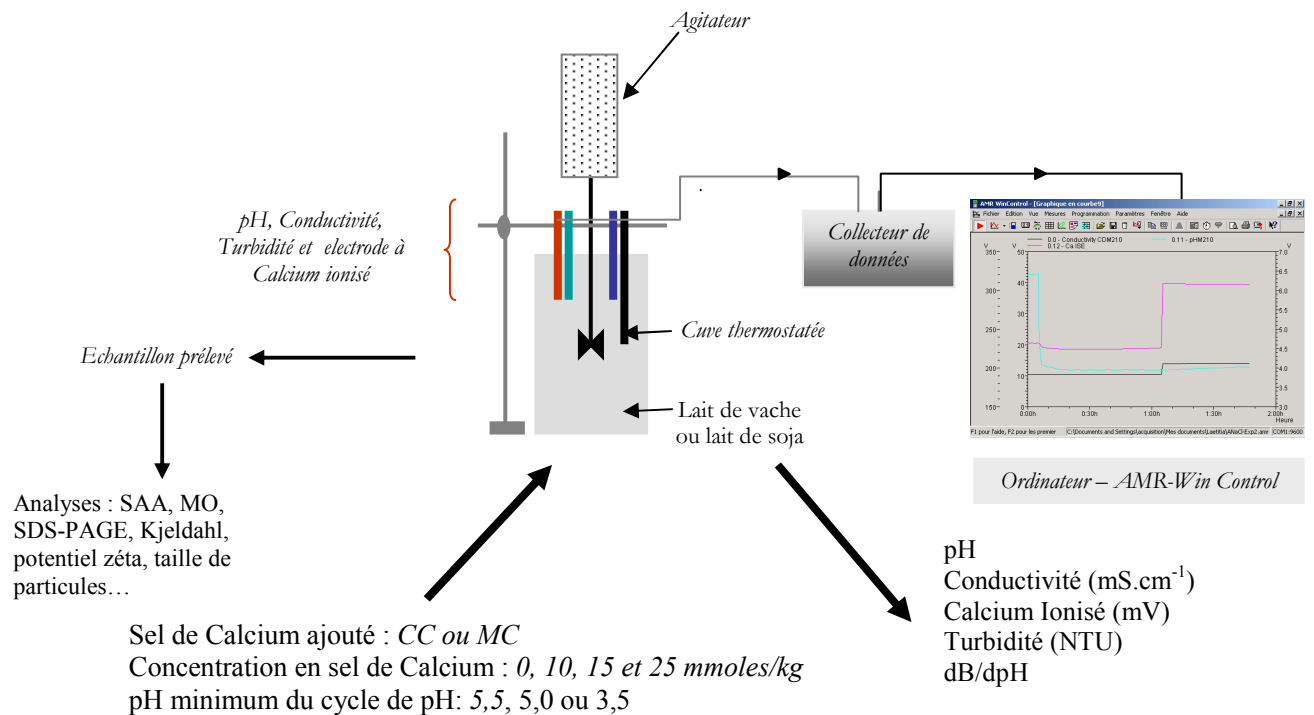


Figure 2. Protocole expérimental utilisé dans l'étude cinétique des équilibres calciques se produisant entre la phase soluble et la phase colloïdale du lait (lait de vache ou lait de soja) soumis à un cycle de pH.

### 1.3.3. Calcul du pouvoir tampon

Les expériences de titration ont été réalisées avec de l'acide chlorhydrique (HCl, 1N) et de l'hydroxide de sodium (NaOH, 1N) ajouté à 1 mL et 0,25 mL par minute, pour le lait de vache et les laits de soja, respectivement. Chaque expérience a été dupliquée. Dans l'étude du pouvoir tampon, les échantillons ont été titrés à partir du pH initial et jusqu'à pH minimal de 3,5, durant l'acidification et la neutralization. L'index du pouvoir tampon (dB/dpH), mesure sans dimension, a été calculé d'après la formule de van Slyke (1922) et tracé en fonction du pH :

$$\frac{dB}{dpH} = \frac{(Vol_{\text{Titrant}}) \times (Normalité)}{(Vol_{\text{Lait}}) \times (\Delta pH)}$$

## **1.4. Comportement en calcium dans les laits supplémentés en calcium et soumis à un cycle de pH**

### **1.4.1. Variations de conductivité**

La concentration totale en ions a été déterminée à l'aide d'un conductimètre CDM 210 (Radiometer analytical, Remiremont, France). Le conductimètre a été calibré quotidiennement avec une solution de KCl à 0,1 M (pour analyse, Riedel-de-Haën, Sigma Aldrich, Seelze, Allemagne).

### **1.4.2. Quantification du calcium par spectrométrie d'absorption atomique**

Pour le lait écrémé de vache, supplémenté en CC ou en MC, le calcium total et le calcium soluble ont été quantifiés pour des cycles de pH à 5,5 et à 5,0, à la fin de chaque étape de la cinétique ( $T_1$  = stabilisation des sondes,  $T_2$  = période d'équilibre en calcium,  $T_3$  = acidification et  $T_4$  = neutralisation).

Le Spectromètre d'Absorption Atomique (SAA, Perkin Elmer, Wellesley, Ma, USA) a été utilisé pour la quantification en calcium d'après la procédure décrite par Brulé et al. (1974) et Dziuba et Muzinska (1998). La lampe de détection du calcium a été réglée à une longueur d'onde de 422,7 nm et une fente de 0,7 nm. Le SAA a été calibré avec des solutions de 0, 4, 6, 8, 10 et 12 mg/L.

Les phases solubles et colloïdales du lait ont été séparées par ultracentrifugation (Sorvall RC M120 GX, les Ulis, France) à 110 000 g durant 1 h (Dziuba et Muzinska, 1998) à 4°C. Le calcium colloïdal a été considéré comme étant la différence entre le calcium total et le calcium soluble. Les ultrafiltrats de lait (phase soluble du lait) obtenus par ultracentrifugation ont été conservés à -20°C jusqu'à l'analyse par SAA.

Pour les échantillons étudiés, 1 mL de lait total ou d'ultrafiltrat de lait a été dilué dans de l'eau distillée contenant du chlorure de lanthane (respectivement 10 ou 5 mL de  $\text{LaCl}_3$ ) pour un volume final de 100 mL ou de 50 mL, respectivement. Ensuite, une dilution appropriée a été effectuée dans de l'eau distillée afin d'atteindre une valeur d'absorbance comprise dans les rangs de calibration. La mesure de calcium a été répétée trois fois, sur des essais expérimentaux qui ont été dupliqués.

Le calcium colloïdal a été calculé d'après l'équation suivante :

$$\text{Ca}_{\text{Colloïdal}} = \text{Ca}_{\text{Total}} - \text{Ca}_{\text{Soluble}}$$



Le calcium complexé contenu dans la phase soluble a été calculé par différence entre le calcium soluble (mesuré par SAA) avec le calcium ionisé contenu dans la phase soluble (mesuré par l'électrode à calcium).

$$Ca_{\text{Complexé}} = Ca_{\text{Soluble}} - Ca_{\text{Ionisé}}$$

### 1.4.3. Variations en calcium ionisé

L'électrode à calcium ionisé (Ca ISE, Sentek, Estate, Braintree, Royaume-Uni) a été utilisée pour évaluer l'ionisation du calcium (Vyas et Tong, 2004 ; Holt et al., 1981 ; Assoumani, 1998 ; Dalgleish et Parker, 1980). La calibration a été menée avec des solutions de CC à  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ , et  $10^{-4}$  M, respectivement. Ces solutions de calibration ont été préparées dans du NaCl à 0,1 M afin d'imiter la force ionique du lait qui est approximativement de 0,085 M (Holt et al., 1981). Durant un cycle de pH, l'électrode à calcium ionisé permet la mesure de la concentration en calcium ionisé par le biais de la mesure du potentiel chimique (mV). L'activité du calcium des solutions de calibration a été calculée d'après l'équation suivante.

$$a_i = \gamma_i * C_i$$

$a_i$  = activité en calcium (M)

$\gamma_i$  = coefficient d'activité du calcium (sans unité) = 0.40 pour une force ionique de 0,1 M (Geerts et al., 1983 ; Van Kreveld et Van Minnen, 1955) mentionné dans : Lucey et al., 1996)

$C_i$  = concentration en calcium ionisé (M)

L'activité du calcium a été calculée d'après l'équation de régression linéaire obtenue à partir des courbes de calibration. Les concentrations en calcium ionisé ont ensuite été calculées d'après l'équation ci-dessus. L'effet de dilution due à l'addition d'acide et de base a été corrigé dans le calcul de la concentration en calcium ionisé.

La concentration en calcium ionisé a été mesurée en fonction du pH. Le pH-mètre et l'électrode en calcium ont été calibrés avant chaque cinétique, à la température à laquelle celle-ci a été effectuée. La calibration du pH-mètre a été effectuée à pH 7.00 et pH 4.00, selon le rang de pH des laits étudiés. La précision de la mesure était de  $\pm 0.02$  unité pH.

## **1.5. Caractérisation de la phase colloïdale protéique des laits**

### **1.5.1. Variation de l'état micellaire**

De légères variations dans la structure des micelles de caséines ont été observées dans le lait écrémé par turbidimétrie (Banon et Hardy, 1992). La méthode de turbidimétrie est basée sur la réflexion d'un faisceau monochromatique dans le proche infra-rouge ( $\lambda = 860$  nm). Le faisceau incident est transmis dans la solution par le biais de fibres optiques et, est réfléchi par toutes les particules en suspension dans la solution étudiée. Le faisceau réfléchi à  $180^\circ$  est ensuite capturé par un autre système de fibres optiques jusqu'à un détecteur électronique qui amplifie le signal reçu et convertit celui-ci en Unité de Turbidité Néphélométrique (UTN). Ce signal de turbidité dépend de trois principaux facteurs : la taille, la concentration et les propriétés optiques des particules colloïdales.

Le turbidimètre (Mc Van Instrument, Mulgrave, Australie) a été calibré deux fois par mois avec deux solutions de valeurs de turbidité connues, à savoir une solution de 0 UTN et une solution de 4000 UTN.

### **1.5.2. Rhéométrie**

Les propriétés colloïdales des laits de soja ont été suivies par rhéométrie. La viscosité apparente des laits de soja a été déterminée à l'aide d'un rhéomètre StressTech (Rheologica, Scheelevägen, Suède). La géométrie utilisée pour les mesures de viscosité apparente est une pale à 4 angles droits, adaptée à une cuve de 18 mL. La vitesse de cisaillement a été fixée à  $100 \text{ s}^{-1}$  et restait constante tout au long de l'expérience ( $T = 25^\circ\text{C}$ ). La viscosité apparente de la solution a été suivie tout au long du cycle de pH ( $\text{pH}_{\text{min}} = 5,5$  ou  $3,5$ ). La viscosité a été mesurée sur le lait de soja durant les 5 premières minutes, afin d'atteindre la stabilité du signal. Ensuite, le lait de soja a été supplémenté en CC, et après 10 minutes de période d'équilibre en calcium, le cycle de pH a débuté. La fréquence d'acquisition des données était fixée à 60 s. Chaque expérience a été dupliquée.

### **1.5.3. Détermination de la taille des particules**

#### **1.5.3.1. Diffusion Dynamique de la Lumière**

Pour l'étude du lait écrémé, la détermination de la taille des particules a été effectuée à l'aide d'un Nanosizer (HPPS 5001, Malvern instrument Ltd, Worcestershire, Royaume-Uni). 75  $\mu\text{L}$  de lait ont été dilués dans 925  $\mu\text{L}$  d'ultrafiltrat. L'ultrafiltrat, obtenu après ultracentrifugation, a été filtré sur un filtre de 0,45  $\mu\text{m}$  (Millipore, Carrigtwohill, Co. Cork, Irlande) avant utilisation.

#### **1.5.3.2. Diffusion Statique de la Lumière**

Pour l'étude du lait de soja, la distribution de taille des particules a été déterminée par DSL, en utilisant un Mastersizer S granulomètre (Malvern Instrument Ltd, Worcestershire, Royaume-Uni). Un faisceau laser à He/Ne (puissance de 5 mW;  $\lambda = 632,8 \text{ nm}$ ) traverse la cellule de mesure, dans laquelle la dispersion est injectée. La lumière laser diffusée par l'échantillon est captée sur 42 photodiodes localisées à différents angles.

Les mesures de taille de particules ont été réalisées pour des laits de soja supplémentés en CC et soumis à un cycle de pH, après stabilisation des sondes ( $T_1$ ), après une période d'équilibre en calcium ( $T_2$ ), après acidification ( $T_3$ ) et après alcalinisation ( $T_4$ ). Les échantillons de lait de soja dilués dans de l'eau distillée à température ambiante ont été introduits dans la cuve de mesure afin d'obtenir une valeur d'obscurité comprise entre 10 et 15%. L'indice de réfraction a été fixé à 1,5295 pour les protéines et à 1,33 pour le solvant (eau distillée) (Strawbridge et al., 1995). La distribution de taille a été mesurée en fonction du volume de distribution des particules (%), variant entre 0,05 et 880  $\mu\text{m}$ . L'acquisition des données a été faite par le logiciel de Malvern (Sizer Sv2.17). Chaque expérience a été dupliquée.

### **1.5.4. Microscopie Optique**

La Microscopie Optique a été effectuée à l'aide d'un microscope à contraste de phase (Leica DMRB, Wetzlar, Allemagne). Le microscope était connecté à un appareil photographique (Kappa optoelectronics GmbH, Allemagne). Le traitement des images a été réalisé par le logiciel Kappa image Base 2.5 (Kappa optoelectronics GmbH, Germany). Les

photographies ont été prises à  $T_1$ ,  $T_2$ ,  $T_3$  et  $T_4$  pour les laits de soja supplémentés en CC et soumis à un cycle de pH ( $\text{pH}_{\text{min}} = 3,5$ ).

### **1.5.5. Variations de potentiel zéta**

Les mesures de mobilité électrophorétique ( $\mu_E$ ) ont été effectuées avec un Nanosizer ZS (HPPS 5001, Malvern instrument Ltd, Worcestershire, Royaume-Uni) par électrophorèse à faisceau Doppler. L'échantillon a été déposé dans une cellule d'électrophorèse capillaire standard, équipée d'électrodes en or. La mesure a été réalisée à 25°C. Le potentiel zéta a été calculé à partir de la mesure de mobilité électrophorétique ( $\mu_E$ ) d'après la loi de Henry. Le potentiel zéta correspond aux charges électriques globales des particules en mouvement et est communément utilisé comme indicateur de la stabilité des dispersions. Si toutes les particules en suspension ont une valeur de potentiel zéta élevée (positive ou négative), les particules vont donc avoir tendance à se repousser et à ne pas rentrer en contact. Cependant, si toutes les particules ont une faible valeur de potentiel zéta, celles-ci vont avoir tendance à s'agréger. Pour des échantillons stabilisés de façon électrostatique, le point où le potentiel zéta est nul est relié à un minimum de stabilité, dû à la faiblesse des forces répulsives entre particules, et se situe près du  $\text{pH}_i$ . Le potentiel zéta varie en fonction du pH, de la force ionique et de la concentration des particules chargées dans le milieu dispersant.

La mobilité électrophorétique a été mesurée pour des échantillons de lait de vache ou de lait de soja soumis à un cycle de pH, au pH initial et pour chaque unité pH tout au long du cycle de pH (pH 6,5, 5,5, 4,5, 3,5), durant l'acidification et la neutralisation. Ce paramètre a également été déterminé dans le lait de vache et dans les laits de soja après supplémentation en CC (25 mmoles/kg).

## **1.6. Caractérisation de la phase soluble des protéines**

### **1.6.1. SDS-PAGE**

Durant des cycles de pH à 5,5, les protéines solubles contenues dans le lait écrémé reconstitué, supplémenté en CC ou en MC, ont été caractérisées à  $T_1$ ,  $T_2$ ,  $T_3$  et  $T_4$ . Les protéines solubles ont d'abord été extraites à partir de la phase totale du lait par ultracentrifugation (Sorvall RC M120 GX, les Ulis, France) durant 1 h à 110000 g et à 4°C (Dziuba et Muzinska, 1998). Les protéines solubles ont ensuite été séparées par

électrophorèse SDS-PAGE à l'aide d'une unité d'électrophorèse Bio-Rad (Laboratoires Bio-Rad, Richmond, CA, U.S.A). Le marqueur protéique (invirogen, Carlsbad, CA, U.S.A) était constitué de 15 protéines de Poids Moléculaires allant de 10 à 220 kDa. La migration a été réalisée sous 50 mV. Le gel a ensuite été coloré par du bleu de Coomassie (USBiological, Swampscott, MA, U.S.A).

### **1.6.2. Analyse Kjeldahl**

La solubilité des protéines de soja Non-Hydrolysées et Hydrolysées a été déterminée dans une solution d'IPS, avant homogénéisation par hautes pressions. L'acidification (HCl, 1N, 0,5 mL/min) a été réalisée à 20°C sous agitation à 200 rpm, dans un réacteur de 1L. Des échantillons de 20 mL ont été prélevés à différentes valeurs de pH: 7,0, 6,0, 5,5, 5,0, 4,5, 3,5 et 3,0. Après centrifugation (Centrifuge Sigma Bioblock, Osterode, Allemagne) à 3034 g durant 35 min à 20°C (FIL, 1995), les surnageants ont été collectés et stockés à -20°C jusqu'au moment de l'analyse Kjeldahl (Vapodest, Gerhardt, Allemagne), afin de déterminer le contenu en azote. La concentration en protéine a ensuite été calculée à partir de la concentration en azote ( $\times 6,25$  pour les protéines de soja).

### **1.7. Analyse statistique et méthode de normalisation**

L'analyse statistique a été réalisée à l'aide du logiciel Kyplot. Des tests paramétriques de comparaisons multiples ont été effectués. La même lettre mentionnée en exposant a été utilisée quand aucune différence entre deux concentrations en calcium mesurées n'a été observée ( $P > 0,05$ ). L'analyse statistique a été effectuée indépendamment pour la phase soluble et pour la phase colloïdale.

La méthode de normalisation Min-Max correspond au ratio de chaque valeur par rapport à la valeur maximale mesurée dans l'expérience correspondante.

## **2. Étude des interactions Calcium-Protéines dans le lait de vache et dans les laits de soja**

### **2.1. Reconstitution des laits pour les expériences de calorimétrie isothermale**

#### **2.1.1. Lait de vache**

Pour l'étude des interactions calcium-protéines, le lait de vache a été reconstitué dans de l'eau miliQ, à partir de la poudre de lait low heat (Ingredia, Arras, France) à 1% (p/p) en concentration protéique. Le lait a été agité à l'aide d'un agitateur magnétique afin de permettre la ré-équilibration des minéraux puis a ensuite été dessalé sur une colonne de dessalage de type PD10 (Amersham Biosciences, Buckinghamshire, Royaume-Uni). Les échantillons ont ensuite été conservés à -20°C jusqu'à ce que les expériences de calorimétrie isothermale soient réalisées.

#### **2.1.2. Lait de soja**

##### **2.1.2.1. Étude d'un échantillon global de protéines de soja**

Les laits de soja Hydrolysés ou Non-Hydrolysés ont été reconstitués dans de l'eau miliQ, à partir de leurs isolats respectifs (SUPRO 760, ISP Non-GM, The Solae Company, Barcelone, Espagne ; ISP 219 Non-GM, Dupont Protein technology, St Louis, MO, USA) à 1% (p/p) en concentration protéique. Les laits de soja ont ensuite été stabilisés par un traitement ultraturax (13500 rpm, 5 min) suivi d'une étape d'homogénéisation (500 bar, 5 cycles). Puis l'étape de dessalage a été effectuée sur une colonne de dessalage de type PD10 (Amersham Biosciences, Buckinghamshire, Royaume-Uni) afin d'éliminer les ions calcium et les ions phytate initialement présents. Les échantillons ont été stockés à -20°C jusqu'à ce que les expériences de calorimétrie isothermale soient réalisées.

##### **2.1.2.2. Étude de protéines solubles de soja**

Dans cette étude concernant les interactions entre le calcium et les protéines solubles de soja, les laits de soja ont été reconstitués dans de l'eau miliQ à 4,2% (p/p) en concentration protéique. Puis les laits de soja ont été acidifiés (HCl, 1N, Riedel-de Haën, Seelze, Sigma Aldrich, Allemagne) dans un réacteur thermostaté ( $T_{\text{exp}} = 20^{\circ}\text{C}$ , 150 rpm) jusqu'à atteindre le pH isoélectrique des protéines de soja ( $\text{pH}_i = 4,5$ ). Le lait acidifié a ensuite été centrifugé

(Centrifuge Sigma Bioblock, Osterode, Allemagne) à 3034 g, 35 min, 20°C (FIL, 1995). Le surnageant contenant les protéines de soja solubles a été collecté puis neutralisé (NaOH, 1N, Riedel-de Haën, Seelze, Sigma Aldrich, Allemagne) jusqu'à atteindre le pH initial des laits de soja (~7,1-7,2). L'étape de dessalage a ensuite été réalisée sur une colonne de dessalage de type PD10 (Amersham Biosciences, Buckinghamshire, UK). Les échantillons ont été stockés à -20°C jusqu'au moment de l'analyse.

## **2.2. Détermination de la concentration en protéines**

La concentration en protéines a été déterminée par dosage de Bradford. La courbe de calibration a été réalisée avec une solution d'Albumine Sérique Bovine à 1,35 mg.mL<sup>-1</sup>. Différentes concentrations allant de 0 à 1,35 mg.mL<sup>-1</sup> ont été réalisées. Puis les protéines ont été colorées à l'aide du réactif de Bradford, dilué préalablement 5 fois (G250 100 mg, Bio-Rad S.A, Marne La Coquette, France ; éthanol 50 mL ; Acide phosphorique 85% 100 mL, Labosi, Elancourt, France ; eau distillée 100 mL). A 20 µL de chaque point de la courbe de calibration et des échantillons étudiés, 1 mL de réactif de Bradford dilué 5 fois a été ajouté. Le temps d'incubation était de l'ordre de 2 à 3 minutes. Après agitation, l'absorbance a été lue à une longueur d'onde de 595 nm sur le spectrophotomètre (U.V-160 A, Shimadzu Europa GmbH, Allemagne). La concentration protéique contenue dans chaque échantillon a été déterminée à l'aide d'une courbe de calibration (absorbance mesurée en fonction de la concentration en protéines, mg.mL<sup>-1</sup>).

## **2.3. Protocole expérimental des expériences de calorimétrie isothermale**

Deux cellules sphériques identiques, une cellule de référence et une cellule échantillon, de 1,449 mL sont enfermées dans une enveloppe adiabatique. Avant chaque expérience, les deux solutions ont été dégazées durant 7 minutes afin d'éliminer les bulles d'air susceptibles d'interférer avec la ligne de base. Puis chacune des cellules a été respectivement remplie soit avec une solution protéique de lait de vache ou de lait de soja (dans le cas de la cellule échantillon), soit avec de l'eau miliQ comme solvant, dans le cas de la cellule de référence. Le titrant, une solution de Chlorure de Calcium (CC, CaCl<sub>2</sub>.2H<sub>2</sub>O, Carl Roth GmbH, Karlsruhe, Allemagne), a été préparé dans de l'eau miliQ et injecté pas à pas dans la cellule échantillon, agitée continuellement à 300 rpm. La mesure a été effectuée à température constante. De

petits aliquots de CC (typiquement 2 à 5  $\mu\text{L}$ ) ont été successivement injectés dans la solution protéique.

Les cinq premières injections étaient usuellement fixées à un volume de 2  $\mu\text{L}$ . Le nombre total d'injections variaient selon l'expérience de sorte à atteindre la saturation. Deux injections consécutives se produisaient à 300 s d'intervalle. A cause d'un possible effet de dilution lors de la période d'équilibration précédent la première mesure, la première injection a été ignorée dans l'analyse des données.

Puisqu'aucune information concernant les chaleurs de chacun des systèmes étudiés n'est disponible dans la littérature, la puissance de référence de l'instrument a été fixée à 15  $\mu\text{cal.s}^{-1}$ . Les conditions expérimentales de chaque expérience de calorimétrie sont résumées dans les tableaux 2 et 3 pour les échantillons de protéines globales et pour les échantillons de protéines solubles, respectivement. L'échantillon global de protéines a été étudié dans le cas du lait de vache et du lait de soja. Les échantillons de protéines solubles ont été étudiés dans le cas des protéines de soja uniquement. Chaque expérience a été dupliquée.

Tableau 2. Paramètres de titration utilisés dans les expériences de calorimétrie isothermale dans l'étude des interactions entre les protéines globales et le calcium (NH : Non Hydrolysé ; H : Hydrolysé ; T = 25°C)

Echantillon	[CC] mM	Nombre d'injections	Titration
Lait de vache	50	65	2 $\mu\text{L}$ *10 and 5 $\mu\text{L}$ *55
Lait de soja NH	25	32	2 $\mu\text{L}$ *5 and 5 $\mu\text{L}$ *27
Lait de soja H	25	32	2 $\mu\text{L}$ *5 and 5 $\mu\text{L}$ *27

Tableau 3. Paramètres de titration utilisés dans les expériences de calorimétrie isothermale dans l'étude des interactions entre les protéines solubles et le calcium (NH : Non Hydrolysé ; H : Hydrolysé ; T = 25°C)

Echantillon	T <sub>exp</sub> (°)	[CC] mM	Nombre d'injections	Titration
Protéines solubles - NH	4	5	50	2 $\mu\text{L}$ *5 and 5 $\mu\text{L}$ *45
	25	5	50	2 $\mu\text{L}$ *5 and 5 $\mu\text{L}$ *45
	50	5	50	2 $\mu\text{L}$ *5 and 5 $\mu\text{L}$ *45
Protéines solubles - H	4	20	50	2 $\mu\text{L}$ *5 and 5 $\mu\text{L}$ *45
	25	20	40	2 $\mu\text{L}$ *5 and 5 $\mu\text{L}$ *35
	50	25	50	2 $\mu\text{L}$ *5 and 5 $\mu\text{L}$ *45

Dans l'analyse des résultats de calorimétrie isothermale, la titration de solution de calcium dans de l'eau miliQ a été étudiée en tant que référence (à savoir la solution de ligand ajoutée dans le solvant, sans la présence de macromolécules). Cette expérience précédente a permis de déterminer la chaleur de dilution du ligand (CC). Ce signal référence, mené dans



des conditions expérimentales similaires à la titration de l'échantillon protéique par le CC, a été retranché au signal obtenu pour l'échantillon. La référence correspond au signal entre des injections consécutives lorsque aucun changement dans le flux de chaleur ne se produit. Une fois la soustraction réalisée, les données d'enthalpie ( $\Delta H$ ) ont été traitées à l'aide du logiciel ORIGIN, en utilisant un modèle à un seul site de fixation. Les cycles de « fit » ont été répétés jusqu'à ce que la valeur du  $\chi^2$  ne soit plus réduite. A cette étape, les paramètres de fit ( $N$ ,  $K$ ,  $\Delta H$ ,  $\Delta S$ ) ont été retenus.

Dans l'étude réalisée avec les protéines solubles de soja uniquement, les agrégats de protéines de soja ont été éliminés par précipitation au point isoélectrique (4,5). Puis les protéines solubles collectées ont été neutralisées par alcalinisation jusqu'au pH initial (~7,1-7,2). Les expériences ont été menées à trois températures différentes (4, 25 et 50°C). En effet, la mesure des isothermes de liaison à deux températures au moins, permet la détermination additionnelle de la chaleur latente de la liaison ( $\Delta C_p$ ), à travers la relation suivante :

$$\Delta C_p = \frac{\Delta H^0_{T_2} - \Delta H^0_{T_1}}{T_2 - T_1}$$

Ce paramètre thermodynamique est un indicateur des changements dans l'exposition des résidus polaires et non-polaires.

La courbe de liaison usuelle tracée en kcal/mol d'injectant en fonction du ratio molaire (mole Ca/ mole de protéines) est présentée dans cette étude en fonction du ratio massique (g Ca/ g de protéines). En effet, cette représentation est plus appropriée pour un mélange complexe de protéines, ce qui est le cas de nos échantillons.

## 2.4. Les isothermes de liaison du calcium

Les isothermes de liaison du calcium ont été mesurés à l'aide d'une électrode sensible aux ions calcium (Ca ISE, Sentek, Estate, Braintree, Royaume-Uni). La calibration a été effectuée avec des solutions de CC à  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ , et  $10^{-4}$ M préparées dans du NaCl à 0,1M. Des expériences similaires à celles réalisées en calorimétrie isothermale sur des échantillons de protéines globales ont été produites sur de plus grands volumes de lait de vache ou de lait de soja. Les volumes de CC titrés sont proportionnels aux volumes de lait. Dans les isothermes de liaison du calcium, le rapport de concentration de calcium Libre/Lié a été tracé en fonction de la concentration de CC (mM) ajouté.

## **2.5. Mobilité électrophorétique et variations du potentiel zéta**

Des expériences similaires à celles réalisées en calorimétrie isothermale sur des échantillons de protéines globales ont été reproduites sur du lait de vache ou des laits de soja afin de déterminer la mobilité électrophorétique et les variations de potentiel zéta lors de la titration de CC. Ces paramètres précédents ont été déterminés par Zéta Sizer (Malvern instrument Ltd, Worcestershire, Royaume-Uni).

## **2.6. Protocole expérimental utilisé pour les expériences en Infra-Rouge à Transformée de Fourier**

Pour les analyses Infra-Rouge, le lait de vache et les laits de soja ont été reconstitués à 4,2% (p/p) en concentration protéique. Les laits de soja ont été stabilisés par ultraturrax (13500 rpm, 5 min) suivi d'un traitement d'homogénéisation par hautes pressions (500 bar, 5 cycles).

Les expériences ont été menées par spectrométrie Infra-Rouge, en Réflection Totale Atténuée (IR-TF-RTA) à température ambiante, en utilisant un spectromètre Tensor 27 moyen-IRFT Bruker (Brüker, Karlsruhe, Allemagne) équipé avec une cellule optique RTA et un détecteur RT-DTGS (mercure-cadmium-Tellurium, Brüker, Karlsruhe, Allemagne). Le diaphragme a été réglé à 4 mm et la vitesse de balayage fixée à 20 kHz. 256 scans ont été acquis pour chaque échantillon et chaque référence, entre 4000 et 900  $\text{cm}^{-1}$ , avec une résolution spectrale de 2  $\text{cm}^{-1}$ . Chaque expérience a été dupliquée. Les spectres bruts ont été traités en utilisant le logiciel OPUS/IR (Brücker, Wissemburg, France).

Chaque spectre brut a d'abord été corrigé par rapport à la contribution du  $\text{CO}_2$  et de l' $\text{H}_2\text{O}$  atmosphérique, lissé sur une fenêtre de 9 points en utilisant un algorithme de Savitzky-Golay. La ligne de base a été corrigée sur 4 points. Les bandes vibrationnelles ont ensuite été normalisées vectoriellement et une dérivée seconde a été appliquée. En effet, ce dernier traitement améliore la résolution spectrale à travers la détection d'épaulements présents lors de chevauchement spectral importants. Le signe de la dérivée seconde est opposé à celui du spectre d'origine et de ce fait, orienté vers le bas.

## **RÉSULTATS ET DISCUSSION**



## 1. Comportement de la phase protéique et équilibres en calcium entre la phase soluble et la phase colloïdale dans le lait de vache soumis à un cycle de pH

Les équilibres en calcium entre la phase soluble et la phase colloïdale ont été étudiés dans du lait de vache, supplémenté en calcium et soumis à un cycle de pH. Ces équilibres calciques ont été reliés aux variations de la phase protéique colloïdale.

La supplémentation en calcium (0-25 mmoles/kg) avec du Chlorure de Calcium (CC) ou une source de calcium naturel provenant du lait (Calcium du lait, MC) a été suivie par un cycle de pH ( $\text{pH}_{\min}$  5,5, 5,0 ou 3,5). Le pH, le calcium ionisé ( $\text{Ca}^{2+}$ ) et les variations de turbidité ont été enregistrés *in situ* et la phase protéique a été caractérisée par Diffusion Dynamique de la Lumière (DLS) et par électrophorèse (SDS-PAGE) à des temps spécifiques du cycle du pH.

Pour des cycles de pH modérés ( $\text{pH}_{\min}$  5,5 ou 5,0), la référence et le lait supplémenté en MC ont montré des tendances similaires.

La concentration en calcium ionisé augmente et diminue respectivement lors de l'acidification et de l'alcalinisation, ce qui peut être relié aux variations de l'état de minéralisation des micelles de caséines.

La taille des particules et la turbidité diminuent tout au long du cycle du pH jusqu'à atteindre un état avancé de destructuration de la phase colloïdale pour des cycles de pH à 5,0. Cependant, le cycle de pH n'est pas réversible ni sur les variations de calcium ionisé ni sur les variations de la phase protéique.

Dans le lait de vache supplémenté en CC, la taille des particules et la turbidité augmentent lors de la supplémentation en CC. L'acidification entraîne une diminution modérée de la turbidité, ce qui pourrait être expliqué par une légère destructuration des micelles de caséines. Néanmoins, les micelles de caséines restent plus minéralisées lors de l'acidification dans les cas des laits supplémentés en CC. La supplémentation en CC est supposée stabiliser les monomères de caséines à l'intérieur des micelles de caséines. La

solubilisation du matériel micellaire est réversible lors de l'alcalinisation. Ceci indique que les micelles de caséines sont moins déstabilisées lors de cycle de pH lorsque le lait est préalablement supplémenté en CC. Cette réversibilité des phénomènes pourrait être due à la capture préalable du calcium, impliquant un renforcement des micelles de caséines par le biais d'interactions entre les ions calcium et les protéines de lait de vache, de nature électrostatique.

Les cycles de pH à 3,5, montrent des phénomènes irréversibles. Les variations de turbidité et de calcium ionisé conduisent à des profils plus complexes. Les valeurs extrêmes impliquant une large destruction micellaire (minimum de turbidité et maximum de calcium ionisé) sont atteintes dans les environs du pH isoélectrique des caséines.

## 2. Comportement de la phase protéique et équilibres en calcium entre la phase soluble et la phase colloïdale dans le lait de soja soumis à un cycle de pH

Les équilibres calciques entre la phase soluble et la phase colloïdale ont été étudiés dans du lait de soja, supplémenté en Chlorure de Calcium (CC, 25 mmoles/kg) lors de cycles de pH ( $\text{pH}_{\text{min}}$  5,5 ou 3,5). Les variations de calcium ionisé ( $\text{Ca}^{2+}$ ) ont été reliées aux variations de la phase protéique ( $\eta_{\text{app}}$ ,  $D_{4,3}$ , potentiel zéta).

Dans les laits de soja reconstitués (Non-Hydrolysé, NH, ou Hydrolysé, H), la concentration initiale en calcium ionisé est négligeable. Après supplémentation en CC, la concentration en calcium ionisé augmente durant l'acidification, due à la régression de l'état d'ionisation des protéines, impliquant une solubilisation consécutive du calcium ionisé. La neutralisation par alcalinisation mène à une diminution de la concentration en calcium ionisé.

**Pour des cycles de pH à 5,5 ( $> \text{pH}_i$ ),** la viscosité apparente ( $\eta_{\text{app}}$ ) des laits de soja-NH augmente durant l'acidification puis diminue lors de la neutralisation sans être restaurée, contrairement au lait de soja-H. Le diamètre volumique moyen ( $D_{4,3}$ ) des agrégats constitués de protéines de soja, est plus élevé dans le cas du lait de soja-NH. Il augmente puis diminue lors de l'acidification et lors de la neutralisation par alcalinisation respectivement.

**Pour des cycles de pH à 3,5 ( $< \text{pH}_i$ ),** la concentration en calcium ionisé solubilisé durant la phase d'acidification atteint un maximum aux environs du point isoélectrique ( $\text{pH}_i$ ). En dessous du  $\text{pH}_i$ , le calcium ionisé ( $\text{Ca}^{2+}$ ) est supposé interagir avec des anions contenus dans la phase soluble. La viscosité apparente ( $\eta_{\text{app}}$ ) montre des variations similaires aux cycles obtenus à pH 5,5, avec un maximum dans la région du point isoélectrique. Pour les deux types de laits de soja, l'augmentation de taille des particules est partiellement restaurée lors de la neutralisation par alcalinisation.

**Lors de la supplémentation en CC,** la viscosité apparente ( $\eta_{\text{app}}$ ) et le diamètre volumique moyen ( $D_{4,3}$ ) augmentent, ce qui reflète l'agrégation des protéines de soja en présence d'ions calcium.

Pour des cycles de pH à 5,5, l'agrégation protéique induite par l'acidification est partiellement voire complètement réversible lors de l'alcalinisation, pour les laits de soja Non Hydrolysé et Hydrolysé, respectivement. Pour des cycles de pH à 3,5, les phénomènes observés sont irréversibles.



### **3. Étude des interactions calcium-protéines dans le lait de vache et dans les laits de soja**

Les interactions calcium-protéines ont été thermodynamiquement caractérisées par Calorimétrie Isothermale de Titration. Les protéines de lait de vache, les protéines de soja (Hydrolysées ou Non-Hydrolysées) ont été étudiées.

En parallèle, le potentiel zéta a été mesuré afin d'estimer la part des interactions électrostatiques impliquées dans les interactions entre les ions calcium et les protéines étudiées (protéines de vache ou de soja).

La spectrométrie par Infra-Rouge à Transformée de Fourier a été utilisée afin d'obtenir une meilleure caractérisation des sites de fixation des ions calcium.

Les signaux thermodynamiques globaux, obtenus lors de la titration des protéines de vache ou des protéines de soja par du Chlorure de Calcium (CC) ont montré des phénomènes de nature endothermique.

Les courbes de titration ont été fittées à l'aide d'un modèle à un seul site de fixation afin de déterminer les paramètres thermodynamiques ( $N$ ,  $K$ ,  $\Delta H$ ,  $\Delta S$ ).

Les protéines de vache pouvaient lier jusqu'à 8 mg de Ca par g de protéines une fois la saturation atteinte.

Les protéines de soja Non-Hydrolysées pouvaient lier plus de calcium que dans le cas des protéines de soja Hydrolysées (~52 et ~2 mg de Ca par g de protéines, respectivement).

La constante d'association  $K$ , a diminué respectivement des protéines de soja Hydrolysées, aux protéines de vache puis aux protéines de soja Non-Hydrolysées, ce qui indique une plus grande facilité de liaison du calcium sur les protéines de soja Hydrolysées.

Les changements de la chaleur latente ( $\Delta C_p$ ), déterminés pour les interactions entre les ions calcium et les protéines solubles de soja uniquement, ont indiqué des changements dans

les interactions hydrophobiques et/ou dans les liaisons hydrogènes. Quelque soit la nature des protéines de soja (Hydrolysées ou Non-Hydrolysées), une valeur positive du  $\Delta C_p$  est indicative de la réduction dans le nombre de résidus polaires exposés, accessibles en surface. Cela pourrait être dû au camouflage du ligand chargé (l'ion calcium) et des groupes protéiques chargés, à l'intérieur du site de fixation.

Les variations de potentiel zéta produites lors de la titration des protéines de vache ou des protéines de soja (NH ou H) indiquaient l'existence d'interactions entre les ions calcium et ces protéines de nature électrostatique.

De ce fait, le signal exothermique habituellement observé lors des interactions électrostatiques est complètement caché par le signal endothermique de forte amplitude. Cet important signal endothermique pourrait provenir du relargage de molécules d'eau, soit de la coque d'hydratation des ions calcium, soit de la déshydratation du cœur hydrophobe des protéines.

La liaison du calcium sur les protéines devrait plutôt être décrite comme un échange entre les protons ( $H^+$ ) et les ions calcium ( $Ca^{2+}$ ), ce qui serait plus neutre sur le plan énergétique, en regard des forces électrostatiques impliquées.

Enfin, les expériences réalisées par spectroscopie Infra-Rouge à Transformée de Fourier ont montré une diminution de l'énergie d'absorption dans la région amide I et dans la région amide II, ainsi que dans la région carboxylate, lors de la supplémentation en CC. Ces variations observées étaient d'autant plus importantes dans les laits de soja, en comparaison à celles observées dans le lait de vache.

## **CONCLUSIONS ET PERSPECTIVES**



## Conclusions générales

Le contexte industriel de cette étude était de comprendre et de comparer les interactions calcium-protéines dans le lait de vache (vecteur naturel du calcium), et dans le lait de soja dans un objectif final de supplémentation en calcium de lait de soja. Le but scientifique de cette étude était donc de comprendre les équilibres en calcium dans le lait de vache, dans le lait de soja, puis de comparer ces deux systèmes lactés.

Dans une première partie de ce travail de thèse, la méthodologie expérimentale a été mise au point en travaillant sur du lait de vache écrémé, étant donné que ce système biologique a déjà été largement étudié dans la littérature.

Le lait de vache ou le lait de soja (Non-Hydrolysé ou Hydrolysé) ont été supplémentés en calcium (0-25 mmoles/kg) puis soumis à un cycle de pH ( $\text{pH}_{\text{min}}$  5,5, 5,0 ou 3,5). Les expériences ont été menées dans un réacteur thermostaté, équipé d'une sonde à pH, d'un turbidimètre, d'un conductimètre et d'une électrode à calcium ionisé. A la fin de chaque étape spécifique de la cinétique (stabilisation des sondes, période d'équilibre en calcium, acidification et alcalinisation), des échantillons ont été prélevés pour une caractérisation ultérieure de la phase protéique (mesure de la taille des particules, mesure du potentiel zéta...). Quelque soit le type de système lacté (lait écrémé de vache, lait de soja Non-Hydrolysé ou Hydrolysé), les variations en calcium ionisé mesurées lors d'un cycle de pH ont été reliées aux variations de la phase protéique.

- **Variations en calcium ionisé**

Initialement, la concentration en calcium ionisé mesuré était de l'ordre de 2 mM dans le lait écrémé de vache alors que cette valeur était pratiquement négligeable dans le cas des laits de soja.

Durant un cycle de pH, les variations en calcium ionisé présentaient des tendances similaires pour les deux types de lait (lait de vache ou lait de soja), et ce, quelque soit le pH minimum atteint pour un cycle de pH donné (pH 5,5 ou 3,5).

Les profils dans leur ensemble peuvent être divisés en trois phases principales, correspondant à la stabilisation de la concentration en calcium ionisé après supplémentation en Chlorure de Calcium entre la phase soluble et la phase colloïdale, puis aux étapes d'acidification et d'alcalinisation.

L'acidification a entraîné la solubilisation du calcium à partir de la phase colloïdale, ce qui correspond au phénomène de solubilisation du calcium micellaire se produisant dans le lait de vache, communément décrit dans la littérature.

Lors de l'acidification, la capacité du calcium à se fixer sur les protéines de vache ou les protéines de soja diminue, et résulte en une augmentation du potentiel zéta jusqu'à atteindre une valeur proche de zéro. Cette régression d'ionisation des protéines (de lait de vache ou de lait de soja) a réduit la charge négative des résidus phosphosérines et/ou des groupes carboxyliques des protéines. Au contraire, la neutralisation par alcalinisation a impliqué des variations en calcium inverses à celles observées lors de l'acidification, à savoir une diminution de la concentration en calcium ionisé mesuré dans la phase soluble. Considérant la nature différente du phosphate présent dans le lait de vache ou dans le lait de soja, différents sels de phosphate pourraient être formés lors de l'alcalinisation.

La comparaison des cycles de pH ( $\text{pH}_{\min}$  5,5 ou 3,5) a montré des tendances similaires, quelque soit le système lacté (lait de vache ou lait de soja). Néanmoins, pour des cycles de pH allant jusqu'à pH 3,5, un maximum de calcium ionisé a été atteint dans la région du pH isoélectrique.

Lors d'un cycle de pH ( $\text{pH}_{\min}$  5,5 ou 3,5), un phénomène d'hystérèse a été observé dans le lait de vache mais celui-ci était pratiquement inexistant dans le lait de soja. Cette observation pourrait être reliée à une plus forte capacité du pouvoir tampon dans le lait de vache.

- **Relation entre les variations de calcium ionisé et les variations de la phase protéique lors des cycles de pH**

- **Cycle de pH à 5,5**

**Pour le lait de vache non supplémenté en calcium (lait référence)**, la phase colloïdale a été déstructurée suite à la solubilisation du calcium micellaire durant l'acidification. Lors de l'alcalinisation, le calcium ionisé ( $\text{Ca}^{2+}$ ) a réintégré les micelles de caséines mais de façon insuffisante pour empêcher la désagrégation des micelles de caséines. Plus de monomères de caséines de type  $\alpha_{s1}$  et  $\alpha_{s2}$  ont été détectés dans la phase soluble après un cycle de pH à 5,5, en relation avec la déstructuration des micelles de caséines décrite précédemment.

**Pour les laits de soja non-supplémenté en calcium (laits de soja référence),** le diamètre volumique moyen ( $D_{4,3}$ ) a augmenté, dû à l'agrégation induite par l'acidification, et la viscosité apparente a également augmenté. Lors de l'alcalinisation, l'état d'ionisation des protéines de soja a augmenté de nouveau. La concentration en calcium ionisé ainsi que le diamètre volumique moyen ont diminué de nouveau. L'agrégation protéique a été partiellement et entièrement restaurée pour le lait de soja Non-Hydrolysé et le lait de soja Hydrolysé, respectivement.

**Pour des cycles de pH à 5,5,** la comparaison des systèmes lactés a montré des tendances similaires dans les variations de calcium ionisé, entre le lait de vache et le lait de soja, avec toutefois une différence majeure de l'effet de la variation de calcium ionisé sur la phase protéique.

Les micelles de caséines ont été désagrégées lors de l'acidification, ce qui était principalement dû à la solubilisation du Phosphate de Calcium Micellaire. Au contraire, l'acidification a induit la solubilisation du calcium endogène contenu dans les protéines de soja, entraînant la régression de leur état d'ionisation et leur agrégation consécutive (augmentation du diamètre volumique moyen,  $D_{4,3}$ , et augmentation de la viscosité apparente,  $\eta_{app}$ ). Lors de l'alcalinisation, les micelles de caséines ont continué à se désagréger malgré la réintégration du calcium dans les micelles de caséines, préalablement appauvries en calcium lors de la phase d'acidification.

Dans le lait de soja, lors de l'alcalinisation, l'état d'ionisation des protéines de soja a de nouveau augmenté : le calcium ionisé a réintégré les protéines de soja, entraînant une répulsion électrostatique et la diminution de la taille des agrégats protéiques. Cependant, la viscosité apparente n'a été restaurée que dans le cas du lait de soja Hydrolysé, et non dans le cas du lait de soja Non-Hydrolysé. L'agrégation a été partiellement ou totalement restaurée pour le lait de soja-NH et le lait de soja-H, respectivement.

**Pour le lait de vache supplémenté en CC (Lait CC),** la turbidité a augmenté lors de la supplémentation en Chlorure de Calcium et serait reliée à l'augmentation de densité micellaire, due à l'association des ions calcium avec les micelles de caséines. Simultanément, la concentration en caséines de type  $\alpha_{s1}$  et  $\alpha_{s2}$  a diminué dans la phase soluble lors de la supplémentation en CC. Puis la turbidité a diminué lors de l'acidification, ce qui correspond à la destructuration de la phase colloïdale et au relargage de monomères de caséines  $\alpha_s$  suivant

la solubilisation du Phosphate de Calcium Micellaire. Lors de l'alcalinisation, la turbidité a diminué et puis a augmenté de nouveau, ce qui correspond respectivement à la destruction et à la restructuration des particules colloïdales à base de caséines. Ceci reflèterait les interactions inverses des monomères de caséines de type  $\alpha_s$  avec les protéines de la phase colloïdale via des interactions avec des ions calcium.

**Pour les laits de soja supplémentés en CC (Lait de soja-CC)**, la concentration en calcium ionisé a augmenté lors de l'addition de CC. Dans le même temps, le potentiel zéta, le diamètre volumique moyen et la viscosité apparente ont également augmenté. La variation concomitante de ces trois paramètres indique que les interactions entre les ions calcium et les protéines de soja sont de nature électrostatique, et implique la formation d'agrégats de protéines de soja. Lors de l'acidification, le calcium lié aux protéines ou à d'autres anions a été libéré dans la phase soluble. Une diminution de la viscosité apparente a été mesurée mais la taille des agrégats protéiques de soja est restée pratiquement inchangée. Durant l'alcalinisation, les ions calcium ont été réabsorbés par la phase colloïdale protéique. Une fois la supplémentation en CC, la viscosité apparente a augmenté et aucune autre variation significative n'a été mesurée sur le diamètre volumique ( $D_{4,3}$ ), lors du cycle de pH. Dans le lait de soja, l'agrégation induite par la supplémentation en calcium est irréversible, et le cycle de pH consécutif n'induit seulement qu'un effet global mineur.

### ➤ Cycle de pH à 3,5

**Pour le lait de vache**, un maximum de turbidité et un maximum de calcium ionisé ont été mesurés aux environs de la zone de pH isoélectrique, durant l'acidification et l'alcalinisation ; ceci correspond à un phénomène d'agrégation maximal. Durant l'alcalinisation, la turbidité a diminué jusqu'à atteindre un minimum, et ensuite a de nouveau augmenté. La différence majeure entre le lait de vache non supplémenté en CC (lait référence) et le lait de vache supplémenté en CC a été observée durant l'étape de neutralisation par alcalinisation : le minimum de turbidité apparaissait plus tôt et était également plus élevé pour le lait supplémenté. Ceci confirme l'hypothèse d'un renforcement initial de la phase protéique colloïdale lors de la supplémentation en CC. Le taux de restructuration était plus élevé dans le lait supplémenté, en comparaison au lait non supplémenté en CC. Néanmoins, le cycle de pH à 3,5 n'était pas réversible quelque soit le lait vache (supplémenté ou non).



**Pour les laits de soja**, tous les paramètres étudiés ( $\text{Ca}^{2+}$ ,  $\eta_{\text{app}}$ , et le potentiel zéta) ont montré deux étapes distinctes durant l'acidification (et les phénomènes inverses lors de l'alcalinisation) lors du passage de la région du pH isoélectrique. Pour chacun des paramètres étudiés, le maximum a été atteint dans la région de pH isoélectrique, où le potentiel zéta est nul et où le maximum d'agrégation est observé.

**Avec la supplémentation en CC**, la taille des particules ( $D_{4,3}$ ) a augmenté et était irréversible quelque soit le type de lait de soja (Non-Hydrolysé ou Hydrolysé). Les agrégats de protéines de soja induits par l'ajout de calcium ont montré seulement quelques variations de taille, alors que la concentration en calcium ionisé, la viscosité apparente et le pouvoir tampon ont continué de varier tout au long du cycle de pH. Ainsi, la densité en calcium contenue dans les agrégats de protéines de soja pourrait probablement varier. Pour conclure, les agrégats formés lors de la supplémentation en calcium seraient plus forts que les agrégats formés lors de l'acidification (plus grande viscosité apparente,  $\eta_{\text{app}}$ , et plus grand diamètre volumique moyen,  $D_{4,3}$ ). Il se peut que des interactions plus fortes soient impliquées dans le premier cas.

- **Comparaison du pouvoir tampon dans le lait de vache et dans le lait de soja ( $\text{pH}_{\text{min}} 3,5$ )**

Lors de l'acidification, le maximum de pouvoir tampon a été mesuré à une valeur de pH de l'ordre de 5,2 pour le lait de vache et était essentiellement due à la solubilisation du Phosphate de Calcium Micellaire. Cependant, lors de la neutralisation par alcalinisation, le maximum de pouvoir tampon a été mesuré à pH 6,6 du fait de la formation de phosphate de calcium accompagnant le relargage de protons. Lors de la supplémentation en CC, le pouvoir tampon a également subi une augmentation.

Le pouvoir tampon (dB/dpH) était plus élevé dans le lait de soja Hydrolysé, en comparaison au lait de soja Non-Hydrolysé, en absence ou en présence de CC ajouté.

D'après l'ensemble des résultats, puisque les protéines de soja NH lient plus de calcium que les protéines de soja H et que de plus, la concentration en calcium ionisé était pratiquement similaire entre les deux types de lait de soja, cette plus forte valeur de pouvoir

tampon dans les laits Hydrolysés pourrait être dûe à une plus grande proportion de calcium complexé dans le lait de soja Hydrolysé. En effet, il est probable que ce calcium complexé à d'autres anions présents dans la phase soluble, contribue plus efficacement et plus facilement au pouvoir tampon que le calcium lié aux protéines de soja.

Quelque soit le type de lait de soja et similairement au lait de vache, le pouvoir tampon a été déplacé vers des valeurs plus élevées lors de la supplémentation en CC, et un pic de pouvoir tampon, centré sur des valeurs de pH allant de 4,5 à 5,0, apparaît de façon distincte. Similairement au lait de vache, le maximum de pouvoir tampon a été observé dans le lait de soja dans un même rang de pH (4,5-5,0). Ceci pourrait être le fait de calcium initialement absorbé par les protéines de soja, agrégées en présence de CC, et ensuite solubilisé durant l'acidification.

La supplémentation en calcium a amélioré le pouvoir tampon des laits de soja. Ce paramètre était maximal dans des rangs de pH où le maximum de calcium a été solubilisé et où le maximum d'agrégation induite par acidification s'est produit. Lors de l'agrégation, plus d'interactions existent au sein des agrégats de soja. De fait, ces précédents agrégats ont été renforcés, et sont par conséquent plus résistants aux variations de pH.

Dans une seconde partie de la thèse, les interactions calcium-protéines ont été caractérisées thermodynamiquement en utilisant la calorimétrie isothermale et les sites de fixation du calcium ont été identifiés par spectroscopie Infra-Rouge à Transformée de Fourier. Les paramètres thermodynamiques, tels que  $N$ ,  $K$ ,  $\Delta H$ ,  $\Delta S$ ,  $\Delta G$  ont été déterminés après avoir fitté les données expérimentales à l'aide d'un modèle à un seul site de fixation. Les interactions protéines-calcium ont été étudiées dans un échantillon de protéines globales de vache ou de soja. Dans les laits de soja, les interactions calcium-protéines ont aussi été étudiées pour des échantillons de protéines solubles à différentes température (4, 25 et 50°C) afin de déterminer la valeur de la chaleur latente ( $\Delta C_p$ ).

- **Caractérisation thermodynamique des interactions calcium-protéines**

Les profils thermodynamiques des interactions calcium-protéines étaient similaires dans les échantillons de protéines globales de lait de vache, de lait de soja Hydrolysé ou Non-Hydrolysé. Des enthalpies de liaison de nature endothermique ont été observées dans chacun des cas. Les données expérimentales obtenues pour les deux types de système lacté (lait de vache ou lait de soja) ont été fittées à l'aide d'un modèle à un seul site de fixation, ce qui signifie que les sites de fixation de calcium sur les protéines (de vache ou de soja) sont équivalents et indépendants. L'échantillon global des protéines de lait de vache pouvait lier jusqu'à 8 mg de calcium par g de protéine à saturation. En comparaison, les protéines de soja Non-Hydrolysées et Hydrolysées pouvait lier jusqu'à 51,7 et 2,2 mg de calcium par g de protéines, respectivement.

La liaison du calcium sur les protéines ne pouvait pas être expliqué exclusivement par les interactions électrostatiques. En effet, dans ce cas, le signal observé serait strictement exothermique. Néanmoins, la part exothermique du signal thermodynamique due aux interactions électrostatiques a été complètement cachée par un fort signal endothermique provenant du relargage de molécules d'eau, provenant de la coque d'hydratation des ions calcium et/ou de la déshydratation du cœur hydrophobe des protéines de vache ou de soja.

Quelque soit le type de système lacté, la liaison du calcium sur les protéines (de vache ou de soja) devrait plutôt être décrite comme un échange entre les protons et les ions calcium, ce qui serait plus neutre sur le plan énergétique, au regard des forces électrostatiques impliquées.

- **Identification des sites de fixation du calcium**

Dans le lait de vache ou dans les laits de soja, des variations se sont produites lors de la supplémentation en CC dans les bandes amide I et II. La première bande amide correspond principalement aux vibrations d'élongation concernant les groupements carbonyle C=O, couplées avec des vibrations de déformation des N-H, spécifiques à la fonction amide I. La région amide II correspond principalement aux vibrations de déformation N-H couplées avec des vibrations d'élongation C-N. Les variations d'intensité de ces bandes se produisant lors de la supplémentation en CC sont plus importantes dans les laits de soja, en comparaison au lait de vache, où seule une légère diminution de l'intensité de ces bandes a été observée.

Dans les laits de soja, en plus des variations de ces bandes amides, des variations se sont produites dans deux autres bandes spécifiques, centrées sur  $1450\text{ cm}^{-1}$  et  $1400\text{ cm}^{-1}$ . Ces deux bandes précédentes correspondent respectivement aux vibrations de déformation des C-H des protéines et aux vibrations d'élongation des O-C-O des groupes carboxylates.

La diminution de l'intensité de ces deux bandes amides et de la bande des C-H, pourrait être indirectement reliée à l'interaction des ions calcium sur les protéines. En effet, l'interactions des ions calcium sur les protéines de lait de vache ou de soja pourrait modifier l'environnement de ces deux fonctions amides, entraînant le renforcement et une plus grande rigidité de la structure primaire des protéines, et par conséquent, une diminution de l'énergie de vibration de ces deux bandes amides. La diminution de l'intensité des énergies de vibration d'élongation de groupes O-C-O pourrait être directement reliée aux interactions des ions calcium sur les groupes carboxylates des protéines de soja, due à une forte proportion en acides aminés acides (acide glutamique et acide aspartique) dans les protéines de soja.

## PERSPECTIVES

Cette étude a été proposée afin de mieux comprendre les interactions se produisant entre les ions calcium et les protéines, de vache ou de soja. En effet, le contexte industriel était de comparer ces deux systèmes lactés. Le lait de vache, en tant que vecteur naturel du calcium, a été choisi comme modèle biologique, à imiter, en vue d'une supplémentation ultérieure en calcium dans le lait de soja. Afin d'obtenir des informations utiles, le choix d'un mélange complexe de protéines préparées de façon industrielle a été fait afin de travailler au plus près des phénomènes qui se produisent dans la réalité.

Cependant, afin d'aboutir à une meilleure interprétation des résultats concernant les équilibres en calcium dans le lait de vache ou dans le lait de soja, l'étude d'espèces minérales autres que les ions calcium, et plus particulièrement les ions citrate, phosphate et magnésium pourrait apporter des informations intéressantes.

Afin d'avoir une compréhension plus profonde des interactions calcium-protéines se produisant dans les protéines de vache ou de soja, d'autres expériences de calorimétrie isothermale devraient être réalisées. Le même type d'expériences que celles présentées dans ce travail de thèse pourraient être menées avec des fractions protéiques purifiées contenues dans le lait de vache (telles que les caséines  $\alpha_{s1}$ ,  $\alpha_{s2}$ , la  $\beta$ -lactoglobuline, l' $\alpha$ -lactalbumine...) ou celles contenues dans le lait de soja (protéines 7S ou 11S). En effet, à l'aide de ces expériences complémentaires, nous pourrions déterminer avec précision les paramètres thermodynamiques  $N$ ,  $K$ ,  $\Delta H$ ,  $\Delta S$  et  $\Delta G$  pour chaque fraction protéique purifiée. Ainsi, nous pourrions déterminer quelles sont les protéines les plus efficaces dans la fixation du calcium.

Similairement à l'étude des protéines solubles de soja, les mêmes expériences pourraient être réalisées sur des protéines solubles de lait de vache afin de déterminer l'effet de la température sur l'enthalpie mesurée. Ainsi, la chaleur latente pourrait aussi être déterminée pour des échantillons de protéines de lait de vache, et une comparaison complète du profil thermodynamique des interactions calcium-protéines pourrait être réalisée.

Les expériences de calorimétrie isothermale pourraient être couplées à d'autres techniques telles que le zéta-sizer afin d'obtenir plus d'informations sur les événements se produisant lors des interactions calcium-protéines. Similairement à Pochard et al. (1999), la

conductimétrie et la mesure de turbidimétrie pourraient être utilisées en parallèle des expériences menées en calorimétrie. La complexation des ions calcium sur des protéines de vache ou de soja complètement neutralisées pourrait être suivie par la méthode de conductimétrie décrite par Pochard et al. (1998) et la turbidimétrie pourrait être suivie afin de détecter la limite de solubilité des protéines de vache ou des protéines de soja en présence de calcium.

Nous pourrions aussi utiliser la spectrométrie en UV-Visible par balayage de températures afin d'élucider les effets de la liaison des ions calcium sur la stabilité des protéines, ainsi que la spectrométrie par Dichroïsme Circulaire afin de déterminer les changements structuraux des protéines de vache ou de soja lors de la fixation du calcium (Saboury et al., 2005). La combinaison des résultats obtenus en calorimétrie avec des données structurales permettrait de calculer et de quantifier le changement de surfaces non polaires accessibles à l'eau. Les changements conformationnels protéiques lors de la titration par le Chlorure de Calcium pourraient être étudiés par RMN (Wintrode et Privalov, 1997). Penfold et al. (1998) et Matulis et al. (2000) ont utilisé des modèles de liaison électrostatique pour l'étude de la fixation du calcium sur les protéines. L'utilisation de tels modèles pourrait quantifier la part de contribution des interactions électrostatiques sur le signal global d'enthalpie mesuré.

De plus, dans le cadre d'une approche plus industrielle, l'efficacité des complexants utilisés couramment dans l'industrie agro-alimentaire pourrait aussi faire l'objet d'une étude scientifique.

Dans cette étude sur les interactions calcium-protéines, l'approche Infra-Rouge a apporté des informations sur les variations de structures secondaires se produisant lors de la supplémentation en calcium sur les protéines de soja ou des protéines de vache. Afin de mener une investigation plus approfondie des modifications structurales des protéines en interactions avec les ions calcium, il serait utile de simplifier les systèmes en travaillant sur des fractions protéiques purifiées. Ainsi, les deux types d'études menées en parallèle, sur des systèmes réels et sur des systèmes simplifiés pourraient être la meilleure façon de comprendre entièrement les interactions calcium-protéines.

## **I. INTRODUCTION**





Calcium is a mineral essential for humans particularly during certain phases of life such as growth for bone constitution (Bonjour et al., 2005), lactation and during old-age to act against osteoporosis (Renner, 1994; Nordin, 1997; Heaney, 2000, 2001; O'Connell and Stamm, 2004). Yet, specific population at risk do not satisfy the Recommended Daily Intake of calcium. Recent studies on consumer habits reported that 50 to 75% of girl teenagers, women older than 55 years old, men older than 65 years old and elderly people do not satisfy the calcium Recommended Daily Allowances (Gueguen, 2000). According to an INCA survey (Volatier et al., 2000), even in western countries these specific populations at risk do not satisfy the RDA for calcium. Several authors concluded that an appropriate calcium intake during childhood would prevent from osteoporosis later in life (Chan, 1991). More, apart from the effect of calcium on osteoporosis prevention, calcium is also recognised as a factor in the prevention of colon cancer (Wallace et al., 2004), hypertension (Tsuda et al., 2001) and a number of other disorders (Miller et al., 2001; McCarron and Heaney, 2004)...

Authorities took into consideration the importance to meet calcium needs. Hence most of the governments and health organization are emphasizing for calcium enrichment of food and today calcium fortification is a growing trend in the food industry. With an ageing population, especially in Western countries, the expenses of health care associated with osteoporosis fracture are considerable. Thus prevention of osteoporosis and its complications is an essential socio-economic priority. This nutritional deficiency in calcium presents a world-wide health stake and can be overcome by calcium supplementation of food.

Dairy products, naturally rich in calcium and with a high calcium bioavailability, are appropriate foods for calcium supplementation. As a natural source of calcium, with casein micelles as nano-vector, cow milk constitutes an appropriate food for calcium supplementation strategy. More milk and dairy products have a healthy image and are ideal vehicles for delivery of additional calcium. Nevertheless, for various nutritional, ethical, religious or economical reasons, cow milk can not be consumed by specific populations.

Hence soy "milk" constitutes an alternative to cow milk, especially for population suffering of Cow Milk protein Allergy (Exl and Fritsché, 2001) and/or lactose intolerance (Swagerty et al., 2002). This former imitation milk has a similar nutritive value than cow milk (Desikachar and Subrahmanyam, 1946; Ciquel, INRA, 1995) and is free of cholesterol and lactose. Yet soy "milk" contains only one fifth to one eighth of calcium than in cow milk according to the literature (Souci et al., 1994; Ciquel, INRA, 1995). Hence calcium supplementation of soy milk at a similar level of cow milk is of specific interest for people

who desire to change their consuming habits with regards to the balance intake in calcium or for specific groups (Lactose intolerance, CMA...).

Up to now, calcium fortification of soy milk at a similar level of cow milk was reported to be successful in presence of calcium complexant and/or stabilizer (Zemel and Shelef, 1986) but in their absence the high sensitivity of soy proteins to calcium induce their aggregation (Saio et al., 1969; Lee and Rha, 1977).

In this context, it seemed useful to understand the calcium natural vector that is present in cow milk, i.e. the casein micelle. Indeed the elaboration of calcium vectors based on soy proteins could be an alternative to calcium complexant and/or stabilizer commonly used in calcium soy milk fortification. The use of pH cycle after calcium supplementation of soy milk would be an interesting mean to create new soy proteins structures. In a more fundamental approach, calcium-proteins interactions should be characterized. Finally calcium equilibrium between the soluble and the protein phase and calcium-proteins interactions should be compared between cow skim milk and soy milk.

This Ph.D. report is constituted of three chapters: a state of the art, the material and methods then the results and discussion.

The bibliography chapter relates first the importance of calcium mineral element on human health and more specifically world-wide health stake of calcium food supplementation. Then an overview is proposed on calcium equilibrium in cow skim milk system submitted to variations of physico-chemical parameters (i.e., pH, temperature, ionic strength...). The last bibliography part focuses on soy milk and the influence of calcium supplementation on calcium equilibrium in this latter system; the influence of various physico-chemical parameters such as pH, temperature, calcium supplementation, on soy proteins is also reported.

The material and methods used for the study of calcium equilibrium between the soluble and the colloidal phase and calcium-protein interactions in cow skim milk and in soy milks are described in the **second chapter** of the Ph.D thesis report. In the **third chapter**, results are presented and discussed. Calcium equilibrium between soluble and colloidal protein phase are studied in calcium supplemented milky systems during pH cycle (**part 1** for cow milk and **part 2** for soy “milk”). The calcium-protein interactions into cow and soy milks (**part 3**) are characterized mainly by Isothermal Titration Calorimetry and Fourier Transform Infra-Red spectroscopy.

Finally, a general conclusion on the comparison of both milky systems is given and enables to make an assessment of the results obtained and to open on the perspectives following this Ph.D. thesis work.



## **II. BIBLIOGRAPHY**



## **1. A worldwide health stake of calcium supplementation of food**

### **1.1. Calcium: a mineral essential for life**

#### **1.1.1. The function of calcium in organism**

Calcium is a mineral essential for life, involved in most metabolic processes. Combined with phosphate salt, calcium provides mechanical rigidity to the skeleton. Present at 1-2% of the body weight of an adult, the major part (99%) is constitutive of bone and teeth.

More, calcium present in the skeleton has the additional role of acting as a reserve supply to meet the body's metabolic needs in case of calcium deficiency. Indeed to ensure the homeostasis, the calcaemia (2.24-2.74 mmol Ca per L<sup>-1</sup> of blood) is maintained constant at the expense of bony calcium. The 1% free-bone calcium participates to other paramount functions, i.e. the neuromuscular excitability, the nervous conduction, the blood coagulation, the biological membrane permeability....

#### **1.1.2. The Recommended Daily Allowance in calcium**

The calcium requirement of adults is defined as the mean intake at which calcium intake and output are equaled. Nevertheless this mean value does not take the individual variations into consideration. On the contrary, the Recommended Daily Allowance (**RDA**) is an intake calculated to meet the requirement of the majority of the population and is therefore a higher figure than the calculated requirement.

The mean requirement, calculated from balance studies, was defined as about 800 mg/day on western diets, implying an allowance of 1000 mg/day or more. New guidelines consistently advocate higher daily intakes up to 1500 mg/day (NIH consensus conference, 1994; Murray and Bowyer, 1996; Masi and Bilezikian, 1997; Reid, 1997), which may be difficult to reach for many individuals.

RDA was defined taking into account the net balance for the maintenance and the actual absorption coefficient (Guégen, 1981). Corresponding allowance have been calculated for pregnancy, period of lactation, children and adolescents, considering the additional needs of the foetus, milk production and growth, respectively. The RDA in calcium are summarized in table 1 for each class of French population.

Table 1. Recommended Daily Allowance (RDA) in mg/day (Guéguen, 2001)

Class of Population	Calcium (mg/day)
Children 1-3 yrs old	500
Children 4-9 yrs old	800
Teenagers 10-14 yrs old	1200
Teenagers 15-18 yrs old	1200
Adults > 18 yrs old	900
Women > 55 yrs old	1200
Men > 65 yrs old	1200
Pregnant women third term	1000
Lactating women	1000
Post-lactating women	1000

### 1.1.3. The effect of calcium deficiency

Calcium is a mineral essential for humans, particularly during certain phases of life such as growth (Cashman and Flynn, 1999; Flynn, 2003), pregnancy, lactation (Krebs et al., 1997; Thomas and Weisman, 2006) and during old age. Yet, calcium deficiency is easily induced because of the obligatory losses of calcium via the bowel, kidneys and skin. Adequate intake of calcium has been demonstrated to reduce the risk of chronic diseases such as osteoporosis, hypertension (Tsuda et al., 2001), colon cancer (Wallace et al., 2004), breast cancer, kidney stones, polycystic ovary syndrome, ovarian cancer, and a number of other disorders (Miller et al., 2001; Nicklas, 2003; McCarron and Heaney, 2004).

During childhood and adolescence, calcium deficiency may impair growth or delay consolidation of the skeleton and can even lead to rickets, which is due to a simultaneous vitamin D deficiency (Pediatrics, 1999).

Several studies report the effect of calcium deficiency on osteoporosis (Nordin, 1997; Prentice, 1997; Heaney, 2000). Indeed, in adults, calcium deficiency causes the mobilization of bone and leads latter to osteoporosis, “a condition of skeletal fragility, characterized by decreased bone mass and microarchitectural deterioration of bone tissue, with a consequent increase of fracture”. Osteoporosis, a common feature of ageing, will take on increasing significance as people live longer and as the world’s population continues to increase in number (European Commission, 1998). Loss of bone starts in women at the time of the menopause (~55 years old) and in men at about the age 65. Osteoporosis leads to an increase in fracture rates in both sexes, the individual risk of fracture being inversely related to bone



density. Hence the calcium RDA is increased with age to minimize the age-related bone loss and consequently reduce fracture risk (Heaney, 2001).

It is generally accepted that the increase of calcium intake, especially through milk and dairy products, during childhood and adolescence, can promote a higher peak bone mass in adults (Sandler et al., 1985; Chan, 1991; Sentipal et al., 1991; Tylavsky et al., 1992; Ruiz et al., 1995) and thus osteoporosis is prevented (Renner, 1994). Yet, Lanou et al. (2005) reviewed several studies published on the relationship between milk, dairy products on calcium intake and bone mineralization in children and adolescent. Surprisingly, former authors concluded that scant evidence supported nutrition guidelines, which focused specifically on increasing milk and other dairy product intake for promoting child and adolescent bone mineralization.

Moreover, there has been resistance among some nutritionists that calcium deficiency causes osteoporosis in humans. Indeed, several studies (FAO/WHO Group, 1962; Walker, 1972; Hegsted, 1963 and 1986, Kanis and Passmore, 1989a and 1989b) have pointed out to the low calcium intakes of many apparently healthy populations, particularly in developing countries. Morrison et al. (1994) suggested that genetic factors accounted for up to 80% of the bone mass variation in the population.

Recent studies on consumer habits reported that 50 to 75% of girl teenagers, women older than 55 years old, men older than 65 years old and elderly people do not satisfy the calcium RDA (Gueguen, 2000; Volatier, et al., 2000).

## **1.2. The calcium supplementation strategy**

From an economic perspective (European Commission, 1998), the expenses of health care associated with osteoporosis fracture are considerable. Thus osteoporosis prevention and its complication are an essential socio-economical priority. This nutritional deficiency in Ca presents a worldwide health stake and can be overcome by Ca supplementation of food. Besides, in clinical studies carried out in American subjects, the efficiency of dietary calcium fortification has been demonstrated by an improvement in calcium balance when the mineral intake increases (Kohls, 1991; Chan et al., 1995).

Most of the governments and health organizations (ICMR, 1989; NIH consensus conference, 1994; in: Singh et al., 2007) recommend increasing amount of calcium intake for the population. Furthermore, more consumers are made aware of the health benefits of

calcium, and important number of new calcium-fortified products is being marketed (Kuntz, 1998; Flynn and Cashman, 1999; O'Carroll, 2000).

### 1.3. Milk, an appropriate vector for calcium supplementation

#### 1.3.1. Casein micelles: a natural vector of calcium

Currently, approximately 60% of dietary calcium comes from dairy products (in: Wark and Nowson, 2003), although this proportion may fall with the introduction of more calcium-fortified foods (Braun and Weaver, 2006). Nevertheless, milk and dairy products have a healthy image and are ideal vehicles for delivery of additional calcium and other minerals (Potjewijd, 1997; Hamelijnck and Lamers, 1999).

In milk, casein micelles are the natural vector of calcium. These particles are constituted of various caseins:  $\alpha_{s1}$ ,  $\alpha_{s2}$ ,  $\beta$  and  $\kappa$ , contained in a mass ratio of 3.9, 1.1, 3.7 and 1.3, respectively (Holt, 1992). These phosphoproteins contain a high content in acid amino-acid, i.e. aspartic and glutamic acid. With a high proportion of proline in their primary sequences, caseins have a low structure level (few secondary structures) and a high conformational flexibility. Physicochemical properties of caseins are summarized in table 2.

Table 2. Physico-chemical properties of the caseins constitutive of casein micelles (Swaisgood, 1982; Cheftel et al., 1985)

	$\alpha_{s1}$ -casein	$\alpha_{s2}$ -casein	$\beta$ -casein	$\kappa$ -casein
Residue number	199	207	209	169
Molecular Weight (g.mol <sup>-1</sup> )	23612	25228	23980	19005
Isoelectrical pH	6	5.3	5.2	5.6
Total hydrophobicity (kJ.residue <sup>-1</sup> )	4.89	4.64	5.58	5.12

The isoelectrical pH of casein micelles ( $pH_i = 4.6$ ) differs from that of each constitutive casein. This difference expresses the effect of interactions of caseins into the macromolecular micellar structure and reflects specific physicochemical properties of casein micelles.

Casein micelles have an average diameter of 180 nm (Mc Mahon and Brown, 1984), with some variations from 30-600 nm, and a natural hydration of 3.7 to 4 g of water per g of caseins (Payens, 1989; Walstra, 1999). Casein micelle molecular weight varies from  $10^8$  to  $10^9$  g.mol<sup>-1</sup> and is present at particles concentration of  $10^{14}$  to  $10^{16}$  per millilitre of milk (Mc Mahon and Brown, 1984; Ruettimann and Ladish, 1987).

Casein micelles contain 92% of proteins and 8% of minerals. This latter fraction is constituted of 90% of calcium phosphate and 10% of citrate and magnesium ions. Casein micelles properties can vary with several factors such as the season, the period of lactation, the processing and the technique used for measurement (Scher, 1988).

Until now, the structure of casein micelles, which give the milky white colour to milk, remains hypothetical. Several models for casein micelles have been proposed but presently two of them are generally well accepted.

The sub-micellar model composed of sub-units was first described by Moor (1967) and reviewed by Schmidt and Both (1982). This model was later updated by Walstra (1999). In this model, casein micelles would be composed of sub-micelles (average diameter ~10-20 nm) bound each other through hydrophobic and/or ionic interactions. Sub-micelles composition would depend on their respective location inside the casein micelle.  $\alpha_s$  and  $\beta$  caseins were likely to be present more in sub-micelles constitutive of the hydrophobic core of the casein micelle. Inversely, external sub-micelles would rather be composed of  $\alpha_s$  and  $\kappa$  casein, as a hydrophilic layer. Colloidal Calcium Phosphate (CCP) may cement the sub-micelles together, giving the specific structure of the casein micelles. An external hydrophilic hairy layer composed of  $\kappa$ -casein sterically stabilises the casein micelle (Walstra and Jenness, 1984; Holt and Horne, 1996; de Kruif, 1999).

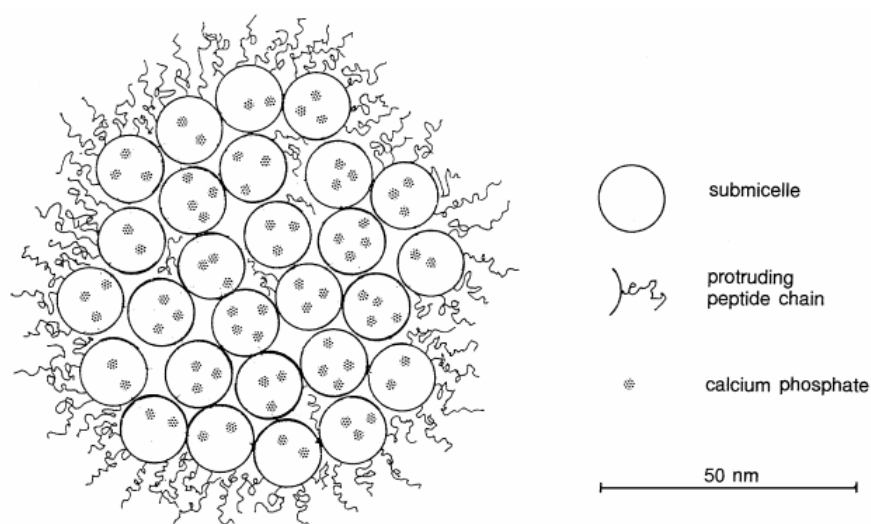


Figure 1. The sub-micellar model described by Walstra (1999)

More recently, another model of casein micelle was proposed in the literature (Holt, 1992; Horne, 1998; de Kruif and Holt, 2003). This open structure (figure 2) is constituted of a network, initiated by the polymerization of caseins via hydrophobic sites and is size limited by electrostatic repulsion strength. A balance between attractive hydrophobic interactions and electrostatic repulsions would maintain the micellar integrity. This network would be stabilized by Colloidal Calcium Phosphate (CCP) linked to anionic clusters of phosphoseryl residues, involving the formation of calcium phosphate nanoclusters of 5 nm diameter (Horne, 2006).

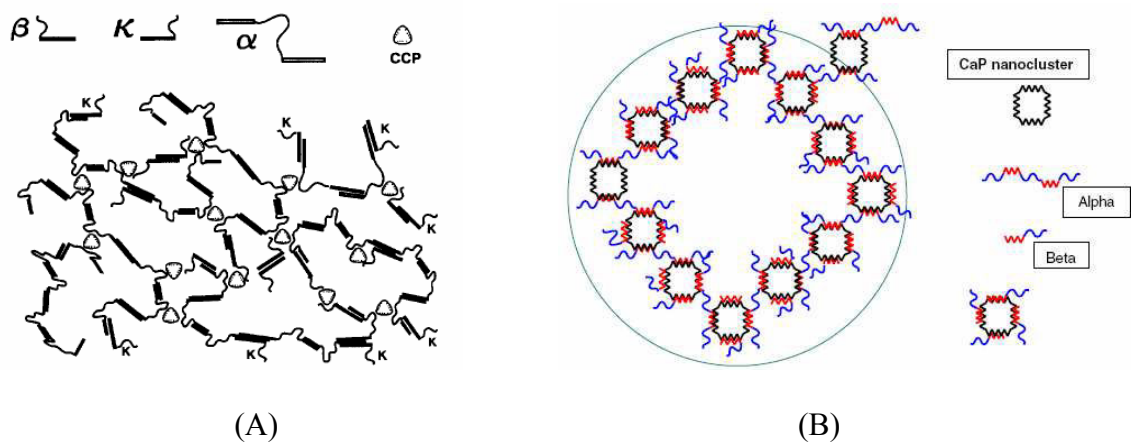


Figure 2. A. Model of open structure of casein micelle (Horne, 1998) with hydrophobic regions (black rectangle) and hydrophilic regions binding CCP.  $\kappa$ -casein is located in surface. B. Model involving the concept of calcium phosphate nanoclusters surrounded by  $\alpha$ - and  $\beta$ -caseins. The dangling hydrophobic regions (blue) of the attached caseins are shown in blue (Horne, 2006)

### 1.3.2. Soy milk, an alternative to cow skim milk

Soy milk has been consumed throughout south-east Asia for centuries but its consumption in western countries has only begun in the XX's, during the second world war II. USA and Brazil are now the world's largest exporters of soybean, representing 42 and 37% of global production respectively (figure 3). This rapid soy expansion in Brazil has created a greater need to protect the important and complex ecosystem of the Amazon. Its production in Europe production is lower than 1%. Inversely, European countries and Japan are the major purchasers of soybean, which are exported mainly from the United States and Brazil.

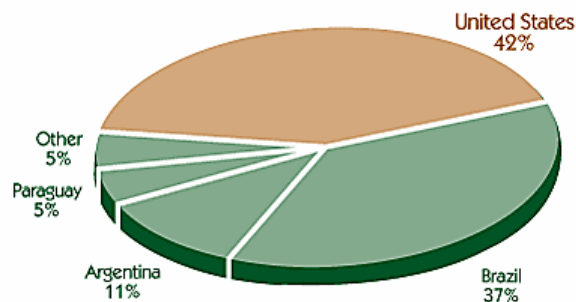


Figure 3. World soybean export (2006) (Soystats, 2007)

Soybean is a legume increasingly consumed for economical and nutritional reasons. At environmental scale, soy production presents several advantages such as the highest vegetal protein yield coupled with a low fertilizer and pesticides utilization. More, soy crab is used for animal feeding. All these characteristics make soy crop very interesting for a rapidly growing world population.

Indeed, soybean products are an important low-cost source of high quality proteins, minerals and vitamins. On a total weight, soybean contains 34% proteins, 19% lipids, 32% carbohydrates, 18% fibers and 4.5% minerals (in: Lecerf and Fressin, 1995).

Soy milk, a vegetable beverage with a milky appearance, is traditionally prepared from soaked soybeans by grinding in water (water to bean ratio between 8:1 to 10:1), filtering and heating (Odo, 2003). Despite its high nutritional qualities, traditional soy milk (Chinese style) has been quite limited at other places than eastern Asia since this former one has an undesirable beany or painty off-flavour and odour, principally due to lipoxygenase action (Wilkens et al., 1967). The modern-style soy milk (reconstituted soy milk) with a dairy like taste and often ultrapasteurized, is more adapted to western countries (the so-called Illinois process described by Nelson et al. (1976).

Nutritional composition of soy milk depends on its way of preparation, especially the volume of water used for extraction. Yet, soy milk, called “*tonyu*” in Japan, is similar to cow milk formula on several aspects.

### 1.3.3. Comparison of cow skim milk versus soy milk

Cow skim milk, with casein micelles as natural vector of calcium, was considered as an appropriate biological system to imitate, for further soy milk calcium supplementation. Before the bibliographical study of calcium equilibrium in these two milky systems, cow skim milk and soy milk are first compared.

#### 1.3.3.1. Nutritional composition

Cow skim milk is a complex food matrix, with a slightly acidic pH, comprised within 6.4 and 6.8. It is composed of proteins, lipids, vitamins and minerals, distributed in 3 phases:

- a proteic colloidal fraction principally composed of casein micelles, which contains two-third of the calcium,
- a soluble phase containing whey proteins, sugar, vitamins and minerals,
- fat globules (less than 1%).

The nutritional compositions of cow milk and soy milk are compared in table 3.

Table 3. Comparison of cow skim milk versus soy milk - Table of composition of Ciquel (INRA, 1995)

Content	Cow skim milk	Soy milk
Water (g/100g)	91	93
Calories (kJ/100g)	145	164
Protein (g/100g)	3.3	3.6
Lipid (g/100g)	0.2 depending	2
Carbohydrates (g/100g)	4.9	1.8
Cholesterol (mg/100g)	2	0
Thiamine (B <sub>1</sub> , mg/100g)	0.05	0.06
Riboflavin (B <sub>2</sub> , mg/100g)	0.16	0.04
Niacin (mg/100g)	0.09	0.05

## 1.3.3.2. Protein composition

Cow skim milk contains a total of 32-35 g.L<sup>-1</sup> of proteins, with 20% of soluble whey proteins (~7 g.L<sup>-1</sup>). Soy milk has a similar content in proteins than cow skim milk (3.6% minimum protein concentration, Mittal et al., 1976).

Cow skim milk and soy milk have about the same nutritive values (Desikachar and Subrahmanyam, 1946; Ciquel, INRA, 1995). Table 4 gives the Essential Amino-Acid (EAA) content in cow skim milk and in soy milk.

Table 4. Comparison of Essential Amino-Acid (EAA) content in cow skim milk (Souci et al., 1994) versus soy milk, (Lecerf and Fressin, 1995; expressed in % total AA)

Amino-Acid	Cow skim milk	Soy milk	Reference protein (FAO-WHO)
Ile	5.7	4.6	4
Leu	8.8	7.9	7
Lys	6.3	6.5	5.5
<b>Met + Cys</b>	<b>2.4</b>	<b>2.6</b>	<b>3.5</b>
Phe + Tyr	6.9	8.2	6
Thr	4.6	3.9	4
Try	0.8	1.3	1
Val	6.9	4.9	5
Sum of the EAA % of the total AA	42.4	39.9	36

FAO: Food and Agriculture Organization  
WHO: World Health Organization

As observed in table 4, cow skim milk and soy milk present a complete source of proteins. Both milks contain all the EAA, except limiting sulphur amino-acids (i.e., Methionine and Cystine). On the contrary, lysine is contained in high quantity which makes soy proteins an excellent complement of cereals (poor source of lysine). The soy biological value is the highest one (73%) among all the leguminous crops. Yet, the proteins quality depends on processing and particularly on heat treatments, which can reduce their digestibility and the bioavailability of amino-acids. Soy protein intake of about 25 g per day reduces the risk of coronary heart disease and lowers blood cholesterol level (in: Odo, 2003).

## 1.3.3.3. Lipid composition

Lipid soy fraction is constituted of a majority of Poly-Unsaturated Fatty Acids (**PUFA**, 62%), such as linoleic and linolenic acids, and Mono-Unsaturated Fatty Acids (24.3%).

Lecithin, an emulsifying phospholipid, is contained in high proportion. Soy also contains vitamin E as gamma tocopherol (in: Dupuy et al., 1994). Compared to bovine milk, the major benefit of soy milk is its absence of cholesterol and its lower concentration in saturated fatty acids (14%). The lipid composition of cow's skim milk is given in appendix 1.

#### 1.3.3.4. Carbohydrate composition

Contrarily to cow milk, soy milk is lactose free. This can be considered as possible inconvenient since lactose improves calcium absorption. Yet, this absence of lactose constitutes an alternative for lactose intolerant people.

According to table 3 presented before, soy milk contains about 1.8 g for 100 g of carbohydrates (up to 2.9 according to the tables of composition).

The soy milk contains carbohydrates, with a low molecular weight, i.e. fructose, arabinose, rhamnose and saccharose. Raffinose and stachyose, two anti-nutritional factors, are eliminated by soaking.

Soy contains 22% dry weight of fibers; among them, 25% are soluble fibers.

#### 1.3.3.5. Vitamin composition

The comparison of vitamin content in cow's milk and soy milk is mentioned in table 5. Soy milk contains more vitamins B<sub>1</sub>, B<sub>2</sub> and folic acid than cow's milk that is conversely richer in panthothenic acid and vitamin C. Equivalent amount of niacin and vitamin B<sub>6</sub> are reported.

Table 5. Comparison of vitamin content in raw cow's milk and soy milk (Kwok and Niranjana, 1995)

Vitamin	Cow's milk ( $\mu\text{g. g}^{-1}$ )	Soy milk ( $\mu\text{g. g}^{-1}$ )
B <sub>1</sub>	0.3	0.6
B <sub>2</sub>	1.7	2.7
B <sub>6</sub>	0.6	0.7
Niacin	1	1
Folic Acid	0.06	0.19
Panthothenic Acid	3.5	1.2
C	10	0



### 1.3.3.6. Mineral composition

Contrarily to dairy foods, soy foods are generally regarded as poor calcium sources because of their fibers contents and other constituents which may bind substantial quantities of mineral (in: Keane et al., 1988). The low content of calcium in soy milk is a disadvantage as compared to cow milk (table 6).

Table 6. Comparison of the mineral composition of cow milk and soy milk

Minerals	Cow's milk <sup>a</sup> (mg/100g)	Cow's milk <sup>a</sup> (mmoles/kg)	Soy milk <sup>b</sup> (mg/100g)	Soy milk <sup>b</sup> (mmoles/kg)	Ratio Cow's/Soy
Sodium	53	23	15	6.52	3.53
Potassium	150	38.36	130	33.25	1.15
Magnesium	14	5.8	18	7.4	0.78
<b>Calcium</b>	<b>123</b>	<b>30.7</b>	<b>15</b>	<b>3.74</b>	<b>8.2</b>
Iron	0.120	0.0215	0.5	0.09	0.24
Phosphorus	97	31.3	45	14.51	2.15
Chloride	100	28.2	0	0	-
TOTAL	537.12	157.38	223.5	65.51	2.40

<sup>a</sup>: Souci et al., 1994 in: Food composition and nutrition tables, 5<sup>th</sup> revised and completed edition

<sup>b</sup>: INRA, 1995. Ciqual., Répertoire général des aliments. Table de composition – 2<sup>ième</sup> édition revue et argumentée

The comparison of mineral content of cow's milk shows three noticeable mineral ratios. Sodium and Calcium are respectively 3.5 and 8.2 times more in cow milk than in soy milk. On the contrary, iron concentration, high in soy milk, is almost absent in cow milk. The total mineral ratio is ~3 times higher in cow skim milk in comparison with soy milk. As the structural organization of soy proteins differs from the micellar state of cow's milk proteins, minerals of soy milk are rather present in the soluble phase.

### 1.3.4. Nutritional interest of soy milk

Soy milk was favoured in western countries due to its nutritional properties and as an alternative of cow milk. Soy proteins were studied in the prevention and treatment of children with Cow Milk Allergy (**CMA**; Businco et al., 1998). This food allergy occurs in less or equal 2% of children in the first three years of life (Host and Halcken, 1990). Soy Protein milk Formulas (**SPF**) were first described as cow-milk substitute (Ruhrah, 1909) but at that time, they were not used for feeding babies with CMA. These formulas were the only available

cow-milk substitute in 1929 and such products ensured a normal life for many children affected by CMA (Naidoo et al., 1981; Cantani et al., 1990). In addition, SPFs had been given to genetically atopic, allergy-prone infants for the prevention of atopic diseases when breast milk was not available. Yet, according to Kahn et al. (1988) soy milk may not be the best alternative to cow milk since up to 5% of allergic infants suffer from soy protein intolerance as well. Indeed, soy proteins can induce enteropathy in young infants with or without cow-milk intolerance. Gastrointestinal symptoms occurred in almost 30% of infants fed with soy milk in the treatment of gastrointestinal cow-milk hypersensitivity. Nevertheless several studies demonstrated that soy proteins are less immunogenic and allergenic than cow milk proteins (Piacentini et al., 1991; Giampietro et al., 1992). According to Eastham et al. (1978), soy proteins are as antigenic as cow milk proteins but as previously pointed out, this antigenicity should not be necessarily regarded as harmful.

SPFs, nutritionally adequate and palatable by most infants, are the preferred food for children with IgE-mediated CMA. Extensively hydrolyzed soy formulas should be used only when there is scientific evidence that a child is allergic to soy.

Soy proteins are also known for their hypocholesterolemic effect compared to cow proteins. Soy phytoestrogens (isoflavones: genistein and daidzein) absorbed onto soy proteins were suggested as the reducing agent of the serum cholesterol level. Moreover, dietary fibers present in soy decrease the cholesterol level of hyperlipidemic subjects (Dupuy et al., 1994).

Another nutritional benefit of soy is the presence of phytoestrogens or isoflavones and their beneficial effects on peri-menopausal women especially to act against breast cancer and osteoporosis. Soy isoflavones may have anticarcinogenic effects and act against menopausal affections. Ho et al. (2003) studied the effect of soy protein/isoflavones intake on bone mass in early postmenopausal Chinese women. Former authors observed a modest but significant association with hip bone mineral density as well as total body bone mineral content. This result was controverted by Spence et al. (2005).

Soy milk also contains anti-nutritional factors such as phytic acid, which binds to certain minerals, and decreases mineral bioavailability, especially calcium (in: Dupuy et al., 1994). Soy Protein Isolate (**SPI**) contains about 1.6 to 2.2% phytic acid on a dry basis. This strong chelating agent, eliminated by an adapted heating treatment, can bind mono and divalent metal ions to form a phytate complex (Erdman, 1979). Hence calcium is better absorbed in

soy milk with low phytic acid concentration. In spite of the chelating effects of phytic acid, soy milk remains a good source of magnesium, potassium, and phosphorus.

Soy milk constitutes an alternative for dairy milk and especially for specific populations which can not consume cow milk for various reasons (vegetarians, lactose intolerance, protein Cow Milk Allergy...). Yet, soy milk (soybeans to water, 1:8 w/v) contains an equal amount of proteins to a comparable amount of cow's milk but only about one-fifth to one-eighth of the calcium. Calcium soy milk supplementation can correct this deficiency. Nevertheless, this is important to prevent calcium-induced aggregation of soy proteins and then to have an overview of calcium equilibrium in cow milk and in soy milk.

## 2. Understanding of mineral equilibrium in cow skim milk

### 2.1. Introduction

Cow skim milk is constituted of two phases, the soluble and the colloidal phase, between which milk minerals are partitioned and in equilibrium (Davies and White, 1960; table 7). In the colloidal dispersed phase, mainly constituted of casein micelles, various caseins are gathering together with minerals. The mineral fraction of milk represents 9% of the average composition of cow's milk and is principally constituted of calcium ( $\text{Ca}^{2+}$ ), phosphorus, magnesium ( $\text{Mg}^{2+}$ ), sodium ( $\text{Na}^+$ ), potassium ( $\text{K}^+$ ) and chloride ( $\text{Cl}^-$ ).

Table 7. Mineral composition and partition in cows milk (Walstra and Jenness, 1984)

Salt Element (MW g/mol)	Composition mmol/kg (mg/kg)	Partition (%)		
		Soluble Phase	Colloidal Phase	Ratio CP /SP
$\text{Na}^+$ (23)	17-28 (391-644)	95	5	0.05
$\text{K}^+$ (39.1)	31-43 (1212-1681)	94	6	0.06
$\text{Cl}^-$ (35.1)	22-34 (772-1207)	100	0	0
<b><math>\text{Ca}^{2+}</math> (40.1)</b>	<b>26-32 (1043-1283)</b>	<b>32</b>	<b>68</b>	<b>2.12</b>
$\text{Mg}^{2+}$ (24.3)	4-6 (97-146)	66	34	0.52
$\text{P}_i$ : $\text{PO}_4$ (95)	19-23 (1805-2185)			
$\text{P}_i$ : P (31)	19-23 (589-713)			
P total: P(31)	30-32 (930-992)	54	46	0.85
Cit (189)	7-11 (1323-2079)	92	8	0.09

CP: Colloidal Phase; SP: Soluble Phase.

In the mineral partition between the soluble and the colloidal phase, monovalent ions (i.e.,  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Cl}^-$ ) are almost present in the soluble phase. On the contrary, calcium, phosphorus and inorganic phosphate are quite more in the colloidal phase. The calcium ratio between the colloidal and the soluble phase is comprised within 2-2.5 (Rajput et al., 1983; De la Fuente, 1998).

The mineral distribution is subjected to chemical equilibria (figure 4), shifted towards the micellar or the soluble phase as a function of various physicochemical parameters (i.e. T, pH, ionic strength...) and technological treatments (Brulé, 1981).

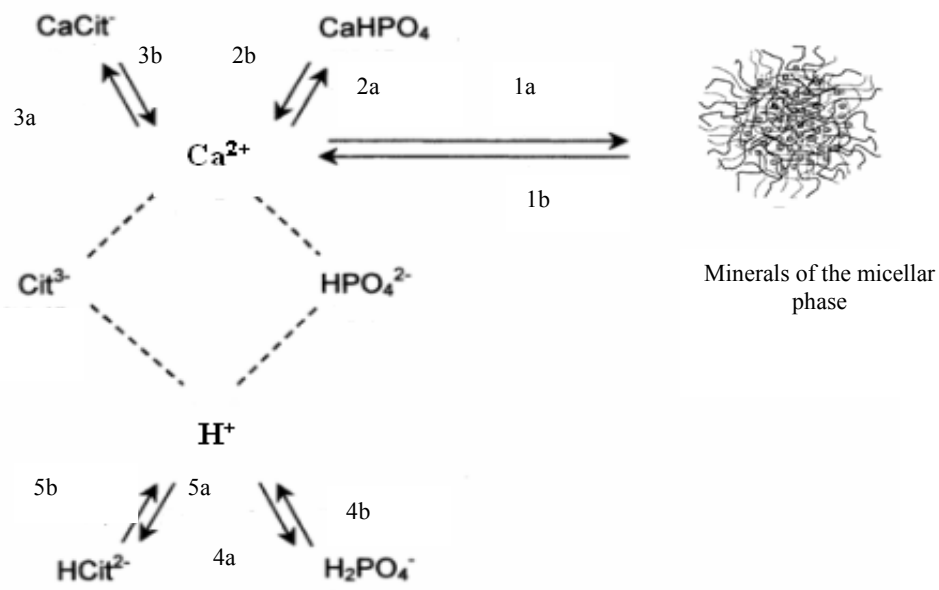


Figure 4. Milk salt equilibria. (Brulé, 1981; in: Gaucheron, 2004)

$\text{H}^+$  and  $\text{Ca}^{2+}$  ions are in equilibrium with their phosphate and citrate salts between the soluble and the dispersed phase. Brulé (1981) presented the calcium mineral equilibrium directly from the micellar phase, whereas Holt et al. (1981) suggested that calcium mineral equilibrium between the soluble and the micellar phase occurs indirectly via  $\text{CaCit}^-$  and  $\text{CaHPO}_4$ . In milk, the soluble phase is saturated in calcium phosphate ( $\text{CaHPO}_4$ , 0.6 mM), which induces its precipitation inside casein micelles.

## 2.2. Caseins-cations interactions

Milk contains ~25-26 g of casein per kg (80% of dairy protein content). These proteins are particularly rich in phosphoserine and glutamic and aspartic acids, the major amino-acids playing a role in caseins-cations interactions.

### 2.2.1. Phosphoserine residues

Only one part of serine residues of caseins is phosphorylated. The phosphorylation rate is different for each casein:  $\alpha_{s2}$ -casein has 11 to 13 phosphoserine residues whereas  $\kappa$ -casein has only 1 or 2. Casein phosphorylation involves enzymatic recognition of a code sequence by casein kinase (Mercier, 1981).

The ionisation state of these residues varies as a function of pH and is characterised by pK values of the first and the second ionisation according to the following acidic-basic equilibrium:



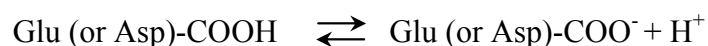
Several parameters influence the second ionisation pK of phosphoserine residues, i.e. the protein environment, the localization of these residues inside the casein and the ionic environment (in: Gaucheron, 2004). The pK value of the free phosphoserine residue is close to 5.9 (Sleigh et al., 1983) whereas the pK value of phosphoserine residues of caseins vary within 6.3-6.9 (Humphrey and Jolley, 1982; Baumy et al., 1989).

The ionised forms of phosphoserine residues are potential electrostatic interaction sites for cations such as  $\text{Ca}^{2+}$ .

### 2.2.2. Acidic residues

Caseins are relatively rich in glutamic and aspartic acids, present in different proportions according to the caseins nature.  $\alpha_{s1}$ -casein and  $\kappa$ -casein count 31 and 16 acidic residues, respectively. Besides phosphoserine and acidic residues, there exist probably minor interaction sites for calcium (Gaucheron, 2004).

Acidic residues are also in a variable ionization state as a function of pH. The ionization state is characterised by the pK value of the following acidic-basic equilibrium:



This pK value is close to pH 4.5-4.7 and this function is almost entirely ionised at neutral pH. Hence the negative charges of these acidic residues enable potential interactions for cations onto caseins.

### 2.2.3. Cations binding capacity of caseins

The charge or ionization state of caseins depends on several parameters such as the casein nature (due to the amino-acid composition), the ionic strength and the pH. Due to the clustered localization of phosphoserine and acidic residues, the negative charge distribution on caseins is heterogeneous.

The ions binding onto caseins involve electrostatic interactions which depend on pH and ionic strength. When the ionic strength increases, repulsion forces between negatively charged casein molecules are screened. Hence the isoelectrical pH ( $pH_i$ ) of the protein decreases due to the charge screening effect.

Similarly, interactions between charged species can be drawn according to the pH range:

- Near neutral pH and above their  $pH_i$ , caseins are negatively charged ( $pH_i$  of individual casein ~5.3)
- Near their  $pH_i$ , caseins are electrically neutral and electrostatic interactions are limited. On the contrary, hydrophobic protein-protein interactions are favoured rather than protein-ions or protein-solvent interactions. Aggregation can be observed.
- At acidic pH and below their  $pH_i$ , caseins are positively charged and can be found in the soluble phase. Interactions occur more likely with anions.

With respect to the charge neutrality and at neutral pH, casein-cation interactions occur in different ways (in: Gaucheron, 2004):

- with a single phosphoserine residue,
- with two close phosphoserine residues inside one casein molecule (intramolecular bridge),
- with two different phosphoserine residues of different caseins (intermolecular bridge),
- with carboxylic group of acidic residues.

The ability of caseins to bind cations is influenced by the casein nature and its phosphorylation state. The ability to bind cations is higher with a higher number of phosphoserine residues. Hence casein dephosphorylation induces a decrease of the ability of cations binding in cow's milk.

The cation nature has also a noteworthy effect on its interaction with caseins, due to its specific physicochemical properties, i.e. its size, its hydration and electronegativity. For  $Ca^{2+}$ , these parameters are 0.99 Å for the ionic radius, 4.12 Å for the hydrated radius (Nightingale, 1959) and 1.0 for electronegativity (Pauling scale).

### 2.3. Effect of physicochemical treatments on ionic equilibria

Mineral equilibria vary significantly with physicochemical conditions such as thermal treatment, pH and ionic strength variations, addition of salt and chelating agents or proteins...

#### 2.3.1. Temperature effect

##### 2.3.1.1. Cooling effect

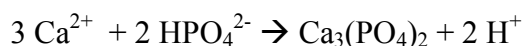
During cooling to 4°C, Micellar Calcium Phosphate (**MCP**; Brulé and Fauquant, 1981) is solubilized with an increase of calcium ion activity (arrow 1b, figure 4). CaHPO<sub>4</sub> and CaCit<sup>-</sup> complexes are ionized into Ca<sup>2+</sup>, HPO<sub>4</sub><sup>2-</sup> and Cit<sup>3-</sup> (arrows 2b and 3b, figure 4). The liberated Cit<sup>3-</sup> and HPO<sub>4</sub><sup>2-</sup> are then able to complex H<sup>+</sup> and an ionisation regression of phosphate ions and citrate ions present in the soluble phase occurs (arrows 4a and 5a, figure 4). Hence, calcium phosphate solubility is increased.

In the meantime, solubilization of caseins (Dalglish and Law, 1989) and especially of β-caseins is observed (Dziuba and Muzinska, 1998). This could be due to hydrophobic bond weakness upon cooling combined to the solubilization of colloidal minerals. The casein micelle hydration increases. Cooling effects are reversible and relatively slow (Davies and White, 1960; Dziuba and Muzinska, 1998).

Physicochemical changes are amplified with slower freezing. Indeed, a mineral concentration step occurs in the aqueous phase following water freezing (the so-called cryoconcentration phenomenon), pH decreases and mineral equilibrium alterations occur, which induce micelle destabilization.

##### 2.3.1.2. Heat treatment

Heating causes the reverse effects of cooling on mineral equilibrium. H<sub>2</sub>PO<sub>4</sub><sup>-</sup> and HCit<sup>2-</sup> are ionized (arrows 4b and 5b, figure 4). The dissociated HPO<sub>4</sub><sup>2-</sup> and Cit<sup>3-</sup> are then available to complex Ca<sup>2+</sup> and form CaHPO<sub>4</sub> and CaCit<sup>-</sup> respectively (arrows 2a and 3a, figure 4). CaHPO<sub>4</sub> is shifted from the soluble to the micellar phase. Calcium phosphate precipitation would occur as the following reaction (Walstra and Jenness, 1984):



#### 2.3.1.2.1. Moderate heating treatment

The mineral equilibrium shift is a function of the intensity of the heating treatment and can be more or less reversible. Heating treatment of low intensity ( $T < 90^{\circ}\text{C}$ , few seconds or few minutes) does not affect a lot the mineral fraction of milk.

Pouliot et al. (1988) studied the heating effect from 4 to  $90^{\circ}\text{C}$  on the salt balance changes in milk. The amount of material transferred from the soluble to the micellar phase increased with temperature; Ca and P represented the main part ( $>80\%$ ). A two-step mechanism was observed for Ca, P, and pH. An initial sharp decrease in concentration occurred in the first minutes and was followed by a slower reaction, temperature-independent (similar slopes). The similarities observed between pH, Ca and  $\text{P}_i$  curves strongly suggested that calcium phosphate precipitation is the major cause of pH decrease in heated milk. The extent of this precipitation to a micellar form is proportional to the severity of the heating treatment.

For low heating treatments, the bound calcium/protein ratio was higher with higher temperature (Brulé and Fauquant, 1981; Parker and Dalgleish, 1981). More, Brulé and Fauquant (1981) showed that the higher the amount of protein in retentate the lower is the ratio of bound  $\text{Ca}^{2+}$  to protein at any one temperature.

The reversibility of the heat-induced precipitation on cooling is dependent on the intensity of the heat treatment ( $60\text{-}94^{\circ}\text{C}$ ). The effects of low heating treatment are partially reversible upon cooling (Pouliot et al., 1989a; Law, 1996). Indeed the milk permeate was slightly depleted in calcium (5-10%) and in phosphate (1-7%) depending on the cooling temperature (Pouliot et al., 1989b).

#### 2.3.1.2.2. High heating treatment

With higher heating treatment ( $T > 90^{\circ}\text{C}$ , few seconds or minutes), mineral equilibria are strongly disturbed. Precipitation mechanism occurs in two steps as previously reported for moderate temperature (Pouliot et al., 1989a). The second step could correspond to a conversion of initially precipitated calcium phosphate into a more alkaline form with a higher Ca/ $\text{P}_i$  ratio. This calcium phosphate could be tricalcium Phosphate  $\text{Ca}_3(\text{PO}_4)_2$ , Hydroxyapatite  $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$  or Amorphous Calcium Phosphate, or dicalcium Phosphate  $\text{CaHPO}_4$ . Pouliot et al., (1989b) suggested that the precipitation of dicalcium phosphate could occur together with some tricalcium citrate  $\text{Ca}_3\text{Cit}_2$  precipitation.



## 2.3.2. pH effect

### 2.3.2.1. Acidification

Acidification involves a micellar mineral solubilization with regard to calcium, magnesium, inorganic phosphate and citrate ions (Brulé et al., 1974; Dalgleish and Law, 1989; Le Graët and Brulé, 1993; Famelart et al., 1996; Le Graët and Gaucheron, 1999). Reducing pH imply a decrease of interaction between phosphate and calcium ions (arrows 4a and 5a, arrows 2b and 3b, arrow 1b, figure 4). The pH-dependent mineral solubilization (figure 5) induces an increase of ionic strength from 0.075 M (pH 6.7) to 0.13 M (pH 4.6), which causes a decrease of activity coefficient and then a higher calcium phosphate solubility (Gaucheron, 2004).

Electrostatic bonds are sensitive to pH effect as well. Hence, during acidification, the mineral ability to bind to caseins decreases. Indeed the ionization regression reduces the negative charge of phosphoserine residues and carboxylic group of proteins. When the pH tends to the casein  $pH_i$ , the various ions do not interact with  $\beta$ -casein anymore (Gaucheron et al., 1997). Similarly, Dalgleish and Parker (1980) reported that the higher the pH (from pH 7 to pH 6), the higher the calcium ions bound to  $\alpha_{s1}$ -casein.

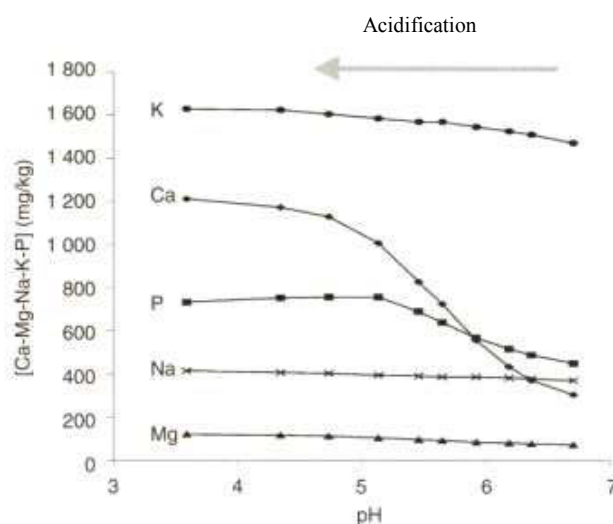


Figure 5. Mineral solubilization during milk acidification (Le Graët and Brulé, 1993)

The micellar solubilization was studied as a function of pH, from pH 6.7 to pH 4.9 by Dalgleish and Law (1989). These authors obtained the same sigmoidal curves than presented in figure 6. At low concentration in  $P_i$ , they expected the existence of two components in the dissociation pattern since the plot was clearly non-linear (similar to Van Hooydonk et al., 1986).

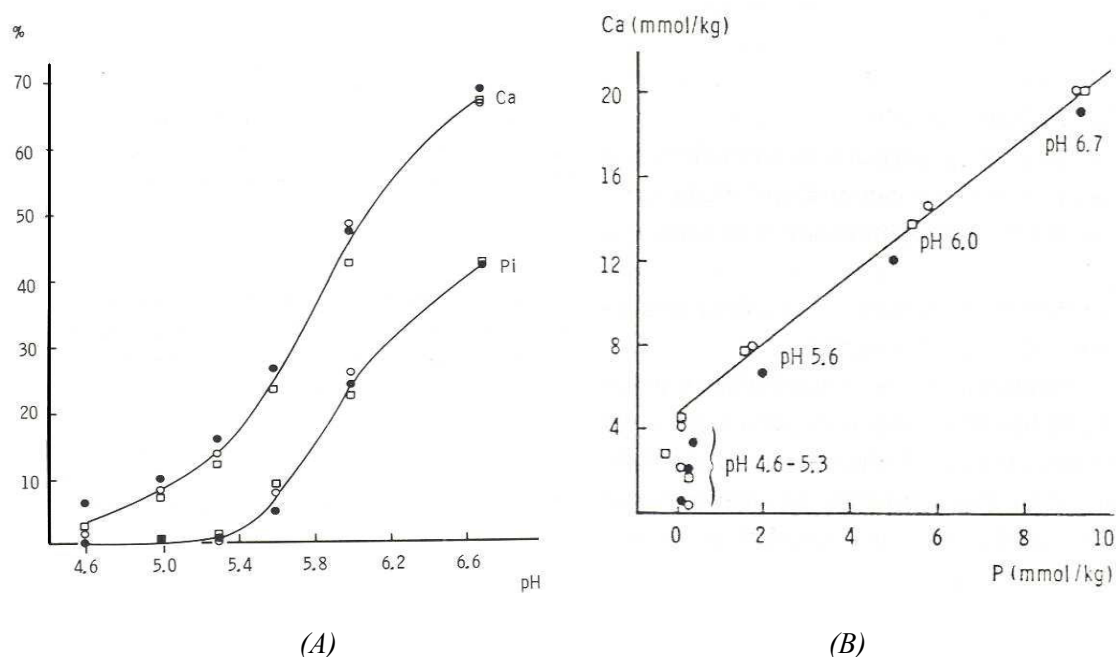


Figure 6 A. Micellar calcium and inorganic phosphate at 30°C. Three experiments: each symbol represents the average of skim milk and the same skim milk after rennet-treatment.

Percentage of total concentration in milk (Van Hooydonk et al., 1986)

B. Relationship between micellar calcium and inorganic phosphate in the pH range 4.6-6.7 at 30°C. Equal symbols refer to the same milk sample (Van Hooydonk et al., 1986)

Le Graët and Brulé (1993) studied the combined effect of pH (from 6.7 to 3.5) and ionic strength (from 0.08 to 0.34 M) on mineral solubilization. The amount of calcium transferred from the micellar to the soluble phase increased with higher ionic strength. On the contrary, no effect was observed on phosphorus.

The combined effect of casein concentration (27, 55, 83 and 144 g/kg) and ionic strength (0, 10 and 20g/kg NaCl added) were investigated during acidification (Le Graët and Gaucheron, 1999). Calcium solubilization upon acidification depended on casein concentration. Casein concentration in the soluble phase of casein micelle suspensions was higher with higher casein concentrations upon acidification. Nevertheless for a same pH value, calcium solubilized (% of total) was higher for lower casein concentration.

Increasing ionic strength by adding NaCl had no effect on acid-induced mineral solubilisation. Indeed, addition of NaCl to the casein micelle suspension increased the  $Ca^{2+}$

concentration before acidification but during acidification step itself, the added NaCl did not affect the  $\text{Ca}^{2+}$  solubilization and the figures were very similar. Same results were obtained with  $\text{P}_i$ .

#### 2.3.2.2. Alkalinization

Milk alkalinization shows the reverse effect of acidification previously described:

- An ionisation (deprotonation) of phosphate and citrate ions (arrows 4b and 5b, figure 4),
- An association with calcium ions to form calcium phosphate and citrate salts rather insoluble.

Until pH 8.4, MCP evolves quantitatively but remains constant in its composition (Chaplin, 1984). Van Dijk (1991, 1992) related important shifts in mineral equilibria due to alkalinization, notably a decrease of inorganic phosphate and calcium of the soluble phase.

Van Dijk (1991) showed that  $\text{P}_{ic}/\text{P}_o$  (Colloidal Inorganic Phosphate/ Organic phosphate) ratios were markedly larger than 2 for pH higher than ~8. Yet, when formaldehyde was added at an equal or higher pH, a lower  $\text{P}_{ic}/\text{P}_o$  ratio was obtained. Indeed, formaldehyde is known to lower the concentration of free lysine residues. Hence, Van Dijk concluded that calcium phosphate combines also with lysine residues certainly above pH 8. Above pH 9, the turbidity decreased substantially and the milk slowly became as transparent as whey ( $\tau < 0.5$  at 600-900 nm; Van Dijk, 1992). In the pH range 9 to 11, the turbidity decrease was faster at higher pH (figure 7a). This suggests that micellar structure is strongly affected and this is in relation with a micellar size decrease down to casein micelle disappearance. Addition of small amounts of NaCl (e.g. 17 mM) before alkalinization slowed down the process (figure 7b, solid line), while addition of large amounts of NaCl 18h after alkalinization (i.e. 0.17 or 1M) led to a partial restoration of the original  $\tau$  so to the back formation of pseudo casein micelles (figure 7b, broken line).

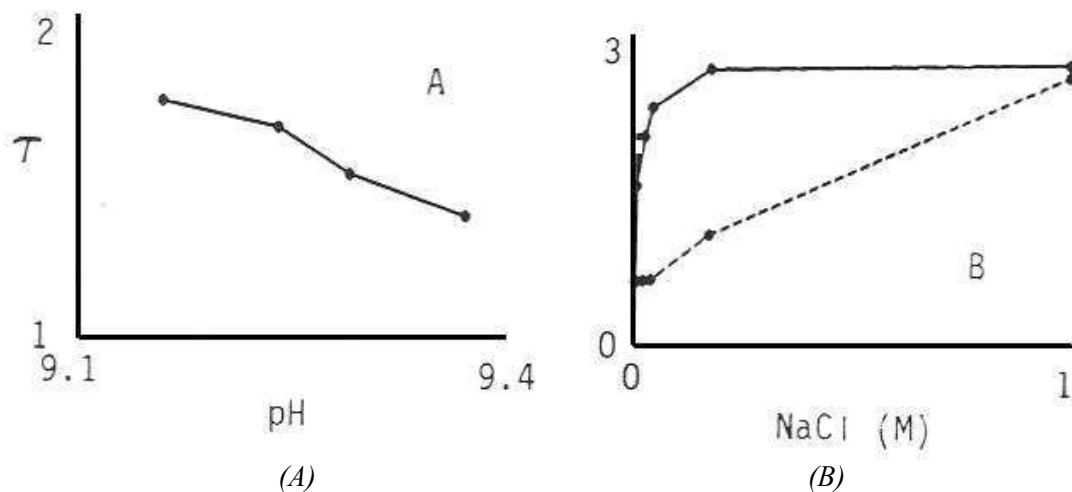


Figure 7. A. Turbidity ( $\tau$ ) at 700 nm and 1 h after addition of 4M NaOH vs. pH, B. Turbidity ( $\tau$ ) vs. added NaCl (incubation period 18h, B solide line) or 1 h after addition of NaCl to milk which was alkalinized 18 h before as a function of added NaCl (B broken line). In the last experiment, the pH was restored immediately after addition of NaCl to the value before this addition (i.e. 8.86).

### 2.3.2.3. Buffering capacity of dairy products and mineral composition

The buffering capacity of milk influences many of its physicochemical properties, by controlling pH during processing. Mineral solubilization during acidification can be indirectly followed by the buffering capacity of the suspension. The buffering capacity (dB/dpH) was calculated from Van Slyke's formula (Van Slyke, 1922).

$$\frac{dB}{dpH} = \frac{(Vol)_A * (Normality)}{(Vol)_{milk} * (\Delta pH)} \quad \text{Equation 1}$$

with  $Vol_A$  = Volume of acid or alkali

and Normality: the normality of acid or alkali solutions used

The relationship of buffering capacity as a function of pH through titration curves enables the determination of the product ability to be acidified and is directly reliable with the composition of the studied product. It increases with the presence of proteins, inorganic phosphate, carbonate and citrate. Each molecule has a maximum buffering capacity at its demi-dissociation pH or pKa (Walstra and Jenness, 1984). pKa values depend on certain parameters such as the ionic strength and the calcium ion concentration in the medium.

### 2.3.3. Ionic strength effect

Salt addition modifies the ionic environment and mineral equilibrium. The nature of added salt and its concentration have also an effect on these variations. Adding salts increases the ionic strength, which reduces the activity coefficient and increases the solubility of calcium citrate and calcium phosphate. Indeed, the bonds are weakened and ions dissociation increases with ionic strength.

Electrostatic interactions between cations and caseins (e.g. for  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Na}^+$  and  $\text{K}^+$ ) are sensitive to ionic strength. The ionic strength increase induces a decrease of casein  $\text{pH}_i$  due to charge screening and as a consequence, the cation ability to interact with casein is reduced.

In 1996, Famelart et al. reported that an increase in ionic strength of the aqueous phase led to higher diffusible calcium and phosphorus, to greater micelle voluminosity and to greater casein and mineral solubilization during acidification.

#### 2.3.3.1. Sodium chloride effect

The ionic strength increase through NaCl addition (up to 500 mM; Grufferty and Fox, 1985) involved a slight pH decrease probably due to an exchange  $\text{Na}^+/\text{H}^+$  from caseins. An increase of the soluble calcium concentration without effect on the soluble  $\text{P}_i$  content suggested that casein-bound calcium rather than CCP is solubilized after NaCl addition. An exchange of 2  $\text{Na}^+$  for 1  $\text{Ca}^{2+}$ , directly attached to the caseins was proposed.

Up to 400 mM NaCl, the micellar hydration increases from 2.2 to 2.4 g  $\text{H}_2\text{O}/\text{g}$  protein. The  $\alpha_{s1}$ - and  $\beta$ -casein binding capacity of  $\text{Ca}^{2+}$  was studied as a function of ionic strength (NaCl added from 0.05 M to 0.2 M; Dalgleish and Parker, 1980; Parker and Dalgleish, 1981). It was observed that the higher the ionic strength, the lower was  $\text{Ca}^{2+}$  bound to proteins.

#### 2.3.3.2. Calcium chloride addition

The calcium chloride addition induces  $\text{Ca}^{2+}$  association with citrate and phosphate leading to calcium citrate and calcium phosphate salts formation in milk. These salts are a little or not soluble at all (arrows 2a and 3a, figure 4). Thus inorganic phosphate and citrate ions concentrations in the aqueous phase decrease (Philippe et al., 2003). Simultaneously the

pH decrease (Le Ray, et al., 1998; Philippe et al., 2003) indicates that a part of the added calcium bind onto the casein micelles (arrow 1a, figure 4). In addition, the higher is free  $\text{Ca}^{2+}$  concentration, the higher is mole of  $\text{Ca}^{2+}$  bound per mole of proteins is expected (Dalglish and Parker, 1980; Parker and Dalglish, 1981).

As a consequence of mineral equilibrium modification, casein micelles undergo physicochemical changes such as hydration and charge decrease, hydrophobicity increase; milk appearance gains in whiteness (Philippe et al., 2003). Thermal stability of milk supplemented in calcium is strongly affected.

#### **2.3.4. Complexant addition**

The presence of chelating agents (citrate, EDTA) at a minimal concentration significantly decreases the binding between cations and caseins.

Citrate addition in milk or micellar suspension causes solubilization of micellar calcium (Brulé and Fauquant, 1981) and inorganic phosphate (arrow 1b, figure 4). Micellar calcium solubilization due to citrate addition enables free  $\text{H}^+$  to bind the newly free phosphoserine residues. This involves a pH increase. Following calcium complexation, strong physicochemical changes can be observed such as micellar destructuring, decrease of whiteness up to transparization and a viscosity increase. Thanks to its complexing properties, citrate is used to limit thermal precipitations.

Similar results are obtained after EDTA addition. In 1981, Pierre and Brulé observed that natural variations of the calcium equilibrium in milk during cold storage may also be affected by addition of certain chemicals. In comparison to cold storage treatment alone, more Ca was removed by the supplemented effect of EDTA. Contrarily to NaCl addition effect, both Ca and P were released upon EDTA addition.

In presence of inorganic phosphate at the same concentration than organic phosphate of  $\beta$ -casein, calcium binds preferentially to phosphoserine residues, due to their clustered localization on casein and, as a consequence, the important concentration of local negative charges.

#### **2.3.5. Conclusion**

Calcium equilibria in milk are summarized in figure 8 together with the exchanges of material between the colloidal, i.e. micelle of caseins, and the soluble phases.

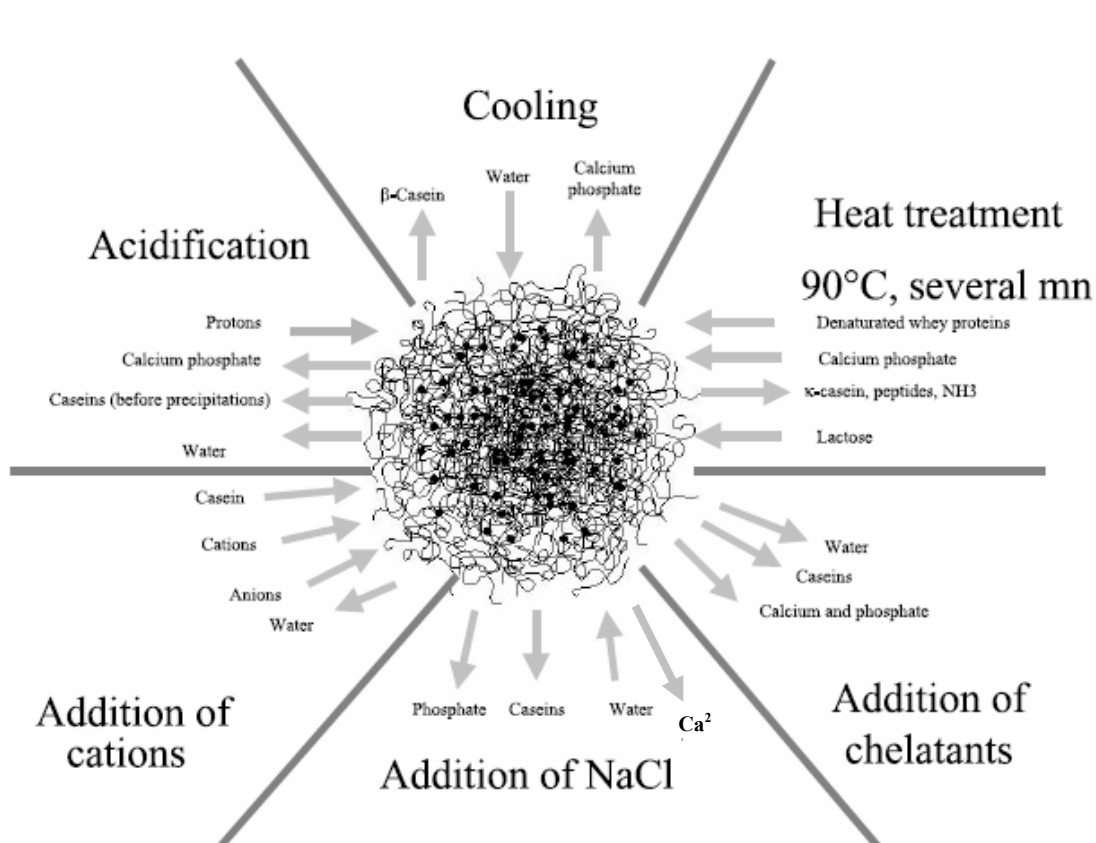


Figure 8. Modification of salt equilibrium in different physico-chemical conditions (Gaucheron, 2005)

### 3. Soy milk proteins, a potential vector for calcium supplementation

#### 3.1. Structure and properties of soy proteins

The seed proteins of soybean are albumins and globulins. Globulins, the major storage proteins, account for about 50-90% of proteins. These proteins were initially classified according to their ultracentrifugal pattern into 2S, 7S, 11S and 15S fractions (Naismith, 1955; Wolf and Briggs, 1956). The major protein fraction is constituted of 7S and 11S proteins and the ratio 7S-11S varies among soy cultivars.

Soy proteins are soluble at neutral pH and as a consequence extractible by wet extraction. Their precipitations occur at pH 4.5, the isoelectrical pH.

Soy products of various protein concentrations can be prepared. Soy Protein concentrates (SPC) are obtained from defatted soy flour. For better protein purification, alkaline solubilization must be performed and insoluble part discarded by centrifugation. Soy Protein Isolates (SPI) are then obtained by precipitation at pH 4.5. Soy flour, SPC and SPI contain respectively around 50%, 70% and 95% of protein (w/w). Soy protein purification causes physical and chemical changes that affect their functional properties (Kinsella, 1979).

SPI are composed of two minor fractions (2S and 15S) and are mostly composed of globulins especially glycinin (11S) and  $\beta$ -conglycinin (7S) as major fractions (figure 9).

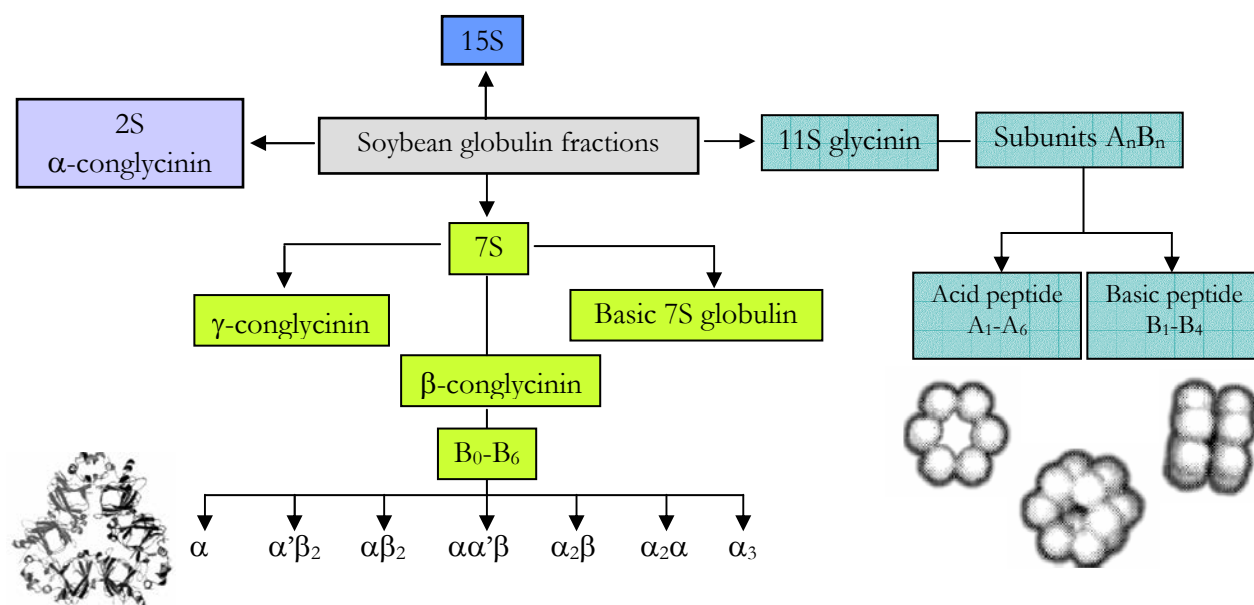


Figure 9. Soy protein classification



The 2S fraction has been reported to contain from 8 to 22% of the extractable soybean proteins and consists of a number of enzymes. The 2S fraction has been assigned an average molecular weight of 26000 Da, but in fact, it is composed of a number of proteins with a variety of molecular weight. Probably, the most studied protein of the 2S fraction is the trypsin inhibitor. The smallest of these inhibitors has been termed as the Bowman-Birk inhibitor. The other main trypsin inhibitor of soybean is the Kunitz inhibitor. These antinutritional factors can be inactivated almost completely by commercial heat treatment.

The 15S fraction is the final fraction of the extractable soy proteins. This material comprises about 5% of the total extractable protein. It is poorly characterised only and is thought to be composed of polymers of the other soy proteins.

11S and 7S proteins have different structures and molecular properties and consequently different functional properties (Saio and Watanabe, 1978). These two globular proteins have a very compact structure at pH 7.6 (in: Yuan et al., 2002).

11S protein is a hexamer. As a constituent subunit, five subunits are identified. Each subunit is composed of an acidic and a basic polypeptide. The acidic and basic polypeptides are linked together via a disulphide bond. According to the literature, 11S protein has an estimated Molecular Weight (MW, kDa) comprised between 309 and 373 kDa with the acidic (36-40 kDa) and the basic (18-20 kDa) polypeptide chains differing in their Molecular Weight (Catsimpoolas et al., 1969; Nielsen, 1985).

Koshiyama and Fukushima (1973) determined by Optical Rotary Dispersion that the proportion of  $\alpha$ -helix,  $\beta$ -structure and random coil in 11S protein were approximately 5, 35 and 60%, respectively. Similar results were obtained by Yamaushi et al. (1979; 6, 40 and 55%, respectively) whereas Ishino and Kudo (1980) estimated 20, 17 and 63% for  $\alpha$ -helix,  $\beta$ -structure and random coil. Nevertheless, all authors agreed that 11S protein fraction has a relatively low content of ordered structure and a high content of random coil structure.

11S amino-acid composition is low in methionine but high in lysine (Catsimpoolas et al, 1971). 23.5 and 46.7% of amino-acids are hydrophobic and hydrophilic, respectively (Takagi et al, 1979). Acid subunits have a higher content of glutamic acid, proline and half cystine than in basic subunits. Basic subunits have a higher hydrophobic amino-acid content than in acidic subunits. Isoelectrical point of 11S protein was determined with more accuracy at pH 4.64 by Koshiyama (1972).

The 7S fraction is classified into three major fractions with different physicochemical properties, designated as  $\beta$ -conglycinin,  $\gamma$ -conglycinin and basic 7S globulins.  $\beta$ -conglycinin is the most prevalent of these fractions whereas  $\gamma$ -conglycinin and basic 7S globulins account for less than a few percent. 7S protein has a trimeric structure having a molecular weight of 140-200 kDa and consists of subunits  $\alpha'$  (83-57 kDa),  $\alpha$  (76-57 kDa) and  $\beta$  (53-42 kDa) (Brooks and Morr, 1985).

The conventional method for 7S and 11S proteins purification is based on their different precipitation behaviour with calcium salt (Saio et al, 1973). Several methods of 11S protein preparation were reviewed by Peng et al. (1984).

The soy globulins (11S and 7S) contain different proportions of cystine residues in their sequence, glycinin (11S) being the richest fraction (38-42 residues/mol). These sulfhydryl groups (SH) and disulfide (SS) bonds are important in protein conformation since they provide to the macromolecule structural rigidity and stability. Both trimer 7S and hexamer 11S are formed under the action of electrostatic and hydrophobic forces.

## **3.2. Effect of physicochemical treatments on soy proteins**

### **3.2.1. Temperature effect**

Heat treatment is one of the most commonly used treatments to modify the functional properties of proteins. Yet, in the case of soy proteins, one of the factors limiting their use as a highly functional food protein is their poor heat stability.

During heat treatment two essentially independent thermal events occur. First, oligomeric structures are disrupted. Then, monomers of globular proteins are unfolded by denaturation, exposing hydrophobic residues to the surface, initiating further rearrangements and/or aggregation.

Hydrophobic interactions play a substantial role in the stabilization of the tertiary structure and in protein-protein interactions. Sorgentini et al. (1995) observed that heating treatment of purified soy protein fraction caused their denaturation and an increase of surface hydrophobicity for 7S and 11S globulins.

Thermal denaturation of soy proteins affects interaction with water and promotes aggregation phenomena (Catsimpoolas and Meyer, 1970). Indeed, extensive disulfide bridging occurs within the major globulin proteins 11S (Kinsella, 1979).

German et al. (1982) studied the thermal dissociation and association behaviour of soy proteins. At temperature higher than 90°C, isolated glycinin 11S formed insoluble aggregates that consisted exclusively of the basic subunits peptides, which spontaneously coagulate via hydrophobic interactions. On the contrary, the absence of aggregation in whole soy milk suggested an interaction between 11S and 7S following heating, resulting either in stabilization of the 11S structure or solubilization of the basic subunits following dissociation. Reversible cross-linking experiments suggest that heating 11S in the presence of 7S results in complex formation between dissociated 7S subunits and 11S basic subunits.

Wagner and Anon (1990) studied thermal transitions of soybean proteins by Differential Scanning Calorimetry (DSC). The  $\Delta H$  value measured with DSC represents a valuable parameter in assessing the degree of denaturation of plant proteins. Hermansson (1978) determined the endothermic transition of 7S and 11S proteins to be respectively 74°C and 83°C whereas German et al. (1982) obtained higher values (77°C and 92°C, respectively). The same trends were more recently obtained by Sorgentini et al. (1995), who observed that heat-induced denaturation of proteins was followed by an irreversible process such as aggregation.

At each temperature, a specific equilibrium exists between proteins in the native and in the denatured state. However on (partial) unfolding of soy proteins, functional groups and hydrophobic groups become exposed and immediately interact each other leading to an irreversible protein aggregation and gelation. It is known that formation of intermolecular disulfide bonds via thiol (-SH) – disulfide (SS) interchange and oxidation reactions plays a role in aggregation of soy protein.

Catsimpoilas et al. (1970) studied the thermal aggregation of 11S subunits by kinetic scattering absorbance measurement (320 m $\mu$ ). Above 70°C, 11S protein dissociated into their subunits. The rate and extent of aggregation increased with low ionic strength, similarly to Petrucelli and Anon (1994), who observed the stabilizing effect produced by the ionic strength increase.

On the contrary, the thermal aggregation of the subunits decreased at extreme pH values (acidic or alkaline) and at high ionic strength. Maximum aggregation rate occurred between pH 4.0 and 6.0. Hence involvement of ionic and hydrophobic bonds in the aggregation process was strongly suggested. Complete dissociation of proteins into subunits, by cleavage of disulphide bonds with mercaptoethanol, appeared to be one of the conditions favouring aggregation. Thermal aggregation, involving 11S protein dissociation into subunits, could be

distinguished from intermolecular aggregation of protein at ambient temperatures as the result of pH adjustment in the region of the isoelectrical point.

The freezing effect was also studied. Upon freezing, protein molecules become partially insoluble due to the intermolecular disulphide bonds formation (Hashizume et al., 1971).

### **3.2.2. pH effect**

Both 7S and 11S proteins have complex oligomeric structures. Their association/dissociation state and thermal stability is known to be highly dependent on pH and on ionic strength. Indeed, pH is the principal factor that affects the protein charge. Yuan et al. (2002) studied the aggregation/dispersion state of soy proteins as a function of pH using spectrophotometry. As the pH approached the  $pH_i$ , soy proteins aggregation resulted in precipitation due to strong protein-protein interaction. The electrostatic charge can be used to predict soy protein dispersion and association. Indeed proteins precipitate at their  $pH_i$ . Far from this pH value, proteins disperse in suspension because of electrostatic repulsion. 7S globulin is precipitated in a much narrower interval of pH values than 11S globulin because of the greater van der Waals and hydrophobic forces among 11S globulins. 11S proteins dissociate into subunits, which then re-associated at acidic (pH 2) and alkaline pH (pH 11). Acid denaturation of protein and subsequent denaturation starts at pH 3.75 and reaches a maximum at pH 2.0. 11S protein has been reported to associate or dissociate easily by changing pH or ionic strength (in: Sakakibara and Noguchi, 1977).

### **3.2.3. High Pressure effect**

Several studies report the high pressure effect on soy proteins modifications (Zhang et al., 2003, 2005). Similarly to heat treatment, a surface hydrophobicity increase was observed after high-pressure treatment by spectrofluorimetry for both purified 11S and 7S globulins (Zhang et al., 2003). In the meantime, electrophoretic analysis clearly showed change of soy proteins and indicated that soy proteins were dissociated by high pressure into subunits, some of which associated to aggregate and became insoluble. More, the soy milk viscosity increased with increasing high pressure treatment. High pressure denaturation occurred at 300 MPa for  $\beta$ -conglycinin (7S) and 400 MPa for glycinin (11S). All these changes indicated that

soy proteins were modified to form colloidal structures. Indeed soy proteins were denatured and dissociated by high pressure into subunits, some of which may associate to aggregate and become insoluble.

The homogenization effect (50-1200 bars) increased the soy milk stability (Durand and Hosken, 1999). Durand et al. (2003) reported that the most stable milks were related to the smallest particles size.

### **3.2.4. Ionic strength effect**

NaCl addition (0.1-0.2M) seems to facilitate association and aggregation of soy proteins while decreasing their solubility (Shen, 1976; Hermansson, 1978). With higher ionic strength, hydrophobicity increases, due to the charge screening effect and a subsequent repulsion forces decrease, would explain the lower solubility of proteins (Damodaran and Kinsella, 1982).

Yuan et al. (2002) studied the precipitation equilibria of 7S and 11S soy proteins in presence of  $\text{Ca}^{2+}$  ( $\text{CaCl}_2$ ) and  $\text{Na}^+$  ( $\text{NaCl}$ , 0-0.3M). Ions such as  $\text{Ca}^{2+}$  or  $\text{Na}^+$  neutralize the electrical charges of negatively charged proteins and induce protein aggregation, whereas the hydration repulsion further enhances the protein solubility due to an electrical double layer. With an ionic strength increase, a larger amount of precipitant is required and the protein solubility is higher. Due to the double electrical layer formed by  $\text{Na}^+$  addition, the number of  $\text{Ca}^{2+}$  ions needed is increased from 164 to 1000 to precipitate a mole of protein for 7S, and from 79 to 435 for 11S.

### **3.2.5. Calcium effect**

#### **3.2.5.1. Calcium-soy protein interactions**

Several techniques have been used to determine ion-protein interactions. Appurao and Narasinga Rao (1975) used equilibrium dialysis (method introduced by Hummel and Dryer, 1962) to determine the amount of  $\text{Ca}^{2+}$  ions bound by 11S proteins.  $\text{Ca}^{2+}$  binding was negligible at pH 5.5 but not at pH 7.8 and the maximum number of bound calcium ions was 17 moles  $\text{Ca}^{2+}/10^5$  g protein at this pH. The authors expected that the binding sites could be the imidazole groups of the histidine residues. They also observed that the ionic strength increase (NaCl 0.5M) or EDTA treatment decreased  $\text{Ca}^{2+}$  binding by the 11S fraction. NaCl

addition and EDTA treatment reduced the binding affinity by a factor 20 and a factor 2, respectively.

The interaction of 11S soy protein with calcium ions was also studied by dilatometry (Sakakibara and Noguchi, 1977). The volume change measured was associated with the  $\text{Ca}^{2+}$ -11S protein interaction. With calcium chloride (CC) addition to 11S protein solution, the volume increased up to a constant saturated value of 0.04M of CC and then decreased. At pH 8, the number of  $\text{Ca}^{2+}$  bound to proteins was estimated to be  $11 \pm 2$  mole  $\text{Ca}^{2+}/10^5$  g proteins. On the contrary, the authors observed that  $\text{Ca}^{2+}$  did not bind to 11S protein at pH 6 and pH 7. The binding of  $\text{Ca}^{2+}$  to the protein was dependent on pH and on  $\text{Ca}^{2+}$  concentration. The binding behaviour observed as a function of pH involves several phenomena. First, the electrostatic binding is due to the increase of the negative charges on the protein surface with increasing pH. Then the chelation of calcium ion by carboxyl group and imidazole group of amino groups occurs. The last parameter is the change of protein conformation due to the dissociation-association reaction.

Appurao and Narasinga Rao (1976) studied the binding of  $\text{Ca}^{2+}$  by the 7S fraction of soy proteins. They observed that the binding site of calcium occurred at the imidazole groups of the histidine residues of protein at pH 7.8. Rate of proteolysis, fluorescence, Optical Rotary Dispersion and Circular Dichroism measurements did not indicate any conformational change in the protein due to calcium binding. Once more, ionic strength increase (NaCl 0.5 M) suppressed protein precipitation by calcium ions.

Scilingo and Anon (2004) studied the effect of calcium on soy protein isolate. The addition of small amounts of calcium (1.23-5.0 mg/g protein) induced the formation of 7S  $\alpha$  and  $\alpha'$ soluble aggregates whereas larger amounts (5.0-9.73 mg/g protein) involved the selective insolubilization of the 11S fraction. Calcium addition produced thermal stabilization of the soy proteins, mainly the 11S fraction, and increased the denaturation enthalpy. A decrease in the surface hydrophobicity of proteins with increasing calcium content was also observed. Indeed, calcium promotes the formation of aggregates that are initially soluble, in part because they are stabilized by hydrophobic interactions. Then the size increase renders the aggregate insoluble, leading to precipitation. The results obtained suggested the existence of specific calcium-soy protein interactions, especially with 11S fraction.

Using calcium ion-selective electrode, Kroll (1984) studied the pH effect on calcium ions binding by soy proteins. Calcium ion-soy protein interactions are affected by pH. Indeed, hydrogen ions ( $H^+$ ) compete with calcium ions for the same binding sites on proteins. When hydrogen titration curves made in the absence and in the presence of calcium ions are compared, a difference will be seen in the position of the curves if calcium ions are bound to the protein molecule at the same binding sites where hydrogen ions are bound. The difference is that at such given pH, more hydrogen ions will be dissociated in the presence of calcium ions than in the absence of calcium ions. Kroll identified these binding sites as the side-chain carboxyl groups of the aspartic and glutamic acid residues and as the imidazole group of the histidine residues. The number of calcium ions binding sites was determined at 43 per  $10^5$  g of low phytate content proteins. The affinity of the binding sites for calcium ions was shown to increase as pH increased over the pH range 4-9 (the binding constant increased with increasing pH). Below pH 3, no calcium ion binding occurred whereas between pH 3 and 7, a small change in pH resulted in a large change of bound calcium ions. Thus, a critical pH range was found that corresponds to the ionization (deprotonation) of the side-chain carboxyl and imidazole groups. According to the linear Scatchard plot (Ca bound/free Ca vs. Ca bound), the binding sites were determined equivalent (same intrinsic association constant  $K$ ) and independent (non-interacting sites).

More generally, Molina and Wagner (1999) studied the effects of divalent cations in presence of phosphate, citrate and chloride on SPI aggregation. Phosphate became incorporated into  $Ca^{2+}$ -induced aggregates whereas citrate and chloride ions inhibited aggregation and contributed to the dissociation of these aggregates. Even though aggregation was induced by divalent cations, competition between various species could modify the aggregates properties.

Ono et al. (1993), considering that soy proteins consisted of soluble and colloidal fractions with particles ranging from 40 to 200 nm, investigated the effect of calcium and pH on the protein solubility of each protein fraction. They reported that colloidal protein particles precipitated at lower calcium concentration than soluble proteins. More, precipitation occurred at higher pH in presence of calcium.

### 3.2.5.2. Phytate-calcium-soy protein interactions

Soybeans contain several antinutritional factors such as phytate, oxalate and tanins, which interfere with minerals absorption. Heaney et al. (1991) demonstrated that calcium fraction absorbed was  $0.310\pm 0.070$ ,  $0.414\pm 0.074$  and  $0.377\pm 0.056$  for high phytate soybeans, low-phytate soybeans and milk, respectively. Hence, phytate content influenced absorbability. Weaver et al. (1993) observed that the absorbability of calcium decreased with higher phytate content.

In 1989, Grynspan and Cheryan studied the solubility of mixtures of SPI, calcium and phytate as a function of pH and molar ratio of the components to determine phytate-calcium interaction with soy proteins. Below the isoelectrical point, phytate (negatively charged) and protein (positively charged) solubility profiles paralleled each other, indicating some type of protein-phytate interactions. Moreover, phytate addition shifted the isoelectrical pH and the minimum solubility to lower pH values. Between the isoelectrical pH and pH 6.5, the complex apparently dissociated. Phytate addition resulted in an increase in the maximum solubility of phosphorus and protein, as well as a shift in the solubility profiles. Calcium had no apparent effect on protein solubility in this pH region. Higher pH (>6.5) resulted in the formation of ternary protein-calcium-phytate complexes and a significant drop in calcium and phosphorus solubility, probably due to the formation of insoluble calcium-phytate salt.

### 3.3. Soy milk calcium fortification and stabilization

Soy milk has much lower calcium content than cow milk (12 and 120 mg/100 mL, respectively). Taking into account the economical and the nutritional interest of soy milk, calcium fortification and stabilization of this beverage is of major interest for people that can not consume dairy products for various medical reasons (lactose intolerance, cow milk protein allergy) or those who avoid milk for reasons such as taste, preference, religious or ethical considerations. In addition a majority of the world-wide population does not satisfy the RDA for calcium. Hence calcium fortification of soy milk would be an alternative to overcome this deficiency.

Mostly, efforts to fortify soy milk with calcium have been largely unsuccessful. Calcium tends to coagulate the proteins, forming precipitates and causing gelation of the soy milk



during storage. The use of highly ionized calcium salts, such as calcium chloride caused immediate precipitation at concentration levels required for fortification (Saio et al., 1969). Similarly, the thickening of soy protein suspensions (1-7% w/w) upon calcium supplementation, indicative of coagulation, was observed by Lee and Rha (1977).

Yet this coagulation phenomenon can be overcome under carefully controlled conditions and several authors reported their ability to fortify soy milk with calcium.

In 1983, Weingartner et al. formulated a soy protein beverage (6% soy solids) supplemented at a similar calcium level of cow's milk (25-30 mM) with calcium citrate and calcium phosphate. These authors reported that this calcium fortified and pasteurized beverage had acceptable sensory properties and that calcium salts did not adversely affect protein stability. Soy milk remained stable for 6 months when stored at 1°C or at room temperature.

Hirotsuka et al. (1984) fortified soy milk with calcium by means of calcium-lecithin liposome system. Calcium ions were enveloped with a membrane before addition to soy protein in order to prevent coagulation and precipitation. Calcium-lecithin liposomes were then added to soy protein solution. By this way, authors reported that it was possible to prepare calcium fortified soy milk containing more calcium than in cow milk: precipitation and coagulation was not observed in the soy protein-liposome system containing 60 mM  $\text{Ca}^{2+}$ .

Zemel and Shelef (1986) fortified soy milk with calcium using an alkali metal polyphosphate salt as chelating agent to suppress aggregation. Authors reported that soy milk fortification increased calcium content to the same or higher level of calcium than in cow milk. The patented (0195167) product had a refrigerated shelf-life of three weeks.

Calcium sulphate and calcium lactate were studied to increase the calcium content of soy milk in presence of sodium citrate as chelating agent (Prabharaksa et al., 1989). Soy milk fortified with calcium sulphate was very viscous and the viscosity increased with higher concentration; therefore no further attempt was made to use calcium sulphate. Yet, the viscosity of soy milk decreased as the sodium citrate concentration increased. The maximum amount of calcium lactate added to soy milk without coagulation was 75 mg calcium/100 mL soy milk (approximately 60% of calcium in cow milk). Calcium lactate could be added to soy

milk up to 0.45% (w/v) without coagulation when 0.4% (w/v) sodium citrate was simultaneously added. According to the former authors, calcium content can not be increased to the level found in cow's milk since coagulation occurred with higher calcium lactate concentrations.

The stabilization of soy milk fortified with calcium gluconate (same or higher level of calcium than in cow milk) by Sodium HexaMetaPhosphate (**SHMP**) as a sequestering agent and calcium-D-saccharic acid as a stabilizing agent was studied by Rasyid and Hansen (1991). The soy milk obtained had high calcium content and satisfactory heat stability. Hence, SHMP and calcium-D-saccharic acid were effective for reduction of the calcium ion activity of soy milk. Sequestering and stabilizing agent may act in synergy.

Yazici et al. (1997) reported calcium fortification of soy milk (200mg/100g) with calcium lactogluconate (1.55% w/w) and various amounts (0-1.50% w/w) of SHMP or potassium citrate. The best soy milk stability was observed at 1.25% potassium citrate. For successful calcium fortification, previous authors recommended to maintain a calcium-to-protein ratio lower than 38 mg/g and to use an appropriate sequestering agent at a molar ratio of 0.8 per mole of calcium.

More recently soy milk was fortified with calcium carbonate and tri-calcium phosphate at similar level of calcium to cow milk and calcium bioavailability evaluated by an *in vitro* Miller's method that involved a simulated human gastrointestinal digestion followed by measurement of dialyzable calcium (Chaiwanon et al., 2000). The following calcium ranked dialysis percentage was: calcium carbonate fortified soy milk (19±0.7%)> cow's milk (17±0.8%)> tri-calcium phosphate fortified soy milk (15±0.7%)> non-fortified soy milk (11±2%). The various Ca/P ratios in fortified soy milks may be explained by the formation of different calcium phosphate salts upon calcium fortification.

## 4. Synthesis and positioning of the study

With a similar nutritive value as cow milk, devoid of cholesterol and lactose, soy milk constitutes an alternative to cow milk, especially for population suffering of Cow Milk protein Allergy or lactose intolerance. Yet soy milk contains only one fifth to one eighth of calcium level found in cow milk; hence calcium supplementation of soy milk at a level similar to cow milk is of specific interest.

Up to now, calcium fortification of soy milk was reported to be successful in presence of calcium complexant and/or stabilizer. In their absence, the high sensitivity of soy proteins to calcium induced their aggregation.

In this context, it seemed useful to understand the natural calcium vector of cow milk, i.e. casein micelle, in order to try to reproduce a vector of calcium based on soy proteins.

This could be an alternative to the use of calcium complexant and/or stabilizer, commonly used in calcium soy milk fortification.

In cow skim milk, calcium equilibrium and their subsequent effects on soluble and colloidal proteins were studied as a function of various parameters, i.e. pH, temperature, ionic strength and addition of various calcium types. In order to study the calcium-protein interactions, cow milk and calcium supplemented cow milk were submitted to pH cycle. Acidification and alkalization modified the ionized states of proteins and of minerals in the systems and this permitted to follow the exchange of calcium between the colloidal and soluble phases (**Part 1**). Two calcium sources were studied and compared: calcium chloride, as a highly soluble calcium salt commonly used in food industry, and milk calcium, which represents a natural and healthy image.

The second experimental step of this Ph.D. work focused on calcium equilibrium in calcium supplemented soy milk subjected to pH cycle (**Part 2**). The global methodology used in the study of calcium equilibrium in calcium supplemented skim milk was adapted to the soy milk system.

For better understanding, calcium-protein interactions were characterized thermodynamically using Isothermal Titration Calorimetry (**ITC**). The influence of calcium binding to milk and soy proteins was observed by the mean of Fourier Transformed Infra-Red spectroscopy (**Part 3**). In conclusion, both milky systems are compared and perspectives of this Ph.D. thesis work are proposed. Figure 10 gives a general overview of the thesis in terms of its main aspects and tools used.

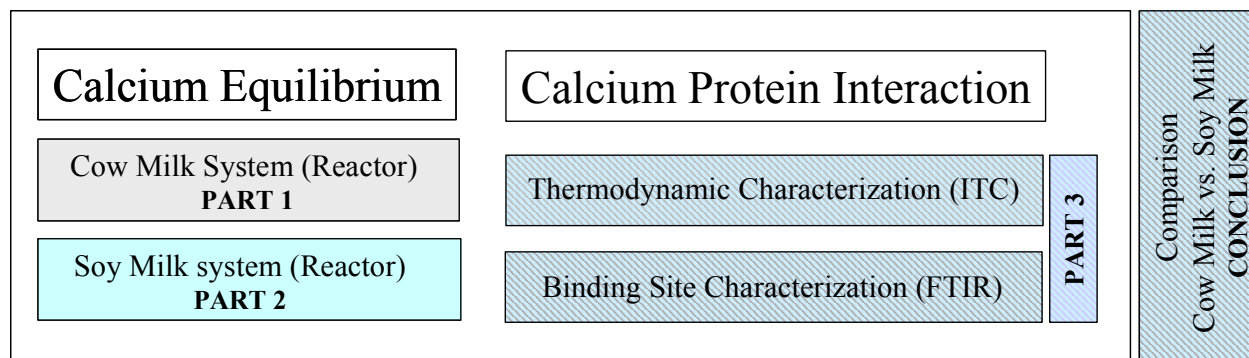


Figure 10. General overview of thesis in terms of its main aspects and tool used

### **III. MATERIALS AND METHODS**



# 1. Study of calcium equilibrium between soluble and colloidal phase in pH-treated milks

## 1.1. Milks preparation

### 1.1.1. Cow skim milk

Low heat skim milk powder (Ingredia, Arras, France) was reconstituted at 12% (w/w) in distilled water and stirred for 3 h to allow mineral re-equilibration (Anema and Li, 2003). Then reconstituted skim milk was equilibrated at 4°C overnight before use. Two kg of reconstituted skim milk were prepared for each experiment. In these conditions, the protein level of reconstituted cow skim milk was of 4.2% (w/w).

### 1.1.2. Soy milks

#### 1.1.2.1. Soy Protein Isolates

Two commercial Soy Protein Isolates (**SPI**) were used for soy milk reconstitution. Non Hydrolysed (**NH**) SPI (SUPRO 760, Non-GM Isolated Soy Protein, The Solae company, Barcelona, Spain) and Hydrolysed (**H**) SPI (Non-GM Isolated Soy Protein 219, Dupont Protein technology, St Louis, MO, USA) contained 88.2 and 86% of protein concentration (w/w), respectively. Tables 8 and 9 give the amino-acid contents of non-hydrolysed and hydrolysed SPI and their respective isoelectrical pH ( $pH_i$ ) and Reactive pK ( $pK_R$ ).

Table 8. Amino-acid content of non hydrolysed (NH) and hydrolysed (H) Soy Protein Isolate (SPI) (more details given in Appendix 2)

Amino Acids	g AA/100 g product		g AA/100 g protein	
	NH SPI (88.2% protein)	H SPI (86% protein)	NH SPI	H SPI
Alanine	3.8	3.7	4.3	4.3
Arginine	6.7	6.5	7.6	7.6
<b>Aspartic Acid</b>	<b>10.2</b>	<b>10.0</b>	<b>11.6</b>	<b>11.6</b>
Cysteine	1.1	1.1	1.3	1.3
<b>Glutamic Acid</b>	<b>16.8</b>	<b>16.5</b>	<b>19.1</b>	<b>19.1</b>
Glycine	3.7	3.6	4.2	4.2
Histidine	2.3	2.3	2.6	2.6
Isoleucine	4.3	4.2	4.9	4.9
Leucine	7.2	7.0	8.2	8.2
Lysine	5.6	5.4	6.3	6.3
Methionine	1.1	1.1	1.3	1.3
Phenylalanine	4.6	4.5	5.2	5.2
Proline	4.5	4.4	5.1	5.1
Serine	4.6	4.5	5.2	5.2
Threonine	3.4	3.3	3.7	3.7
Tryptophan	1.2	1.1	1.4	1.4
Tyrosine	3.3	3.3	3.8	3.8
Valine	4.4	4.3	5.0	5.0
Total Sulfur AA	2.3	2.2	2.6	2.6
Total Aromatic AA	7.9	7.7	9.0	9.0



Table 9. Isoelectrical pH ( $pH_i$ ) and reactive pK ( $pK_R$ ) of amino acid (CRC handbook of Chemistry and Physics, 88<sup>th</sup> edition; Weast, 1970)

Amino Acids	$pK_R$	$pH_i$
Alanine	any	6.1
Arginine	12.48	10.76
Aspartic Acid	3.86	2.98
Cysteine	10.78	5.02
Glutamic Acid	4.25	3.08
Glycine	any	6.064
Histidine	6.0	7.64
Isoleucine	any	6.038
Leucine	any	6.036
Lysine	10.53	9.47
Methionine	any	5.74
Phenylalanine	any	5.91
Proline	any	6.3
Serine	any	5.68
Threonine	any	6.53
Tryptophan	any	5.88
Tyrosine	10.07	5.63
Valine	any	6.002

N.B.:  $pK_R$  does exist only for amino-acid with a ionizable radical

#### 1.1.2.2. Soy milk reconstitution and stabilization

Non hydrolysed and hydrolysed soy milks were reconstituted from their respective SPI in distilled water at 4.2% proteins concentration (w/w) similarly to the protein level of reconstituted cow skim milk.

The presence of protein aggregates in soy isolates led to large instability of reconstituted suspensions as it can be seen in figure 11.

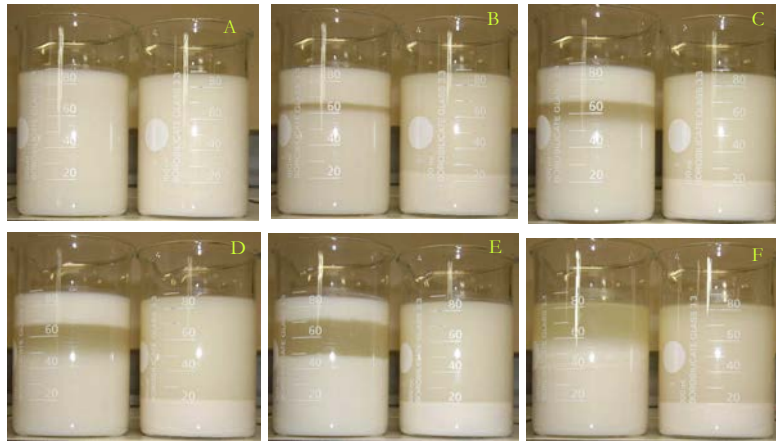


Figure 11. Soy Protein Isolate solutions (4.2% protein w/w) ageing as a function of time

For each picture, Left sample: non hydrolysed SPI; Right sample: hydrolysed SPI. A: Just after preparation and stirring; B: 1h after; C: 3h after; D: 4h after; E: after one night; F: after two nights

To resolve the physical instability of SPI preparations, an appropriate stabilization treatment was applied by shearing at 13500 rpm during 5 min (ultraturrax, Ultralightnin, Bioblock, Advantech, Strasbourg, France) then by high pressure homogenizing at 500 bars during 5 cycles (EmusiFlex-C3, Sodexim S.A, Muizon, France; figure 12).

For the purposes of clarity, non stabilized and stabilized soy milks will be referred to SPI solution and soy milk in the following, respectively.

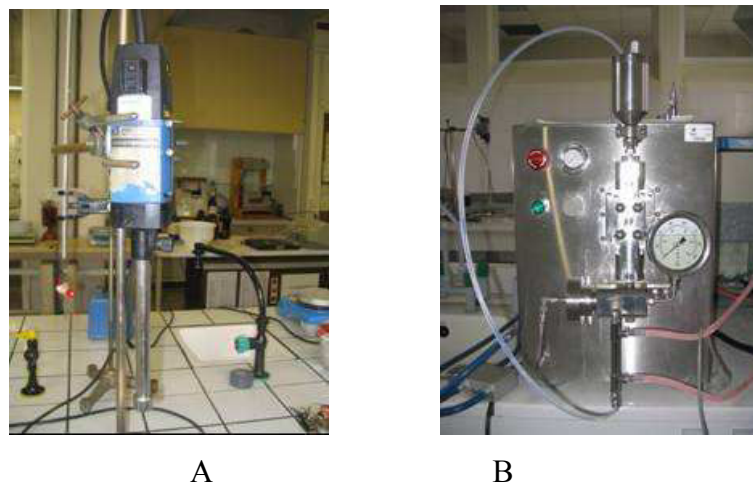


Figure 12. Ultraturrax (A) and high pressure homogenizer (B) equipment used for soy milk stabilization

## 1.2. Calcium salt supplementation

### 1.2.1. Cow skim milk supplementation

Calcium salt supplementation was performed with Calcium Chloride powder (**CC**,  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , analytical grade, Carl Roth GmbH, Karlsruhe, Germany) or Milk Calcium powder (**MC**, Fieldgate natural dairy calcium, First district association, Minnesota, USA). CC was chosen for its high solubility and its common use, and MC was studied as a natural source of calcium. Milk calcium, mainly constituted of calcium phosphate contained calcium (28% w/w) but also other compounds such as lactose and minerals (table 10). This calcium percentage was taken into account for further supplementation.

Table 10. Milk calcium (Fieldgate natural dairy calcium, First district association, Minnesota, USA) specification

Calcium dry basis %:	26.0-29.0%
Phosphorus dry basis %:	14% Minimum
Free moisture %:	7% Maximum
Phosphate ( $\text{PO}_4$ ) %:	40% Minimum
Protein dry basis %:	4% Maximum
Fat dry basis %:	0.04% Maximum
Ash %:	75.0% Minimum
Lactose:	7.0% Maximum
pH:	6.0-8.0

Two kg of reconstituted skim milk were supplemented with CC or MC. For each calcium salt, calcium concentrations were chosen at 10, 15 and 25 mmoles/kg of skim milk according to the concentrations range commonly mentioned in the literature (Guillaume et al., 2002). Unsupplemented skim milk constituted the reference.

After a calcium equilibrium period (2 h), supplemented skim milk was subjected to pH cycle. In each experiment, equilibrium time after calcium supplementation was kept constant to enable further kinetics comparison, whatever the calcium salt studied.

### 1.2.2. Soy milks supplementation

After homogenization, reconstituted soy milk was supplemented with 25 mmoles of  $\text{CaCl}_2$  (**CC**, Carl Roth GmbH, Karlsruhe, Germany) per kg of soy milk in order to reach a

calcium supplementation level similar to cow skim milk. After a 2 hours equilibrium period, soy milk was subjected to pH cycle procedure.

### 1.3. pH cycle procedure

#### 1.3.1. General procedure

The pH cycle procedure was carried out using a titrator (Multiburette 2S, Crison, La Souterraine, France). The vessel was equipped with a four bladed 45° impeller (R 100 impeller: 8 cm-diameter) in constant rotation (Lightnin LabMaster™ Mixer). The temperature was kept constant by using a double wall jacket vessel.

Acidification was performed by addition of HCl 1N (Riedel-de Haën, Seelze, Sigma Aldrich, Germany) in skim or in soy milk until reaching a minimal pH value ( $\text{pH}_{\min}$ ). Then directly after acidification, neutralization was performed by addition of NaOH 1N (Riedel-de Haën, Seelze, Sigma Aldrich, Germany). Acid and base were both added to the medium at a determined rate. pH cycle was stopped when the initial pH of milk system was reached. All titration parameters, summarized in table 11, were kept proportional with the reactor volume.

Table 11. Titration parameters

	Cow skim milk	Soy milk
Vat Volume	2L	500 mL
Stirring	250 rpm	150 rpm
Temperature	4°C	25°C
Initial pH	6.9	7.1-7.2
$\text{pH}_{\min}$ of pH cycle	5.5, 5.0, or 3.5	5.5 or 3.5
Rate of acid or base addition	1 mL/min	0.25 mL/min

Dairy system was thermostated at 4°C in order to minimize aggregation and gelation of proteins.

At the end of each step of the kinetic ( $T_1$  = probes stabilization,  $T_2$  = Ca equilibrium period,  $T_3$  = acidification and  $T_4$  = alkalization), samples were collected for further analysis. Each experiment was duplicated.

The kinetic of this experiment is presented in figure 13.

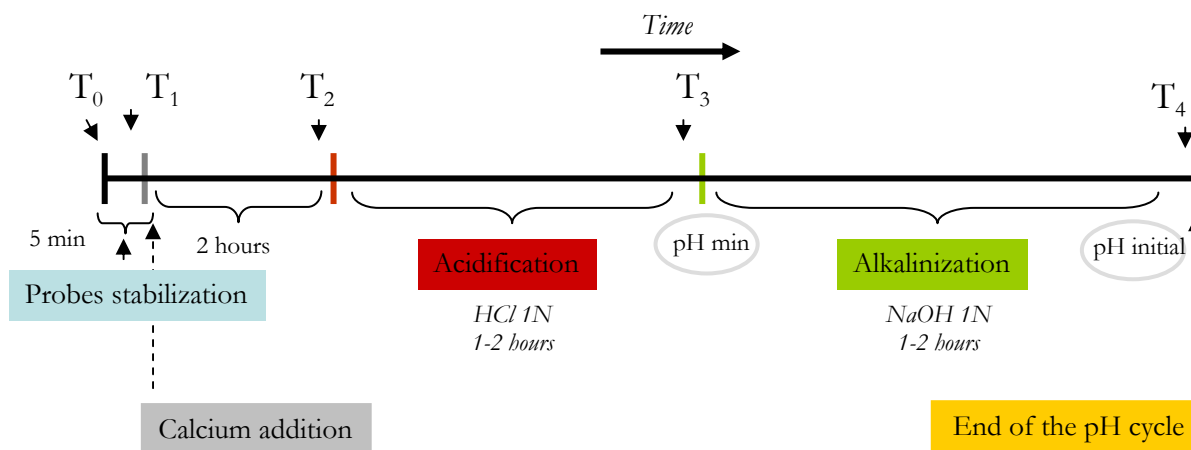


Figure 13. pH-cycle experiment on cow and soy milks as a function of time:  $T_1$  = probes stabilization,  $T_2$  = Ca equilibrium period,  $T_3$  = acidification and  $T_4$  = alkalinization.

### 1.3.2. Experimental set up

The whole kinetic (calcium supplementation and pH cycle) was followed by various probes immersed into the liquid: pH-meter (Radiometer analytical, Remiremont, France), conductivity-meter (CDM 210, Radiometer analytical, Remiremont, France) and Calcium Ion Selective Electrode (Ca ISE, Sentek, Estate, Braintree, United Kingdom). A turbidity sensor (Analite NEP 160, McVan Instruments, Mulgrave, Australia) was placed through the vessel wall to avoid disturbances during stirring.

The continuous monitoring (Almemo 8990-8 V5, Ahlborn, Holzkirchen, Germany) of pH, conductivity, ionized calcium and turbidity was performed using a data logger coupled with a PC equipped with software (AMR WinControl for Almemo, Akrobit<sup>®</sup>, Gera, Germany; figure 14). Electrodes calibration was performed daily.

This experimental set-up was previously described by Gaiani et al. (2005) in a kinetic study on dairy powders rehydration.

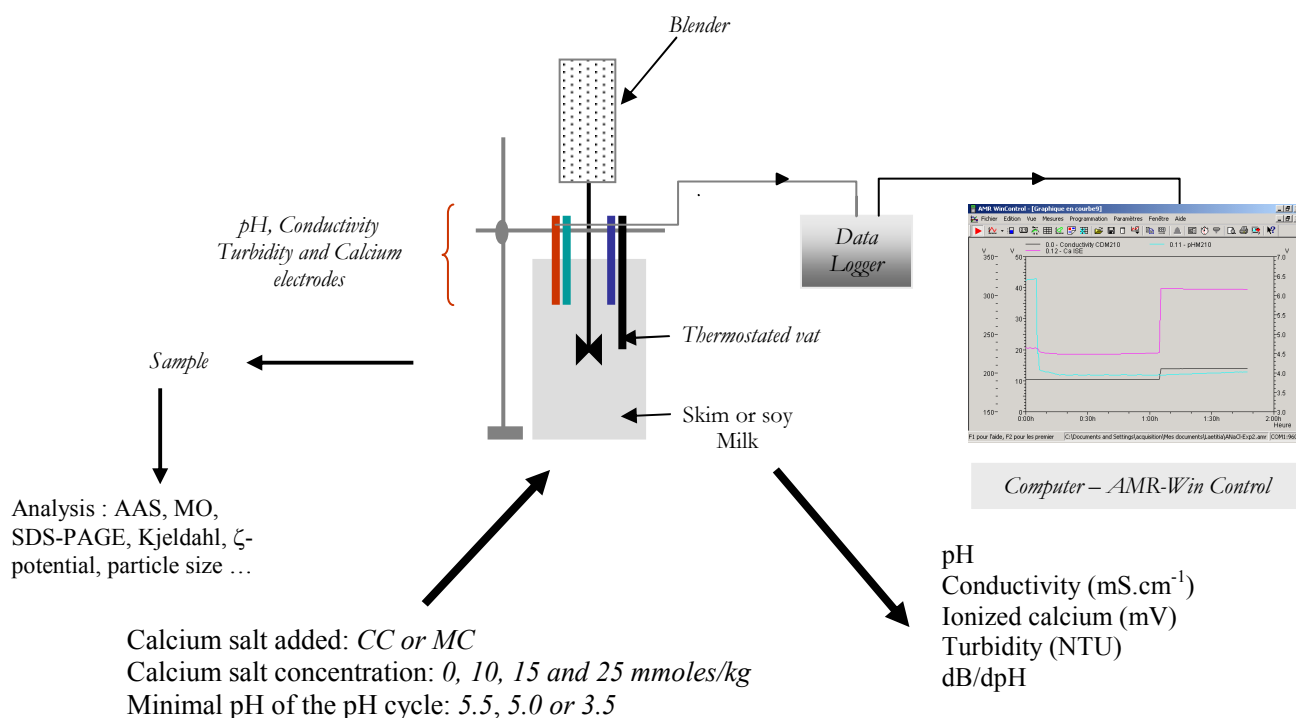


Figure 14. The experimental set up used for kinetic study of calcium equilibrium in calcium supplemented skim milk or soy milks subjected to pH cycle

### 1.3.3. Buffering index calculation

Titration were performed in duplicate for each experiment on reconstituted skim milk and in soy milks with hydrochloric acid (HCl, 1N) and sodium hydroxide (NaOH, 1N) added in 1 mL and 0.25 mL increment at 1 min interval, respectively. The samples were titrated from initial pH to pH 3.5, during acidification and back alkalization. Buffering index values (dB/dpH), essentially dimensionless, were calculated according to the van Slyke formula (1922) and plotted as a function of pH:

$$\frac{dB}{dpH} = \frac{(Vol_{\text{Titrant}}) \times (Normality)}{(Vol_{\text{Milk}}) \times (\Delta pH)} \quad \text{Equation 2}$$

## 1.4. Calcium behavior in calcium supplemented and pH treated milks

### 1.4.1. Conductivity variations

Total ion concentration was determined with conductivity-meter CDM 210 (Radiometer analytical, Remiremont, France). Conductivity-meter was calibrated daily with KCl 0.1 M (analytical grade, Riedel-de-Haën, Sigma Aldrich, Seelze, Germany).

### 1.4.2. Calcium quantification by AAS

For skim milk, CC and MC supplemented, total and soluble calcium were quantified for pH cycles to 5.5 and 5.0, at the end of each step of the kinetic ( $T_1$  = probes stabilization,  $T_2$  = calcium salt equilibrium,  $T_3$  = acidification and  $T_4$  = neutralization).

Atomic Absorption Spectrometer (AAS, Perkin Elmer, Wellesley, Ma, USA) was used for calcium quantification according to the procedure described by Brulé et al., (1974) and Dziuba and Muzinska (1998). Calcium detection lamp was set at a wavelength of 422.7 nm with a slit of 0.7 nm. AAS apparatus was calibrated with calcium solutions of 0, 4, 6, 8, 10 and 12 mg/L.

Colloidal and soluble phases of milk were separated by ultracentrifugation (Sorvall RC M120 GX, les Ulis, France) set at 110,000 g during 1 h (Dziuba and Muzinska, 1998) at 4°C. Colloidal calcium was considered as the difference between total calcium and soluble calcium (equation 3). Milk ultrafiltrates (soluble phase of milk) obtained by ultracentrifugation were stored at – 20°C until AAS analysis.

For the studied samples, 1 mL of total milk or milk ultrafiltrate was diluted in distilled water supplemented with lanthanum chloride (respectively 10 or 5 mL of  $\text{LaCl}_3$ ) for a final volume of 100 mL or 50 mL respectively. Then dilution was performed in distilled water until reaching an absorbance value comprised within the calibration range. Calcium was quantified in triplicate, on duplicate experimental trials.

Colloidal calcium was calculated from the following equation:

$$\text{Ca}_{\text{Colloidal}} = \text{Ca}_{\text{Total}} - \text{Ca}_{\text{Soluble}} \quad \text{Equation 3}$$

Complexed calcium from the soluble phase was calculated as the difference of soluble calcium (measured by AAS) with ionized calcium of the soluble phase (measured by Ca ISE).

$$Ca_{\text{Complexed}} = Ca_{\text{Soluble}} - Ca_{\text{Ionized}} \quad \text{Equation 4}$$

Determination of the calcium concentration in each phase of milk is presented in figure 15.

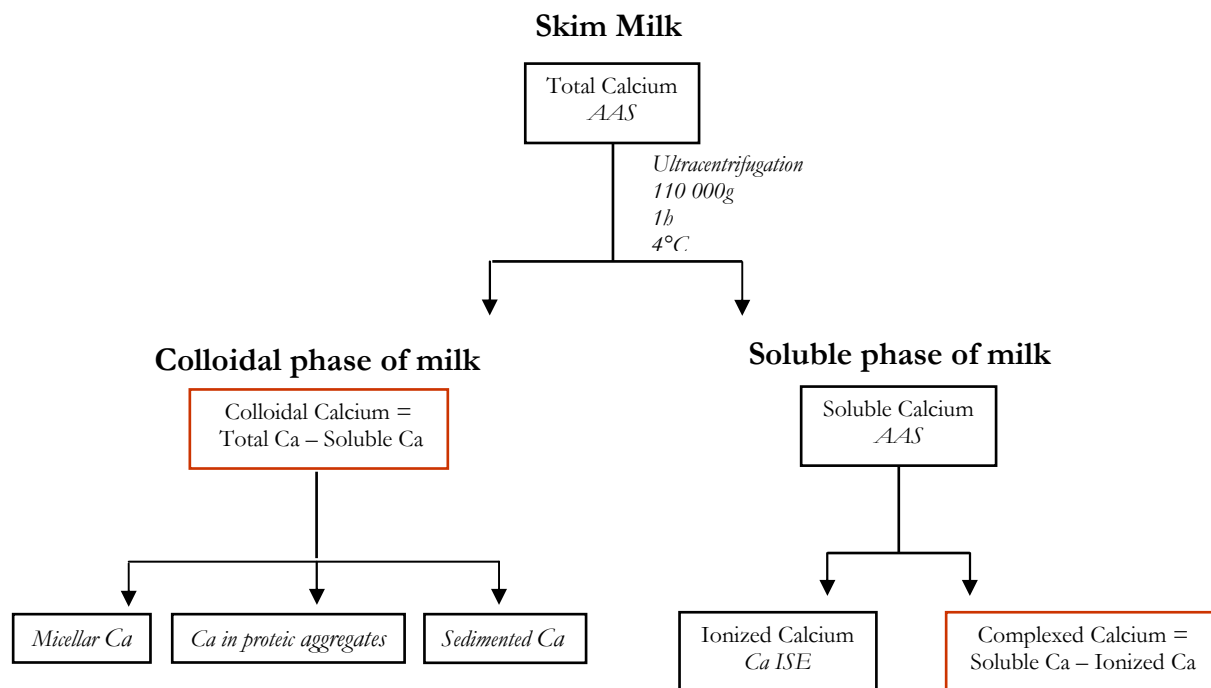


Figure 15. Determination of calcium concentration in each phase of skim milk (in red rectangle: calculated calcium concentration)

### 1.4.3. Ionized calcium variations

Calcium Ion Selective Electrode (Ca ISE, Sentek, Estate, Braintree, United Kingdom) was used to evaluate calcium ionisation (Dalglish and Parker, 1980; Holt et al., 1981; Assoumani, 1998; Vyas and Tong, 2004). Calibration was done with CC calibrating solutions of  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ , and  $10^{-4}$  M, respectively. These calibrating solutions were prepared in NaCl 0.1 M to mimic milk ionic strength value, which is around 0.085 M (Holt et al., 1981).

During pH cycle, Ca ISE measured ionized calcium concentration through a chemical potential measurement (mV). Calcium activity of calibrating solutions was calculated according to the following equation:



$$a_i = \gamma_i * C_i \quad \text{Equation 5}$$

$a_i$  = calcium activity ( $M$ )

$\gamma_i$  = activity coefficient (no unit) = 0.40 for an ionic strength value of 0.1  $M$  (Geerts et al., 1983; Van Kreveld and Van Minnen, 1955, mentioned in: Lucey et al., 1996), N.B = some other authors used 0.485 as activity coefficient.

$C_i$  = ionized calcium concentration ( $M$ )

Ca ISE calibration curves (Potential (mV) plotted versus Calcium activity ( $M$ )) and potential measurements allowed CC activity determination and as a consequence ionized calcium ( $Ca^{2+}$ ) measurements. Figure 16 presents the calibration curve obtained for a given experiment and its corresponding logarithmic linear regression equation.

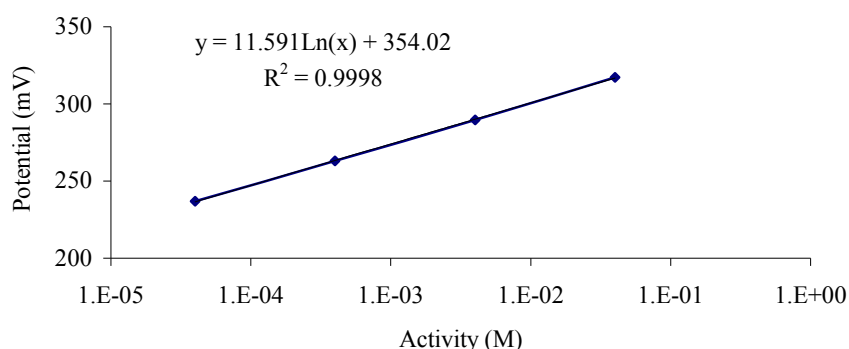


Figure 16. Example of calibration curve obtained from calcium ion selective electrode (Sentek, Estate, Braintree, United Kingdom)

Calcium activities were calculated from logarithmic linear regression equation of calibration curves. Ionized calcium concentrations were then calculated from equation 5. Dilution effect due to acid and base addition was corrected in ionized calcium concentration calculation.

Ionized calcium concentration was measured as a function of pH. pH meter and Ca ISE were daily calibrated at the experimental temperature. pH-meter calibration was performed at pH 7.00 and pH 4.00, according to the pH range of skim milk and soy milk and the accuracy of the measurement was of  $\pm 0.02$  pH unit.

## **1.5. Colloidal protein phase characterization of milks**

### **1.5.1. Micellar state variations**

Slight variations in casein micelle structure were observed in skim milk by turbidimetry (Banon and Hardy, 1992). Turbidimetric method is based on light reflection of a monochromatic beam in near infra-red ( $\lambda = 860$  nm). The incident beam is transmitted in the solution by the mean of optical fibers and is reflected back by particles in suspension in the studied solution. The beam reflected at  $180^\circ$  is captured by another system of optical fibers up to an electronic sensor which amplifies the received signal and converts it in Nephelometric Turbidity Unit (NTU). The turbidity signal is known to depend on three main factors: size, concentration and optical properties of colloidal particles.

Turbidity-meter (Mc Van Instrument, Mulgrave, Australia) was calibrated twice a month with 2 solutions of known turbidity values, i.e. 0 NTU and 4000 NTU. This latter solution was a mix in equal amount of a sulphate hydrazine solution (10 g dissolved for 1 L of filtered distilled water) and a hexamethylen tetramin solution (100 g for 1 L of filtered distilled water). Both products were purchased at VWR international Prolabo (Fontenay sous bois, France). The 4000 NTU-solution remained at rest 24 h before calibration.

### **1.5.2. Rheometry**

Colloidal properties of soy milks were followed by rheometry. Apparent viscosity of soy milks was determined with a StressTech rheometer (Rheologica, Scheelevägen, Sweden). The geometry used for apparent viscosity measurement is a custom built paddle stirrer adapted to particulate system, for a 18 mL-vat. This is a four pales right angle paddle (figure 17).

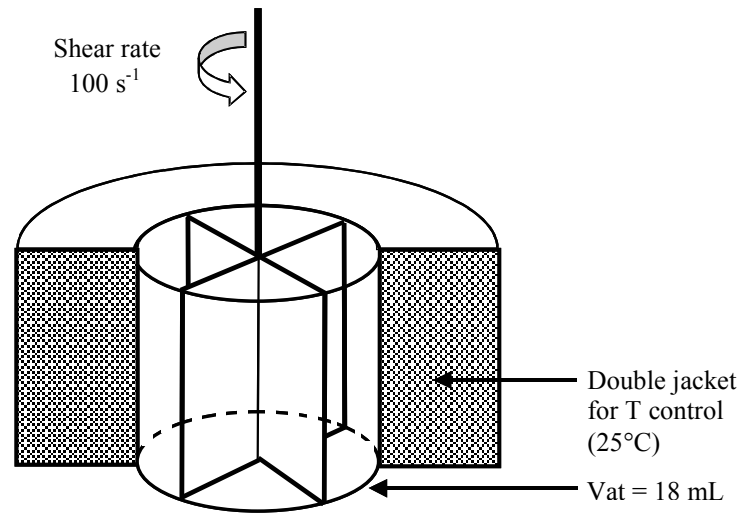


Figure 17. Scheme of the module used for viscosity measurement during pH cycle of milks

The shear rate was set at  $100 \text{ s}^{-1}$  and remained constant all over the experiments ( $T = 25^\circ\text{C}$ ). The apparent viscosity of the solution was followed all over the pH cycle ( $\text{pH}_{\text{min}} = 5.5$  or  $3.5$ ). During the first 5 minutes, viscosity was measured on soy milk to reach stability. Then, soy milk was supplemented with CC and after 10 min of calcium equilibrium period, pH cycle began. Data acquisition frequency was set to 60 s. Each experiment was duplicated.

### 1.5.3. Particle size determination

#### 1.5.3.1. Dynamic Light Scattering

For skim milk study, particles size determination was performed by the mean of a Nanosizer (HPPS 5001, Malvern instrument Ltd, Worcestershire, UK). Two kinds of samples were studied: ultrafiltrate and skim milk diluted in ultrafiltrate ( $75 \mu\text{L}$  of skim milk in  $925 \mu\text{L}$  of ultrafiltrate). Ultrafiltrate obtained after ultracentrifugation ( $110\,000g$ ,  $1\text{h}$ ,  $4^\circ\text{C}$ ) was then filtered through  $0.45 \mu\text{m}$ -filters (Millipore, Carrigtwohill, Co. Cork, Ireland) before use.

#### 1.5.3.2. Static Light Scattering

For soy milk study, the size distribution of particles was determined by SLS, using a Mastersizer S granulometer (Malvern Instrument Ltd, Worcestershire, UK). A He/Ne laser ( $5 \text{ mW}$  power;  $\lambda = 632.8 \text{ nm}$ ) passed through a  $0.5\text{-mm}$ -width measurement cell, in which the mixed dispersion was injected. The laser light scattered by the sample was converged by a

Reverse Fourier 300-mm focusing lens (0.45-mm focal distance) and directed onto 42 photodiodes localized at various angles. The laser light is scattered by a particle with an angle inversely proportional to its size. Indeed contrarily to small particles, big particles scatter a large quantity of light with a small angle.

Particle size measurements were carried out for CC supplemented and pH cycled soy milks, after probe stabilization ( $T_1$ ), calcium equilibrium period ( $T_2$ ), acidification ( $T_3$ ) and alkalization ( $T_4$ ). Soy milk sample diluted in distilled water at ambient temperature was introduced in the small volume dispersion unit and fitted with an obscuration value comprised within 10-15%. The refractive index was set at 1.5295 for proteins and 1.33 for the solvent (distilled water) (Strawbridge et al., 1995).

The size distribution was measured versus the volume distribution of particles (%). The distributions of particle size obtained varied in theory between 0.05 and 880  $\mu\text{m}$ . The data acquisition was made by Malvern software (Sizer Sv2.17). Each experiment was duplicated.

#### **1.5.4. Optical Microscopy**

Optical microscopy was obtained with a contrast phase microscope (Leica DMRB, Wetzlar, Germany). The microscope was connected to a camera (Kappa optoelectronics GmbH, Germany). Pictures treatment was made by Kappa image Base 2.5 software (Kappa optoelectronics GmbH, Germany). Pictures were taken at  $T_1$ ,  $T_2$ ,  $T_3$  and  $T_4$  for CC-supplemented and pH cycled soy milks ( $\text{pH}_{\text{min}} = 3.5$ ).

#### **1.5.5. Zeta potential variations**

The electrophoretic mobility measurements ( $\mu_E$ ) were performed with a Nanosizer ZS (HPPS 5001, Malvern instrument Ltd, Worcestershire, UK) by means of laser Doppler electrophoresis. The sample was put in a standard capillary electrophoresis cell equipped with gold electrodes. The measurement was performed at 25°C.

The zeta potential was calculated from the electrophoretic mobility measurement ( $\mu_E$ ) according to the Henry's law given hereafter; electrophoretic mobility units ( $\mu\text{m}\cdot\text{cm}\cdot\text{s}^{-1}\cdot\text{V}^{-1}$ ) will be referred to e.m.u. in the following.

$$\mu_E = \frac{\varepsilon \zeta f(K\alpha)}{6\pi\lambda} \quad \text{Equation 6}$$

with:

$\zeta$  = Zeta potential (mV)

$\mu_E$  = Electrophoretic mobility (e.m.u.)

$\varepsilon$  = dielectric constant ( $\varepsilon = 78.5$  for water)

$\lambda$  = viscosity (Pa.s)

The electrophoretic mobility measurements were realized in aqueous medium with a low electrolyte concentration. In such conditions we can consider  $f(K\alpha)$  equal to 1.5 value. Hence the simplified expression of zeta potential is at 25°C:

$$\zeta_{(mV)} = 12.85\mu_{E(mV)} \quad \text{Equation 7}$$

The calculated zeta potential reflects the global electrical charges of the particles in motion in an aqueous medium and is commonly used as an indicator of dispersion stability. If all the particles in suspension have a large negative or positive zeta potential then they will tend to repel each other and there will be no tendency for the particles to come together. However, if the particles have low zeta potential values, they would tend to aggregate. For the electrostatically stabilized sample, the point of zero zeta potential is related to the point of minimal stability due to the weakness of repulsive forces between particles: it is around their isoelectrical point. The zeta potential is influenced by pH, ionic strength (through the concentration and the type of ions present) and the concentration of any charged particles in the dispersant.

The electrophoretic mobility was measured in pH cycled skim milk and soy milks, for initial pH and each pH unit all over the pH cycle (pH 6.5, 5.5, 4.5, 3.5), during acidification and back neutralization. These parameters were also determined in skim and soy milks after CC supplementation (25 mmol/kg). Reconstituted skim milk and soy milks were diluted in filtered ultrapure water (pore size 0.22  $\mu\text{m}$ ).

## 1.6. Soluble protein phase characterization

### 1.6.1. SDS-PAGE

During pH cycle to 5.5, soluble proteins contained in reconstituted skim milk, CC or MC supplemented, were characterized at T<sub>1</sub>, T<sub>2</sub>, T<sub>3</sub> and T<sub>4</sub>. Soluble proteins were first extracted from total phase of milk by ultracentrifugation (Sorvall RC M120 GX, les Ulis, France) during 1 h at 110,000 g at 4°C (Dziuba and Muzinska, 1998). Then soluble proteins were separated by Sodium Dodecyl Sulphate PolyAcrylamide Gel electrophoresis (**SDS-PAGE**) using a Bio-Rad mini-gel slab electrophoresis unit (Bio-Rad Laboratories, Richmond, CA, U.S.A). Bench Mark protein ladder (invitrogen, Carlsbad, CA, U.S.A) constituted of 15 engineered proteins ranging from 10 to 220 kDa was used as Molecular Weight indicators (**MW**). Migration occurred under 50 mV. Gel was stained with Coomassie Brilliant Blue R (USBiological, Swampscott, MA, U.S.A).

### 1.6.2. Kjeldahl analysis

The solubility of non hydrolysed and hydrolysed soy proteins was determined in reconstituted SPI solution before homogenization. Acidification (HCl, 1N, 0.5 mL/min) was performed at 20°C under stirring at 200 rpm, directly in a 1L-reactor. 20 mL-samples were taken at the following pH values: 7.0, 6.0, 5.5, 5.0, 4.5, 3.5 and 3.0. After centrifugation (Centrifuge Sigma Bioblock, Osterode, Germany) at 3034 g during 35 min at 20°C (FIL, 1995), supernatants were collected and stored at -20°C until nitrogen content determination by Kjeldahl analysis (Vapodest, Gerhardt, Germany). The soluble nitrogen content was calculated according to equation 8 with correction of dilution effect due to acidification. Protein content in the supernatant was calculated from nitrogen content (×6.25 for soy proteins). Five references were done with distilled water and each sample was measured in triplicate. Soluble nitrogen content was calculated according to the following formula:

$$N_2 = \frac{(V - V_0) \times 0.14}{0.6} \quad \text{Equation 8}$$

With:

N<sub>2</sub>, soluble nitrogen content (g/L),

V, Volume of H<sub>2</sub>SO<sub>4</sub> N/100 titrant (mL),

V<sub>0</sub>, volume of H<sub>2</sub>SO<sub>4</sub> N/100 titrant for the reference,

0.14 (mg/mL) fits with the fact that 1 mL of H<sub>2</sub>SO<sub>4</sub> N/100 reacts with 0.14 mg of N<sub>2</sub>,

0.6 (mL) fits with the volume of supernatant titrated with H<sub>2</sub>SO<sub>4</sub>

### **1.7. Statistical analysis and normalization method**

Statistical analysis was performed using Kyplot freeware. Multiple comparison parametric tests were performed. The same superscript letter was used when no differences between 2 calcium concentrations measured were observed ( $P > 0.05$ ). Statistical analysis was performed independently for the colloidal and the soluble phase.

The Min-Max normalisation method corresponds to the ratio of each value to the maximal value measured during the corresponding experiment.

## **2. Study of calcium-protein interactions in cow skim milk and in soy milks**

### **2.1. Isothermal Titration Calorimetry**

#### **2.1.1. Calcium-protein interactions studied by ITC**

Isothermal Titration Calorimetry (ITC) is a technique of choice for studying the biomolecular interactions and especially mineral-protein interactions (Ladbury and Chowdhry, 1996). Several studies reported the use of ITC for the thermodynamic characterization of calcium binding with proteins such as  $\alpha$ -lactalbumin, calmoduline, lysozyme... (Kuroki et al., 1992; Gopal et al., 1997; Hendrix et al., 2000; Saboury and Karbassi, 2000; Nielsen et al., 2003; Saboury et al., 2005; Yamniuk and Vogel, 2005; Saboury et al., 2006). ITC has not yet been applied to the study of calcium interactions into complex systems as milk proteins or soy proteins suspensions.

By ITC, thermodynamic characterization of the interactions is obtained in addition to the molar ratio of mineral binding with proteins and the binding constants (in: Vanhooren et al., 2002). ITC measures the binding equilibrium directly by determining the heat involved on association of a ligand with its binding partner. In a single experiment, the value of the binding constant ( $K_a$ ), the stoichiometry ( $N$ ) and the binding enthalpy ( $\Delta H$ ) are determined. The free energy ( $\Delta G$ ) and entropy ( $\Delta S$ ) are determined from the binding constant. The enthalpy of the reaction upon calcium binding with protein is either endothermic ( $\Delta H > 0$ ) or exothermic ( $\Delta H < 0$ ) and can be related to the nature of the reaction. An exothermic reaction

is reported to characterize attractive interaction, mainly of electrostatic nature (Sinn et al., 2004), and would be expected in the study of calcium-protein interaction. Unexpectedly, endothermic reactions are often observed upon calcium binding. Hence not Coulomb interactions, but rather liberation of water molecules from the hydration shells of the component is expected as the driven energy source for the binding of multivalent ions with polyelectrolytes (Sinn et al., 2004). The temperature dependence of the  $\Delta H$  parameter, measured by performing the titration at varying temperatures, describes the heat capacity ( $\Delta C_p$ ). Both the hydrophobic and the polar residues can be exposed to water, but contribution of the latter to the  $\Delta C_p$  is smaller, and additionally can be cancelled by the burial of the polar groups in the protein binding center (in: Niedzwiecka et al., 2002). The  $\Delta C_p$  is closely related to changes in the exposure of hydrophobic surfaces occurring upon protein (un)folding (Privalov and Makhatadze, 1992; Oobataka and Ooi, 1993; Freire, 1995). A decrease in  $\Delta C_p$ , observed in protein folding and other self assembly processes involving proteins (Livingstone et al., 1991), indicates a decrease in exposure of hydrophobic surface (Makhatadze and Privalov, 1990). The extensive hydration and a reduction in the number of surface-exposed polar residues (presumably the burial of charged ligand and protein polar groups in the binding site) can make the  $\Delta C_p$  sign positive (Hileman et al., 1998; Niedzwiecka et al., 2002). A positive  $\Delta C_p$  is relevant to the protein unfolding. Finally the stronger the binding, the less positive the  $\Delta C_p$  is. Therefore, the  $\Delta C_p$  values offer direct information on the extent of the conformational changes of proteins (Griko, 1999, 2000). The binding titration curve and the appropriate fit of experimental data determine whether there is one or more calcium binding sites, independent or not, involving positive or negative cooperativity phenomenon. More, enthalpy-entropy compensation is often observed in some proteins due to the linkage system involving either conformational changes or change of state. Kuroki et al. (1992) reported that enthalpy-entropy compensation observed upon binding of  $\text{Ca}^{2+}$  could be attributed to protein folding. Hence, in combination with structural information, the energetics of binding can provide a complete profile of the interaction.



## 2.1.2. Theory

### 2.1.2.1. General principle

Isothermal Titration Calorimetry (**ITC**) is a thermodynamic technique used for monitoring any chemical reaction initiated by the addition of a binding component. It has become the method of choice for characterizing biomolecular interactions. When two molecules interact, heat is either released or absorbed. Measurement of this heat allows accurate determination of binding constants ( $K_B$ ), reaction stoichiometry ( $N$ ), enthalpy ( $\Delta H$ ) and entropy ( $\Delta S$ ), thereby providing a complete thermodynamic profile of the molecular interaction in a single experiment. In a typical ITC experiment, a syringe containing a “ligand” solution is titrated into a cell containing a solution of the “macromolecule” kept at constant temperature. VP-ITC microcalorimeter from MicroCal (North-Hampton, MA, U.S.A) was used in this study (figure 18).

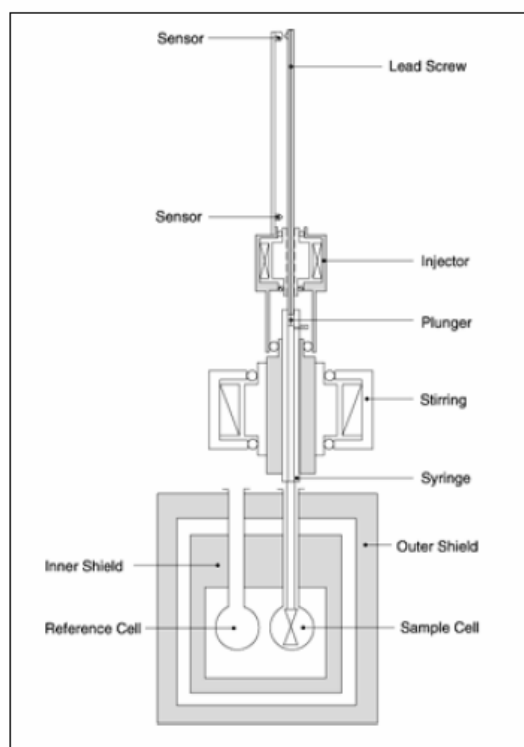


Figure 18. The VP-ITC apparatus (MicroCal, North-Hampton, MA, U.S.A)

When ligand is injected into the cell, the two materials interact. The heat ( $\Delta H$ ), released or absorbed, is directly proportional to the amount of binding occurring over the time (figure 19, top panel). As the macromolecule in the cell becomes saturated with ligand, the heat signal diminishes until only background heat of dilution is observed. A binding curve and its

specific binding parameters ( $N$ ,  $K$ ,  $\Delta H$ ,  $\Delta S$ ) are then obtained after integration from a plot of the heats of binding obtained from each injection against the ratio of ligand (figure 19, lower panel).

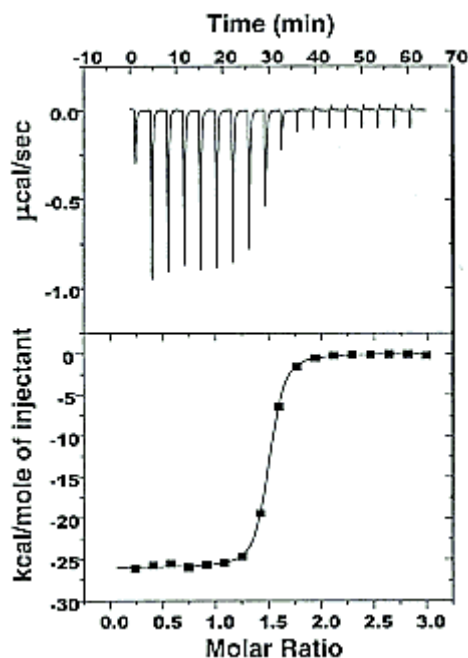


Figure 19. Example of an ITC experiment. Heat of binding (top panel) and the corresponding binding curve (lower panel)

An exothermic reaction yields a negative peak signal because the released heat in the sample cell is no longer required from the resistive heater of the instrument. On the contrary, endothermic reactions cause a positive peak.

This amount of heat ( $q_i$ ) is proportional both to the amount of ligand that binds to the protein in a particular injection ( $v \times \Delta L_i$ ) and to the characteristic binding enthalpy ( $\Delta H$ ) for the reaction as described in equation hereafter.

$$q_i = v \times \Delta L_i \times \Delta H \quad \text{Equation 9}$$

Where  $v$  is the volume of the reaction cell and  $\Delta L_i$  is the increase of concentration of bound ligand after the  $i^{\text{th}}$  injection.

A modern ITC instrument operates on the heat compensation principle: the instrumental response (measured signal) is the amount of power ( $DP$ ,  $\mu\text{cal/sec}$ ) necessary to maintain the temperature difference between the reaction and the reference cells. The heat ( $\Delta H$ ,  $\text{kcal/mole}$

of injectant) after each injection is therefore obtained by integration of the area under each peak. Because the amount of uncomplexed protein available progressively decreases after each injection, the magnitude of the peak becomes progressively smaller until complete saturation is achieved. Once this situation is reached, subsequent injections produce similar peaks corresponding to dilution or mechanical effects that need to be subtracted from the entire injection peaks before analysis.

#### 2.1.2.2. Equations used for fitting ITC binding data

Origin software provides three built-in curve fitting models: one set of sites, two sets of sites and sequential binding sites. Each fitting model has a unique set of fitting parameters. Only the two first fitting models will be further detailed since they are discussed in this study. The last model of sequential binding sites will not be discussed here.

The corrected area under each peak is given by the equation 10, which is used to analyse the data. The quantity  $\Delta L_i$  is the difference between the concentration of bound ligand in the  $i^{\text{th}}$  and  $(i-1)^{\text{th}}$  injections, and its functional form depends on the specific binding model.

##### 2.1.2.2.1. One set of sites model

Each fitting model has a unique set of fitting parameters. For the one set of sites model, these are **N** (number of sites in molar ratio: moles of ligand per moles of protein), **K** (binding constant in  $M^{-1}$ ) and  $\Delta H$  (heat change in cal/mole). For the simplest case, in which the protein has one binding site, equation becomes:

$$q_i = v \times \Delta H \times [P] \times \left( \frac{K_a [L]_i}{1 + K_a [L]_i} - \frac{K_a [L]_{i-1}}{1 + K_a [L]_{i-1}} \right) \quad \text{Equation 10}$$

Where [P] is the protein concentration,  $K_a$  is the binding constant and [L] is the free ligand concentration.

As the known experimental quantity is the total concentration, rather than the free ligand concentration, the equation above needs to be rewritten in terms of the total ligand concentration. The solution for this has been published by Wiseman et al. (1989) and Sinn et al. (2006). The whole mathematical equations used for one set of sites are given in appendix 3.

#### 2.1.2.2.2. Two set of sites model

The model for one set of sites discussed just above can be used for any number of sites  $N$ , if all the sites have the same  $K$  and  $\Delta H$ . If a macromolecule has two types of sites with different values of  $K$  and/or  $\Delta H$ , then the model with two sets of sites must be used. The whole mathematical equations used for fitting the two set of sites models are given in appendix 3.

### 2.1.3. ITC Procedure

Two identical spherical cells, a reference cell and a sample cell, both with a volume of 1.449 mL, are enclosed in an adiabatic jacket. Before each experiment, both solutions were degassed during 7 min to eliminate air bubbles which could falsify the baseline. Then, the working cell was filled with the milk or soy protein solution, and the reference cell was filled with the solvent used to prepare the sample solution (miliQ water). The titrant, a Calcium Chloride solution (CC,  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , Carl Roth GmbH, Karlsruhe, Germany) prepared in miliQ water, was injected stepwise into the working cell. The sample cell was constantly stirred (300 rpm). The measurement was performed at a constant temperature. Small aliquots of titrant (typically 2 or 5  $\mu\text{L}$ ) were successively injected into the solution of the working cell. The five first injections were usually set to a volume of 2  $\mu\text{L}$ . The total number of injections varied according to the experiment in order to reach saturation; the spacing time was of 300 s. Because of possible dilution during the equilibration time preceding the measurement, the first injection was ignored in the analysis of the data. Each injection produces a characteristic peak in the heat flow due to the released or absorbed heat. Since we had little information about the expected heats of our systems, the reference power of the ITC instrument was set at 15  $\mu\text{cal} \cdot \text{s}^{-1}$ . The experimental conditions of each ITC experiment are summarized in table 12 and table 13 for the whole protein sample and the soluble protein sample study respectively. The whole protein samples were studied in the case of both milks. The soluble protein sample was studied for soy proteins only. Each experiment was duplicated.

Table 12. ITC experimental parameters for the study of calcium-protein interaction in the whole protein fraction (NH: Non Hydrolysed; H: Hydrolysed) performed at 25°C

Sample	[CC] mM	Number of injections	Titration
Cow skim milk	50	65	2 $\mu\text{L}$ *10 and 5 $\mu\text{L}$ *55
NH- Soy milk	25	32	2 $\mu\text{L}$ *5 and 5 $\mu\text{L}$ *27
H-Soy milk	25	32	2 $\mu\text{L}$ *5 and 5 $\mu\text{L}$ *27

Table 13. ITC experimental parameters for the study of calcium-soy protein interaction in the soluble protein fraction (NH: Non Hydrolysed; H: Hydrolysed)

Sample	T <sub>exp</sub> (°)	[CC] mM	Number of injections	Titration
NH- Soluble proteins	4	5	50	2 μL*5 and 5 μL*45
	25	5	50	2 μL*5 and 5 μL*45
	50	5	50	2 μL*5 and 5 μL*45
H- Soluble proteins	4	20	50	2 μL*5 and 5 μL*45
	25	20	40	2 μL*5 and 5 μL*35
	50	25	50	2 μL*5 and 5 μL*45

In the analysis, the titration of calcium solution into miliQ water was studied as the reference experiment (i.e. ligand solution added to buffer in cell without the presence of macromolecule). This former experiment determined the heat of dilution of ligand (CC). This reference experiment, carried out in the same way as the titration with proteic sample was subtracted from the sample data. Reference corresponds to the signal between consecutive injections when no change in the heat flow was detected. Before subtracting the reference, the molar ratio was set similar for both set of data. Once subtraction performed, the Area Data ( $\Delta H$ ) were fitted by ORIGIN software, using the “One set of sites” built-in curve fitting model. Fitting cycles were repeated until Chi<sup>2</sup> value was not reduced anymore and fitting parameters (N, K,  $\Delta H$ ,  $\Delta S$ ) were obtained.

In the study of soluble proteins fraction, soy proteins aggregates were discarded by precipitation at the isoelectrical pH (4.5) and soluble proteins collected were back neutralized up to reach the initial pH (~7.1-7.2). Experiments were carried out at three different temperatures (4, 25 and 50°C). Indeed measuring the binding isotherm at least for two temperatures allows additional determination of the change in heat capacity ( $\Delta C_p$ ) of binding through the following relation:

$$\Delta C_p = \frac{\Delta H^0_{T_2} - \Delta H^0_{T_1}}{T_2 - T_1} \quad \text{Equation 11}$$

This thermodynamic parameter is a good indicator of changes in exposure of polar and non-polar residues.

The usual binding curves plotted in kcal/mol of injectant vs. the molar ratio (mole Ca/ mole of protein) will be presented in this study versus the mass ratio (g Ca/ g of protein). Indeed due to the complex mixture of proteins sample, this presentation will be more appropriate.

## 2.1.4. Milks reconstitution for ITC

### 2.1.4.1. Cow skim milk

For calcium-protein interaction study, cow skim milk was reconstituted in miliQ water from low heat skim milk powder (Ingredia, Arras, France) at 1% (w/w) protein concentration. Milk was magnetically stirred during 3 hours to enable mineral re-equilibration and then desalted on PD10 desalting column (Amersham Biosciences, Buckinghamshire, UK). According to their specification, former column removed up to 95% of salts. Samples were stored at -20°C until Isothermal Titration Calorimetry analysis.

### 2.1.4.2. Soy milk

#### 2.1.4.2.1. Study on whole soy proteins

For the study of calcium-soy protein interactions in whole soy proteins fraction, non hydrolysed and hydrolysed soy milks were reconstituted in miliQ water, from their respective SPI (SUPRO 760, Non-GM Isolated Soy Protein, The Solae Company, Barcelona, Spain; Non-GM Isolated Soy Protein 219, Dupont Protein technology, St Louis, MO, USA) at 1% (w/w) protein concentration. Soy milks were stabilized by ultraturrax treatment (13500 rpm, 5 min) followed by homogenization (500 bar, 5 cycles). Then desalting step was performed on PD10 desalting column (Amersham Biosciences, Buckinghamshire, UK) to remove calcium and phytate ions initially present. Samples were stored at -20°C until ITC analysis.

#### 2.1.4.2.2. Study on soluble soy proteins

For the study of calcium-soy protein interactions in soluble proteins fraction, soy milks were reconstituted in miliQ water at 4.2% (w/w) protein concentration. Then soy milks were acidified (HCl, 1N, Riedel-de Haën, Seelze, Sigma Aldrich, Germany) in a reactor vat ( $T_{\text{exp}} = 20^{\circ}\text{C}$ , 150 rpm) up to reach the isoelectrical pH of soy proteins ( $\text{pH}_i = 4.5$ ). Acidified milk was centrifuged (Centrifuge Sigma Bioblock, Osterode, Germany) at 3034 g, 35 min, 20°C (FIL, 1995). The supernatant containing soluble soy proteins was collected and back neutralized (NaOH, 1N, Riedel-de Haën, Seelze, Sigma Aldrich, Germany) up to reach the

initial pH of soy milk (~7.1-7.2). Then desalting step was performed on PD10 desalting column (Amersham Biosciences, Buckinghamshire, UK). Samples were stored at -20°C until ITC analysis.

## **2.2. Protein concentration determination**

Protein concentration was determined by Bradford quantification. Calibration curve was performed with Bovine Serum Albumin solution at 1.35 mg.mL<sup>-1</sup>. Various concentrations ranging from 0 to 1.35 mg.mL<sup>-1</sup> were prepared. Then proteins were dyed with Bradford reagent, previously diluted 5-times (G250 100 mg, Bio-Rad S.A, Marne La Coquette, France; ethanol 50 mL; Phosphoric acid 85% 100 mL, Labosi, Elancourt, France; distilled water 100 mL). At 20 µL of each point of the calibration curve and of studied samples, 1 mL of diluted Bradford reagent was added. The incubation time lasted around 2-3 min. After agitation, absorbance was read at a wavelength of 595 nm on spectrophotometer (U.V-160 A, Shimadzu Europa GmbH, Germany). The proteic concentration contained in each sample was determined by the mean of calibration curve (absorbance measured versus protein concentration, mg.mL<sup>-1</sup>).

## **2.3. Calcium binding isotherms**

Calcium binding isotherms were measured using the Calcium Ion Selective Electrode (Ca ISE, Sentek, Estate, Braintree, United Kingdom). Calibration was carried out with CC calibrating solutions of 10<sup>-1</sup>, 10<sup>-2</sup>, 10<sup>-3</sup>, and 10<sup>-4</sup>M prepared in NaCl 0.1M. Same experiments than ITC performed on whole protein fraction were done on a larger volume of cow skim or soy milk sample. CC volume added was kept proportional with the volume of milk. In the calcium binding isotherms, concentration of free/bound Ca<sup>2+</sup> (mM) was plotted versus the concentration of added Ca<sup>2+</sup> (mM).

## **2.4. Electrophoretic mobility and zeta-potential variations**

Similar experiments than ITC performed on whole protein fraction were reproduced on cow skim milk or soy milks with higher volume of sample in order to determine the electrophoretic mobility and the zeta potential variations upon CC titration. The former parameters were determined by Zeta Sizer (Malvern instrument Ltd, Worcestershire, UK).

Volumes of milks and CC titrated were scaled up to keep proportionality with ITC reaction cell.

## 2.5. Fourier Transformed Infra-Red spectroscopy

### 2.5.1. Theory

Fourier Transformed Infra-Red spectroscopy (**FTIR**) is used for identifying the types of chemical bonds (functional groups) in a molecule by producing a characteristic infrared absorption spectrum (“a molecular fingerprint”). This is based on the absorption of an infrared radiation by the analysed material.

Infra-red spectroscopy enables the measurement of the vibration energy of molecular interactions. The effect of an electromagnetic infra-red radiation on molecular bonds induces dipoles formation. The former ones are then able to absorb the energy when oscillatory frequency of the chemical bond is equal to the one of the issued wave. Several vibration modes exist. Stretching vibrations, symmetric or anti-symmetric, affect the length of atomic interaction. On the contrary, bending vibrations occur in the molecular plan or out-of-plan.

Spectra present the sample absorption versus the wavenumber  $\nu$  ( $\text{cm}^{-1}$ ), the inverse number of the wavelength  $\lambda$  (cm). Stretching wavenumbers are always higher than bending ones since less energy is needed to bend than to stretch a molecule. Infra-red is composed of three ranges: the Near Infra-Red ( $14283\text{-}4000\text{ cm}^{-1}$ ), the Medium Infra-Red used in this study ( $4000\text{-}400\text{ cm}^{-1}$ ) and the Far-Infra-Red ( $400\text{-}10\text{ cm}^{-1}$ ).

In transmission Infra-Red spectrometry, a monochromatic laser beam of specific wavelength passes through the analysed sample. Due to the light absorption by the sample, the beam intensity is attenuated from  $I_0$  down to  $I_T$  (with  $I_T < I_0$ ). The principle remains the same for the refraction mode used in this study.

When the surface of the lit up sample is bright, light is back reflected according to the classic principle of geometric optic. The reflected waves propagate in the same plane than the incident waves and their direction form an angle with the normal, which is equal to the incident angle (Bertrand and Dufour, 2006).

The critical angle is defined by its sine (equation 12):

$$\text{Sine}\phi = \frac{n_2}{n_1} \quad \text{Equation 12}$$

With  $n_1$ , the refraction index of the solid medium on which is the sample and  $n_2$ , the refraction index of the studied sample.



Coming from the denser medium and for incident angles higher than  $\phi$ , the beam undergoes a total reflection inside the medium of higher density (figure 20). Attenuated Total Reflection (ATR) comes from the fact that the reflected light is partially attenuated by the sample absorption.

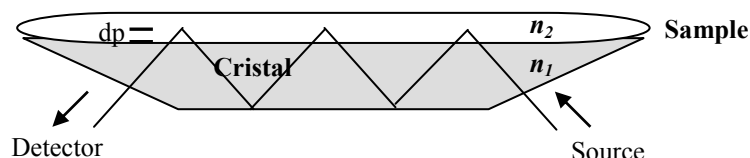


Figure 20. Process of Attenuated Total Reflection (ATR) occurring at the higher surface of a flat ATR crystal (refraction index  $n_1$ ) and sample (refraction index  $n_2$ ) in which the evanescent waves penetrate up to a depth ( $dp$ )

The depth of penetration of the evanescent waves inside the sample can be calculated by equation 13:

$$dp = \frac{\lambda}{\sqrt{2\pi n_1 * (\sin^2 \phi - (\frac{n_2}{n_1})^2)}} \quad \text{Equation 13}$$

By detecting characteristic vibrations of chemical bonds, FTIR measures their respective absorption energy and enables the determination of chemical functions (alcohol, amide, keton...) content in the sample. Moreover, in an absorption spectrum, the absorption intensity of each peak is directly proportional to the concentration of the chemical bond. The absorption wavelength depends of the type of chemical bond but on the molecular environment as well. Table 14 gives the characteristic spectra of various functional groups.

Table 14. Characteristic bands of various functional groups (Bertrand and Dufour, 2006)

Vibration Mode	Functional group	Band position (cm <sup>-1</sup> )
Stretching vibration	O-H	3590-3650
	C-H	2850-3300
	C-O	900-1200
	C-Cl	700
	C-F	1100
	C=C	1600-1680
	C=O	1700-1750
	C≡C	2000-2200
	C≡N	2200-2300
Bending vibration	-CH <sub>2</sub>	1300-1600
	C=CH <sub>2</sub>	890
	HC=CH cis	690
	HC=CH trans	965
	C=CH trisubstituted	790-840

### 2.5.2. Procedure

For Infra-Red analysis, cow skim milk and soy milks were reconstituted at 4.2% (w/w) protein concentration. Soy milks were stabilized by ultraturrax treatment (13500 rpm, 5 min) followed by homogenization by high pressure (500 bar, 5 cycles).

Experiments were done with Fourier Transformed Infra-Red spectrometry, in Attenuated Total Reflection (ATR-FTIR) at room temperature using a Tensor 27 mid-FTIR Bruker spectrometer (Brüker, Karlsruhe, Germany) equipped with an ATR optic cell and a RT-DTGS detector (Quick silver-cadmium-Tellurium, Brüker, Karlsruhe, Germany). The diaphragm was set at 4 mm and the scanning speed was of 20 kHz. 256 scans were acquired for each sample and each reference between 4000 and 900 cm<sup>-1</sup>, with a spectral resolution of 2 cm<sup>-1</sup>. Each experiment was duplicated. Raw spectra were treated using OPUS/IR software (Brücker, Wissemburg, France).

Each raw spectrum was first corrected for the atmospheric CO<sub>2</sub> and H<sub>2</sub>O contribution, smoothed over 9 points using a Savitzky-Golay algorithm and each baseline was corrected over 4 points. Then specific vibrational bands were vectorially normalized and a second derivative was applied. This former treatment improves the spectral resolution through the

detection of shoulders that are present for important spectral overlapping. The sign of second derivative is opposite to the original spectrum and is oriented downward.



## **IV. RESULTS AND DISCUSSION**



## 1. Protein phase behaviour and calcium equilibrium between soluble and colloidal phase in cow skim milk supplemented in calcium and subjected to pH cycle

**ABSTRACT.** Calcium equilibrium between soluble and colloidal phases was studied in Ca-supplemented cow milk upon pH cycle and related to protein phase variations. Ca-supplementation (0-25 mmol/kg) with Calcium Chloride (CC) or Milk Calcium (MC) was followed by pH cycle ( $\text{pH}_{\min}$  5.5, 5.0 and 3.5). pH, ionized calcium and turbidity variations were recorded *in situ* and protein phase was characterized by Dynamic Light Scattering (DLS) and electrophoresis (SDS-PAGE) at specific times of the pH cycle.

For moderate pH cycles ( $\text{pH}_{\min}$  5.5 or 5.0), reference and MC-milk showed similar trends. Ionized Ca increased and decreased during acidification and alkalization respectively, related to micellar mineralization variation. Particle size and turbidity decreased all over the pH cycle until it reached an advanced state of colloidal phase destructuring for pH cycle to 5.0. Yet pH cycle was reversible neither on ionized calcium variations nor on protein phase variations. In milk supplemented with CC, moderate pH cycle had less influence than in reference and MC milk. The particle size and turbidity increased upon CC-supplementation. Then, acidification gave also a moderate turbidity decrease that could be explained by casein micelle slight destructuring. Yet micelles remained more mineralized during acidification and this is supposed to stabilize casein monomers inside. Micellar material solubilization was reversible upon alkalization indicating that casein micelles were less destabilized upon pH cycle when supplemented with CC. Reversibility could be due to the previous capture of Ca, involving casein micelles reinforcement through Ca-protein interactions of electrostatic nature.

The pH cycles to 3.5 led to irreversible phenomena. Turbidity and ionized calcium variations showed more complex profiles. Extreme values implying large micelle destructuring (minimum in turbidity and maxima in ionized calcium) were reached at around the  $\text{pH}_i$  of caseins.

## 1.1. Introduction

In this chapter, the effect of calcium salt supplementation is studied during pH cycle in reconstituted skim milk. The aim of the present study was to determine the solubilization of two different calcium salts commonly used in food industry for further milky systems enrichment. Two calcium salts were compared for their distinct physicochemical properties: Calcium Chloride (CC) for its high solubility and its common use and Milk Calcium (MC) for its potential use as natural source of calcium for enrichment of dairy products. Indeed, MC is derived from milk and offers a healthy image in labelling (EDM, 1998). With a good sensorial profile, this calcium source is used in a wide variety of products at relatively high concentrations without causing off-flavour.

Calcium addition was analyzed in relation to calcium distribution between the soluble and the colloidal phases in milk subjected to pH cycle.

Moderate acidification was performed in a first part of the study with two minimal pH values chosen at pH 5.5 or 5.0. This narrow pH range was reported to be of interest on reversible variation of casein micellar structure (Gastaldi et al., 1996). Calcium salt concentrations added were 0, 10, 15 and 25 mmoles/kg of skim milk.

In a second part of this study, pH cycle to pH 3.5 was studied in order to characterize the phenomena occurring below isoelectrical pH of caseins ( $\text{pH}_i \sim 4.6$ ). The experimental set-up described in a previous work (Gaiani et al., 2005) allowed data acquisition *in situ* of various physicochemical parameters, i.e. pH, ionized calcium and turbidity.

## 1.2. Influence of pH cycle on milk conductivity

### 1.2.1. CC-milk subjected to pH cycle to 5.5 or 5.0

Conductivity was plotted as a function of pH ( $\text{pH}_{\text{min}}$ : 5.5; figure 21), for all the CC concentrations studied (0, 10, 15 and 25 mmoles/kg). The conductivity increases with CC addition, during acidification and alkalization. The more the CC added, the higher the overall conductivity measured. Yet whatever the CC supplementation is, similar rate of conductivity variation is observed especially during acidification. As an acidifying salt, the pH reached after CC addition and equilibrium is lower for higher CC concentration. This is related to the replacement of  $\text{H}^+$  by  $\text{Ca}^{++}$  in the vicinity of proteins. Similar trends were obtained for pH cycle to 5.0.



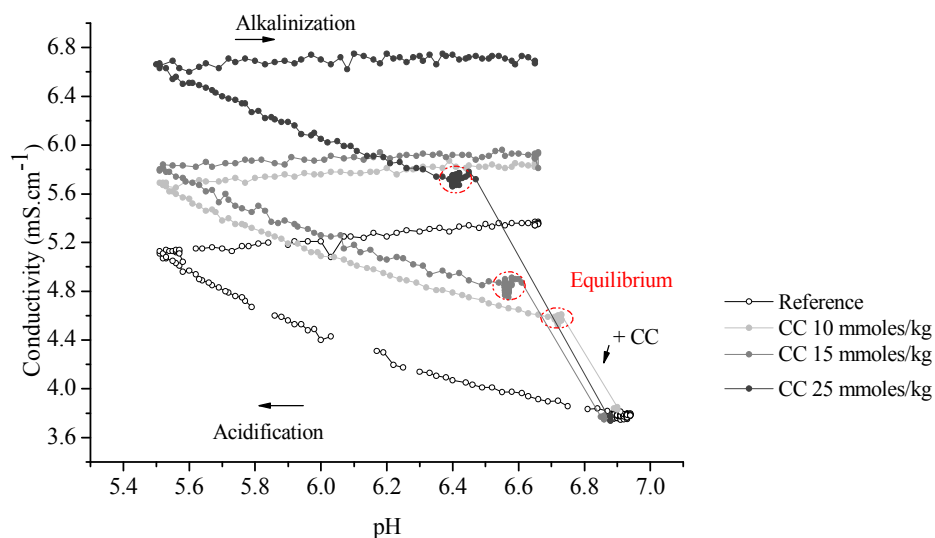


Figure 21. Conductivity ( $\text{mS}\cdot\text{cm}^{-1}$ ) as a function of pH for CC-milk subjected to pH cycle to 5.5 ( $T_{\text{exp}} = 4^{\circ}\text{C}$ )

### 1.2.2. MC-milk subjected to pH cycle to 5.5 or 5.0

Reconstituted skim milk was MC supplemented (0, 10, 15 and 25 mmol/kg) and subjected to pH cycle to 5.5. Conductivity ( $\text{mS}\cdot\text{cm}^{-1}$ ) was plotted as a function of pH (figure 22). Contrarily to CC supplementation, the similar conductivity signal to the reference milk is observed upon pH cycle whatever the MC supplementation. This would suggest that MC is inert and does not dissociate in the medium whatever the concentration added. More, conductivity increases all over the pH cycle and the rate of conductivity increase is higher during acidification than during alkalinization.

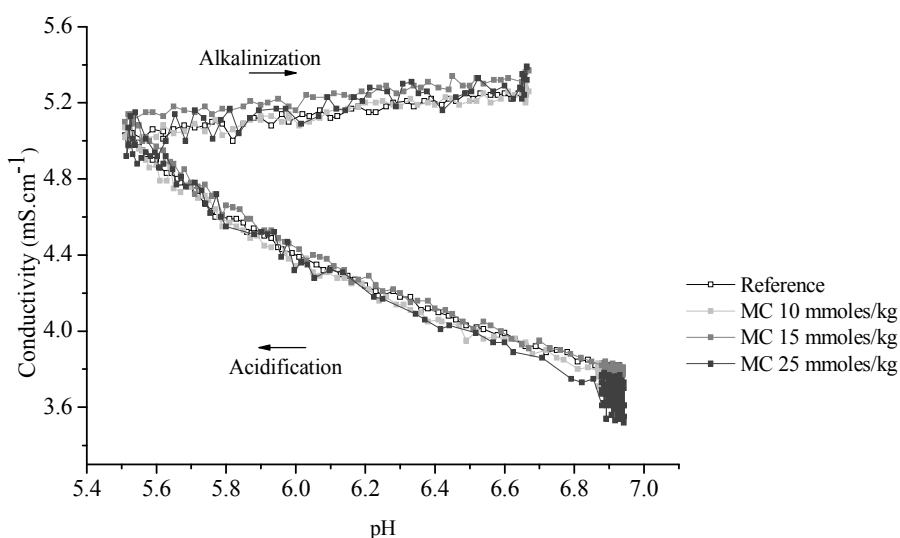


Figure 22. Conductivity ( $\text{mS}\cdot\text{cm}^{-1}$ ) as a function of pH for MC-milk subjected to pH cycle to 5.5 ( $T_{\text{exp}} = 4^{\circ}\text{C}$ )

Skim milk was supplemented with MC (0, 10 or 25 mmol/kg) and subjected to pH cycle to pH 5.0. Similar conductivity signal was observed as a function of pH, whatever MC supplementation (results not shown). This absence of variation after MC supplementation would suggest that MC remains inert and does not react with the medium even for pH cycle to 5.0. Conductivity reached at the end of pH cycle to 5.0 was higher than former pH cycle ( $\sim + 1 \text{ mS.cm}^{-1}$ ).

### **1.3. Calcium variations kinetics during pH cycle**

#### **1.3.1. Calcium variations during pH cycle to 5.5 or to 5.0**

Around 1.8 mM of ionized calcium was measured in skim milk at pH 6.9 and 4°C similarly to the 2 mM found by Philippe et al. (2003) at pH 6.65 and 25°C and in accordance with the low influence of temperature on mineral solubilization (Dalglish and Law, 1989).

In order to facilitate comparison between skim milk and calcium salt supplemented milks (CC-milk and MC-milk), normalized ionized calcium concentration variations are presented as a function of normalized time in figure 23.

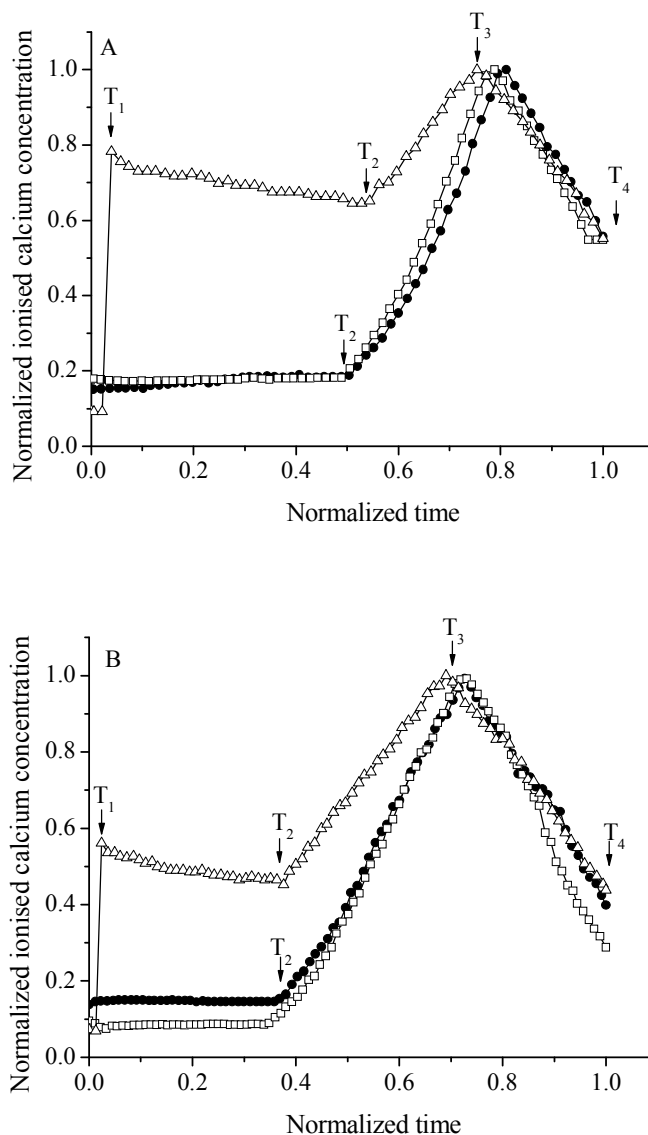


Figure 23. Normalized ionized calcium concentration (M) as a function of normalized time for skim milk (-●-), and for MC-milk (-□-) and CC-milk (-Δ-) subjected to pH cycle (A: pH<sub>min</sub> 5.5, B: pH<sub>min</sub> 5.0). T<sub>1</sub> = end of probes stabilization; T<sub>2</sub> = end of calcium salt equilibrium; T<sub>3</sub> = end of acidification; T<sub>4</sub> = end of neutralization. T<sub>exp</sub> = 4°C.

The profiles as a whole can be divided into three phases: the stabilization of ionized calcium after calcium salts addition, the increase of ionized calcium concentration with acidification and the decrease of ionized calcium concentration with neutralization.

From figure 23, distinct behaviours are observed for CC and MC supplemented milks. Ionized calcium concentration increases just after CC supplementation whereas no significant variation occurs with MC supplementation. In the meantime, CC addition reduces milk pH from pH 6.9 to pH 6.4 displacing protons from proteins. During the stabilization period

following salts addition ( $T_1$  to  $T_2$ ), a decrease in ionized calcium concentration for CC supplemented milk is observed from 16 mM to around 13 mM. The ionized calcium concentration variations in CC milk could be related to calcium ions equilibrium between the soluble and colloidal phases of milk. Then, the decrease should correspond either to the formation of complexes with soluble anions, i.e. citrate or phosphate, or to the absorption of calcium ions into casein micelles. No variations were observed in the case of MC addition. Ionized calcium variations observed after MC or CC addition fit with the solubility of calcium salts, commonly reported in the literature, i.e. high for CC and poor for MC. Moreover, experiments performed with pure calcium phosphate ( $\text{CaHPO}_4$ , calcium initially present in milk and in MC) showed similar behaviour than MC supplementation (results not shown).

For the three dairy systems in figure 23, ionized calcium concentration increases and decreases during acidification ( $T_2$  to  $T_3$ ) and neutralization ( $T_3$  to  $T_4$ ), respectively. This reflects the micellar pH-dependent demineralization described by many authors. For the reference skim milk, this would especially fit with the solubilization of Colloidal Calcium Phosphate (CCP), when milk pH is reduced (Brulé and Fauquant, 1981; Pierre and Brulé, 1981; Chaplin, 1984; Van Hooydonk et al., 1986; Dalgleish and Law, 1989; Banon and Hardy, 1992).

Calcium ionized during acidification step may be endogenous (initially present in the colloidal phase before calcium supplementation) or exogenous (supplemented calcium) or both. Le Graët and Brulé (1993) reported that, in milk, Colloidal Calcium Phosphate would be first solubilized when pH is decreased up to 5.2 because of concomitant solubilization of calcium and phosphate ions. Below pH 5.2, only calcium bound to phosphoserines and carboxyl casein groups was solubilized. Dalgleish and Parker (1980) demonstrated that the strength of binding of calcium ions to  $\alpha_{s1}$ -caseins was decreased with decreasing pH. The decrease in cations binding by caseins on reducing pH reflects the decrease in ionization state of the phosphoseryl, glutamyl, aspartyl, tyrosyl and histidyl residues of proteins and indicates that the interactions between casein molecules and cations are of electrostatic nature.

Philippe et al. (2003) studied the acidification of CC-supplemented skim milk (0-13.5 mmol of CC / kg of milk) in the pH range 6.7 to 3.5. On the contrary, these authors only observed the solubilization of milk CCP since whatever the added calcium concentration, solubilization curves of Ca and Pi vs. pH were similar. These authors concluded that whatever

the added calcium concentration, the behaviour of CCP seemed to be unchanged upon acidification.

In our experiment, a hysteresis phenomenon was observed upon pH cycle. Indeed, acidification and alkalization phases were not superimposed even though same normality (1N) and addition rate (1 mL/min) were used for acid/base addition.

During pH cycles, normalized profiles of ionized calcium versus time (figure 23) vary largely and similarly for reference and MC-milk compared with the lower variations measured for CC-milk. During acidification to pH 5.5 and pH 5.0, up to 80 and 90% ionized calcium is solubilized with reference or MC-milk, respectively. In the meantime, 35% (at pH 5.5) and 55% (at pH 5.0) of calcium is ionized from CC-milk. The calcium released from the colloidal phase to the soluble phase (compared with reference and MC-milk) is reduced in CC-milk. This can be related to a higher concentration in ionized calcium measured in the soluble phase after CC supplementation and hence a lower electromotric strength, decreasing the transfer of calcium from the colloidal to the soluble phase.

During neutralization phase, ionized calcium concentration is restored only for CC-milk indicating the reversibility of calcium ionization in this dairy system.

The concentrations of soluble and colloidal calcium are indicated in tables 15 and 16 for pH cycle to pH 5.5 and to pH 5.0, respectively. Colloidal calcium refers either to micellar calcium or to insoluble calcium particles. Soluble calcium represents an ionized form ( $\text{Ca}^{2+}$ ) or complexed form with anions such as citrate and phosphate.

Table 15. Colloidal and soluble calcium concentrations (mmoles/kg) during milk pH cycle to pH 5.5<sup>1</sup>

	T <sub>1</sub> Mean	SD	T <sub>2</sub> Mean	SD	T <sub>3</sub> Mean	SD	T <sub>4</sub> Mean	SD
<i>Colloidal calcium</i>								
Skim milk	26 <sup>a</sup>	2	25 <sup>ac</sup>	0.7	9	5.5	18 <sup>cd</sup>	2.6
MC milk	24 <sup>ad</sup>	3	48 <sup>b</sup>	1.3	36 <sup>e</sup>	4.6	42 <sup>be</sup>	2.2
CC milk	28 <sup>a</sup>	7	42 <sup>be</sup>	2	26 <sup>acd</sup>	7	39 <sup>e</sup>	4
<i>Soluble calcium</i>								
Skim milk	10 <sup>f</sup>	0.6	11 <sup>f</sup>	1	25 <sup>g</sup>	0.4	19 <sup>h</sup>	0.4
MC milk	10 <sup>f</sup>	1	10 <sup>f</sup>	0.5	23 <sup>g</sup>	1.7	18 <sup>h</sup>	1
CC milk	11 <sup>f</sup>	0.2	26 <sup>g</sup>	4	39	2	24 <sup>g</sup>	2.7

<sup>a-h</sup> The same superscript letter was used when no differences between 2 calcium concentrations measured were observed ( $P > 0.05$ ).

<sup>1</sup>Statistical analysis was performed independently for colloidal and soluble phase. SD = Standard Deviation. T<sub>1</sub> = end of probes stabilization; T<sub>2</sub> = end of calcium salt equilibrium; T<sub>3</sub> = end of acidification; T<sub>4</sub> = end of neutralization.

MC = Milk Calcium supplemented milk; CC = Calcium Chloride supplemented milk

Table 16. Colloidal and soluble calcium concentrations (mmoles/kg) during milk pH cycle to pH 5.0<sup>1</sup>

	T <sub>1</sub>		T <sub>2</sub>		T <sub>3</sub>		T <sub>4</sub>	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
<i>Colloidal calcium</i>								
Skim milk	25 <sup>af</sup>	1.1	27 <sup>ag</sup>	3	2.8	0.1	12 <sup>c</sup>	3
MC milk	27 <sup>a</sup>	1.6	46 <sup>b</sup>	0.6	18 <sup>cf</sup>	5.2	41 <sup>bd</sup>	2.3
CC milk	25 <sup>af</sup>	1.2	40 <sup>be</sup>	3.8	19 <sup>ac</sup>	11.7	35 <sup>deg</sup>	2
<i>Soluble Calcium</i>								
Skim milk	11 <sup>h</sup>	0.5	11 <sup>h</sup>	0.7	34 <sup>i</sup>	1.5	24 <sup>k</sup>	2.3
MC milk	11 <sup>h</sup>	0.5	11 <sup>h</sup>	0.2	37 <sup>ij</sup>	1.8	20 <sup>k</sup>	0.5
CC milk	11 <sup>h</sup>	1.1	26 <sup>k</sup>	0.4	40 <sup>j</sup>	10	25 <sup>k</sup>	0.7

<sup>a-k</sup>The same superscript letter was used when no differences between 2 calcium concentrations measured were observed ( $P > 0.05$ ).

<sup>1</sup>Statistical analysis was performed independently for colloidal and soluble phase.

SD = Standard Deviation. T<sub>1</sub> = end of probes stabilization; T<sub>2</sub> = end of calcium salt equilibrium; T<sub>3</sub> = end of acidification; T<sub>4</sub> = end of neutralization.

MC = Milk Calcium supplemented milk; CC = Calcium Chloride supplemented milk

From tables 15 and 16, it appears that MC supplementation of milk leads to a higher colloidal calcium concentration, as compared to CC-milk and skim milk. Because MC solubility is very poor compared to the CC one, we can expect that a significant amount of MC powder added in skim milk is still insoluble in the colloidal fraction. Additionally, CC addition is accompanied by an increase in soluble calcium, contrarily to skim milk and MC-milk. Soluble calcium concentrations are similar for skim milk and MC-milk.

For the three dairy systems, pH cycles induce an increase in soluble calcium during acidification (from T<sub>2</sub> to T<sub>3</sub>), related mainly to micellar demineralization, and a decrease in soluble calcium during neutralization due to a reverse micellar mineralization (from T<sub>3</sub> to T<sub>4</sub>). The reversibility of calcium solubilization is only obtained for CC-milk as it was observed for the ionized calcium fractions in figure 23.

### 1.3.2. Calcium variations during pH cycle to 3.5

Ionized calcium variations were also studied for pH cycle to 3.5. Normalized ionized calcium variations are plotted versus time in figure 24.

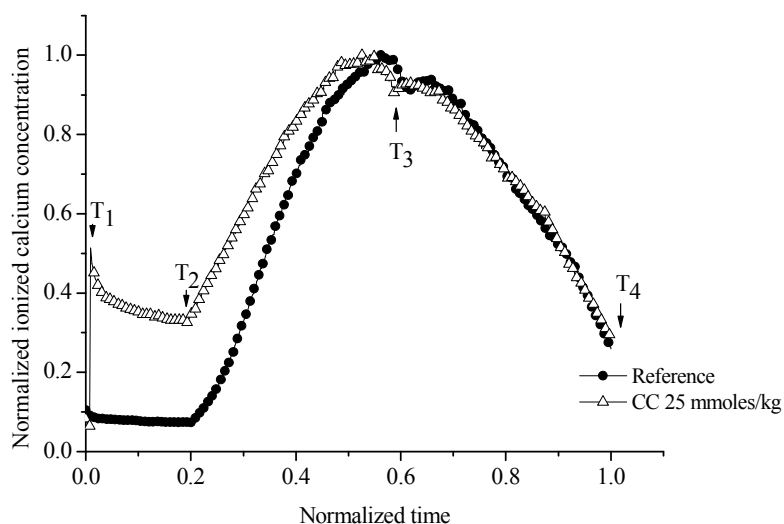


Figure 24. Normalized ionized calcium variations as a function of normalized time for CC supplemented milk subjected to pH cycle to 3.5 (Min-Max method).  $T_{\text{exp}} = 4^{\circ}\text{C}$ .

The major variations of ionized calcium were similar to those observed for pH cycle to 5.5 or to 5.0: ionized calcium concentration increased first upon CC supplementation, then with acidification, and it decreased upon alkalization. For pH cycle to 3.5, we can notice the presence of two maxima. The two successive passages through isoelectrical casein pH (pH 4.6) upon acidification and alkalization explain the presence of two maxima. Ionized calcium concentration increased during the first phase of acidification up to a maximum at pH 4.6 and then decreased slightly down to pH 3.5. Reverse phenomenon occurred during alkalization.

## 1.4. pH-dependent calcium variations

### 1.4.1. Effect of calcium salt concentration

#### 1.4.1.1. CC-supplementation

Ionized calcium concentration measured in CC-milk subjected to pH cycle was plotted as a function of pH for various studied concentrations (figure 25). It was observed that the higher the CC supplementation the more calcium ions ( $\text{Ca}^{2+}$ ) were present in the medium.

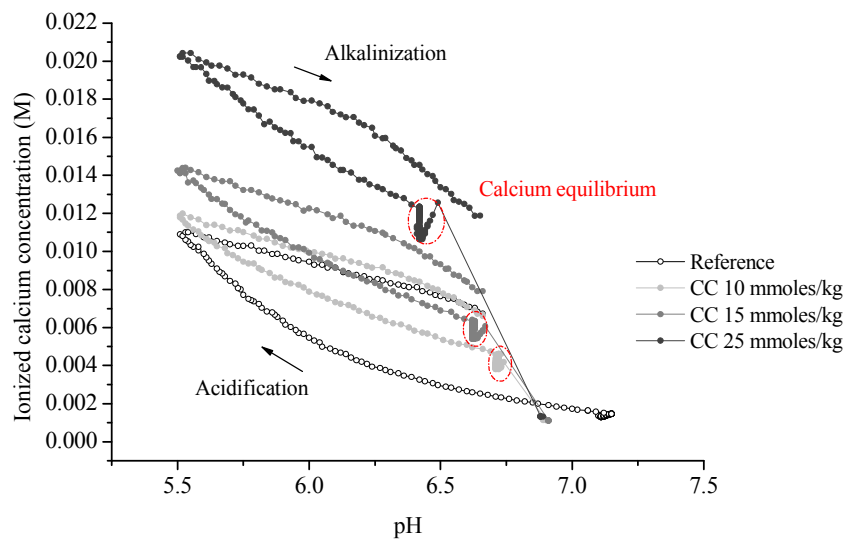


Figure 25. Ionized calcium concentration (M) as a function of pH for CC-milk (0, 10, 15 and 25 mmol/kg) subjected to pH cycle to 5.5.  $T_{\text{exp}} = 4^{\circ}\text{C}$ .

The Min-Max normalization of ionized calcium concentration as a function of pH (figure 26) shows two behaviours: the reference milk and the CC-milks. The proportion of ionized calcium at the end of the pH cycle is more important in reference than in supplemented milks. For all CC concentrations studied, the alkalinization phase of each curve is exactly superimposable contrarily to the acidification phase.

In proportion, normalized ionized calcium hysteresis area decreases with higher CC concentration added. This could be interpreted as a lower ionized calcium concentration exchange during pH cycle for higher CC supplementation. This result can be related to the observation of Gastaldi et al. (1994) who reported that micelles in CC-supplemented milk remained more mineralized than those of unsupplemented milk during acidification. We could expect that the electromotric strength acts against  $\text{Ca}^{2+}$  solubilization during acidification. Indeed the more the CC added, the less the fraction of  $\text{Ca}^{2+}$  is during acidification as it can be seen from the slope in figure 26.



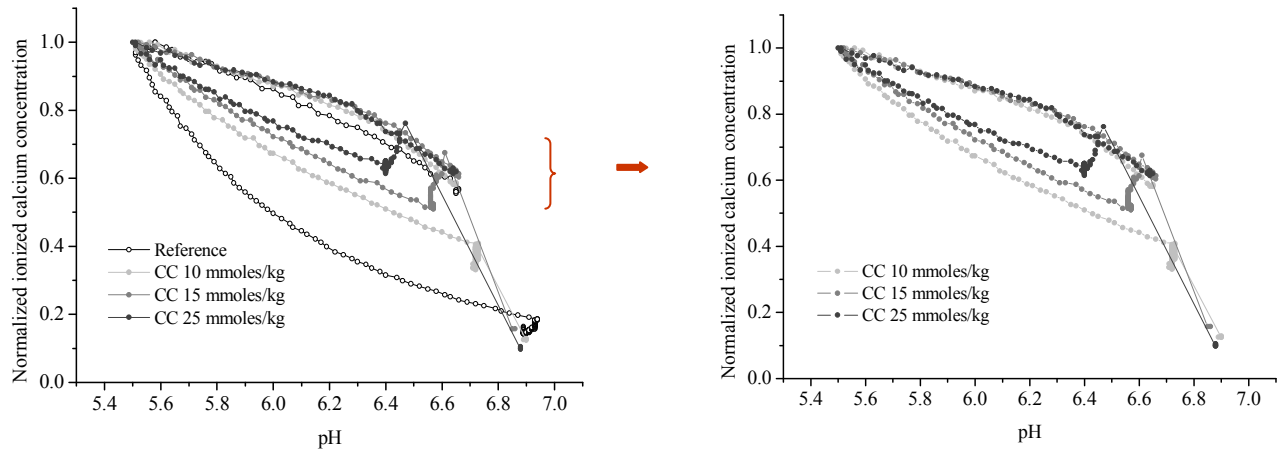


Figure 26. Normalized ionized calcium concentration as a function of pH (Min-Max normalization) for CC-supplemented skim milk subjected to pH cycle to 5.5.  $T_{\text{exp}} = 4^{\circ}\text{C}$ .

#### 1.4.1.2. MC-supplementation

Normalized ionized calcium concentration (Min-Max normalization) was plotted as a function of pH, for various MC concentrations added (figure 27).

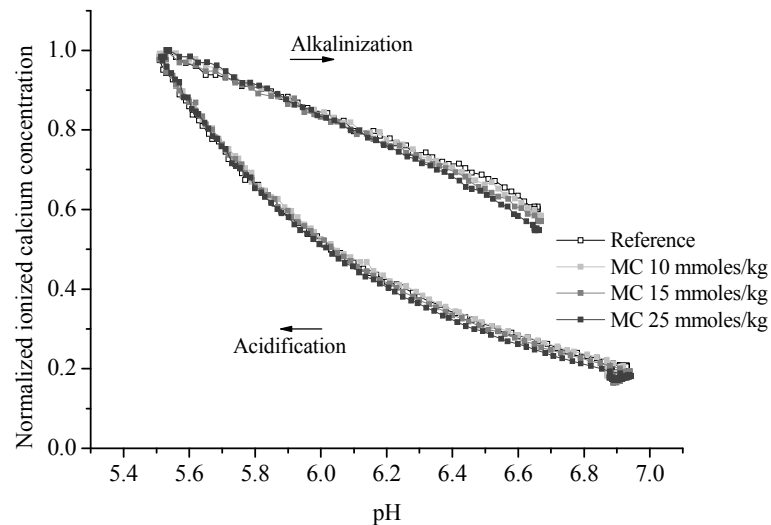


Figure 27. Normalized ionized calcium concentration as a function of pH (Min-Max normalization) for MC-supplemented milk subjected to pH cycle to 5.5.  $T_{\text{exp}} = 4^{\circ}\text{C}$ .

Whatever the MC concentration added, no difference of normalized ionized calcium concentration is observed (similar to conductivity results). This indicates the non ionization of MC in our experimental conditions since all the curves are superimposable. Hence normalized ionized calcium concentration variations are only due to endogenous calcium coming from the colloidal phase of milk (CCP).

Similarly normalized ionized calcium concentration (Min-Max normalization) was plotted as a function of pH for pH cycle to 5.0 (figure 28).

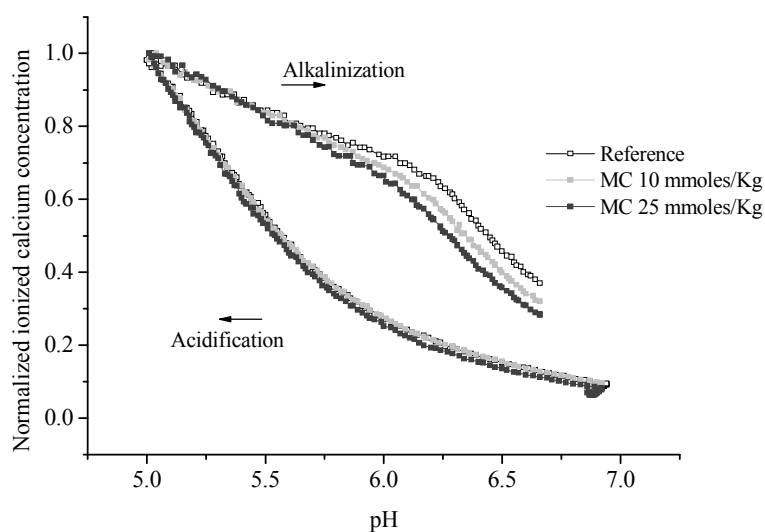


Figure 28. Normalized ionized calcium concentration (Min-Max normalization) as a function of pH for MC-supplemented milk subjected to pH cycle 5.0.  $T_{\text{exp}} = 4^{\circ}\text{C}$ .

Whatever the MC concentration added, no difference of normalized ionized calcium concentration is observed during acidification and the first phase of alkalization (up to pH 5.75). This result is in accordance with the insolubility of MC reported previously.

During the second phase of alkalization ( $\text{pH} > 5.75$ ), the curves are not superimposable anymore. With higher MC supplementation, ionized calcium concentration measured decreases faster in proportion. This decrease would suggest a more important absorption of ionized calcium by colloidal phase at higher MC concentration.

#### 1.4.2. Comparison of calcium salts

During acidification and neutralization, normalized ionized calcium concentration increases and decreases respectively and hysteresis phenomenon is observed. Whatever the minimal pH, i.e. pH 5.5 or pH 5.0, skim milk (figure 29A) and MC-milk (figure 29B) show similar trends contrarily to CC-milk (figure 29C). The hysteresis area is reduced for CC-milk (figure 29C), which means that the reversibility of micellar mineral solubilization is better in these conditions. This could be related to the previous capture of calcium cations into the micelles during the equilibrium phase ( $T_1$  to  $T_2$ ) involving a reinforcement of casein micelles

(higher calcium density, higher strength of interactions) and then less variations during pH cycle.

Normalized ionized calcium variations plotted versus pH profiles for the 2 pH cycles (i.e. to pH 5.5 or pH 5.0) show some differences during the neutralization phase. Indeed, for pH cycle to pH 5.0, an inflexion point around pH 6.0 to 6.2 is noticeable during neutralization from which, ionized calcium concentration decreases faster to its initial value. When pH is decreased up to pH 5.0, micellar calcium phosphate is supposed to be solubilized (similar to pH cycle to 5.5) but also calcium bound to phosphoserines and carboxyl groups of caseins (Le Graët and Brulé, 1993). These differences in the nature of calcium interactions affected during pH cycles should explain differences between pH cycles.

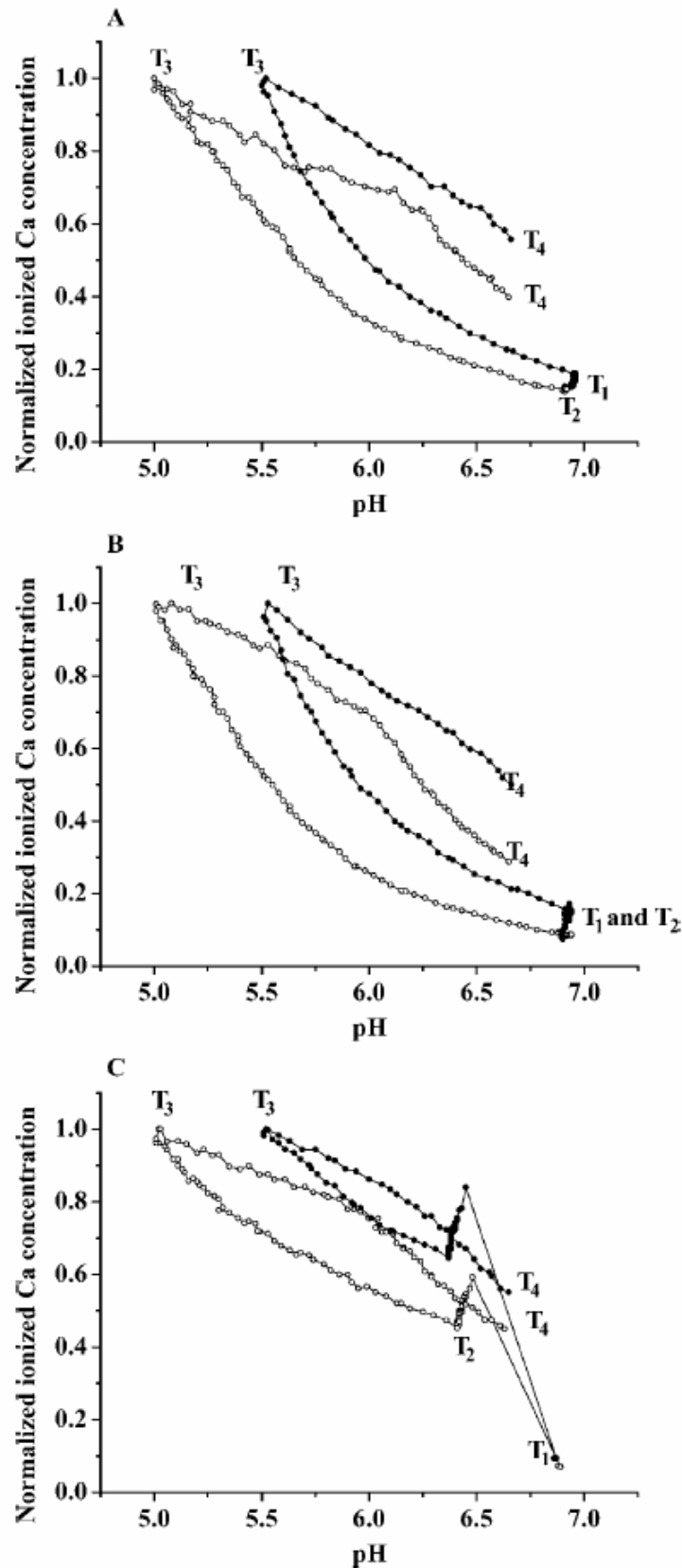


Figure 29. Min-Max normalized ionized calcium measured during pH cycles as a function of pH (A: Skim milk; B: MC-milk; C: CC-milk)

-○- :  $\text{pH}_{\min} = 5.0$ ; -●- :  $\text{pH}_{\min} = 5.5$ .  $T_{\text{exp}} = 4^\circ\text{C}$ .

$T_1$  = end of probes stabilization;  $T_2$  = end of calcium salt equilibrium;  $T_3$  = end of acidification;  $T_4$  = end of neutralization

### 1.4.3. Effect of minimal pH value during pH cycle on the ionized calcium

For pH cycle to 3.5, a characteristic ionized calcium profile was obtained. Normalized ionized calcium variations are plotted versus pH in figure 30.

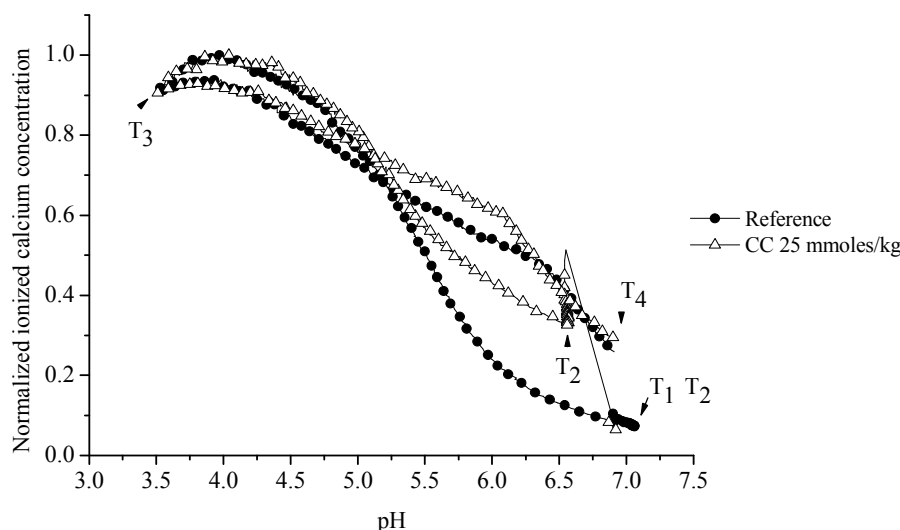


Figure 30. Normalized ionized calcium variations versus pH cycle to 3.5 (Min-Max normalization).  $T_{\text{exp}} = 4^{\circ}\text{C}$ .

Calcium is solubilized upon acidification (similar to Pyne, 1962; Brulé et al., 1974; Rajput et al., 1983; Van Hooydonk et al., 1986; Visser et al., 1986; Dalglish and Law, 1989; Le Graët and Brulé, 1993; Famelart et al., 1996; Gastaldi et al., 1996; Law, 1996) with a maximum of ionized calcium reached around the isoelectrical pH of milk proteins ( $\sim$  pH 4.6). A reversed phenomenon occurs during alkalinization.

According to the mineral equilibriums in milk proposed by Brulé (1981) (figure 4), the protons ions ( $\text{H}^+$ , coming from HCl) complex citrate ions and inorganic phosphate contained in the soluble phase. This protonation mechanism, consequently involves the ionization of calcium citrate and calcium phosphate into calcium, citrate and phosphate ions: hence the ionized calcium concentration increases. This mineral equilibrium induces in turn calcium phosphate solubilization from the micellar phase. In this pH range, milk proteins are globally positively charged. According to Walstra and Jenness (1984), the pKa values of phosphate ions contained in milk are respectively 6.6, 5.8 and 3. Hence calcium ions bind anions contained in the soluble phase and more particularly phosphate.

Contrarily to the study of Le Graët and Brulé (1993), we observed 4 phases upon calcium solubilization during acidification up to 3.5 whereas former authors described the 3 following phases during calcium solubilization:

- From initial pH of milk to pH 6.0,
- From pH 6.2 to 5.2: in this pH range calcium solubilization is linear (similar to our results),
- Then, from pH 5.2 to 3.5.

During these 3 steps, the authors quantified that 22%, 50% and 28% calcium were respectively solubilized. When comparing our results (figure 30) with the study of Le Graët and Brulé (1993), we can distinguish 4 phases: from pH 6.7 to pH 6.0 (phase 1) and from pH 6.2 to pH 5.2 (phase 2) as mentioned above, then from pH 5.2 to pH 4.0 (phase 3), and finally from pH 4.0 to pH 3.5 (phase 4).

During the 3 first steps we determined that 13.6%, 44.8% and 31.2% were solubilized respectively, 10% of calcium being initially under ionized form. During the last phase (from pH 4 to pH 3.5), ionized calcium decreased and certainly complexed up to 9.7% with citrate and phosphate anions.

The differences we observed with the results of Le Graët and Brulé certainly come from the measurements we made on ionized calcium and not on soluble calcium, which both includes calcium complexed with other minerals and ionized calcium. Since at such pH range milk proteins are positively charged, calcium ions are preferentially complexed with anions contained in the soluble phase of milk. Hence the soluble calcium remains identical after pH 4.0 and our results are similar to those of Le Graët and Brulé.

## **1.5. Relationship between particle size and protein colloidal phase variations**

### **1.5.1. Reference skim milk subjected to pH-cycle**

#### **1.5.1.1. pH cycle to 5.5**

Turbidity results for reference skim milk subjected to pH cycle to 5.5 are presented in figure 31. These variations are related to the average size of particles contained in skim milk (colloidal particles), measured at specific times of the kinetic ( $T_1$  = end of probes stabilization;  $T_2$  = end of calcium salt equilibrium;  $T_3$  = end of acidification;  $T_4$  = end of neutralization; table 17).

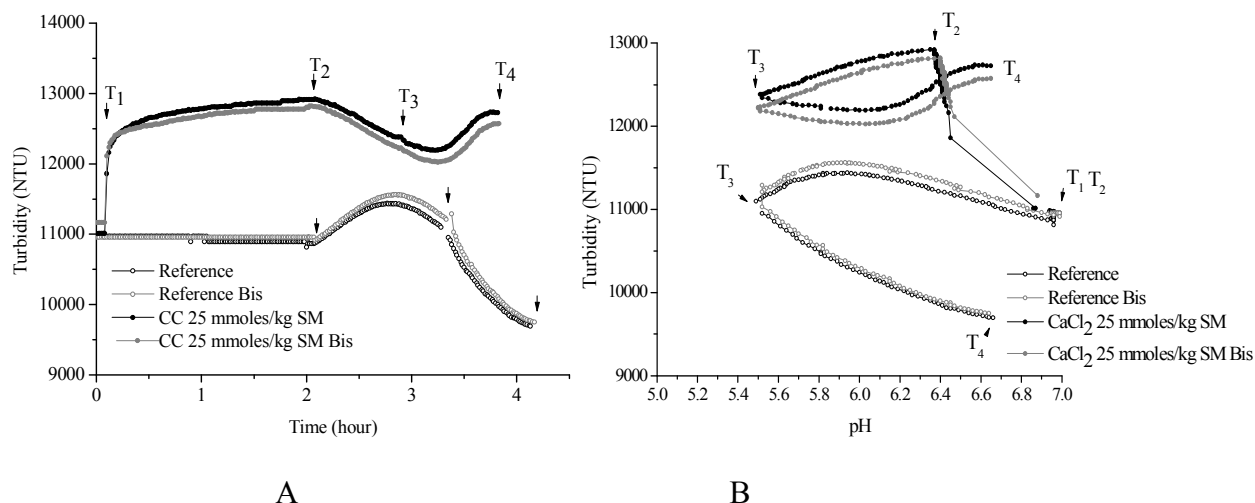


Figure 31. Turbidity (NTU) plotted as a function of time (A) and as a function of pH (B) for a minimal pH value of 5.5.  $T_{\text{exp}} = 4^{\circ}\text{C}$ .

Table 17. Average size (nm) of colloidal particles (skim milk diluted in UF) and turbidity (skim milk, NTU) measured at specific times.  $[\text{CC}] = 0$  mmole/kg of Skim Milk -  $\text{pH}_{\text{min}} = 5.5$

Time	Average size (nm)	$\sigma$ (nm)	Turbidity (NTU)
T <sub>1</sub>	283	$\pm 3$	10694
T <sub>2</sub>	292	$\pm 7$	10903
T <sub>3</sub>	270	$\pm 3$	11154
T <sub>4</sub>	240	$\pm 4$	9722

T<sub>1</sub>= End of probes stabilization

T<sub>2</sub>= End of calcium equilibrium phase

T<sub>3</sub>= End of acidification

T<sub>4</sub>= End of alkalization

UF= Ultrafiltrate

SM= Skim Milk

For reference skim milk, turbidity variations are composed of two phases during acidification: first, turbidity slightly increases and then decreases. When minimal pH is reached (end of acidification, T<sub>3</sub>), turbidity is slightly higher than at the end of equilibrium phase (T<sub>2</sub>). Yet the slope of turbidity variation is negative in this part of the curve (figure 31). At the end of acidification, the turbidity increase is not related to an average size increase of milk colloidal particles (table 17) but rather a higher concentration of particles in the medium and/or a variation in their optical properties. There may exist a shift with time between turbidity and colloidal particle average size, with turbidity variations being slower than average size variations.

## 1.5.1.2. pH cycle to 5.0

Turbidity and average size of colloidal particles, measured at specific times of the kinetic, are presented for reference skim milk subjected to pH cycle to 5.0 in table 18. The average size and turbidity variations in reference skim milk subjected to pH cycle show a more important decrease for pH cycle to 5.0 than for pH cycle to 5.5, both during acidification and alkalization.

Table 18. Average size (nm) of colloidal particles (skim milk diluted in UF) and turbidity (skim milk, NTU) measured at specific times - [CC] = 0 mmole/kg of Skim Milk -  $\text{pH}_{\min} = 5.0$

Time	Average size (nm)	$\sigma$ (nm)	Turbidity (NTU)
T <sub>1</sub>	255	± 5	11230
T <sub>2</sub>	265	± 4	11188
T <sub>3</sub>	242	± 5	10437
T <sub>4</sub>	203	± 3	6592

T<sub>1</sub>= End of probes stabilization  
 T<sub>2</sub>= End of calcium equilibrium phase  
 T<sub>3</sub>= End of acidification  
 T<sub>4</sub>= End of alkalization

UF= Ultrafiltrate  
 SM= Skim Milk

## 1.5.2. CC-milk subjected to pH cycle to 5.5

Turbidity and average size of colloidal particles, measured at specific times of the kinetic, are presented for CC-milk subjected to pH cycle to 5.5 in table 19.

Table 19. Average size (nm) of colloidal particles (skim milk diluted in UF) and turbidity (skim milk, NTU) measured at specific times - [CC] = 25 mmoles/kg of skim milk -  $\text{pH}_{\min} = 5.5$

Time	Average size (nm)	$\sigma$ (nm)	Turbidity (NTU)
T <sub>1</sub>	293	± 6	11088
T <sub>2</sub>	321	± 5	12868
T <sub>3</sub>	283	± 2	12301
T <sub>4</sub>	320	± 7	12650

T<sub>1</sub>= End of probes stabilization  
 T<sub>2</sub>= End of calcium equilibrium phase  
 T<sub>3</sub>= End of acidification  
 T<sub>4</sub>= End of alkalization

UF= Ultrafiltrate  
 SM= Skim Milk

Upon CC supplementation (from T<sub>1</sub> to T<sub>2</sub>, table 19), average size of particles in milk (~ + 30 nm) and turbidity increase (+ 1780 NTU). The association of soluble proteins onto the casein micelles through calcium bridges would explain that the average size of particles



increases in milk. During acidification and alkalization, colloidal particles size and turbidity decrease ( $\Delta d = - 38 \text{ nm}$ ;  $\Delta \tau = - 570 \text{ NTU}$ ) and increase ( $\Delta d = + 37 \text{ nm}$ ;  $\Delta \tau = + 350 \text{ NTU}$ ), respectively.

In milk, particle size/turbidity relationship does exist but is not proportional. The variations of concentration and optical properties of colloidal particles surely have a noticeable effect on turbidity variations as well.

### **1.5.3. Effect of CC-supplementation in skim milk subjected to pH cycle to 5.5**

In reference milk, particles size and turbidity decrease all over the pH cycle and show a probable delay of turbidity on the size variations for pH cycle to 5.5. pH cycle effect is reversible neither on particles size nor on turbidity measured.

In CC-milk, particles size (especially for casein micelles) and turbidity increase with CC-supplementation. Both parameters decrease and then increase again during acidification and alkalization respectively, involving a negligible effect of pH cycle on average size measured.

Upon pH cycle turbidity variations occur in a quicker way in CC-milk, compared to reference. Casein micelles reinforcement in CC-milk may involve weaker calcium-proteins interactions than those naturally existing in casein micelles.

## **1.6. Relationship between ionized calcium and protein colloidal phase**

### **1.6.1. Effect of calcium salt concentration**

#### **1.6.1.1. CC-supplementation**

Turbidity (mainly related to micellar casein structure variation) and normalized turbidity are presented versus pH in figures 32 and 33 respectively. Two groups of curves are noticeable, the reference and the CC-supplemented milks.

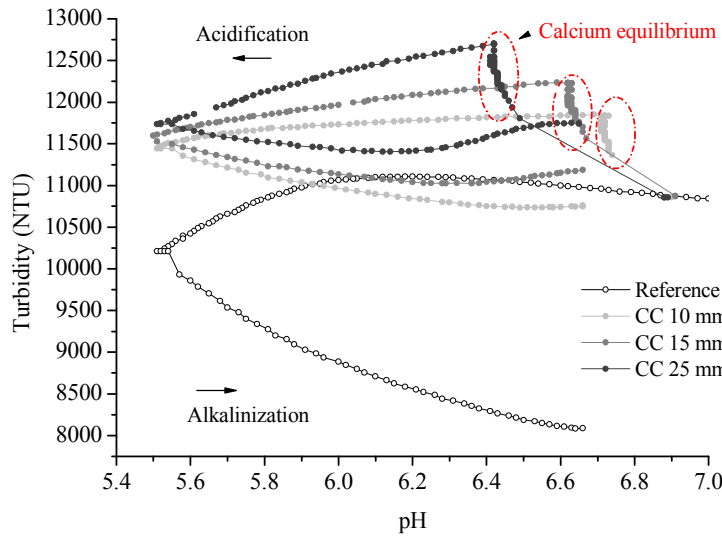


Figure 32. Turbidity ( $\tau$ , NTU) as a function of pH for CC-supplemented milk subjected to pH cycle to 5.5.  $T_{\text{exp}} = 4^{\circ}\text{C}$ .

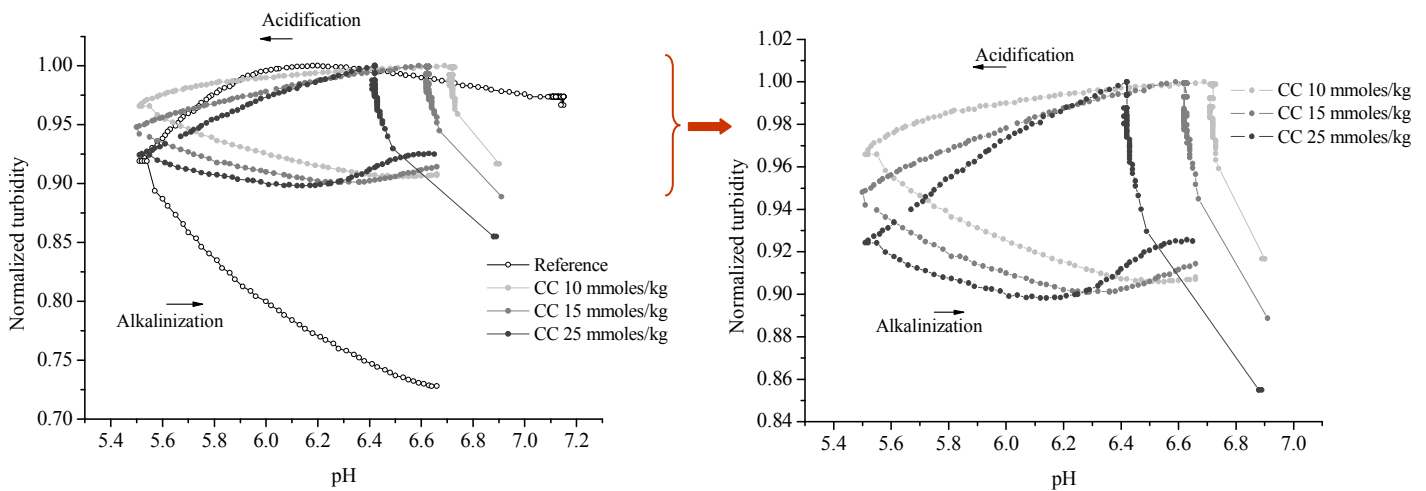


Figure 33. Normalized turbidity (Min-Max normalization) as a function of pH for CC-supplemented milk subjected to pH cycle to 5.5.  $T_{\text{exp}} = 4^{\circ}\text{C}$ .

For the reference, a slight turbidity increase is observed during the first part of acidification (similar to Banon and Hardy, 1991), which would be related to micellar voluminosity variation (Snoeren et al., 1984). Then turbidity decreases both during acidification and alkalinization and would be linked to colloidal phase destructuring (Dalglish and Law, 1988, and 1989).

In CC-milks, turbidity increases with CC supplementation, and in a more important way with higher CC concentration added. This result is in accordance with the observations of Green (1982), who reported a strong turbidity increase with the addition of any cationic additives. Similarly, Philippe et al. (2003) observed this turbidity increase in CC-milk by the mean of a spectrophotometer ( $\lambda=500$  nm). From the variation of other parameters (milk lightness increase, average diameter constant and hydrodynamic diameter constant), these authors related the turbidity increase to a micellar density increase.

Philippe et al. (2005) studied casein micelles suspended in milk ultrafiltrate and supplemented in calcium (2.5-8 mmoles of CC/kg). They observed that calcium associated with casein micelles (similar to Gaucheron, 2004). At the same time, the casein content increased and the water content decreased in the casein micelles.

Then turbidity decreases during acidification, which would be linked to colloidal phase destructuring. Indeed, as the pH is decreased from that of normal milk ( $\sim 6.7$ ), CCP is solubilized and is not able to maintain the integrity of micelles anymore (Pyne and McGann, 1960): colloidal caseins monomers, and especially  $\beta$ -caseins, are solubilized. This phenomenon is indirectly observed through turbidity measurement (figure 33).

Two successive phenomena are observed during alkalization. First, turbidity decreases faster for lower CC concentration (Casein-based particles destructuring) and then increases sooner for higher CC concentration added (Casein-based particles restructuring).

At the end of alkalization a plateau value is reached for the milk supplemented with 25 mmoles CC/kg. A similar plateau would certainly be reached for lower CC supplementation but at a more alkaline final pH.

All over the pH cycle, turbidity decreases in reference but not in CC-milks that show less turbidity variations. This could be due to a reinforcement of casein micelles. Supplemented skim milk would maintain a higher micellar association than unsupplemented milk.

Turbidity was plotted as a function of ionized calcium (figure 34). During equilibrium phase, turbidity increase is related to ionized calcium concentration decrease. As mentioned above, this should reflect intake of calcium ions by the colloidal phase. Acidification is characterized by two phases, the first one is an increase of turbidity and ionized calcium then the second one corresponds to a turbidity decrease with ionized calcium concentration increase. The ionized calcium-dependent variations of turbidity appear linear for the highest levels of added calcium. This could be explained by calcium ions solubilization from casein

micelles and concomitant destructuring of micelles. During alkalization, reverse phenomena occurred in two phases implying that ionized calcium partially come back into the micelles and led to partial casein micelles restructuring. The reverse character is higher with higher calcium levels added to milk.

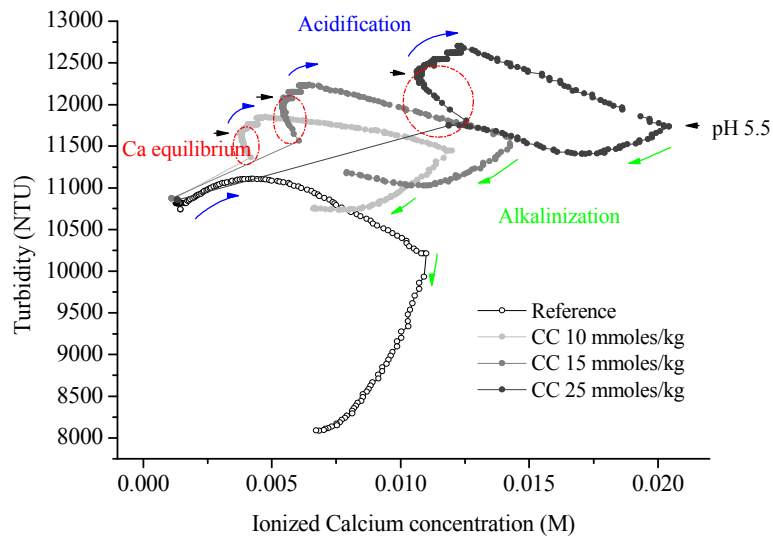


Figure 34. Turbidity ( $\tau$ , NTU) as a function of ionized calcium concentration (M) for CC-supplemented milk subjected to pH cycle to 5.5.  $T_{\text{exp}} = 4^{\circ}\text{C}$ .

#### 1.6.1.2. MC-supplementation

Turbidity (figure 35A) and normalized turbidity (figure 35B) were plotted versus pH. With higher MC concentration, the same turbidity variation trend is observed (figure 35A), and turbidity curves are only shifted upward (exact superimposition figure 35B). Hence, turbidity upward shift is only due to the presence of inert MC present as colloidal particles.

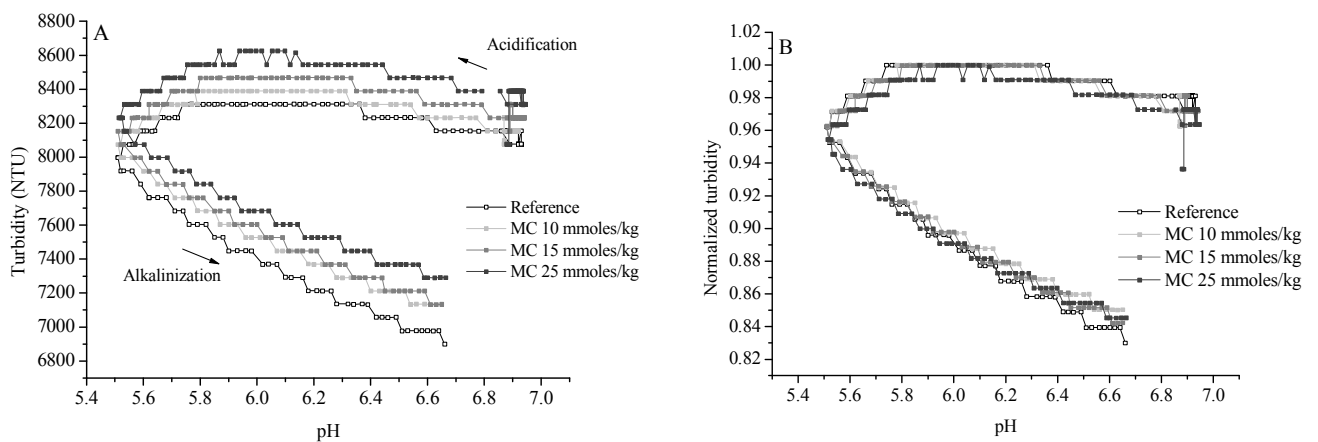


Figure 35. Turbidity (NTU) and normalized turbidity (Min-Max normalization) as a function of pH (panel A and B respectively) for MC-supplemented milk subjected to pH cycle to 5.5

Normalized turbidity (Min-Max normalization) was plotted as a function of pH for MC-supplemented milk subjected to pH cycle to 5.0 (figure 36).

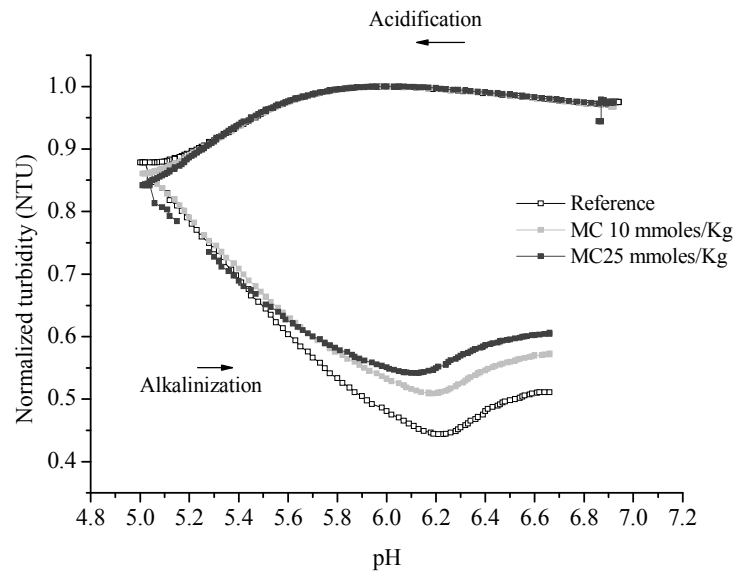


Figure 36. Normalized turbidity (Min-Max normalization) as a function of pH for MC-supplemented milk subjected to pH cycle to 5.0.  $T_{\text{exp}} = 4^{\circ}\text{C}$ .

Whatever the MC concentration added, exact superposition of curve is observed during acidification and during the first phase of alkalization. During the second phase of alkalization, all the turbidity curves and their respective minima are shifted upward with higher MC concentration added. We could expect that colloidal phase restructuring (due to protein aggregation?) is favoured with higher MC concentration.

Normalized turbidity was also plotted as a function of normalized ionized calcium concentration for MC-supplemented milk subjected to pH cycle to 5.0 (figure 37). In the first phase of acidification, both turbidity and ionized calcium concentration increase. This fits with the beginning of micellar solubilization. Then, acidification involves a turbidity decrease while ionized calcium concentration measured increases: micellar calcium solubilization goes with casein micelle destructuring which in turn involves turbidity decrease. During the first phase of alkalization, ionized calcium concentration is decreased while turbidity goes on to decrease until reaching a minimal value. Then, ionized calcium concentration decreases again whereas turbidity increases. This could be interpreted as casein micelles restructuring. At the end of pH cycle, the turbidity is higher for higher MC supplementation but turbidity has not reached its initial value.

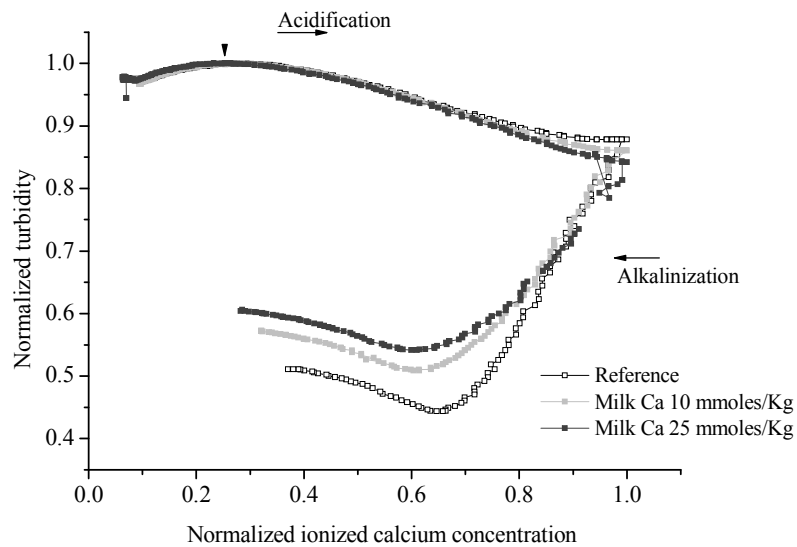


Figure 37. Normalized turbidity (Min-Max normalization) as a function of normalized ionized calcium concentration for MC-supplemented skim milk subjected to pH cycle to 5.0.  $T_{\text{exp}} = 4^{\circ}\text{C}$ .

### 1.6.2. Soluble proteins patterns in reference milk and supplemented milks during pH cycle

The soluble proteins were studied for calcium salt supplemented milks subjected to pH cycle to 5.5 by SDS-PAGE electrophoresis (figure 38).

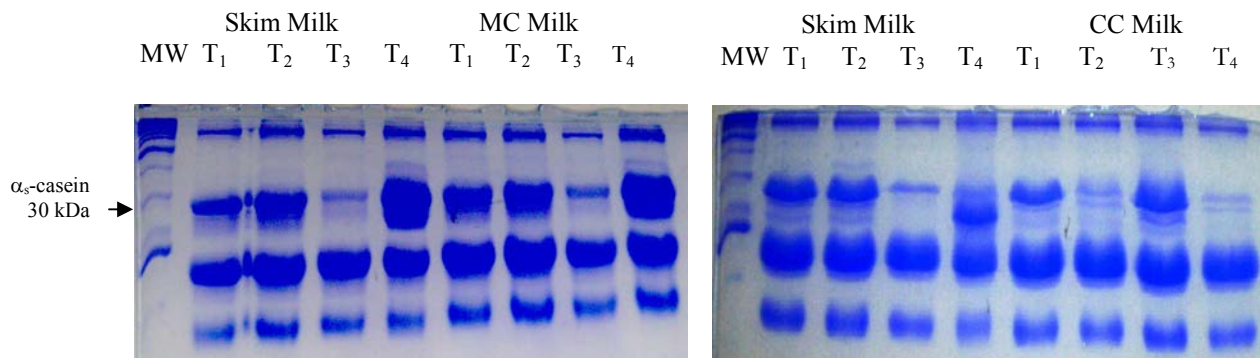


Figure 38. SDS-PAGE of skim milk, MC-milk and CC-milk supernatant ( $T_1$  = end of probe stabilization;  $T_2$  = end of calcium equilibrium;  $T_3$  = end of acidification;  $T_4$  = end of alkalinization)

In accordance with the results of ionized calcium variations and turbidity variations upon pH cycle ( $\text{pH}_{\text{min}} 5.5$ ), it appears that electrophoresis profiles from reference skim milk and MC-milk are similar. None significant variations in soluble protein phase is observed by MC addition to skim milk. It behaves like reference milk: more soluble  $\alpha_s$ -casein monomers

after pH cycle ( $T_4$ ) are detected, in relation with colloidal casein micelle solubilization. By comparison of CC-milk and reference skim milk, the main variation occurs for proteins of molecular weight around 30 kDa. According to the molecular weight of cow milk proteins reported in the literature, this molecular range fits with  $\alpha_{s1}$ - and  $\alpha_{s2}$ -caseins monomers. In CC-milk, the 30 kDa-band intensity decreases between  $T_1$  and  $T_2$ . We propose that absorption of calcium by casein micelles as previously suggested permits to soluble  $\alpha_s$ -caseins monomers to interact with the colloidal phase. During acidification (until  $T_3$ ), the increase of soluble 30 kDa-band fits with the casein monomers loss from colloidal phase. During alkalization (to  $T_4$ ), the 30 kDa-band decrease should reflect reverse interactions of casein monomers with colloidal proteins via calcium interactions.

### 1.6.3. Effect of minimal pH of pH cycle

Relationship between turbidity variations and ionized calcium variations can be drawn for pH cycle to 3.5 (figure 39).

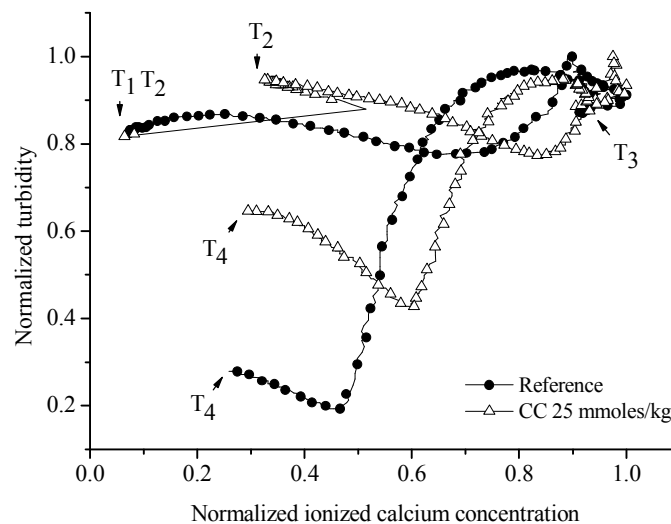


Figure 39. Min-Max normalized turbidity (NTU) as a function of normalized ionized calcium concentration for pH cycle to 3.5.

$T_1$  = end of probes stabilization;  $T_2$  = end of calcium salt equilibrium;  $T_3$  = end of acidification;  $T_4$  = end of neutralization

For reference, normalized turbidity slightly increases with ionized calcium concentration during the first phase of acidification (similar phenomenon than for pH cycle to 5.5 or to 5.0). Then turbidity decreases up to reach a first minimum while ionized calcium concentration goes on to increase. We would expect that this turbidity decrease would fit with micellar casein destructuring in relation with monomers, especially  $\beta$ -caseins, solubilization (Rose, 1968; Downey and Murphy, 1970; Creamer et al., 1977; Dalgleish and Law, 1988). This first minimum of turbidity is set at pH value around 5.1.

Then, turbidity increases again up to reach a maximum peak of turbidity at pH 4.5. According to the  $\zeta$ -potential variations measured for the reference skim milk upon pH cycle to 3.5, this turbidity increase can be explained by an accelerated aggregation phenomenon in this part of pH cycle due to a regression of ionization state of proteins (table 20). Indeed, from initial pH of skim milk (6.72) to pH 5.5, the  $\zeta$ -potential value increases by about 3.6 mV whereas between pH 5.5 and pH 4.5, the  $\zeta$ -potential value increases by 16.3 mV.

Table 20. Electrophoretic mobility (e.m.u.) and  $\zeta$ -potential variations (mV) in reference skim milk subjected to pH cycle to 3.5

pH	$\mu$ (e.m.u.)	$\sigma$ (e.m.u.)	Mean $\zeta$ (mV)	$\sigma$ (mV)
6.72	-2.113	0.05	-26.9	0.6
6.5	-2.157	0.05	-27.5	0.6
5.5	-1.870	0.04	-23.9	0.5
4.5	-0.595	0.03	-7.6	0.4
3.5	1.840	0.06	23.5	0.8
4.5	-0.174	0.03	-2.2	0.4
5.5	-1.600	0.05	-20.4	0.7
6.5	-1.993	0.04	-25.4	0.5
6.72	-2.263	0.06	-28.9	0.8

This first peak of turbidity fits with the maximal aggregation occurring in the isoelectrical pH region ( $\zeta$ -potential = 0). According to table 20, the isoelectrical pH is set at pH value comprised within pH range from pH 4.5 and 3.5.

Far and below this  $pH_i$  region, the global charges of proteins become positive and more important in absolute magnitude (table 20, between pH 4.5 and pH 3.5). This induces in turn the desegregation of protein aggregates due to electrostatic repulsion ( $\tau$  decrease).

A lag between the maximum of turbidity and the maximum of ionized calcium concentration of 0.5 pH unit is observed. Hence, the maximum of ionized calcium concentration is obtained after the maximum of aggregation and would be due to decomplexation of calcium from calcium salts, i.e. calcium citrate and calcium phosphate.



Then during back neutralization, reversed phenomenon occurs. A second maximum of turbidity is reached at the isoelectrical pH region, after which turbidity decreases. This fits with colloidal phase destructuring up to reach a minimum of turbidity at pH 6.33, which fits with the highest state of protein phase desegregation. Then turbidity increases again and would fit with protein colloidal phase restructuring. Ionized calcium concentration decreases all over the alkalization.

Similar signal is observed for CC-milk. Yet some differences are noticed. First, turbidity increases just after CC supplementation and ionized calcium concentration decreases during calcium equilibrium period.

The electrophoretic mobility was measured in reference milk and in CC-milk just after CC supplementation (table 21).

Table 21. Electrophoretic mobility (e.m.u.) and  $\zeta$ -potential variations (mV) of reference skim milk and CC-milk

Sample	$\mu$ (e.m.u.)	$\sigma$ (e.m.u.)	$\zeta$ (mV)	$\sigma$ (mV)
Skim milk	-2.113	0.05	-26.9	0.6
Skim milk + CC	-1.680	0.05	-21.4	0.6

The electrophoretic mobility and the corresponding calculated  $\zeta$ -potential decreased upon CC supplementation. According to this result, we can conclude that the protein colloidal phase reinforcement occurring upon CC supplementation involves CC-milk proteins interactions of electrostatic nature.

CC supplementation involves a certain lag, compared to the reference milk, to reach the first minimum of turbidity.

The electrophoretic mobility (figure 40) was determined at the end of each step of the kinetic ( $T_1$  = probes stabilization,  $T_2$  = calcium equilibrium,  $T_3$  = acidification,  $T_4$  = alkalization) for reference skim milk and CC-milk subjected to pH cycle to 3.5.

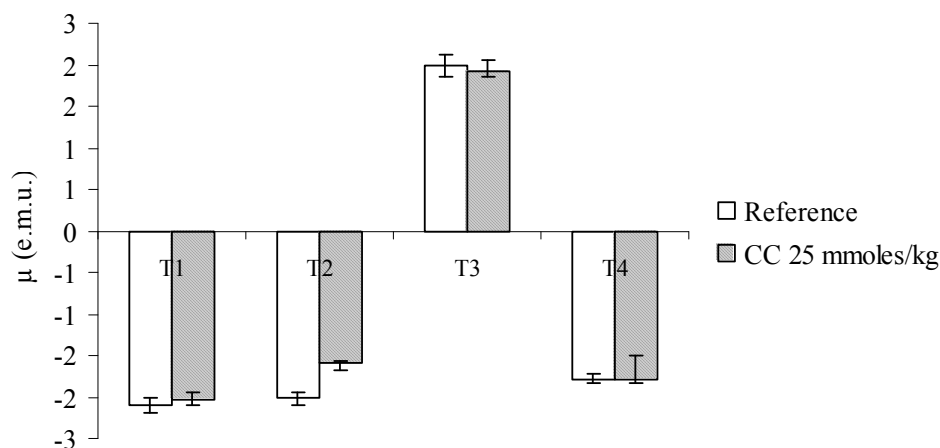


Figure 40. Electrophoretic mobility measured in reference milk and in CC-milk subjected to pH cycle to 3.5. T<sub>1</sub> = end of probes stabilization; T<sub>2</sub> = end of calcium salt equilibrium; T<sub>3</sub> = end of acidification; T<sub>4</sub> = end of neutralization.

According to figure 40, the only significant difference in electrophoretic mobility, between reference milk and CC-milk, is observed at the end of the calcium equilibrium (T<sub>2</sub>). The major difference between reference skim milk and CC-milk can be observed during back neutralization. First the minimum of turbidity appears sooner (at pH value of 6.1) and is higher for CC-milk which confirms the hypothesis of an initial reinforcement of colloidal protein phase upon CC supplementation.

### 1.7. Buffering index variations upon pH cycle to 3.5

The buffering capacity of milk products corresponds to the ability of the product to be acidified or alkalinized. This parameter depends on several compositional factors including small constituents (inorganic phosphate, citrate, organic acids) and milk proteins (caseins and whey proteins). The effects of natural and induced variations in the composition of milk on the buffering capacity were reviewed by Salaün et al. (2005). Physico-chemical changes such as calcium salt addition result in a buffering capacity specific to the transformed product, i.e. the CC-milk.

The buffering capacity was calculated according to Van Slyke formula (1922) for reference skim milk and CC milk subjected to pH cycle to 3.5. The buffering index dB/dpH is plotted vs. pH (figure 41).

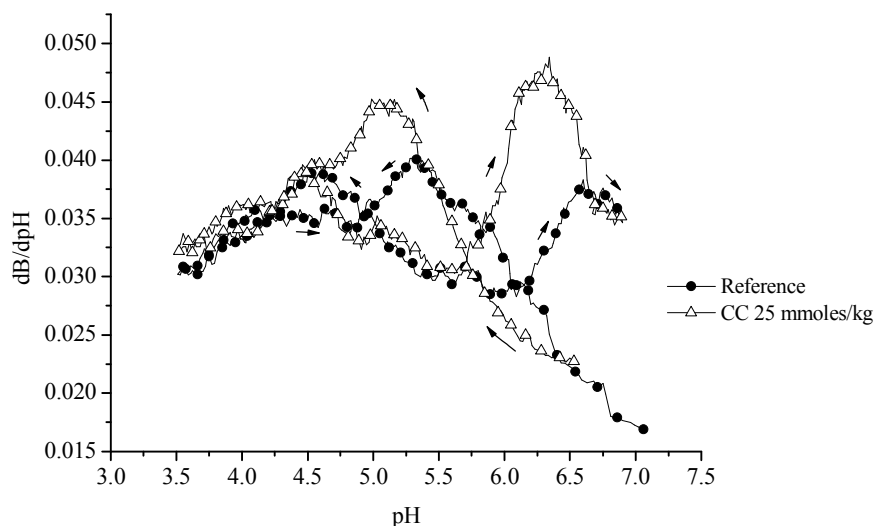


Figure 41. Buffering index variations in reference milk and in CC-milk subjected to pH cycle to 3.5.  $T_{\text{exp}} = 4^{\circ}\text{C}$ .

Acid-base buffering properties were studied by several authors in the literature (Park, 1991; Lucey et al., 1993a, 1993b and 1996; Salaün, 2005; Raouche et al., 2007; Salaün et al., 2007).

In the pH range studied (from pH 6.7 to pH 3.5), we obtained the same buffering trends than Lucey et al. (1993b), who studied the acid-base buffering properties of milk acidified from initial pH (6.7) to pH 2.0 with HCl and back titrated to pH 11 with NaOH. When the reference milk was acidified, maximum buffering occurred at approximately pH value of 5.2. Former authors reported that the maximum buffering occurred at approximately pH 5.1. On the contrary, the buffering curve for acidification of milk was very different from that reported by Park (1991), which had no buffering peak at pH 5.2 and little changes in buffering over the pH range 6 to 3. These differences may come from the titration conditions. Indeed former author used large increments of acid (1.0 mL of 0.5M-HCl for 25 mL sample) and may have missed some pH-dependent changes in buffering. Yet it seems unlikely that this would explain the total absence of buffering maximum at pH ~5.2. Note that similarly to Lucey et al. (1993b), a smaller peak can be noticed at about pH 4.5.

When acidified milk was back titrated with base, there was low buffering index value at pH 5.2 and the maximum of buffering occurred at pH 6.6. Lucey et al. (1993b) found this second maximum of buffering at pH 6.3. This shift could be induced by the various conditions of titration (0.1 mL of 0.5M-NaOH for 30 mL sample).

The maximum in the buffering curve at pH ~5.2 can be explained as follows (Lucey et al., 1993b). The buffering capacity of milk in the pH range 4.5-5.5 is essentially due to Micellar Calcium Phosphate (**MCP**, Salaün et al., 2005), especially the CCP. Indeed the CCP of milk is dissolved on acidification, especially below pH 5.6 and is complete at pH ~ 5.2 (Pierre et al., 1983; Van Hooydonk et al., 1986; Dalgleish and Law, 1989).

Upon acidification, CCP solubilization results in the formation of phosphate ions ( $\text{HPO}_4^{2-}$ ), which combined to protons ( $\text{H}^+$ ), resulting in buffering. During back neutralization, the buffering index measured at pH 5.2 is low because all the CCP was already solubilized and the maximum buffering occurs at pH 6.6. This is due to the formation of calcium phosphate with the release of protons (from  $\text{HPO}_4^{2-}$  and  $\text{H}_2\text{PO}_4^-$ ). The strong buffering effect in the pH range 6-7 arising from the formation of calcium phosphate was reported by several authors (Tobias, 1960; Visser et al., 1986). The buffering index increases with CC supplementation (figure 41). Maximum peak is shifted from about 0.040 to 0.044 and from 0.038 to 0.046, upon acidification and alkalinization respectively. Similarly, Guillaume et al. (2002) reported that the addition of calcium up to 17 mmol/kg results in an increase in buffering capacity between pH 4.5 and 5.5. Indeed CC addition to milk induces a decrease of the phosphate and anions concentration in the soluble phase, suggesting the association of calcium phosphate and calcium citrate salts into the micellar phase (Udabage et al., 2000; Philippe et al., 2003). More these associations are responsible for shifts in buffering capacity towards low pH. With CC addition, the maximum buffering capacity peak was shifted towards pH value of 5.1 and towards pH value of 6.3 during acidification and neutralization respectively.

## 1.8. Conclusion

In this chapter, the influence of calcium salt supplementation on calcium equilibrium was studied in skim milk during pH cycle at 4°C in order to inhibit protein aggregation and to focus on ionic exchange.

For moderate pH cycles ( $\text{pH}_{\text{min}}$  5.5 or 5.0), reference and MC-milk showed similar trends. Ionized Ca increased and decreased during acidification and alkalinization respectively, related to micellar demineralization then remineralization. Particle size and turbidity decreased all over the pH cycle implying large colloidal phase destructuring for pH cycle to 5.0. Compared to highly soluble CC, milk calcium salt had few effects on calcium equilibrium in milk.

Addition of CC to milk led to the “reinforcement” of casein micelles as it can be hypothesized from the increase of particle size and turbidity. This was also deduced from the minor and reversible variations observed during pH-cycle of turbidity, size and soluble caseins compared to reference and MC milks.

Differences between moderate pH cycles would be explained by the nature of calcium involved: up to pH 5.5, MCP is supposed to be solubilized and subsequent acidification up to pH 5.0 should also involved calcium bound to phosphoserine and carboxyl groups of caseins. For pH cycle to 3.5, turbidity and ionized calcium variations were larger and dependent also of the fluctuations around the  $pH_i$  of milk proteins. In CC-milk subjected to such pH cycle, phenomena observed were no more reversible.

During acidification, the maximum of buffering capacity was essentially due to MCP solubilization, which resulted in phosphate ion formation. These latter were able to combine protons. On back titration, maximum buffering capacity occurred at pH 6.6 due to the reformation of calcium phosphate and subsequent protons release. Buffering index was increased upon CC supplementation since  $Ca^{2+}$  integrated up to a certain part casein micelles and was then able to exchange with  $H^+$  during acidification.

The study of pH-cycle in cow milk permitted us to acquire experience with kinetic exchange of calcium ions between the colloidal and the soluble phase. The same kinetic approach was used in complex soy protein systems in order to get information about the calcium exchange between soy proteins in a colloidal state and soluble phase.

## 2. Protein phase behaviour and calcium equilibrium between soluble and colloidal phase in soy milks supplemented in calcium and subjected to pH cycle

**ABSTRACT.** Calcium equilibrium between soluble and colloidal phases was studied in CC supplemented soy milk (25 mmol/kg) upon pH cycle ( $\text{pH}_{\min}$  5.5 or 3.5). Ionized calcium variations were related to protein phase variations.

In reconstituted Non-Hydrolysed (NH) or Hydrolysed (H) soy milk, ionized calcium concentration was initially negligible. After CC-supplementation, ionized calcium increased during acidification due to the ionization state regression of proteins involving subsequent  $\text{Ca}^{2+}$  solubilization. Alkalinization led to reverse  $\text{Ca}^{2+}$  reduction.

**For pH cycle to 5.5 ( $> \text{pH}_i$ ),** the apparent viscosity ( $\eta_{\text{app}}$ ) of NH-soy milk increased during acidification and decreased upon alkalinization but was not restored, contrarily to H-soy milk. The volume mean diameter ( $D_{4,3}$ ) of soy proteins based aggregates, higher in NH-soy milk, increased and decreased upon acidification and alkalinization respectively.

**For pH cycle to 3.5 ( $< \text{pH}_i$ ),** ionized calcium solubilized during acidification reached a maximum at about the  $\text{pH}_i$ . Below  $\text{pH}_i$ ,  $\text{Ca}^{2+}$  was supposed to interact with soluble anions.  $\eta_{\text{app}}$  showed similar variations than pH cycle to 5.5, with a maximum in the  $\text{pH}_i$  region. For both soy milks, particle size increase was partially reversible upon alkalinization.

**Upon CC supplementation,**  $\eta_{\text{app}}$  and  $D_{4,3}$  increased irreversibly, involving Ca-soy protein interactions of electrostatic nature.

For pH cycle to 5.5, acid-induced protein aggregation was partially and completely reversible upon alkalinization, for NH and H-soy milks respectively. For pH cycle to 3.5, the irreversibility of phenomena was observed.

## 2.1. SPI solubility characterization

### 2.1.1. SPI solubility versus pH

SPI solubility (4.2% wt/wt) was studied as a function of pH, from initial pH down to pH 3.0. The SPI solubility was expressed as soluble protein concentration ( $\text{g}\cdot\text{L}^{-1}$ ) and in solubility (%) as a function of pH (figures 42A and B). The solubility expressed in percent corresponds to the total proteins that remain in solution after centrifugation (3034g, 35 min, 20°C).

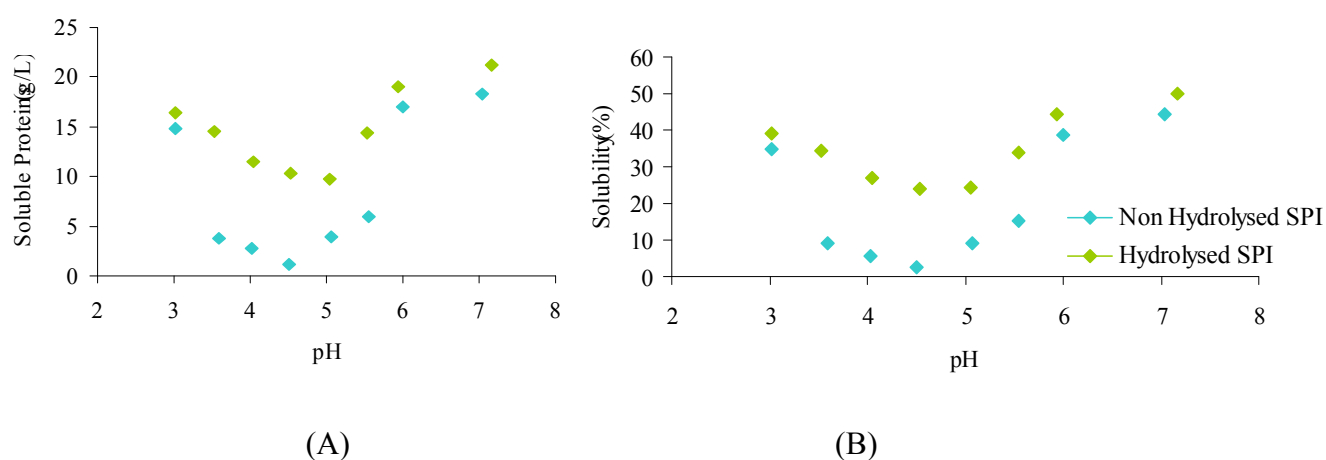


Figure 42. Soluble proteins concentration (g/L, A) and solubility (% , B) versus pH in non-hydrolysed and hydrolysed SPI solutions determined by Kjeldahl after centrifugation (3034g, 35 min, 20°C)

As expected SPI solubility is pH-dependent (figure 42). The minimum of solubility is observed at about pH 4.5 for SPI, which fits with the isoelectrical pH commonly reported in the literature (Shen, 1976; Yuan et al., 2002). All over the pH range studied (~7.2-3.0), hydrolysed soy proteins are more soluble than non hydrolysed soy proteins, especially around the isoelectrical pH ( $\text{pH}_i$ ).

### 2.1.2. Solubility of CC-supplemented SPI versus pH

The SPI solubility was also studied after CC-supplementation (25 mmoles/kg) as a function of acidification (from initial pH ~7.2-7.1 to pH 3.0). The CC concentration added was chosen in order to reach a level of calcium in SPI solution similar to that of cow's milk. Solubility vs. pH is presented in figure 43.

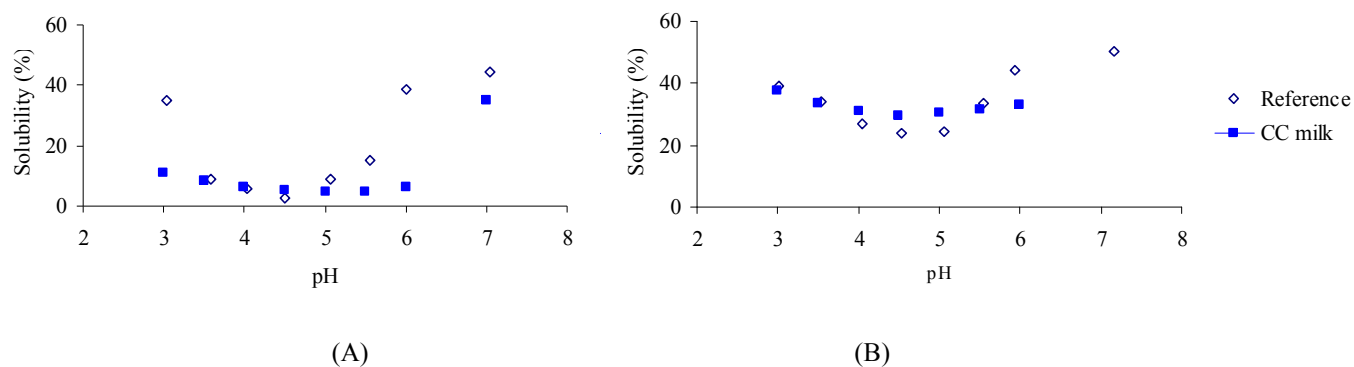


Figure 43. Solubility (%) versus pH in reference and CC-supplemented SPI solution (25 mmoles/kg). A: in Non-hydrolysed SPI solution. B: in hydrolysed SPI solution

According to figure 43, soy protein aggregation is both induced by acidification and by calcium supplementation (similar to Ono et al., 1993). Yet, the pH dependence of CC-supplemented SPI solubility is reduced compared to the reference. This should be related to the instability of soy proteins in presence of calcium as reported previously (Saio et al., 1967; Appurao and Narasinga Rao, 1975 and 1976; Torikata and Yano, 1988; Yuan et al., 2002). Solubility at initial pH is comparable for CC-supplemented and reference. In addition, less aggregation induced by CC occurred for hydrolysed SPI than for non hydrolysed SPI.

Contrary to our results, Grynspan and Cheryan (1989) did not observed any significant changes in solubility profile versus pH (pH range studied 11 to 1) upon CC addition. Yet, former authors used different Ca: Protein ratios (ranging from 0.232 to 0.528% wt/wt) while in our experiments the Ca: Protein ratio was set to 2% (wt/wt).

## 2.2. Ionized calcium variations versus pH cycle

### 2.2.1. pH cycle to 5.5

Ionized calcium variations were studied in soy milks and CC-supplemented soy milks at 25 mmoles/kg subjected to pH cycle to 5.5 ( $T = 25^{\circ}\text{C}$ ).

Regardless the soy milk studied (hydrolysed or non-hydrolysed) similar ionized calcium variations profiles were obtained than in cow skim milk (figures 44A and B).



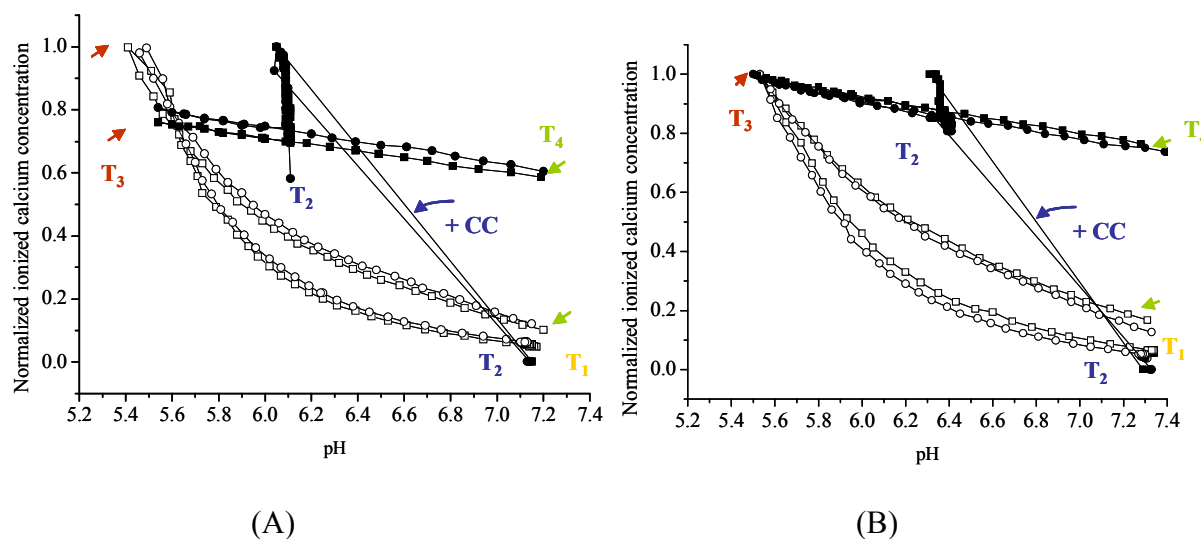


Figure 44. Normalized ionized calcium concentration as a function of pH for non hydrolysed and hydrolyzed soy milks (figure A and B, respectively) CC-supplemented (25 mmoles/kg) and subjected to pH cycle ( $pH_{\min} = 5.5$ ,  $T = 25^{\circ}\text{C}$ )

( $T_1$  = probe stabilization,  $T_2$  = calcium period equilibrium,  $T_3$  = acidification,  $T_4$  = alkalization).

Empty symbol: No CC supplementation; Fill symbol: CC supplementation.

Before CC-supplementation, ionized calcium concentration measured was almost negligible. In reference soy milk, ionized calcium variations were then poorly detectable upon pH cycle.

In CC-supplemented and pH cycled soy milk, ionized calcium concentration increased upon CC supplementation then during acidification up to a minimal pH 5.5. Yet, CC supplementation effect was higher than acidification effect on ionized calcium concentration increase. Moreover, during calcium equilibrium period, ionized calcium concentration measured decreased. This would either fit with the chelation of calcium ions ( $\text{Ca}^{2+}$ ) with anions (i.e. phosphate anions) present in the soluble phase or an interaction of  $\text{Ca}^{2+}$  ions with the negative charge of soy proteins such as carboxyl groups of acid amino-acid. Indeed aspartic acid and glutamic acid content of SPI (non hydrolysed or hydrolysed) are respectively of 11.6 and 19.1% (wt/wt, table 8). During back neutralization, ionized calcium concentrations variations were fully reversed.

### 2.2.2. pH cycle to 3.5

Ionized calcium concentrations variations were plotted as a function of time in figure 45 and as a function of pH in figure 46.

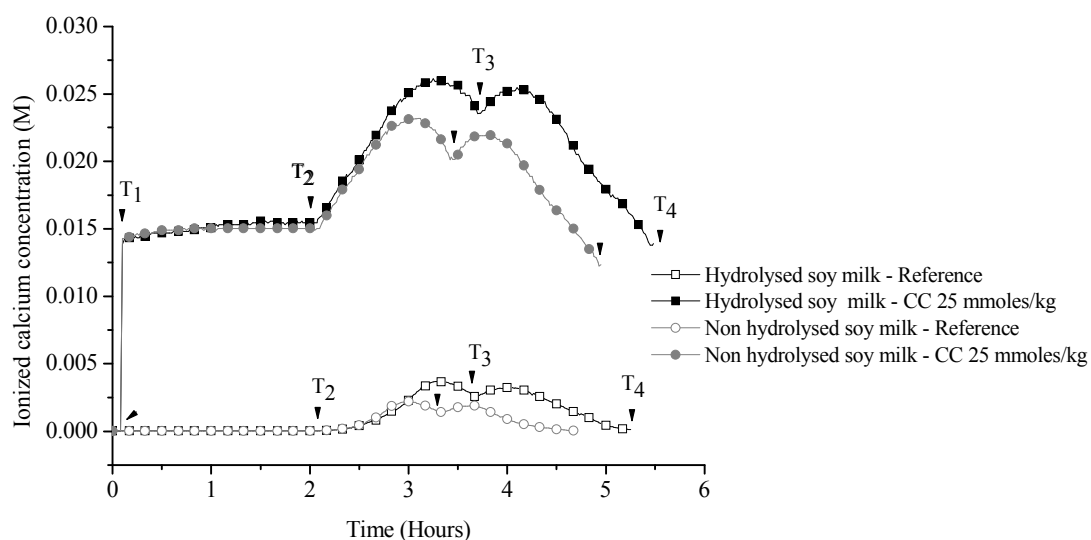


Figure 45. Ionized calcium concentration (M) as a function of time (hours) – Comparison of hydrolysed and non hydrolyzed soy milks CC-supplemented (25 mmoles/kg) and subjected to pH cycle ( $\text{pH}_{\text{min}} = 3.5$ ,  $T = 25^\circ\text{C}$ ) ( $T_1$  = probe stabilization,  $T_2$  = calcium equilibrium period,  $T_3$  = acidification,  $T_4$  = alkalinization)

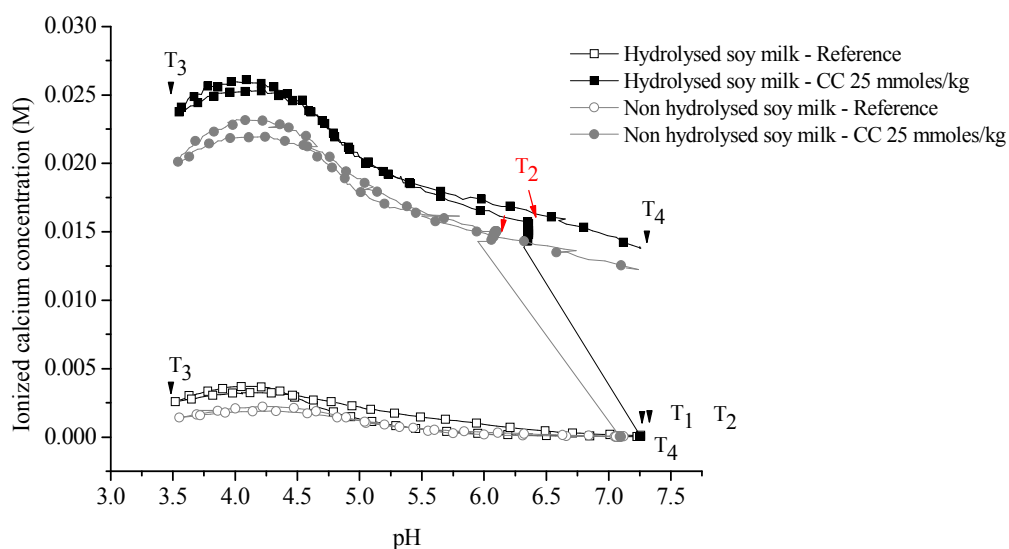


Figure 46. Ionized calcium concentration (M) as a function of pH - Comparison of hydrolysed and non hydrolyzed soy milks, CC-supplemented (25 mmoles/kg) and subjected to pH cycle ( $\text{pH}_{\text{min}} = 3.5$ ,  $T = 25^\circ\text{C}$ ) ( $T_1$  = probe stabilization,  $T_2$  = calcium period equilibrium,  $T_3$  = acidification,  $T_4$  = alkalinization)

Ionized calcium variations are almost similar for both references and for both CC-supplemented soy milks (hydrolysed or non hydrolysed). Yet, a slightly higher value is measured for hydrolysed soy milk.

With CC supplementation, the ionized calcium concentration measured increases in the soluble phase due to CC solubilization into calcium ions ( $\text{Ca}^{2+}$ ) and chloride ions ( $\text{Cl}^-$ ). Yet only 15 mM of ionized calcium are measured in comparison of the 25 mmoles of CC supplemented per kg of soy milk. This former difference can not be explained by a partial solubilization of CC, due to the high solubility of this calcium salt. Ionized calcium interactions either with anions of the soluble phase and/or with soy proteins could rather be expected.

With acidification, from initial pH to isoelectrical pH, soy proteins initially negatively charged undergo a regression of ionization state. This involves a calcium ions release, previously captured upon CC-supplementation. Ionized calcium concentration increases up to reach a maximum value at about the isoelectrical pH region ( $\sim$  pH 4.5), where the global charge of soy protein is null.

Then, for further acidification and below the isoelectrical pH region, the ionized calcium concentration measured decreases. This result is unexpected since proteins are more and more positively charged. Hence, the ionized calcium decrease could rather be explained by calcium ion complexation with anions (such as phosphate or chloride ions...) contained in the soluble phase to form calcium salts. Another explanation would be the calcium ion indirect fixation onto positively charged proteins through anion bridges. Moreover, the pH range studied is at the limit of measurement validity for the Ca ISE (appropriate pH range: 4-9).

With neutralization, inversed phenomena occur. Ionized calcium concentration increases up to isoelectrical pH due surely to calcium salt decomplexation into respective anions and calcium ions. Above isoelectrical pH ( $\sim$ pH 4.5), proteins become more and more negatively charged and are then able to bind calcium ions. The ionized calcium concentration decreases.

### 2.3. Buffering index variations upon pH cycle

The buffering capacity is determined by acid-base equilibrium which attenuates pH changes upon exposure to acid or alkali. The buffering capacity was calculated according to Van Slyke formula (1922) for both CC supplemented soy milks, hydrolysed or non hydrolysed, subjected to pH cycle to pH 3.5.

The buffering index dB/dpH was plotted versus pH (figure 47).

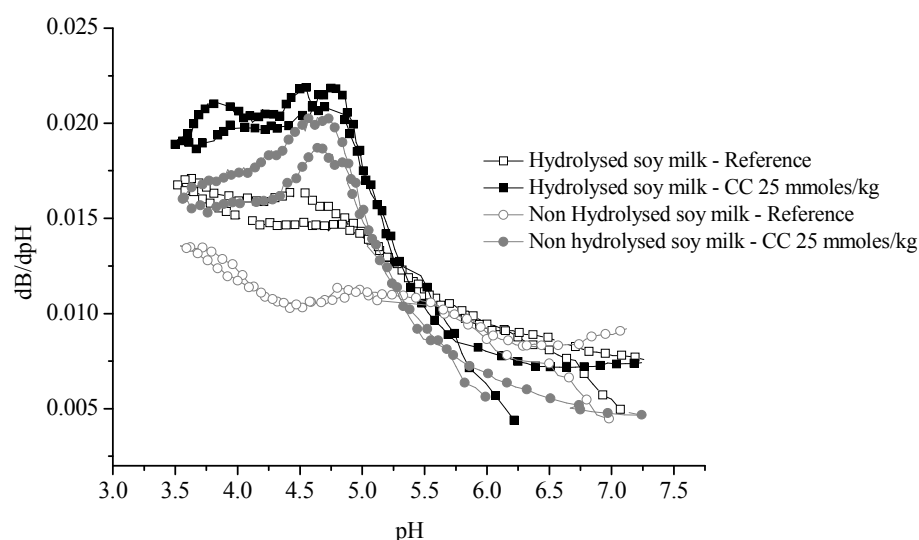


Figure 47. The buffering capacity of non hydrolysed and hydrolysed soy milks, CC-supplemented (25 mmoles/kg) and subjected to pH cycle ( $\text{pH}_{\min} = 3.5$ ). Reference was CC-unsupplemented

The buffering capacity (dB/dpH) profile can be related to ionized calcium profiles in figure 46. The more the ionized calcium, the higher is the buffering capacity. This would imply that the calcium solubilized is replaced by protons acting then on buffering capacity.

Below pH 4.5, the buffering capacity decreases with ionized calcium decrease in supplemented soy milks whereas an increase of buffering capacity is observed with ionized calcium reduction for unsupplemented soy milks. The formation of protein aggregates in supplemented soy milk would be related to this distinct behaviour.

For reference non hydrolysed soy milk, the buffering capacity is reversible all over the pH cycle except for pH higher than 6.0. The maximum of the buffering capacity is determined at the end of pH cycle (pH 3.5) and is set at 0.014. For reference hydrolysed soy milk, the buffering capacity is shifted upward. A first peak is observed at pH 4.5 (dB/dpH = 0.016)

during acidification and a second one is observed at pH 4.8 ( $\text{dB}/\text{dpH} = 0.014$ ) during neutralization. Once more, the maximum buffering capacity is reached at minimal pH 3.5 and is of 0.017.

Park (1991) studied the buffering capacity of various milks including soy based infant formula. When comparing his results in the same pH range with ours, similar results of buffering index capacity are found, between 0.014 and 0.016.

Regardless the soy milk used, hydrolysed or non hydrolysed form, the buffering capacity is shifted upward with CC supplementation (25 mmoles/kg). Moreover a peak of buffering capacity appears clearly in such conditions. The former one is centred at pH 4.5-5.0. Table below summarises the pH value and the corresponding buffering index where the maximum is observed upon acidification and neutralization for both CC-supplemented soy milks.

Table 22. pH range and corresponding buffering index of the peak observed upon acidification and neutralization for CC-supplemented soy milks

Step	Non Hydrolysed soy milk		Hydrolysed soy milk	
	pH	$\text{dB}/\text{dpH}$	pH	$\text{dB}/\text{dpH}$
Acidification	4.5-4.6	0.020	4.5	0.022
Alkalinization	4.6-4.8	0.018	4.7-4.8	0.022

## 2.4. Protein phase characterization versus pH cycle

### 2.4.1. Apparent viscosity variations

#### 2.4.1.1. pH cycle to 5.5

Apparent viscosity profiles are plotted versus the time for reference and CC-supplemented soy milk, subjected to pH cycle to 5.5. Results obtained are presented in figures 48 and 49 for non hydrolysed and hydrolysed soy milks respectively.

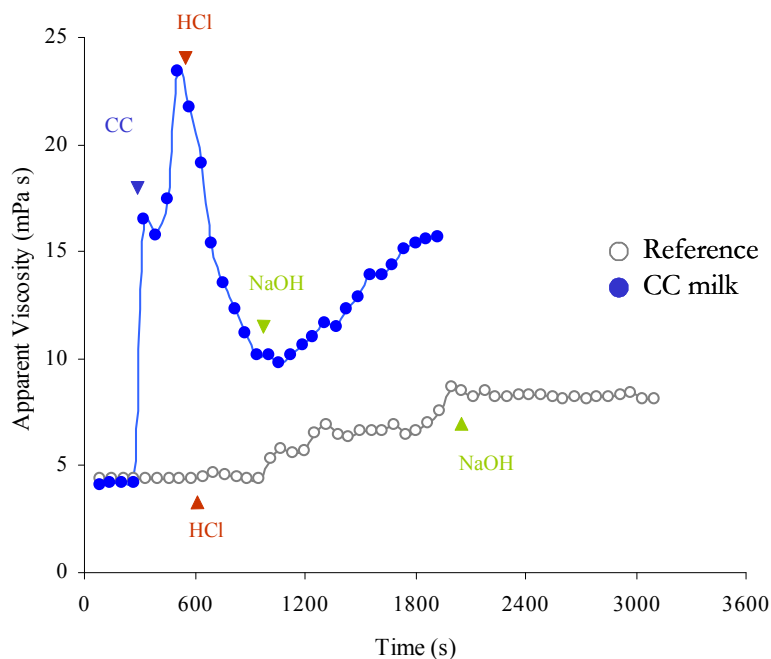


Figure 48. Apparent viscosity measured in reference and CC-supplemented non hydrolysed soy milk (25 mmol/kg), subjected to pH cycle ( $\text{pH}_{\text{min}} = 5.5$ ;  $T = 25^\circ\text{C}$ ) versus the time (shear rate:  $100 \text{ s}^{-1}$ )

The initial apparent viscosity of non hydrolysed soy milk is 4.9 mPa.s. In reference non hydrolysed soy milk, the apparent viscosity increases upon acidification and remains constant upon back neutralization to initial pH. The initial viscosity is not restored upon a pH cycle to 5.5. Hence the interactions formed with acidification are not reversed with alkalization.

In CC-supplemented soy milk, CC addition induces an apparent viscosity increase from 4.9 up to 23 mPa.s as reported by Lee and Rha (1977). Afterwards, acidification down to pH 5.5 induces a global viscosity decrease. The increase of viscosity upon CC addition is consistent with the formation of protein aggregates induced by calcium interactions. The displacement of calcium ions by protons during acidification leads to aggregates disappearance and to a reduction of viscosity. During neutralization to initial pH of non hydrolysed soy milk, the viscosity increases again. Equilibrium of calcium ions between the colloidal and soluble phases inducing protein reaggregation would explained this gain in viscosity.

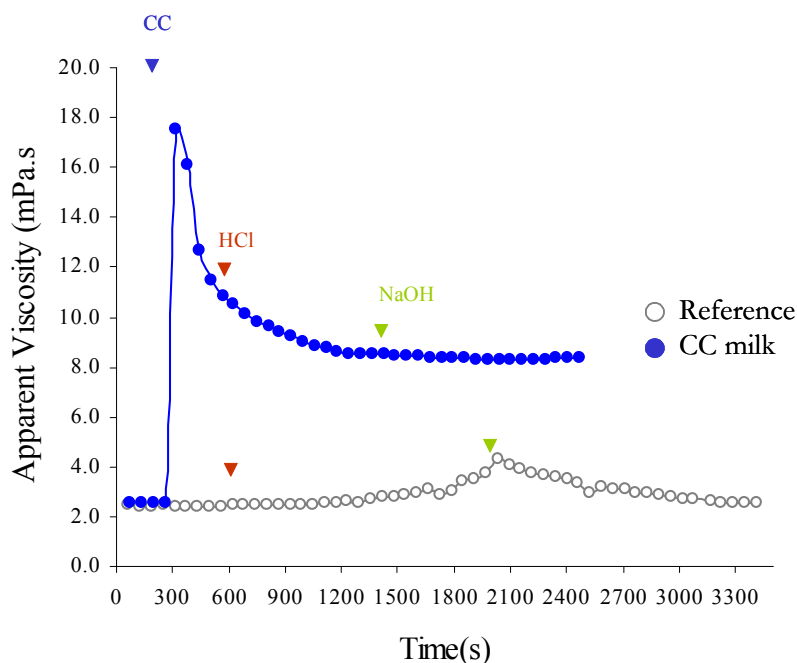


Figure 49. Apparent viscosity measured in reference and CC-supplemented hydrolysed soy milk (25 mmol/kg), subjected to pH cycle ( $\text{pH}_{\min} = 5.5$ ;  $T = 25^\circ\text{C}$ ) versus the time (shear rate:  $100 \text{ s}^{-1}$ )

In reference hydrolysed soy milk, the initial apparent viscosity measured is of  $\sim 2.5$  mPa.s (figure 49). Acidification to pH 5.5 induces a slight viscosity increase. Indeed, the negative charge of soy proteins are neutralised (regression of ionization state of protein for pH closer to  $\text{pH}_i$ ). Protein aggregation is favoured. Initial apparent viscosity is restored upon alkalization ( $\sim 2.7$  mPa.s). During pH cycle, these slight viscosity variations are induced by a small reversible phenomenon aggregation.

As previously observed for non hydrolysed soy milk, CC supplementation induces a rapid increase of hydrolysed soy milk viscosity from 2.5 to 18 mPa.s (figure 49). These changes in viscosity are related to the thickening by aggregation of soy proteins suspensions in presence of calcium. Shearing the system results in a decrease of the viscosity and a plateau value at around 10 mPa.s. Non significant effect is shown when this system is submitted to a pH-cycle down to pH 5.5 (figure 50). The resultant viscosity is around 10 mPa.s.

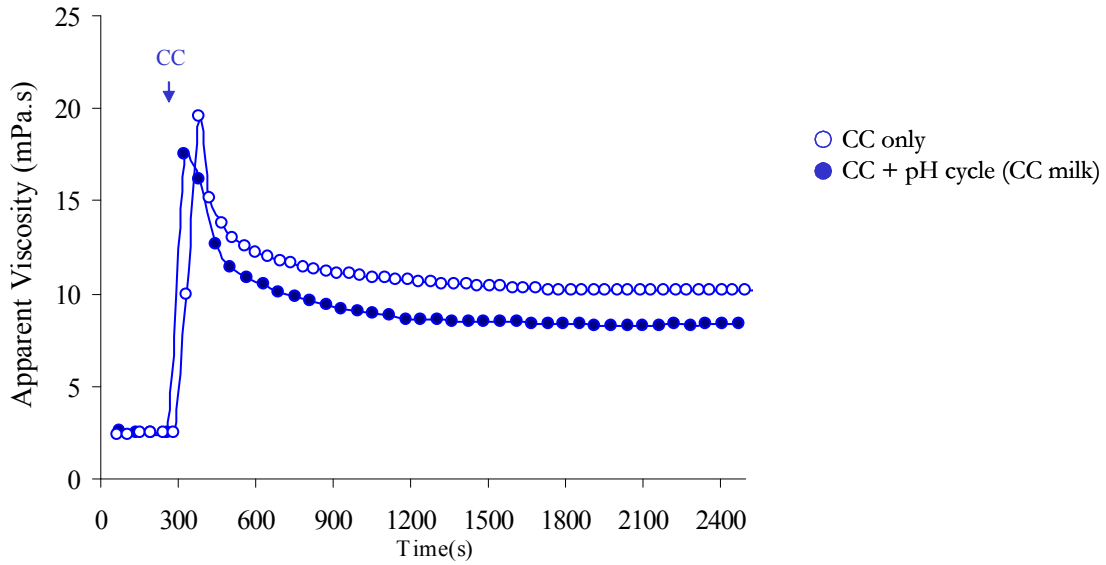


Figure 50. Apparent viscosity measured in CC-supplemented hydrolysed soy milk and CC-supplemented hydrolysed soy milk subjected to pH cycle ( $\text{pH}_{\text{min}} = 5.5$ ;  $T = 25^\circ\text{C}$ ; CC-supplementation 25 mmoles/kg) versus the time

#### 2.4.1.2. pH cycle to 3.5

Apparent viscosity profiles are plotted versus the time for reference and CC-supplemented non hydrolysed soy milk, subjected to pH cycle to 3.5 (figure 51).

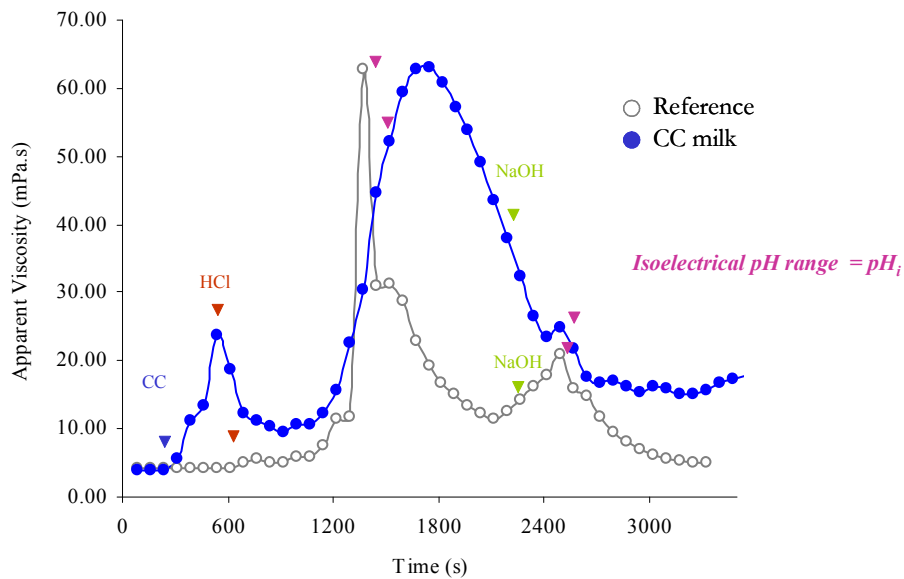


Figure 51. Apparent viscosity measured in reference and CC-supplemented non hydrolysed soy milk (25 mmoles/kg), subjected to pH cycle ( $\text{pH}_{\text{min}} = 3.5$ ;  $T = 25^\circ\text{C}$ ) versus the time



Similarly to pH cycle to 5.5, apparent viscosity globally increases and decreases during acidification and alkalization respectively. For reference non hydrolysed soy milk, acidification from initial pH down to pH 3.5 shows two effects. First, apparent viscosity increases up to a maximal value at around the isoelectrical pH region and then decreases down to pH 3.5. The maximum of apparent viscosity fits with the  $pH_i$  region, where the global charge of protein is null. At such a pH, the strength of repulsion is weak between soy proteins and aggregation occurs. Inverse phenomenon is observed during alkalization, yet with lower variations. A slight difference is measured between the initial (4.5 mPa.s) and the final viscosity (8 mPa.s).

In CC-supplemented soy milk, calcium addition rapidly increases the viscosity of the system. Upon pH cycle, same trends are observed in CC-supplemented sample than in corresponding reference. However, CC-supplemented milk appears more viscous under shearing over the range  $pH_i$ -pH3.5 than reference, suggesting the involvement of stronger interactions once calcium supplementation. At the end of the pH cycle, apparent viscosity is almost twice important than in reference.

Apparent viscosity profiles are plotted versus the time for reference and CC-supplemented hydrolysed soy milk, subjected to pH cycle to 3.5 (figure 52).

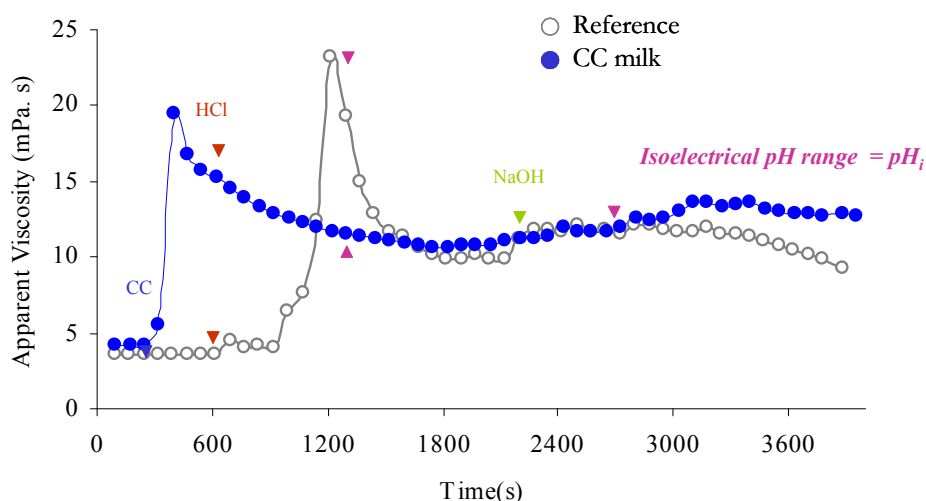


Figure 52. Apparent viscosity measured in reference and CC-supplemented hydrolysed soy milk (25 mmol/kg), subjected to pH cycle ( $pH_{\min} = 3.5$ ;  $T = 25^{\circ}\text{C}$ ) versus the time

For reference, the initial apparent viscosity is of 3.7 mPa.s. Upon pH cycle, same apparent viscosity variations than in non hydrolysed soy milk are observed but fluctuations are smaller. The final viscosity measured after a pH cycle is of 7.1 mPa.s. The interactions formed through pH cycle are not fully reversed.

Once more, CC supplementation induces a rapid increase of viscosity. The effect of pH cycle is negligible compared to the CC-supplementation effect. Since the initial viscosity is not restored, specific calcium-soy protein interactions are expected.

## 2.4.2. Colloidal particle size determination

### 2.4.2.1. pH cycle to 5.5

Figure 53 presents the results of  $D_{4.3}$  ( $\mu\text{m}$ ) measured for non hydrolysed and hydrolysed soy milk after CC supplementation and pH cycle up to 5.5. Unsupplemented soy milk constituted the reference. It can be noticed that the size range is much smaller for hydrolysed soy milk than for non hydrolysed soy milk.

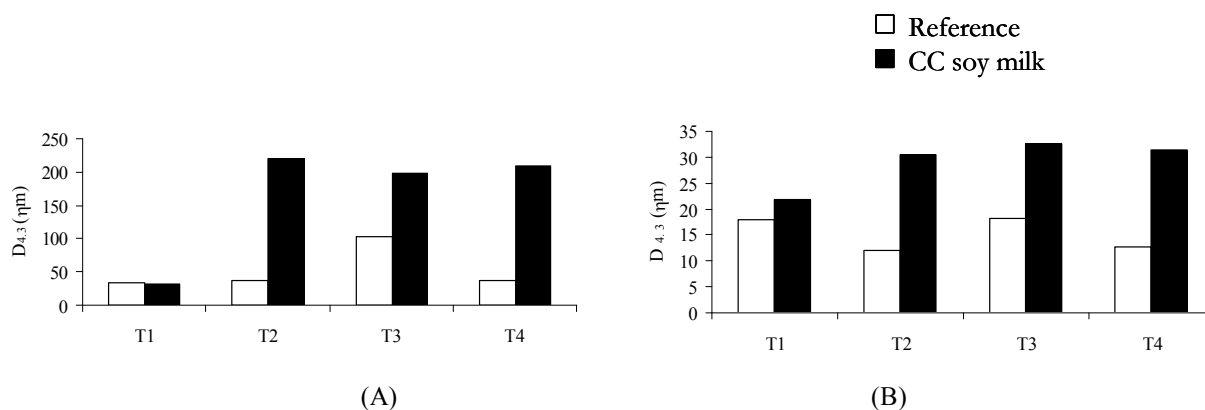


Figure 53. Mean volume diameter  $D_{4.3}$  ( $\mu\text{m}$ ) measured at the end of each step of the kinetic ( $T_1$  = probe stabilization,  $T_2$  = calcium equilibrium,  $T_3$  = acidification,  $T_4$  = alkalization) for non hydrolysed (A) and hydrolysed soy milk (B), CC-supplemented (25 mmoles/kg) and subjected to pH cycle ( $\text{pH}_{\text{min}} = 5.5$ ;  $T = 25^\circ\text{C}$ ). Mean of duplicated experiments.

At  $T_1$ , the initial volume diameter ( $D_{4.3}$ ) is higher in non hydrolysed soy milk compared to hydrolysed soy milk (about 2.5-fold). For reference non hydrolysed soy milk (figure 53 A), the volume diameter ( $D_{4.3}$ ) increases and decreases upon acidification and alkalization, respectively. After CC addition ( $T_2$ , figure 53 A), the particle size in non-hydrolysed soy milk increases 7-fold. Upon subsequent pH cycle, no significant variation of  $D_{4.3}$  is measured.

For hydrolysed soy milk (figure 53 B), CC addition induces also an increase in size from about 20 to 30  $\mu\text{m}$ .

Particle size variations are more important for non hydrolysed soy milk compared to hydrolysed soy milk, especially upon CC supplementation and upon acidification or alkalization for reference soy milks. The heterogeneity of the systems has to be noticed also.

#### 2.4.2.2. pH cycle to 3.5

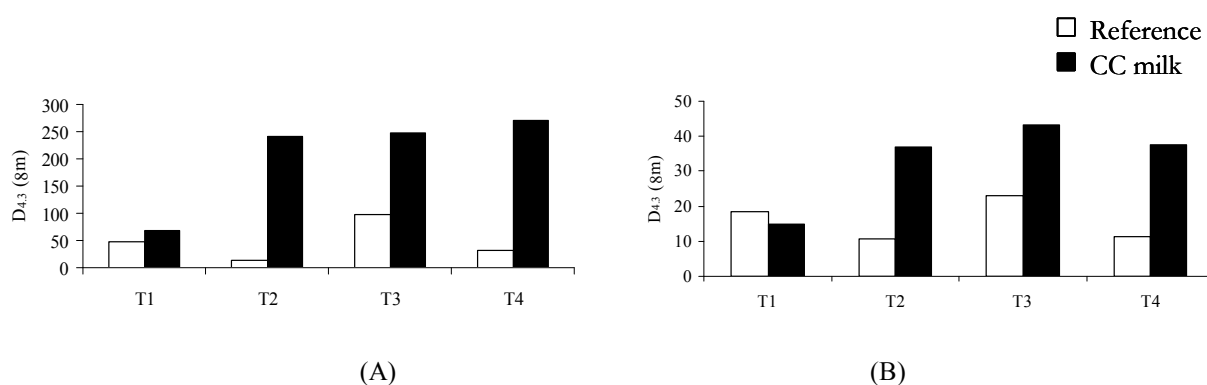


Figure 54. Mean volume diameter  $D_{4.3}$  measured at the end of each specific step of the kinetic ( $T_1$  = probes stabilization,  $T_2$  = calcium equilibrium,  $T_3$  = acidification,  $T_4$  = alkalization) for non hydrolysed (A) and hydrolysed soy milks (B), CC-supplemented (25 mmol/kg) and subjected to pH cycle ( $\text{pH}_{\text{min}} = 3.5$ ;  $T = 25^\circ\text{C}$ )

Similar trends are observed for pH cycle to 3.5 (figure 54) than for pH cycle to 5.5. For reference soy milks, hydrolysed or not, the increase of acid-induced particle size is partially reversible upon alkalization but not reversible after calcium addition.

CC-supplementation (25 mmol/kg) induces a particle size increase ( $T_2$ ) in both soy milks. Yet this particle size increase (from  $T_1$  to  $T_2$ ), related to calcium induced aggregation is commonly reported in literature (Lee and Rha, 1977; Lu et al., 1980; Ono et al., 1993; Molina and Wagner, 1999; Yuan et al., 2002; Scilingo and Anon, 2004). It is higher in non hydrolysed soy milk compared to hydrolysed soy milk (~7-fold and ~3-fold respectively). Indeed the particle size increases from about 50  $\mu\text{m}$  to about 240  $\mu\text{m}$  for non hydrolysed soy milk, and from 20  $\mu\text{m}$  to about 40  $\mu\text{m}$  for hydrolysed soy milk upon CC supplementation.

### 2.4.3. Optical microscopy results

Optical microscopy measurements were performed for pH cycle to 3.5. Pictures were taken at each specific time of the kinetic ( $T_1$ ,  $T_2$ ,  $T_3$ ,  $T_4$ ) for reference and CC-supplemented soy milks subjected to pH cycle (figure 55).

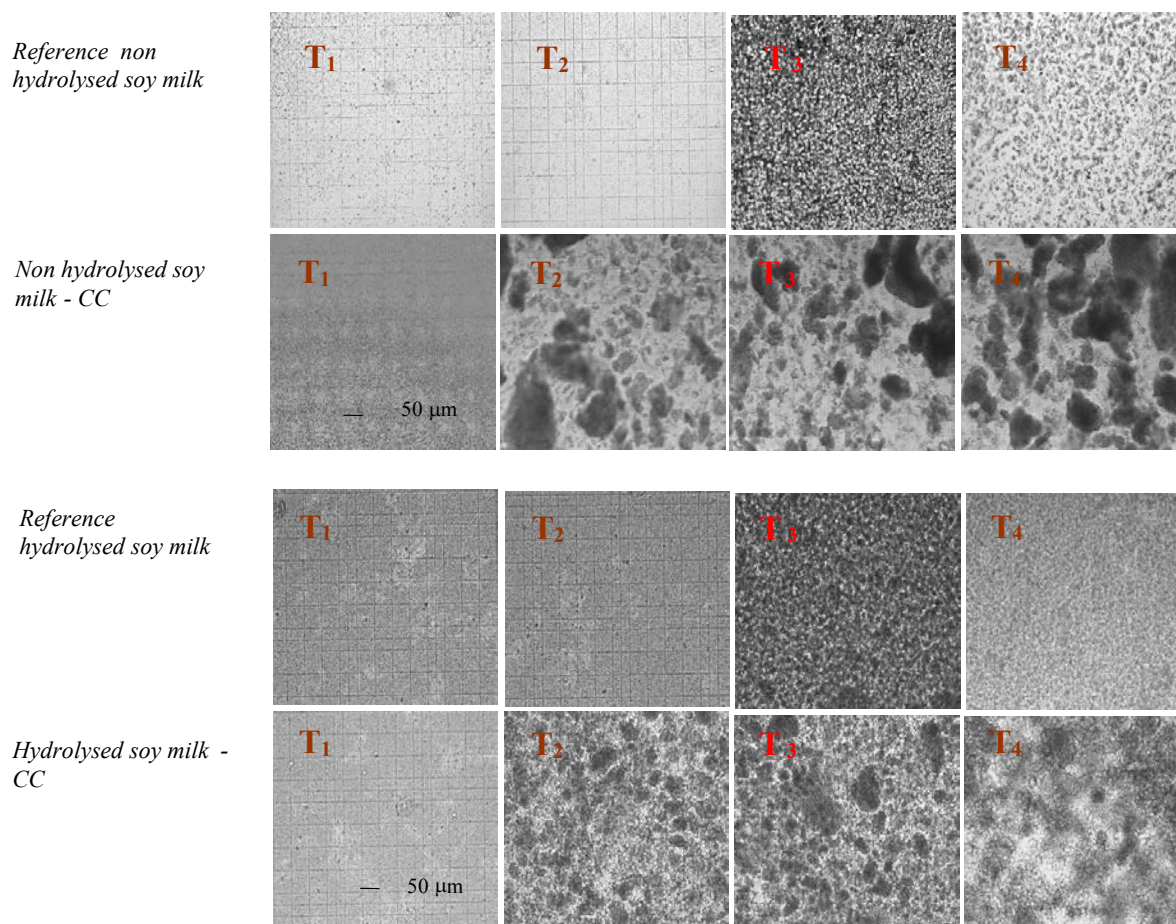


Figure 55. Pictures of non hydrolysed (Up) and hydrolysed soy milk (Down), calcium supplemented (0 or 25 mmoles/kg) and subjected to pH cycle ( $\text{pH}_{\text{min}} = 3.5$ ;  $T = 25^\circ\text{C}$ ) ( $T_1$  = probes stabilization,  $T_2$  = calcium equilibrium,  $T_3$  = acidification,  $T_4$  = alkalinization)

The aggregation induced by calcium addition ( $T_2$ ) is clearly visible from pictures in figure 55 for non-hydrolysed and hydrolysed soy milks. As observed by size distribution measurements, the aggregation induced by acidification ( $T_3$ ) giving a dark appearance to the picture seems partially reversible for control soy milks ( $T_4$ ) whilst irreversible aggregation is evident for CC-supplemented soy milks.

#### 2.4.4. Electrophoretic mobility and zeta-potential

Electrophoretic mobility measurements ( $\mu_E$ ) and subsequent calculated zeta potential values ( $\zeta$ ) are indicated in table 23 for non hydrolysed and hydrolysed soy milks subjected to pH cycle to 3.5.

Table 23. The electrophoretic mobility values ( $\mu$ , e.m.u.) and Zeta potential values ( $\zeta$ , mV) for reference non hydrolysed and hydrolysed soy milk subjected to pH cycle (0 mmoles CC/kg,  $\text{pH}_{\min} = 3.5$ ,  $T = 25^\circ\text{C}$ ).

<i>Non Hydrolyzed soy milk</i>					<i>Hydrolysed soy milk</i>				
pH	$\mu$ (e.m.u.)	$\sigma$ (e.m.u.)	Zeta (mV)	$\sigma$ (mV)	pH	$\mu$ (e.m.u.)	$\sigma$ (e.m.u.)	Zeta (mV)	$\sigma$ (mV)
7.1	-3.217	0.015	-41.0	0.2	7.24	-3.147	0.059	-40.2	0.8
6.35*	-2.670	0.046	-34.1	0.6	6.53	-2.773	0.032	-35.3	0.4
5.5	-1.267	0.194	-16.2	2.5	5.54	-1.800	0.036	-23.0	0.5
4.5	0.284	0.054	3.6	0.7	4.5	-0.265	0.048	-3.4	0.6
3.5	2.727	0.049	34.8	0.6	3.5	0.857	0.039	10.9	0.5
4.5	-0.108	0.035	-1.4	0.5	4.5	-0.146	0.011	-1.9	0.1
5.5	-2.277	0.136	-29.0	1.8	5.5	-1.733	0.100	-22.1	1.2
6.5	-2.367	0.155	-30.2	2.0	6.5	-2.223	0.055	-28.4	0.8
7.1	-3.643	0.170	-46.5	2.2	7.24	-2.713	0.139	-34.6	1.8

\* pH 6.5 was missed

Electrophoretic mobility and zeta potential are negative at initial pH of soy milks: -3.217 e.m.u./-41 mV and -3.147 e.m.u./-40.2 mV for non hydrolysed and hydrolysed soy milk respectively. The former parameters increase with acidification, up to reach a zero value for the isoelectrical pH ( $\text{pH}_i$ ) of soy proteins.  $\text{pH}_i$  is determined just above and just below pH 4.5 for non hydrolysed and hydrolysed soy milk respectively. For pH values lower than  $\text{pH}_i$ , electrophoretic mobility and zeta-potential become positive. At the end of acidification ( $\text{pH}_{\min} = 3.5$ ), non hydrolysed and hydrolysed soy milks have a electrophoretic mobility and a zeta potential of positive value: 2.727 e.m.u./34.8 mV and 0.857 e.m.u./10.9 mV respectively.

With neutralization by alkalization, the reverse phenomenon occurs. The electrophoretic mobility and zeta potential value decrease and become negative to nearly the initial values, i.e. -46.5 mV instead of -41 mV for non hydrolysed soy milk and -34.6 mV instead of -40.2 mV for hydrolysed soy milk and at pH 7.1.

The table 24 gives the electrophoretic mobility and the zeta potential values measured for non hydrolysed and hydrolysed soy milks, before and after CC-supplementation (25 mmoles/kg).

Table 24. The electrophoretic mobility and the zeta potential values for non hydrolysed and hydrolysed soy milk, CC-supplemented (25 mmoles /kg,  $\text{pH}_{\text{initial}} = 7.1$  and  $7.2$  respectively)

Sample	$\mu$ (e.m.u.)	$\sigma$ (e.m.u.)	Zeta (mV)	$\sigma$ (mV)
Non hydrolysed soy milk	-3.217	0.02	-41.0	0.2
Non hydrolysed soy milk + CC	-1.167	0.01	-14.9	0.1
Hydrolysed soy milk	-3.147	0.06	-40.2	0.8
Hydrolysed soy milk + CC	-1.227	0.05	-15.7	0.6

Whatever the soy milk, the electrophoretic mobility and the zeta potential values increase of about +2 e.m.u. and +25 mV with CC supplementation. This increase is significant of the electrostatic nature of calcium-soy protein interactions with the neutralization of negative protein charges by the calcium ions.

This zeta potential variation can explain the calcium induced instability and aggregation of soy milks.

#### 2.4.5. Relationship between soy protein phase behaviour and ionized calcium variations versus pH cycle

##### 2.4.5.1. pH cycle to 5.5

Relationship between ionized calcium variations and protein phase behaviour were drawn in table 25 that summarizes the variation of each studied parameter, i.e.  $[\text{Ca}^{2+}]$ ,  $\eta_{\text{app}}$ ,  $D_{4.3}$ , for both soy milks and pH cycle to 5.5. The slope of the arrows indicated the positive or the negative variations.

Table 25. Variation of  $[\text{Ca}^{2+}]$ ,  $\eta_{\text{app}}$ , and  $D_{4.3}$  for non hydrolysed (NH) and hydrolysed (H) soy milks upon CC-supplementation (25mmoles /kg) and pH cycle to 5.5

Parameter	Sample	CC	Acidification	Alkalinization
$[\text{Ca}^{2+}]$	Reference		↗	↘
	CC milk	↑	↗	↘
$\eta_{\text{app}}$	Reference		↗	↘ → NH
	CC milk	↑	↘	↗ → H
$D_{4.3}$	Reference		↗	↘
	CC milk	↑	↗	↘
$\mu$ and $\zeta$	Reference		↗ -	↘ -
	CC milk	↑ -		

N.B. The grey zone means that there is no measurement

$[\text{Ca}^{2+}]$ : ionized calcium concentration;  $\eta_{\text{app}}$ : apparent viscosity;  $D_{4.3}$ : Volume diameter;  $\mu$ : electrophoretic mobility;  $\zeta$ : zeta-potential (-: negative value)

In reference soy milks, acidification involves endogenous calcium ion solubilization and subsequent regression of the ionization state of soy proteins. The mean volume diameter ( $D_{4.3}$ ) is increased due to acid-induced aggregation and consequently the viscosity is increased. Upon alkalization, the ionization state of soy proteins is increased again. Ionized calcium equilibrium and  $D_{4.3}$  are reversed; yet apparent viscosity is reversed for hydrolysed soy milk only. Hence we can conclude that aggregation is partially or fully reversed for non hydrolysed and hydrolysed soy milks, respectively. Upon alkalization, ionized calcium is either reintegrated inside soy proteins and/or complexed with anions from the bulk (figures 56-57).

With CC-supplementation, ionized calcium concentration is increased but the variations are smaller than expected. In the meantime, the zeta potential, the volume diameter and apparent viscosity are increased as well. The concomitant variations of these 3 parameters indicate calcium-soy protein interactions of electrostatic nature, which induce aggregates formation. During acidification, calcium bound to proteins or to other anions is released in the soluble phase. A decrease in apparent viscosity is measured. During alkalization, calcium ions are reabsorbed by the protein colloidal phase and/or complexed with anions contained in the soluble phase. The consequences are distinct for non hydrolysed and hydrolysed soy milks with an increase and a stability of apparent viscosity observed, respectively. This should be related to the larger size of aggregates induced by calcium addition in non hydrolysed soy milk (250  $\mu\text{m}$ ) compared to hydrolysed soy milk (40  $\mu\text{m}$ ). We can expect a reinforcement of non hydrolysed soy proteins aggregates ( $\uparrow\eta_{\text{app}}$ ) and almost no effect in hydrolysed soy milk. Once CC supplementation, no significant variations are measured on  $D_{4.3}$  for both soy milks (figure 58).

#### 2.4.5.2. pH cycle to 3.5

Relationship between ionized calcium variations and protein phase was drawn for pH cycle to 3.5. Table 26 summarizes the variation of  $[\text{Ca}^{2+}]$ ,  $\eta_{\text{app}}$ ,  $D_{4.3}$ ,  $\text{dB}/\text{dpH}$ ,  $\mu$  and  $\zeta$  for both soy milks, upon CC supplementation and pH cycle to 3.5.

Table 26. Variation of  $[Ca^{2+}]$ ,  $\eta_{app}$ ,  $D_{4.3}$ ,  $dB/dpH$ ,  $\mu$  and  $\zeta$  for non hydrolysed or hydrolysed soy milks upon CC supplementation (25mmoles /kg), acidification and alkalization during pH cycle to 3.5

Parameter	Sample	CC	Acidification		Alkalinization	
			$pH > pH_i$	$pH < pH_i$	$pH < pH_i$	$pH > pH_i$
$[Ca^{2+}]$	Reference					
	CC milk	↑				
$\eta_{app}$	Reference					
	CC milk	↑				
$dB/dpH$	Reference					
	CC milk					
$D_{4.3}$	Reference					
	CC milk	↑				
$\mu$ and $\zeta$	Reference		-	+	+	-
	CC milk	↑ -				

N.B. The grey zone means that there is no measurement

$[Ca^{2+}]$ : ionized calcium concentration;  $\eta_{app}$ : apparent viscosity;  $D_{4.3}$ : Volume diameter;  $\mu$ : electrophoretic mobility;  $\zeta$ : zeta-potential (-: negative value; +: positive value);  $dB/dpH$ : buffering capacity

For pH cycle to 3.5, all the studied parameter ( $[Ca^{2+}]$ ,  $\eta_{app}$ ,  $\mu$  and  $\zeta$ ) show two distinct steps during acidification and the reverse events upon alkalization when the  $pH_i$  region is going through.

For reference soy milks, with acidification, from initial pH to  $pH_i$ , the ionized calcium concentration and the apparent viscosity increase. The electrophoretic mobility and the zeta potential value decrease. The maximum value of each parameter is reached at the isoelectrical  $pH_i$  region, where the zeta potential value is null. With acidification, endogenous and/or exogenous calcium is released due to a regression of ionization state of soy proteins, the zeta potential value decreases from negative to zero value and apparent viscosity increases. The maximum of aggregation is reached at  $pH_i$ . With acidification from  $pH_i$  to pH 3.5 (the minimal pH of the pH cycle), ionized calcium decreases in the soluble phase. Since soy proteins are positively charged below  $pH_i$ , calcium would rather be chelated with anions contained in the soluble phase. Reverse phenomena occur upon alkalization. From pH 3.5 to  $pH_i$ , the ionized calcium increases due to its solubilization from anions. In the meantime, to counteract alkalization and in relation to their buffering properties, soy proteins release protons and become less and less positively charged. Due to the regression of their ionization state, soy protein interactions increase and lead to an elevation of apparent viscosity up to reach a maximum at  $pH_i$ . From  $pH_i$  to initial pH of soy milk, ionized calcium reintegrates soy



protein aggregates. Since apparent viscosity decreases we would expect a restructuring of these soy protein aggregates. pH cycles to 3.5 are not reversible (figures 56-57).

With CC-supplementation, particle size  $D_{4.3}$  increased and was irreversible whatever the soy milk. The calcium-induced aggregation is higher in non hydrolysed than in hydrolysed soy milk ( $D_{4.3}$  is increased  $\sim 7$  and  $\sim 3$  times respectively between  $T_1$  and  $T_2$ ). Hence, this would indicate a higher sensitivity of non hydrolysed soy proteins for calcium ions.

Once CC is supplemented, the formed calcium soy proteins aggregates showed only few variations in size whereas others parameters such as the ionised calcium concentration, the apparent viscosity and the buffering index varied all over the pH cycle (figure 58).

To conclude, the relationships between ionized calcium variations and protein colloidal phase during pH cycle for non hydrolysed and hydrolysed soy milks are schematized in table 27 and figures 56-58.

Table 27. Reversibility (white), partial reversibility (soft grey) and irreversibility (dark grey) of phenomena observed in calcium supplemented and pH cycled soy milks

	Non hydrolysed soy milk		Hydrolysed soy milk	
	Reference	CC milk	Reference	CC milk
pH Cycle to 5.5				
pH Cycle to 3.5				

In figures 56-58, the size of the letter used for describing the physico-chemical parameter is proportional to the phenomenon. A bigger letter means an increase in variation of a given parameter and inversely, a smaller letter fits with a decrease in variation of a given parameter.

For non hydrolysed soy milk, whatever the minimal pH of pH cycle ( $pH_{min} = 5.5$  or 3.5), the phenomena observed were partially reversible (figure 56).

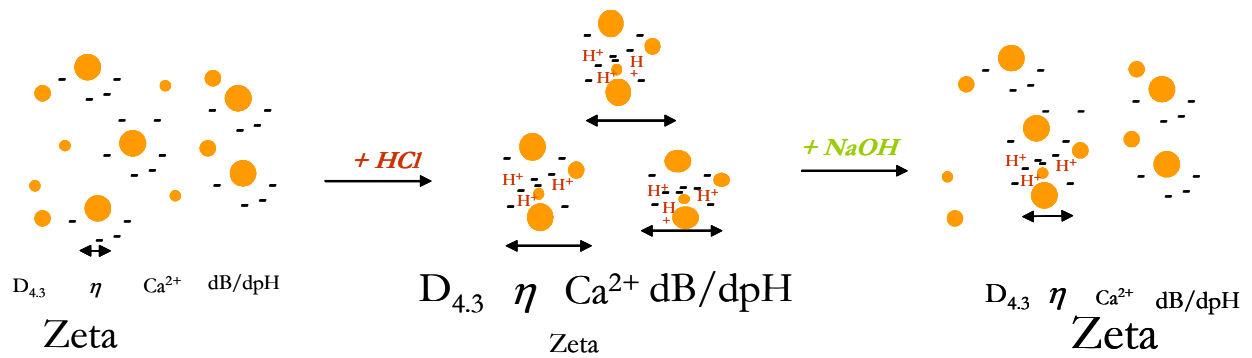


Figure 56. Relationship between ionized calcium variations and protein colloidal phase in non hydrolysed soy milk during pH cycle

For hydrolysed soy milk, the phenomena are completely reversible for pH cycle to 5.5 ( $pH > pH_i$ ) but were irreversible for pH cycle to 3.5 ( $pH < pH_i$ ; figure 57).

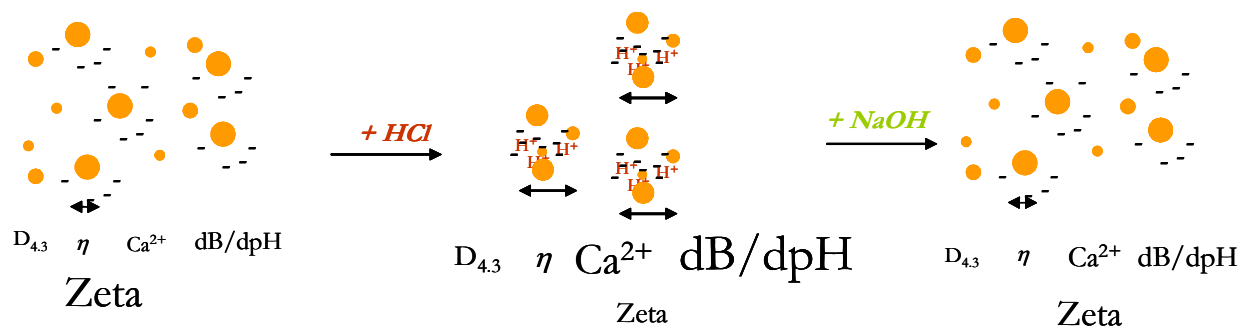


Figure 57. Relationship between ionized calcium variations and protein colloidal phase in hydrolysed soy milk during pH cycle

Whatever the soy milk, non hydrolysed or hydrolysed, the calcium-induced aggregation is irreversible upon pH cycle ( $pH_{min} = 5.5$  or  $3.5$ ; figure 58).

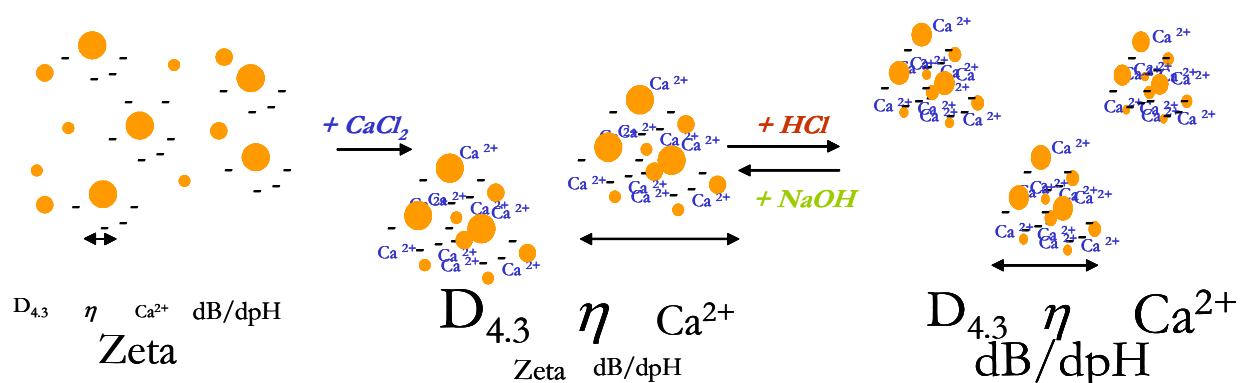


Figure 58. Relationship between ionized calcium variations and protein colloidal phase in calcium supplemented and pH cycled soy milks (hydrolysed or non hydrolysed)

#### 2.4.5.3. Proposition of a model for calcium equilibrium in soy milks

Calcium equilibrium in soy milk during pH cycle is proposed in the following scheme (figure 59). Similarly to the salt equilibrium proposed by Brulé (1981) and Gaucheron (2004) in cow milk, calcium equilibrium exists between calcium ions, the anions contained in the soluble phase and the soy proteins considered as charged entities. Indeed, as mentioned in the specification (Appendix 2), phosphate mineral is present in the soluble phase of reconstituted soy milks. Yet in soy milk, phosphate exists in the majority under phytate form. More, acid-amino acids (contained in high proportion in SPI powders) have a pK value of around 4.5-4.7. Hence for pH higher than pK, aspartic acid and negative acids are negatively charged and can interact with Calcium ions.

During acidification, for pH higher than isoelectrical pH, calcium ions are first in equilibrium with the proteins as a charged entity, calcium is solubilized from soy protein, and hence ionized calcium concentration is increased up to reach the  $pH_i$  (arrows 1). Then, for pH values lower than isoelectrical pH, calcium ions are in equilibrium with anions contained in the soluble phase to form calcium salts (arrows 2), hence ionized calcium concentration is decreased.

During alkalization, for pH below  $pH_i$ , calcium ions are decomplexed from their respective calcium salt, which involves ionized calcium concentration increase (arrows 3). Then, for pH values higher than  $pH_i$ , ionized calcium interact with the soy protein phase, which involves a ionized calcium decrease (arrows 4).

To summarize:

- Above the isoelectrical pH, calcium is in equilibrium both with the proteins as a charged entity and with the free anions as well.
- Below the isoelectrical pH, calcium is in equilibrium with anions contained in the soluble phase only.

Note that the maximum ionized calcium concentration is measured for isoelectrical pH.

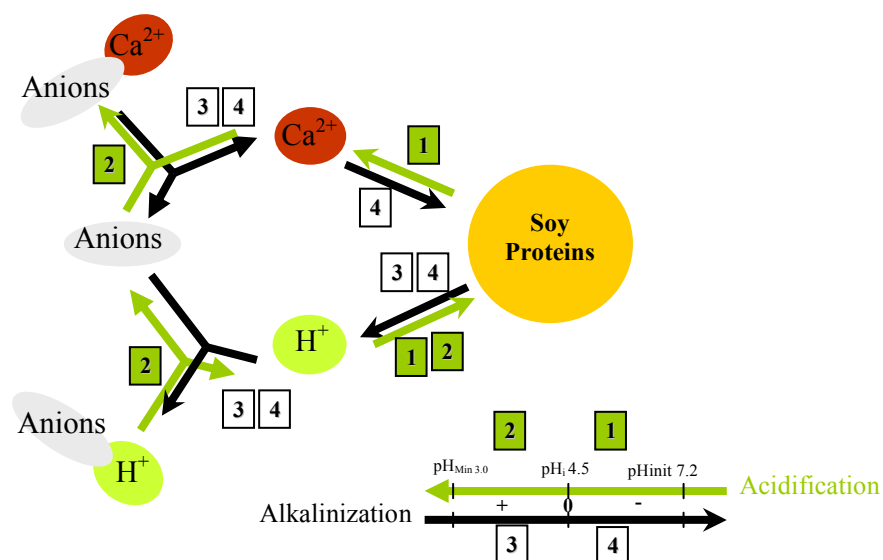


Figure 59. Calcium equilibrium in pH cycled soy milk (Canabady-Rochelle et al., 2008)

## 2.5. Conclusion

The SPI solubility, pH dependent, showed a minimum of solubility at about pH 4.5, the isoelectrical pH, and hydrolysed SPI (H) were more soluble than non hydrolysed SPI (NH). The pH-dependence of SPI solubility was reduced upon CC-supplementation. Less aggregation induced by CC occurred for H-SPI than for NH-SPI.

Calcium equilibrium between soluble and colloidal phases was studied in Ca-supplemented soy milk upon pH cycle and related to protein phase variations. CC-supplementation (0 or 25 mmol/kg) was followed by pH cycle (pH<sub>min</sub> 5.5 or 3.5). Ionized calcium variations were acquired *in situ* and protein phase characterized at specific time of the pH cycle.

Ionized calcium concentration, initially negligible, increased upon CC-supplementation and acidification and decreased during alkalinization.

The apparent viscosity of reference NH-soy milk increased during acidification and was not restored upon alkalinization (pH cycle to 5.5), contrarily to H-soy milk. Once CC supplemented, apparent viscosity increase was irreversible, involving strong Ca-soy protein interactions and following pH cycle effect was negligible.

The initial volume mean diameter, higher in NH-soy milk compared to H-soy milk, increased and decreased upon acidification and alkalinization, respectively. Particle size

variations are more important for NH-soy milk compared to H-soy milk, especially upon CC supplementation (larger size of aggregates induced by CC-supplementation in NH-soy milk).

For pH cycle to 3.5, ionized calcium concentration reached a maximum at about the  $pH_i$ . Above  $pH_i$ , calcium ions bound to negatively charged proteins whereas below this pH value, calcium ions interacted with anions of the soluble phase or with positively charged proteins through anions bridges. Similarly to pH cycle to 5.5, apparent viscosity increased and decreased during acidification and alkalization, respectively, but a maximum of apparent viscosity was observed at the  $pH_i$  region. For such pH cycle reversibility was never observed.

For reference soy milks, the acid-induced particle size increase was only partially reversible upon alkalization and irreversible after CC addition. Optical microscopy confirmed particle size measurements. The irreversibility of pH cycle to 3.5 was also observed through electrophoretic mobility measurements. Such experiment confirmed that the CC-induced aggregation involved Ca-protein interactions of electrostatic nature.

For pH cycle to 5.5, phenomena observed were partially or completely reversible for NH and H-soy milks, respectively. Irreversibility was observed for pH cycle to 3.5 or once CC-supplementation.

Compared to NH-soy milk, the buffering capacity is higher with H-soy milk, and shifted upward upon CC supplementation. The higher proportion of complexed Ca in the latter system is more efficient to counteract pH variation compared to Ca-bound to proteins.

### 3. Study of calcium-protein interactions in cow skim milk and in soy milks

**ABSTRACT.** Calcium-protein interactions were thermodynamically characterized by Isothermal Titration Calorimetry (**ITC**) in milk proteins and in Non-Hydrolysed (**NH**) or Hydrolysed (**H**) soy proteins. In parallel, zeta-potential was measured to estimate the electrostatic part of Ca-protein interactions. Fourier-Transformed Infra-Red spectroscopy (**FTIR**) was used to get a better characterization of Ca-binding sites.

The global thermodynamic signals obtained upon titration of milk proteins and soy proteins with Calcium Chloride (**CC**) were endothermic. Binding titration curves were fitted with the one set of sites model to determine the thermodynamic parameters ( $N$ ,  $K$ ,  $\Delta H$ ,  $\Delta S$ ). Milk proteins bound up to 8 mg Ca per g of proteins at saturation. NH-soy proteins bound more Ca than H-soy proteins (~52 and ~2 mg Ca per g of proteins respectively). Binding constant  $K$  decreased respectively from H-soy proteins, milk proteins and NH-soy proteins and was indicative of the easiest Ca binding onto H-soy protein. The change in heat capacity ( $\Delta C_p$ ), determined for Ca-soy proteins interactions only, indicated changes in hydrophobic interactions and/or hydrogen bonds. Whatever the soy proteins, the positive value of  $\Delta C_p$  indicated a reduction in the number of surface-exposed polar residues and could be due to the burial of charged ligand and protein polar groups in the binding sites.

Zeta-potential variations occurring upon **CC** titration was indicative of Ca-protein interaction of electrostatic nature. Hence, the usual exothermic signal involved by electrostatic interactions was completely hidden by the strong endothermic signal coming from the release of water molecules, either from the hydration shell of the calcium ions and/or dehydration of hydrophobic core of proteins. Ca binding should rather be described as a counterion exchange between  $H^+$  and  $Ca^{2+}$ , which is at least best energetically neutral with respect to the electrostatic forces involved.

Finally, with FTIR spectroscopy, a decrease of the absorption energy in the amide I and II region and in the carboxylate region was observed upon **CC**-addition, with higher variations in soy milks.

### 3.1. Thermodynamic characteristics of calcium-protein interactions

#### 3.1.1. Calcium-milk protein interaction

To the author knowledge few data concerning calcium-milk protein interactions in a complex mixture of proteins is available. Calcium binding with purified milk proteins (i.e.  $\alpha_s$ -casein,  $\beta$ -casein,  $\kappa$ -casein,  $\beta$ -lactoglobulin or  $\alpha$ -lactalbumin) is often more studied in the literature. Hence, whole milk protein solution (prepared from industrial skim milk powder) was titrated with Calcium Chloride (CC, 50 mM) at 25°C to thermodynamically characterize Ca-protein interactions. Three different types of ITC experiments were performed. (i) In the dilution experiments, water was injected into a protein solution. The signal obtained was considered negligible compared to those obtained with the two following experiments. (ii) In the binding titration, CC was injected into the protein solution (sample). (iii) Then CC dilution into miliQ water (reference) was also measured to eliminate possible contribution to this signal, the so-called heat of dilution. In the analysis of our experiments, this later signal was subtracted from the binding titration curve.

Once saturation is achieved (plateau), the binding titration curve ( $\Delta H$ , kcal/mole of CC, versus molar ratio, mole Ca/mole protein; result not shown), was fitted with a one set of sites model. The 4 binding parameters (N, K,  $\Delta H$ ,  $\Delta S$ ) were obtained and the quality of the fit could be evaluated from the  $\chi^2$  value. The one set of sites fitting model matched exactly with the experimental data. As a consequence a very low  $\chi^2$  value, indicative of the fit quality, was obtained (Mean value  $\sim 7.3$ ). In the following binding curve (figure 60), the binding enthalpy ( $\Delta H$ , kcal/mole of CC) is presented as a function of the mass ratio (g Ca/g of protein). The fitting parameters obtained from a “one set of sites” fitting model are summarized in table 28.

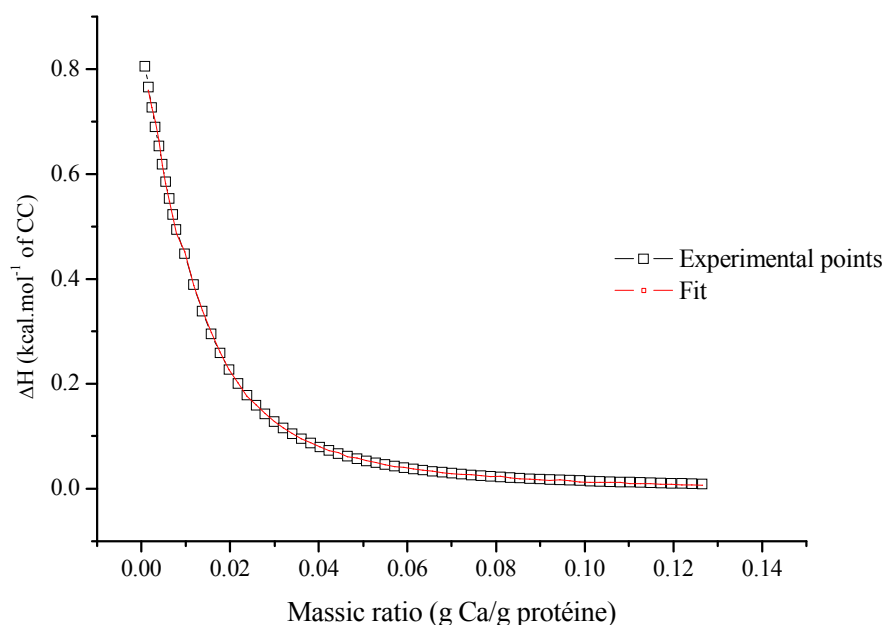


Figure 60. Titration of milk protein solution by CC (50 mM) at 25°C after subtraction of the reference binding curve (CC titration into miliQ water). Duplicated experiments.

Table 28. Binding parameters obtained from a “one set of sites” fitting model – Mean of the duplicated experiments

N	$6 \pm 0.1$	mole Ca/mole Protein
$N$	8	mg Ca/g protein
K	$1070 \pm 15$	$M^{-1}$
$\Delta H$	$1910 \pm 38$	cal.mol <sup>-1</sup> CC
$\Delta S$	20.3	cal.mol <sup>-1</sup> .K <sup>-1</sup>

N: number of sites

K: binding constant, depends only of the titrant (CC) concentration

$\Delta H$ : heat change, depends only of the titrant (CC) concentration

$\Delta S$ : entropy change, calculated from  $\Delta H$  and K

The titration of milk protein solution with CC shows a highly reproducible signal, which is of endothermic nature ( $\Delta H > 0$ ). The enthalpy of dilution of milk proteins is observed once saturation (plateau value) is reached. It provides a measure of the energy changes in the interaction of solvated solute species upon dilution. In our experiment, the enthalpy of dilution is negligible.

From figure 60, N is obtained at half saturation value and K corresponds to the slope of the binding curve. We determined that the whole milk protein fraction could bind up to 8 mg Ca per g of milk protein at saturation. The binding constant (K) is set to  $1.07 \cdot 10^3 M^{-1}$ . The



enthalpy ( $\Delta H$ ) of binding per mole of cation ( $\text{Ca}^{2+}$ ) was endothermic ( $1910 \text{ cal.mol}^{-1} \text{ CC}$ ) and the entropy ( $\Delta S$ ) of binding was of  $20.3 \text{ cal.mol}^{-1}.\text{K}^{-1}$  (table 28). Hence, at  $25^\circ\text{C}$  (equivalent to  $298 \text{ K}$ ), the Gibbs free energy ( $\Delta G$ ) was calculated from the following equation:

$$\Delta G = \Delta H - T\Delta S \quad \text{Equation 14}$$

and was equivalent to  $-4139 \text{ cal.mol}^{-1}$ . As a consequence, the entropy ( $T\Delta S$ ) of this system overcame the binding enthalpy ( $\Delta H > 0$ ) and showed that the calcium ion binding was entropically driven. Since the  $\Delta G$  of the calcium binding reaction was negative, the calcium binding onto milk protein occurred spontaneously.

The results obtained in this study concerning the stoichiometry of calcium-milk protein interactions are compared with other investigations (table 29). In the major part of the studies, the various techniques used (centrifugation, ultrafiltration, dialysis...) separated the colloidal protein phase of the soluble phase. Calcium bound to proteins was calculated as the difference between the total calcium measured in the whole sample and the soluble calcium contained in the soluble phase.

Table 29. Calcium-milk protein interaction determined by Isothermal Titration Calorimetry - Comparison with others investigations

Authors, Year	mole $\text{Ca}^{2+}/10^5$ g proteins	mg Ca/g protein	Sample
This study	20.0	8.0	Whole milk proteins
Pappas and Rothwell, 1991	24.1	9.6	Whole caseins
Imade et al. 1977	16.0	6.4	
Tessier and Rose, 1961	33-36	13.2	
Zittle et al., 1958	29.0	11.6	
Carr, 1953	22.0	8.8	
Chanutin et al., 1942	48.0	19.2	
Pappas and Rothwell, 1991	44.6	17.8	$\alpha_s$ -caseins
Imade et al., 1977	25.0	10.0	
Bingham et al., 1972	42.3	16.9	
Dickson and Perkins, 1971	36.0	14.4	
Noble and Waugh, 1965	46.5	18.6	
Pappas and Rothwell, 1991	34.9	13.9	$\beta$ -caseins
Dickson and Perkins, 1971	20	8	
Pappas and Rothwell, 1991	22.2	8.9	$\kappa$ -caseins
Dickson and Perkins, 1971	11.0	4.4	
Pappas and Rothwell, 1991	35.3	14.1	$\beta$ -lactoglobulin
Jeyarajah and Allen, 1994	8.3	3.3	
Pappas, 1991	31.0	12.4	$\alpha$ -lactalbumin

The results of this study on whole milk proteins are in the range of values (6.4-13.2 mg Ca/ g protein) obtained for other investigations on whole caseins (Carr, 1953; Zittle et al., 1958; Tessier and Rose, 1961; Imade et al., 1977; Pappas and Rothwell, 1991) and more particularly  $\beta$ -caseins (Dickson and Perkins, 1971).

The values of moles Ca bound per  $10^5$  g proteins (N) vary for each protein fraction. Nevertheless the variability for a same protein fraction is not negligible between various investigations. We could expect that differences observed in the value of “N” come from the variations in the method of protein sample preparation and due to the use of different buffers at different ionic strength. Indeed, the effect of the ionic strength increase involves a calcium-protein interaction decrease observed through a partial solubilization of calcium bound to proteins (Le Graët and Brulé, 1993). The variations can also be related to the experimental techniques used to determine the interaction (ITC, Ion Selective Electrode...).

In the literature, calcium binding properties of proteins are often determined through the use of a Scatchard plot (Appendix 4). For a set of equivalent binding sites the plot is a straight line with the intercept on the abscissa giving  $N$  (the maximum amount of bound calcium per  $10^5$  g of protein,  $\text{mole.g}^{-1}$ ), and the slope of the linear Scatchard plot giving  $K$  ( $\text{M}^{-1}$ ). If the binding involves more than one type of binding sites, the plot is nonlinear.

Jeyarajah and Allen (1994) studied the binding of calcium to  $\beta$ -lactoglobulin, the major whey protein. The Scatchard plot obtained from the corresponding curve of calcium binding to  $\beta$ -lactoglobulin showed linearity, which indicated that the calcium- $\beta$ -lactoglobulin interaction fitted to a one-set-of-sites model. This result is similar to what we had obtained in a whole milk proteins fraction. From the Scatchard plot,  $N$  (moles Ca bound/ moles of  $\beta$ -lactoglobulin) and  $K$  (binding constant,  $\text{M}^{-1}$ ) were determined. These former parameters were set at 3 moles Ca bound/ moles of  $\beta$ -lactoglobulin and  $0.13 \text{ mM}^{-1}$ . Taking into account a molecular weight of 36 KDa for the  $\beta$ -lactoglobulin dimer, the number  $N$  can be expressed as 3.3 mg Ca/g of  $\beta$ -lactoglobulin. When comparing with our results, we found a value of  $N$  equal to 8 mg Ca/g of milk protein, which is close to the former study, considering the difference of protein samples. Yet the association constant value is of  $0.13 \text{ mM}^{-1}$ , which is  $8.10^6$  times lower than our results. The association constant  $K$  for  $\text{Ca}^{2+}$ - $\beta$ -lactoglobulin interaction reported by Dufour and Haertlé (1993) is determined at  $3.10^3 \text{ M}^{-1}$  and is closer from our result ( $1.07.10^3 \text{ M}^{-1}$ ).

Tamura et al. (1982) studied the calcium-binding proteins purified from milk. These calcium-binding proteins, clearly identified as different from caseins, had molecular weight of 15 KDa and were expected to be  $\alpha$ -lactalbumin (another whey protein). Contrarily to our results, former authors determined two kinds of binding sites for  $\text{Ca}^{2+}$ , with association constant  $K$  respectively of  $1.7. 10^6$  and  $5.1.10^4 \text{ M}^{-1}$ . The amino-acid analysis performed by the method of Inglis and Liu (1970) showed that these calcium binding proteins were rich in aspartic acid, glutamic acid, leucine and lysine. According to their results, Tamura and co-workers suggested that conformational changes occurred upon calcium binding.

Pappas and Rothwell (1991) determined the total binding calcium capacity of different milk proteins using continuous ultrafiltration in order to reach the binding equilibrium. At such an equilibrium, milk caseins had a high calcium binding capacity due to the high number of calcium binding sites and the order of binding capacity was determined as  $\alpha_{s1}$ -casein  $>$   $\beta$ -casein  $>$   $\kappa$ -casein. For whey proteins the order was set as  $\beta$ -lactoglobulin and  $\alpha$ -lactalbumin. Former authors used the Klotz equation (1950, Appendix 4), to plot another variant of

Scatchard graph, where coordinates change a little, and determine the maximum number (N) of moles of calcium bound to  $10^5$  g of proteins. This representation involves that the plotting of the corresponding curve gives a linear relationship. Yet, the results obtained by former authors deviated from linearity for the whole casein sample. According to Pappas and Rothwell, this may be due to the electrostatic influence of the bound calcium on its subsequent binding and/or the variation in the type and number of calcium binding sites. Yet, similarly to our results, former authors reported that the binding data of whole casein obtained within the range 10-20 mM of CC were in better agreement with linearity.

To evaluate the electrostatic influence upon calcium-milk protein interaction, calcium binding titration was performed in the same conditions that during ITC experiment and followed by zeta-sizer. The CC titration lasted up to reach a similar plateau value of zeta potential, as observed in ITC experiment. The zeta potential of skim milk solution, initially of -25 mV, increased up to reach -20 mV at saturation (results not shown) and was indicative of Ca-protein interactions of electrostatic nature. The zeta-potential variation upon CC titration is low (5 mV) and the binding of calcium on milk protein shows a highly endothermic signal ( $\Delta H > 0$ ). Hence, the exothermic part of the signal involved by electrostatic interactions between  $\text{Ca}^{2+}$  and milk proteins is low and completely hidden by the strong endothermic signal. Sinn and co-workers studied the binding of calcium to polyelectrolytes (2004) and more recently, to negatively charged phosphatidylcholine-phosphatidylserine membranes (2006) by ITC. In both cases, former authors had reported a strong endothermic reaction, enthalpy driven, with a gain in entropy. More Dimova et al. (2003) studied the binding of polymers to calcite crystals in water or calcium ion and also observed an overall endothermic phenomenon for the latter case solely. These results were unexpected since electrostatic interactions are known to be highly exothermic. However, the endothermic character of this process can be understood in terms of the competition between the polymer/ion, polymer/water and ion/water interactions. Similarly to the former authors, we could suggest that not Coulomb interactions, but rather liberation of water molecules, either from the hydration shells of the calcium ion and/or dehydration of hydrophobic core of proteins was the driven energy source for the binding of multivalent ions onto milk proteins. Indeed, the binding of one  $\text{Ca}^{2+}$  ion to the polymer distorts the hydration shells of both binding partners and leads to the liberation of many water molecules from these shells. Before the binding reaction, free  $\text{Ca}^{2+}$  requires at least 6 water molecules to be fully solvated (Enderby, 1981; mentioned in Kuroki et al., 1992). After binding  $\text{Ca}^{2+}$ , at least 7 water molecules are released

from both  $\text{Ca}^{2+}$  (6 water molecules) and the protein (1 water molecule replaced with  $\text{Ca}^{2+}$ ). Thus the increase in entropy upon  $\text{Ca}^{2+}$  binding is considered to be from the release of water molecules and primarily from  $\text{Ca}^{2+}$ . Roughly speaking, the energy gain associated with the binding of the  $\text{Ca}^{2+}$  ion to the polymer is overcompensated by the energy loss arising from the broken hydrogen bonds. This interpretation is in accordance with the study of Garidel and Blume (1999), who reported that calcium ion binding involved a strong dehydration of hydrophobic regions.

As a consequence, binding should be rather described as a counterion  $\text{H}^+/\text{Ca}^{2+}$  exchange, which is more energetically neutral (less exothermic phenomenon) than electrostatic forces.

Simultaneously to zeta potential measurement, the particle size of skim milk was measured by nano-sizer. It was observed that particle size decreased upon calcium supplementation and went on to diminish even when the zeta potential plateau value of -20 mV was already reached. The milk particle size measured was initially of 215 nm and decreased up to stabilize at about 190 nm (result not shown). This retraction of casein micelle could be interpreted as the absorption of calcium added either in the periphery of the casein micelles, i.e. onto the negatively charged hairy layer of  $\kappa$ -casein, or in the core of the casein micelle involving the reinforcement of internal interactions forces. Yet, taking into account the composition in major amino-acids involved in the interaction with calcium (i.e. Asp, Glu, SerP) present in various caseins (table 30), the absorption of calcium would rather occur in the core of casein micelles. Indeed,  $\kappa$ -casein are poorer in these specific amino-acid.

Table 30. Composition in major amino-acids involved in the interaction with calcium present in various caseins (According Eigel et al., 1984)

Amino-acid	$\alpha_{s1}$ -casein	$\alpha_{s2}$ -casein	$\kappa$ -casein	$\beta$ -casein
Aspartic acid (Asp)	7	4	4	4
Glutamic acid (Glu)	24	25	12	18
Phosphoserine (SerP)	8	11	1	5

### 3.1.2. Calcium-soy protein interactions

#### 3.1.2.1. ITC study on whole soy proteins

Whole soy protein solutions (NH or H), prepared from their respective SPI, were titrated by CC (25 mM). The binding titration curves of whole soy protein solution by CC were obtained by ORIGIN integration of injection peak and subsequent subtraction of CC dilution effect (figure 61). In this binding curve, the binding enthalpy ( $\Delta H$ , kcal/mole of injectant) was plotted versus the mass ratio (g Ca/g Soy Protein).

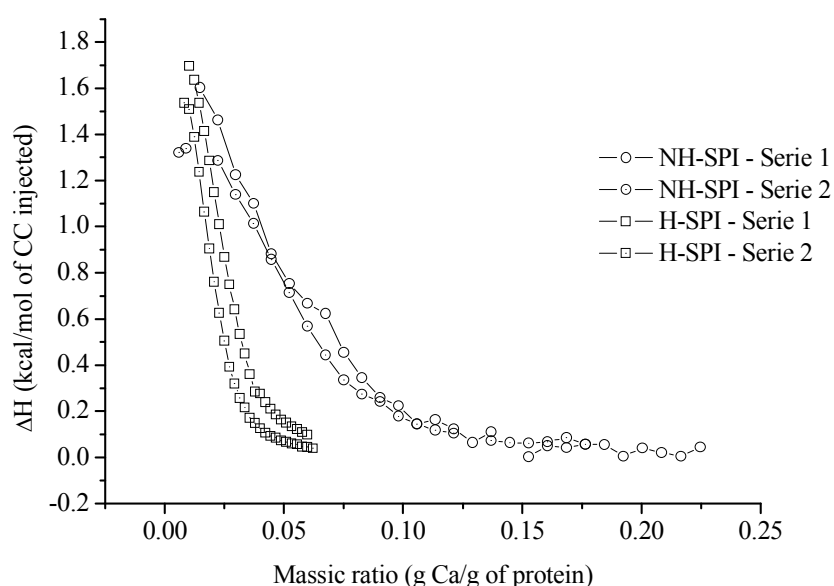


Figure 61. Binding titration curve of soy protein solution by CC (25 mM) after subtraction of the reference signal. SPI: Soy Protein Isolate; NH: Non hydrolysed; H: Hydrolysed.

Binding of  $\text{Ca}^{2+}$  ions to soy proteins showed a highly endothermic process; i.e., the binding seemed solely enthalpy driven ( $\Delta H > 0$ ; figure 61). On the contrary, the dilution enthalpy of CC (25 mM) in miliQ water was negative and consequently exothermic ( $\Delta H < 0$ ; result not shown). Sinn et al (2004), who used the same reference than our, found similar results. This reference signal increased with concentration but was also much smaller in magnitude than the CC-soy protein complexation enthalpy. In this range of concentration, the heat of dilution of soy protein solution was almost constant and could be neglected compared to the heat of titration of CC into soy protein solution.

Upon calcium titration, the binding enthalpy decreases for hydrolysed and non hydrolysed SPI solution down to reach saturation (figure 61). Whatever the SPI solution, the

binding titration curve is characteristic of the one set of sites model, which means that for any number of sites  $n$ , all the sites have the same binding constant value ( $K$ , in  $M^{-1}$ ) and the same enthalpy ( $\Delta H$ ).

Previously, several authors (Appurao and Narasinga Rao, 1975 and 1976; Kroll, 1984; Kumagai et al, 1998 and 2002) characterized the calcium-soy protein interaction and observed a linear relationship on the Scatchard plot. Hence, they concluded that calcium binding sites onto soy protein were equivalent and independent. This is similar to our observations (curve fitting with the one set of sites model).

The binding titration curves vary with the Soy Protein Isolate studied (figure 61). Once the saturation (plateau value) was reached, the mass ratio of calcium bound to soy proteins (g Ca/g proteins) was determined. We can observe that the non hydrolysed soy protein is saturated at much higher calcium loads (plateau value reached latter) than hydrolysed soy proteins. The mean value of calcium ions bound to soy proteins is expressed in table 31 (in mole  $Ca/10^5$  g protein and mg Ca/g of protein respectively) and compared with others investigations on calcium-soy protein interactions.

Table 31. Calcium-soy protein interaction determined by Isothermal Titration Calorimetry Comparison with others investigations

Authors, Year	pH	mole $Ca^{2+}/10^5$ g protein	mg Ca/g protein	Sample
This study	7.2	129	51.7	Non Hydrolysed SPI (S)
		5.5	2.2	Hydrolysed SPI (S)
Kumagai et al., 1998	7.4	986	394.4	Globulin
		707	282.8	Phytate free globulin
Kroll, 1984	6 to 7	22.5-25	9-10	Whole soybean protein
Sakakibara and Nogushi, 1977	6	No Interaction	No Interaction	11S
	7	No Interaction	No Interaction	
	8	3.7-11.5	1.4-4.6 vs. [CC]	
Appurao and Narasinga Rao, 1976	5.5	Negligible	Negligible	7S
	7.8	9 to 10	3.6-4	
Appurao and Narasinga Rao, 1975	5.5	Negligible	Negligible	11S
	7.8	14-17	5.6-6.8	
Saio et al., 1967		24	9.6	CIF

S: stabilized by ultraturrax and high pressure; CIF: Cold Insoluble Fraction  
[CC]: CC concentration added

The values of calcium ions bound to non hydrolysed soy proteins are higher than the values reported in the literature at the exception of the results of Kumagai et al (1998). Indeed

former authors reported the highest values of stoichiometry for calcium-soy protein interactions. On the contrary, the results of calcium ions bound to hydrolysed soy proteins are comprised within the range of values commonly mentioned. These differences of calcium bound onto soy protein can be explained by several reasons such as the kind of soy globulins studied (11S, 7S, or whole soy globulin fraction), the native or aggregated forms, the way of sample preparation on a chemical (pH of extraction, ionic strength, calcium and phytate deionization procedure...) and on mechanical levels (use of ultraturrax and high pressure in this study). Also the number of calcium binding sites may depend on the degree of dissociation of soy globulins and especially on any conformational changes accompanying the dissociation. Therefore, this important difference in the method of protein preparation may account for the difference in the number of calcium ions bound to soy proteins. Finally, differences in the methods of measuring  $\text{Ca}^{2+}$  binding (ITC, Ca ISE, gel filtration, dialysis...) may account for large variation in the quantification of calcium-soy protein interactions.

The huge difference observed with the study of Kumagai and coworker (1998) comes from the fact that they evaluated the calcium bound to one amino-acid of 140 Da on average. We consider that the scientific approach of former authors can be inappropriate. Indeed, each amino-acid contains a carboxylic acid function in  $\text{C}_\alpha$ , which can react under ionized form ( $\text{COO}^-$ ) with calcium ions. In the case of proteins, this carboxylic acid function is involved in an amide bound and consequently less available for interactions.

The comparison of the K value for hydrolysed and non hydrolysed soy protein isolate shows that the hydrolysed soy protein has a higher binding constant affinity (K) (slopes, figure 61). This means that hydrolysed formula binds more easily calcium ions than the non hydrolysed formula does. This may be explained by the fact that as hydrolysed soy proteins are smaller in size, calcium ions binds more easily since there are less conformational modifications and less structural rearrangement upon CC titration. Hence, calcium accessibility is favoured into the calcium binding sites.

In the binding titration curve of soy protein solution by CC (figure 61) the reaction enthalpy ( $\Delta H$ ) decreases with increasing concentration of added calcium. This could be caused by two effects: the enthalpy drops either because the amount of  $\text{Ca}^{2+}$  bound to soy proteins decreases or because the heat per bound  $\text{Ca}^{2+}$  falls. Both effects could also occur simultaneously. In order to answer to this question, the calcium binding isotherm was determined by Ca ISE on similar experiments than ITC. The amount of ionized calcium



bound to soy proteins ( $[AC]$ ) was calculated from differences between concentration of calcium ions in miliQ water after each injection (total ionized calcium,  $[C]_t$ ) and ionized calcium measured in the soy protein solution after each calcium injection (free ionized calcium,  $[C]$ ). This can be summarized by equation 15 hereafter:

$$[AC] = [C]_t - [C] \quad \text{Equation 15}$$

Ionized calcium concentration (figure 62A) and the free/bound calcium ratio (calcium binding isotherm; figure 62B) were plotted versus the concentration of added CC, measured in reference. In figure 63, the ionized calcium concentration (M) was set out as a function of the mass ratio (g Ca/ g of soy proteins).

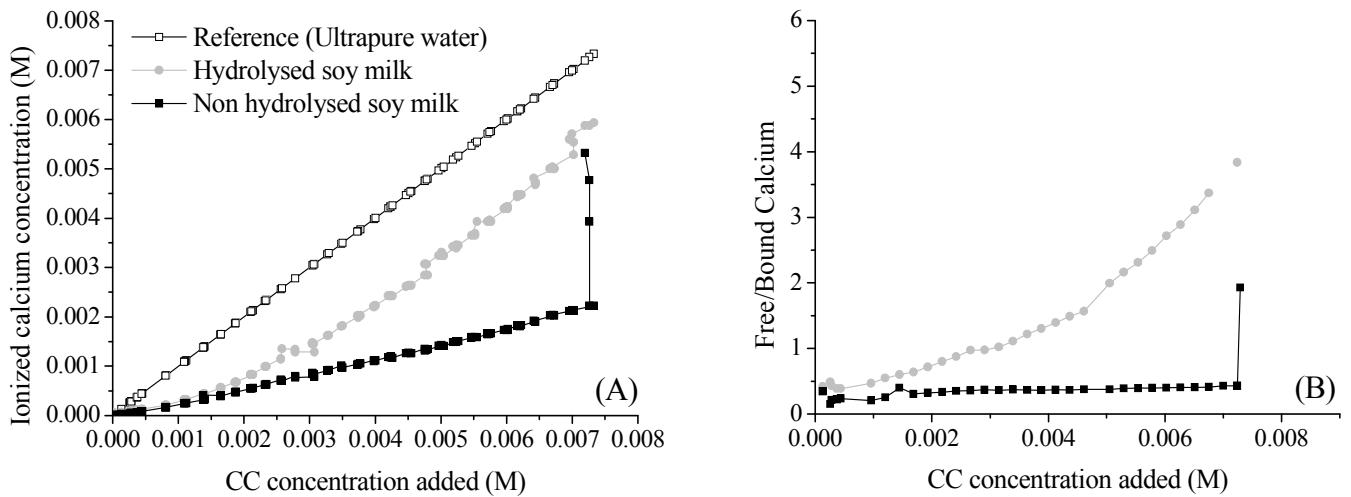


Figure 62. Calcium binding isotherm of soy proteins solution by CC (25 mM). Ionized calcium concentration (A) and Free/Bound calcium (B) as a function of CC concentration added (M)

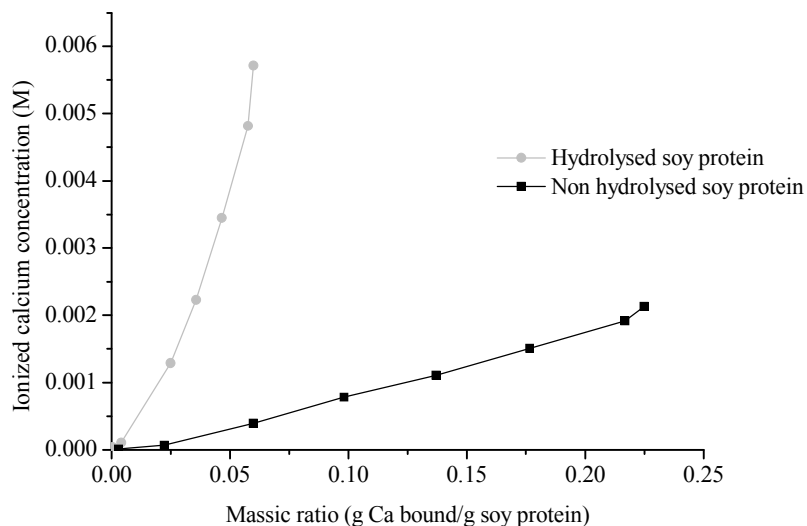


Figure 63. Ionized calcium concentration (M) as a function of mass ratio (g Ca bound/ g soy proteins)

Upon CC titration, the ionized calcium concentration increases in soy protein solutions (figure 62A) but in a smaller way than in the reference (ultrapure water). The ionized calcium concentration measured is higher in hydrolysed soy protein solution compared to the non hydrolysed one, which involves a higher free/bound calcium ratio in the calcium binding isotherm (figure 62B). These results are significant of more important calcium-soy protein interactions in non hydrolysed soy proteins compared to hydrolysed soy proteins whereas there is initially the same concentration in acid amino-acid able to bind calcium ions in both SPI. Hence, we could expect that hydrolysis of soy protein, involves modification of the environment of the calcium-binding sites, which would be then unfavourable for calcium fixation onto the target amino-acid.

Once soy proteins are saturated in calcium, all the supplementary added CC is ionized, which can be related to an important increase of ionized calcium and free/bound calcium ratio. In figure 63, we can observe that hydrolysed soy proteins are saturated at lower mass ratio (g Ca bound/g protein) than non hydrolysed ones. In accordance with ITC results, saturation is reached sooner in hydrolysed soy proteins as compared to non-hydrolysed soy proteins. According to these results, the enthalpy drop observed in ITC experiment (figure 61) is essentially due to the decrease of the amount of  $\text{Ca}^{2+}$  bound to soy proteins (similar to Pochard et al., 1999) and not because the heat per bound  $\text{Ca}^{2+}$  falls as observed in the system studied by Sinn et al (2004).

To evaluate the electrostatic influence upon calcium-soy protein interaction, calcium binding titration was performed in the same conditions that during ITC experiment and followed by zeta-sizer. Indeed, zeta potential variation is related to the electrostatic potential of particles in suspension. The variations of zeta potential (mV) are plotted as a function of mass ratio (g Ca /g of soy protein, figure 64).

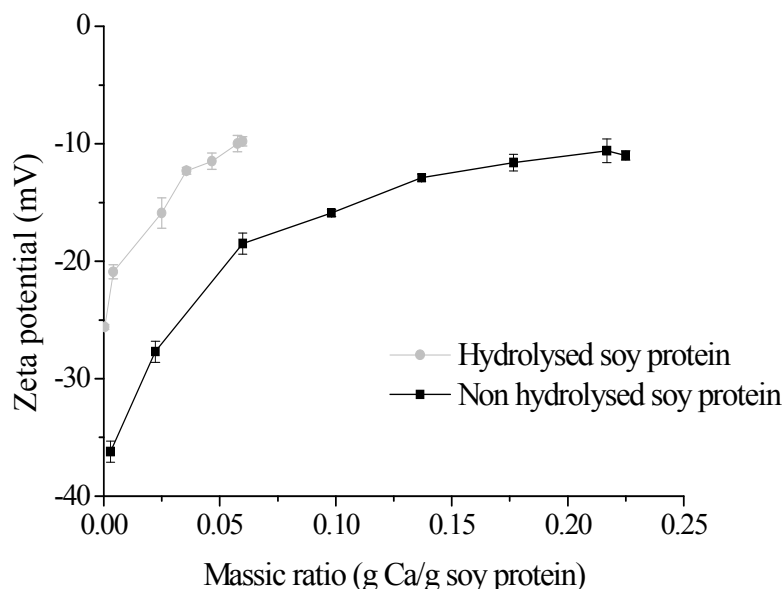


Figure 64. Zeta potential variations (mV) measured as a function of mass ratio (g Ca / g soy protein)

The zeta potential measured in soy protein sample increased upon CC titration (25 mM) up to reach saturation (plateau). This parameter was initially of -36 and -26 mV for non hydrolysed and hydrolysed soy protein samples, respectively, and reached a same plateau value of -10 mV for both soy protein solutions saturated in calcium. These zeta-potential variations are indicative of electrostatic interactions upon calcium binding on soy proteins.

We can notice that the heat of dilution of CC at saturation is set at same plateau value on ITC binding curve (figure 61) and fits to a same final zeta potential for hydrolysed and non hydrolyzed soy protein solution (figure 64). As the non hydrolysed soy proteins bind more calcium ions than the hydrolysed ones, this can partially be explained by a zeta potential value initially (before calcium titration) higher in absolute magnitude (-36 mV) compared to hydrolysed soy proteins (-26 mV). Many factors can positively and negatively influence the zeta-potential of proteins. As in un-desalted soy milks, the same zeta-potential was measured for non hydrolysed soy proteins and hydrolysed ones (~-40 mV), we could suggest that some

charged species would be retained inside the desalting column, modifying the charge distribution around NH and H-soy proteins.

As observed in figure 61, the exothermic part of the thermodynamic signal involved by electrostatic interactions is completely hidden by the strong endothermic signal. Hence, the description of  $\text{Ca}^{2+}$  binding onto polyelectrolytes such as soy proteins by the solely electrostatic interactions would be simply insufficient as this would always lead to an exothermic process. Sakakibara and Noguchi (1977), who studied calcium ions-soy proteins interaction by dilatometry, considered that the positive volume variation accompanying the aggregation of soy protein by calcium ion was the consequence of the release of the ordered water molecules on the soy protein surface at the aggregating sites. Hence similarly to Sinn et al. (2004, 2006), we could suggest that not Coulomb interactions, but rather liberation of water molecules, either from the hydration shells of the calcium ion and/or dehydration of hydrophobic core of soy proteins, was the driven energy source for the binding of multivalent ions onto soy proteins.

Kroll (1984) characterized the calcium binding sites as a function of pH using a calcium ion selective electrode. Former author compared the hydrogen ion titration curve made in absence and in presence of calcium ions. Since a difference was observed between the two titration curves, Kroll concluded that calcium ions bound to the soy protein have the same binding sites than hydrogen ions so that the binding of calcium ion to soy protein was pH dependent.

As a consequence, calcium binding onto soy proteins should be rather described as a counterion  $\text{H}^+/\text{Ca}^{2+}$  exchange, which is more energetically neutral (less exothermic phenomenon) than electrostatic forces involved.

The real reason for the stronger binding of multivalent ions onto soy proteins is therefore not the stronger electrostatic force, but it is simply due to the entropic effects, which keep the free energy of this process negative (as it occurs spontaneously).

Whatever the soy globulin, 7S (Appurao and Narasinga Rao, 1976) and 11S globulins (Appurao and Narasinga Rao, 1975; Sakakibara and Nogushi, 1977), all the investigations concluded that the maximum number of calcium ions bound agreed with the histidine content of soy protein. This would suggest that the imidazole groups of the histidine residues were the probable binding sites, with a higher calcium binding affinity for 11S than 7S fraction. Calcium ions can combine with only the unprotonated imidazole groups since the pK value is

of 6.0. More in the pH range of our study (7.1-7.2), there is still the possibility for the calcium ions to bind the carboxyl groups or phosphate groups.

#### 3.1.2.2. ITC study on soluble soy proteins

Generally, temperature variations generate changes in the conformation and aggregation of proteins and may lead to appreciable changes in their functional and surface properties. Thermal treatment is an important factor, which influences the formation and the stability of protein aggregates during food processing. The temperature effect was studied here on calcium-soluble soy proteins interactions. Indeed, measuring the binding isotherm at two temperatures at least allows additional determination of the change in heat capacity ( $\Delta C_p$ ). This thermodynamic parameter is a key thermal parameter and is generally attributed to the solvent properties. This is a good indicator of changes in hydrophobic interactions and/or hydrogen bonding, as well as in the structuring of water molecules around amino-acid residues.

The binding titrations curves are presented for the different temperatures for non hydrolyzed and hydrolyzed soluble proteins in figures 65.

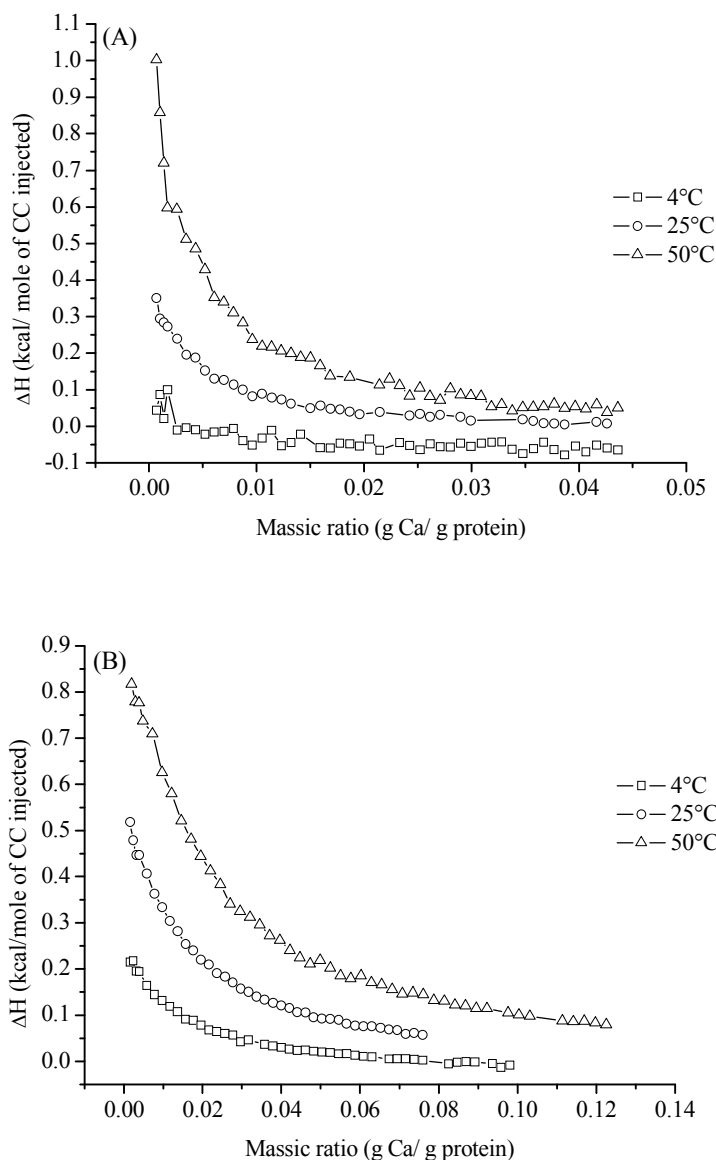


Figure 65. Binding titration curve of soluble non hydrolysed soy protein (A) and soluble hydrolysed soy protein (B) by CC after subtraction of the reference signal (CC titration into miliQ water at corresponding temperature). Duplicated experiments

The binding titration curves of soluble soy proteins (non hydrolyzed or hydrolyzed) with CC show similar trends for all the temperatures studied. Yet enthalpic contributions to the calcium binding reactions ( $\Delta H$ ) are more endothermic at higher temperature.

Binding titration curves were fitted with a one set of sites model and thermodynamic parameters obtained are summarized in table 32. As the experimental data were not perfectly fitted with this former model, the values of  $N$ ,  $K$ ,  $\Delta H$  and  $\Delta S$  must be handled with care and should rather considered as qualitative values. For non hydrolysed soy proteins, the binding

titration at 4°C showed too many discrepancies with the fitting curve obtained with the one set of sites model. Hence, the values were not presented in table 32.

Table 32. Binding parameters obtained after fitting the binding titration curves of the soluble soy proteins with the one set of sites model – Mean values of two duplicated experiments

Sample	T (°C)	K (M <sup>-1</sup> )	$\Delta H$ (kcal/mole)	$\Delta S$	N (g Ca/10 <sup>5</sup> g protein)	N (mg Ca/g protein)
NH-SPI	25	7.3E+03	1.341	22.2	240	2.4
	50	7.0E+03	2.772	26.2	309	3.1
H-SPI	4	2.6E+03	0.430	17.2	1170	11.7
	25	1.3E+03	1.356	18.8	1268	12.7
	50	7.6E+02	2.610	21.3	1607	16.1

NH-SPI: Non Hydrolysed Soy Protein Isolate

H-SPI: Hydrolysed Soy Protein Isolate

The heat capacity ( $\Delta C_p$ ) was determined in order to understand the temperature effect on the thermodynamics of calcium-soluble soy proteins interactions. In figure 66, the evolution of the binding enthalpy ( $\Delta H_{\text{endo}}$ , kcal.mol<sup>-1</sup>.°C<sup>-1</sup>) is plotted as a function of temperature (°C).

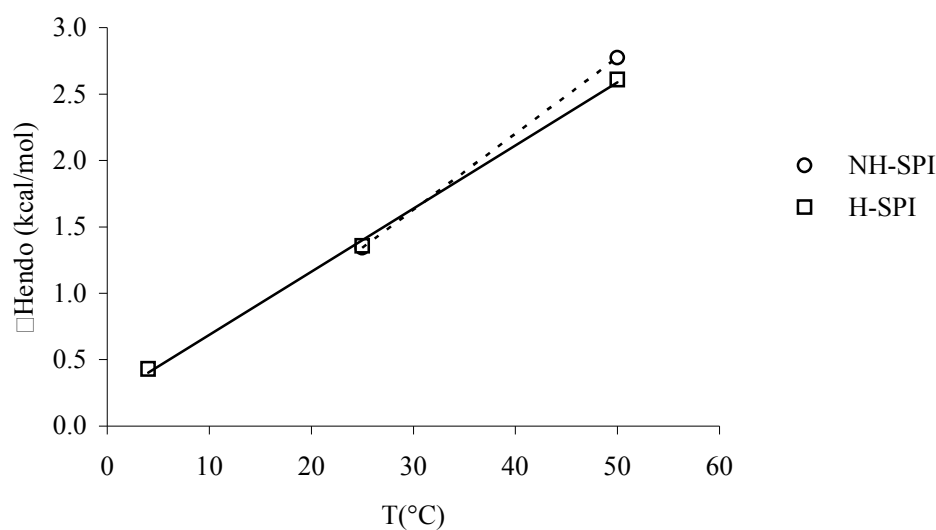


Figure 66. Evolution of  $\Delta H_{\text{endo}}$  (kcal/mol) versus the temperature (°C) during the titration of CC into soluble soy proteins solutions. Lines correspond to a linear fit of data. Each experiment was duplicated. NH: Non Hydrolysed; H: Hydrolysed; SPI: Soy Protein Isolate

From figure 66, we can observe that the binding enthalpy ( $\Delta H_{\text{endo}}$ ) increases with temperature, whatever the soluble soy proteins (hydrolyzed or non hydrolyzed).  $\Delta C_p$  were positive and were set at 57.2 and 47.5  $\text{cal}\cdot\text{mol}^{-1}\cdot\text{C}^{-1}$  for non hydrolysed and hydrolysed soy proteins, respectively. The  $\Delta C_p$  determined for NH-soy proteins must be handled with care since this value was obtained from two distinct temperatures only. Hence, the difference between NH and H-soy protein  $\Delta C_p$  may be non significant.

Such positive values of  $\Delta C_p$  indicate a reduction in the number of surface-exposed polar residues and is relevant to the protein unfolding. The burial of charged ligand and protein polar groups in the binding sites can make the  $\Delta C_p$  positive.

### 3.1.2.3. Comparison of calcium-proteins interactions between soluble and whole soy protein fraction

The binding titration curve of the whole soy protein fraction was compared with the binding titration curve of the soluble soy protein fraction for the NH-soy proteins and H-soy proteins (figure 67A and 67B, respectively). We can conclude from these binding titration curves that, whatever the soy proteins (NH or H), soy proteins contained in the soluble fraction show a lower endothermic signal upon CC titration than the whole protein fraction. Hence fewer interactions are expected between calcium and soy proteins contained in the soluble fraction compared to soy proteins contained in the whole fraction. Soluble soy proteins are less sensitive to calcium than the whole soy proteins.



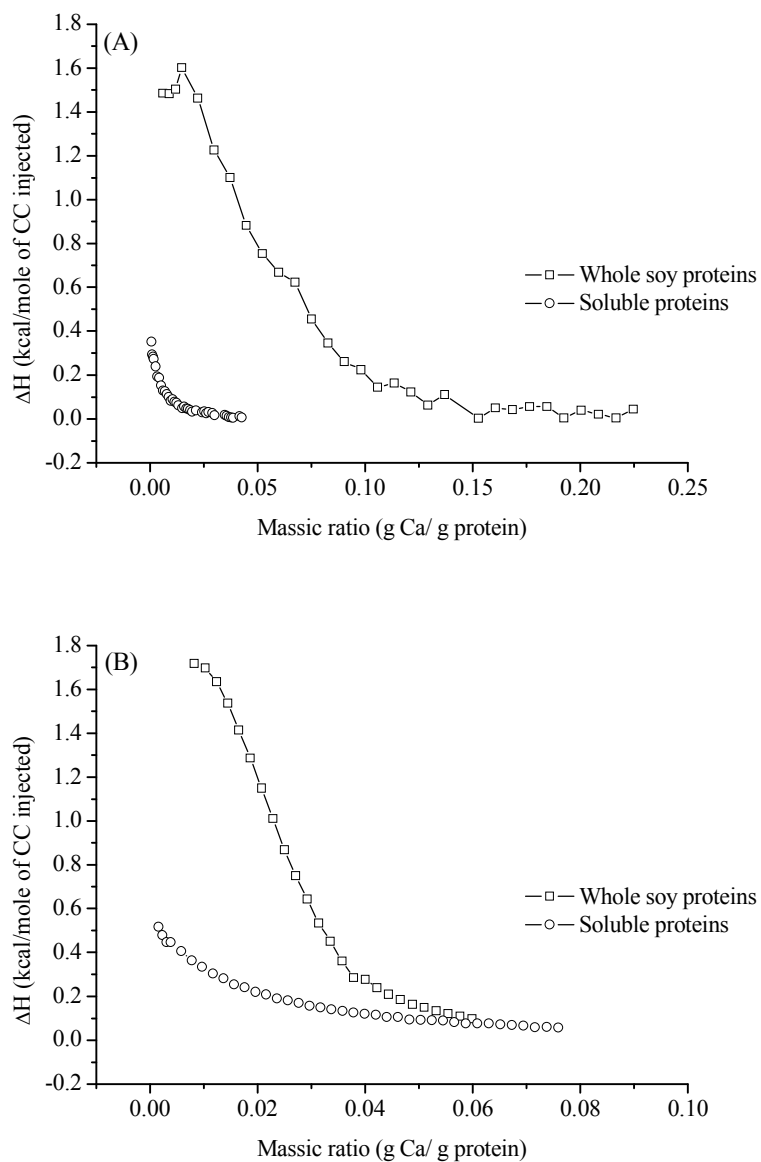


Figure 67. Comparison of the binding titration curve obtained for non hydrolysed (A) and hydrolysed (B) soy proteins in soluble and in whole preparations ( $T_{\text{exp}} = 25^{\circ}\text{C}$ )

## 3.2. Calcium binding site identification by FTIR

### 3.2.1. Calcium-milk protein interaction

Cow skim milk reconstituted in distilled water (4.2% protein concentration w/w), unsupplemented or supplemented in calcium (25 mmol/kg) was examined by Fourier Transformed Infra-Red Spectroscopy at room temperature. As in the calcium equilibrium studies, milk samples were not desalted before calcium addition. Similarly to several works (Dousseau et al., 1989; Dong et al., 1990; Dousseau and Pezolet, 1990), FTIR measurements of proteins were carried out in water and water reference spectrum was subtracted from protein solution spectra. Indeed measurements in aqueous solution are more realistic of events occurring in skim milk upon  $\text{Ca}^{2+}$ -cow proteins interactions.

Figure 68 shows the original spectrum of skim milk reconstituted in distilled water, in presence or in absence of calcium supplementation, from 1800 to 1350  $\text{cm}^{-1}$ .

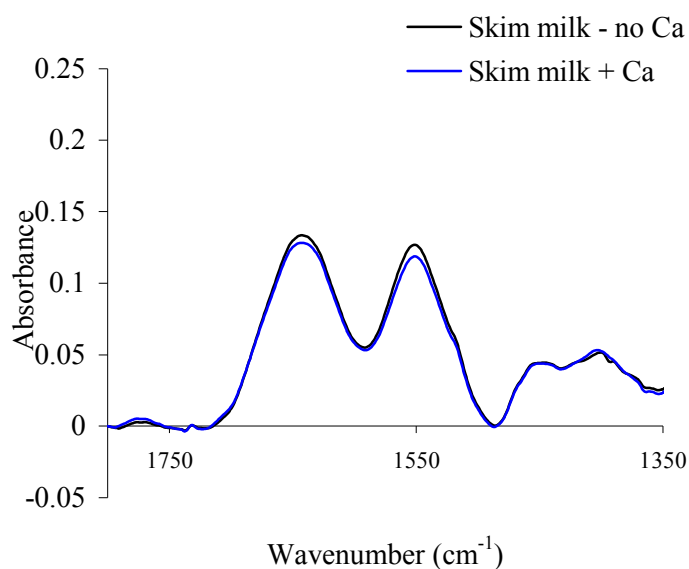


Figure 68. Original FTIR spectrum of skim milk reconstituted in distilled water (4.2% w/w protein concentration), in presence or in absence of calcium supplementation (25 mmol/kg) at room temperature

This spectrum is typical of all spectra obtained for caseins and reported in the literature (Byler et al, 1989; Curley et al., 1998). It consists notably of the amide I region (1700 to 1600  $\text{cm}^{-1}$ ) and of the amide II region (1600-1500  $\text{cm}^{-1}$ ), as usually observed with proteins. The first region corresponds mainly to C=O stretching vibrations coupled with the bending vibrations of N-H, specific of the amide I function (Bertrand and Dufour, 2006). The amide II

region is mainly due to the N-H bending vibration coupled with the C-N stretching modes (Bertrand and Dufour, 2006).

Upon calcium supplementation of milk, a slight decrease of the intensity of these two amide bands is observed (figure 68). We could expect that  $\text{Ca}^{2+}$  interactions onto cow milk proteins modify the environment of these two amide bands, involving the reinforcement and a higher rigidity of the primary structure of proteins and consequently a decrease of the vibration energy of these bands. Moreover, these differences observed in the amide I region are significant of changes occurring in the average relative proportion of the different types of secondary structure present in milk proteins upon calcium supplementation. Indeed when the second-derivative spectra of these two samples are compared for the amide I and the amide II region, i.e. from 1750 to 1500  $\text{cm}^{-1}$  (figure 69), overlapping bands, significant of the variations occurring in the relative proportion of the different types of secondary structure e.g. ( $\alpha$ -helix,  $\beta$ -sheet, disordered structure) can be distinguished.

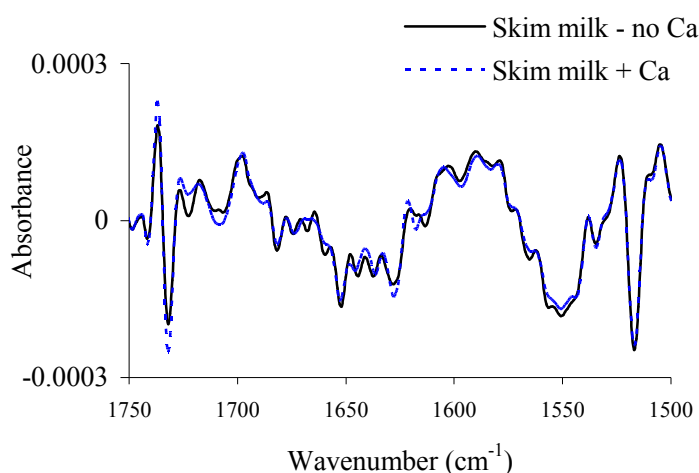


Figure 69. Second-derivative spectra of skim milk reconstituted in distilled water (4.2% w/w protein concentration), in presence or in absence of calcium supplementation (25 mmol/kg) at room temperature, for the amide I and amide II region (1750-1500  $\text{cm}^{-1}$ )

Bands at 1654 and 1645  $\text{cm}^{-1}$  have been shown by previous works (Susi and Byler, 1986; Byler and Susi, 1988) to be associated with  $\alpha$ -helix and disordered or aperiodic structure, respectively. Bands near 1690  $\text{cm}^{-1}$  are usually due to turns while bands below 1635  $\text{cm}^{-1}$  are typically due to the low frequency components of  $\beta$ -chains (Byler et al., 1989).

The spectral band observed at  $1746\text{ cm}^{-1}$  is due to the reactivity of the C=O (the carbonyl function) of carboxylic acids and acid amino-acid residues of proteins (Bertrand and Dufour, 2006), i.e. glutamic and aspartic acid.

The protein conformational changes occurring upon calcium supplementation, observed by FTIR secondary derivate spectra, were previously reported in the study of Dalgleish (1973). The interaction between calcium ions and bovine  $\alpha_{s1}$ -casein were followed using fluorescence spectroscopy at pH 7.0. Former author observed a stepwise increase in the fluorescence of the tryptophan residues of the protein, which could be related to conformational changes occurring upon calcium supplementation. As well, Jeyarajah and Allen (1994) studied the structural changes induced by Calcium binding to  $\beta$ -lactoglobulin by fluorescence spectroscopy. Former authors concluded that calcium binding interaction of  $\beta$ -lactoglobulin induces small structural changes in  $\beta$ -lactoglobulin which lead to increased hydrophobicity. Their results also indicated that hydrophobic interactions play a major role during salt-induced gelation.

### **3.2.2. Calcium-soy protein interactions**

Soy milk reconstituted in distilled water (4.2% protein concentration w/w), unsupplemented or supplemented in calcium (25 mmol/kg) was examined by Fourier Transformed Infra-Red Spectroscopy at room temperature. Figure 70 shows the original spectrum of non-hydrolysed (NH) or hydrolysed (H) soy milks reconstituted in distilled water, in presence or in absence of calcium supplementation, from  $1800$  to  $1350\text{ cm}^{-1}$ .

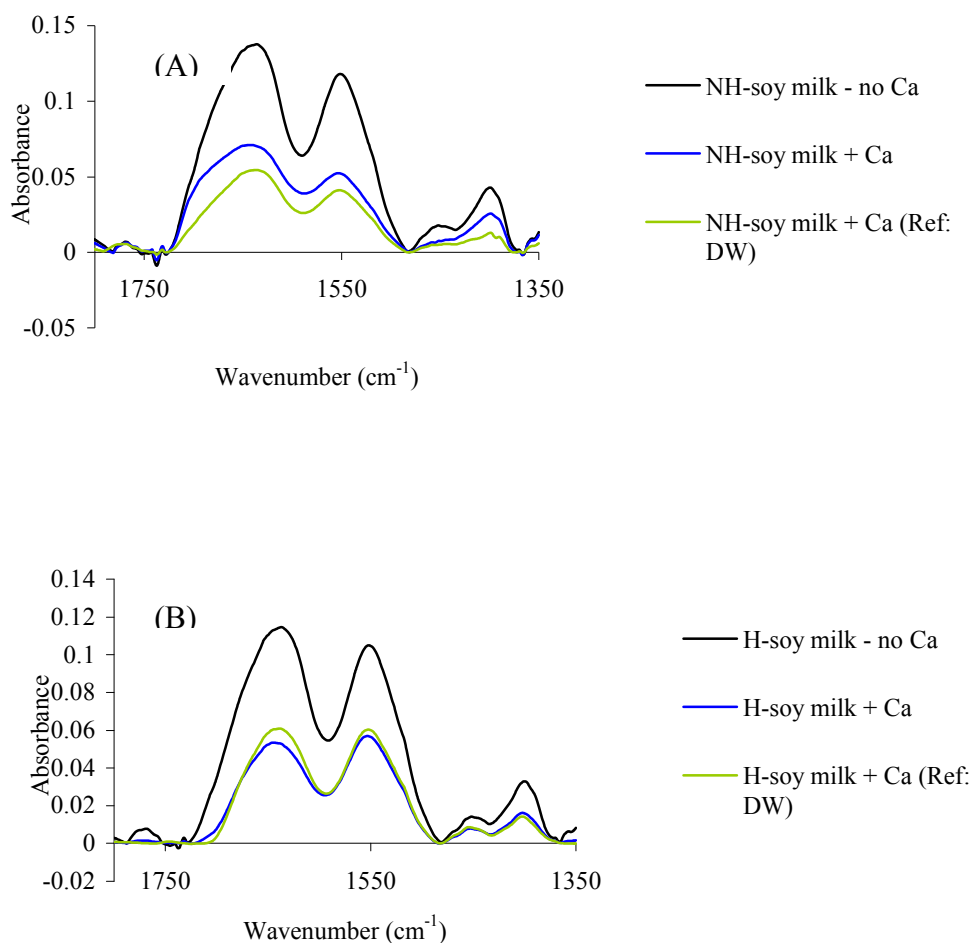


Figure 70. Original FTIR spectrum of non hydrolysed (A) and hydrolysed (B) soy milks reconstituted in distilled water (4.2% w/w protein concentration), in presence or in absence of calcium supplementation (25 mmol/kg) at room temperature

Whatever the soy milk, non-hydrolysed or hydrolysed, the same general trends are observed from 1800 to 1400 cm<sup>-1</sup>, in presence or in absence of calcium supplementation. Four specific bands are localized and centered at:

- 1640 cm<sup>-1</sup>, which corresponds mainly to C=O stretching vibrations coupled with the bending vibrations of N-H specific of the amide I function,
- 1550 cm<sup>-1</sup>, for the amide II band, and is mainly due to the N-H bending and C-N stretching modes,
- 1450 cm<sup>-1</sup>, which fits with the C-H bending vibration of fat and proteins,
- 1400 cm<sup>-1</sup>, for the O-C-O stretching vibrations of carboxylate groups.

As shown in figure 70, the absorbance intensity of these four spectral bands decreases upon calcium supplementation and this decrease is much more important in soy milks as compared to cow skim milk (figure 68). The decrease of the intensity of the two amide bands and of the C-H band ( $1450\text{ cm}^{-1}$ ) could be indirectly related to the  $\text{Ca}^{2+}$  interactions onto soy proteins. Indeed, the environment of these former bands is modified, involving the reinforcement and a higher rigidity of the primary structure of soy proteins. As a consequence, the vibration energy of these bands decreases. On the contrary, the decrease of the intensity of the O-C-O stretching vibrations can be directly related to the  $\text{Ca}^{2+}$  interactions onto carboxylate groups of soy proteins. We can notice that this former band intensity decrease is really visible in the case of soy proteins contrarily to the cow milk proteins. One reason explaining this observation is the high acidic amino-acid content of soy proteins.

The study of each specific band, after vectorial normalization followed by the second derivative leads to a deeper interpretation of FTIR spectra. These bands were characterized according to the CRC handbook Chemistry and Physics. For the non-hydrolysed soy protein spectra, a shoulder was observed at  $1690\text{ cm}^{-1}$ . This band can be related to strong interactions of calcium ions with the carboxylic acids. This shoulder was less visible in the case of hydrolysed soy proteins spectra.

More, in the second derivative spectra of amide II band (result not shown), peaks were observed at  $1549$ ,  $1564$ ,  $1572$  and  $1580\text{ cm}^{-1}$  for NH-soy proteins, at  $1533\text{ cm}^{-1}$  for H-soy proteins and at  $1515\text{ cm}^{-1}$  in both soy proteins. The bands localized at  $1515\text{ cm}^{-1}$  and at  $1533\text{ cm}^{-1}$  both referred to: monosubstituted amid ( $\text{CO-NH-R}$ ), secondary amines with a weak strength of bonding and were assigned to NH bending. Nevertheless, at  $1515\text{ cm}^{-1}$ , the energy of vibration was also due to the C-O at a medium strength of bonding. The bands localized at  $1549$ ,  $1564$ ,  $1572$  and  $1580\text{ cm}^{-1}$  were all related to ionized carboxylic acid with a strong strength of binding, in addition to:

- amides and monosubstituted amides at a medium strength
- secondary amine at a weak strength of binding
- NH assignment.

These differences of bands observed between NH and H soy proteins are in agreement with the ITC data and the respective stoichiometry of calcium ions bound to NH or H soy proteins. Indeed, the bands related to ionized carboxylic acid vibrations, with a strong strength of binding, are only observed in the case of NH-soy proteins. This result partially explains the

higher level of calcium bound in the case of NH-soy proteins as compared to H-soy proteins. More, the strength of binding upon calcium-soy protein interactions are also in agreement with the trend of binding affinity constant ( $K$ ,  $M^{-1}$ ) measured by ITC. Indeed, FTIR spectra analysis shows that stronger strength of binding are involved in NH-soy protein than for H-soy proteins upon Ca-proteins interactions. On the contrary, the binding affinity constant determined with ITC was higher in H-soy proteins compared to NH-soy proteins.

### **3.3. Conclusion**

According to these two complementary techniques (FTIR and ITC), we can conclude that:

- There are more calcium-binding sites onto NH-soy proteins than onto H-soy proteins and there are of more specific nature (compared to H-soy-protein)
- the calcium binds more easily onto H-soy proteins than onto NH-soy proteins (higher binding affinity constant in the former case)
- Yet, the strength of binding is higher for NH-soy proteins.





## **V. Conclusion and Perspectives**



## GENERAL CONCLUSION

The industrial context of this study was to understand and to compare calcium-proteins interactions in cow skim milk, a natural vector of calcium, and in soy milk for further calcium supplementation. The scientific aim of this study was to understand calcium equilibrium in cow skim milk, in soy milk and to compare both milky systems. In the first part of this Ph.D thesis work, the methodology was set up on cow skim milk as a well-known biological system.

Cow skim milk or soy milks (NH or H) were supplemented in calcium (0-25 mmoles/kg) and followed by pH cycle ( $\text{pH}_{\min}$  5.5, 5.0 or 3.5). The experiments were carried out in a thermostated reactor, equipped with pH meter, turbidity meter, conductivity meter and Ca ISE. At the end of each specific step of the kinetic (end of probe stabilization, end of calcium equilibrium period, end of acidification and end of alkalization), samples were taken for further characterization of protein phase (particle size, zeta potential...). Whatever the milky system variations (cow skim milk, NH soy milk or H soy milk), ionized calcium variations upon pH cycle were related to protein phase variations.

- **Ionized calcium variations**

Initially, ionized calcium was around 2 mM in skim milk and almost negligible in soy milks. During pH cycle,  $\text{Ca}^{2+}$  variations had similar trends for skim milk and soy milks, whatever the minimal pH of the pH cycle (5.5 or 3.5). The profile as a whole could be divided into 3 phases corresponding to the stabilization of  $\text{Ca}^{2+}$  concentration after CC supplementation between the soluble and the colloidal phase, the acidification and alkalization steps. Acidification involved calcium solubilization from the protein colloidal phase that corresponds to the well-known micellar calcium solubilization in skim milk. With acidification, the calcium ability to bind milk protein or soy proteins decreased and it resulted in zeta-potential increase to the zero value. This ionization regression of milk and soy proteins reduced the negative charge of phosphoserine residues and/or carboxylic groups of proteins. On the contrary, neutralization by alkalization involved reversed ionized calcium variations, i.e. a decrease of ionized calcium concentration measured in the soluble phase. Considering the different nature of phosphate between skim milk and soy milks, different phosphate salts would be formed upon alkalization.

The comparison of both pH cycles ( $\text{pH}_{\min}$  5.5 or 3.5) showed the same trends, whatever the milky system (cow or soy milk). Nevertheless for pH cycle to 3.5, a maximum of ionized calcium was reached in the isoelectrical pH region.

Upon pH cycle ( $\text{pH}_{\min}$  5.5 or 3.5), a hysteresis phenomenon was observed in cow skim milk but was almost inexistent in soy milks. This could be related to the higher buffering capacity of skim milk.

- **Relationship between ionized calcium variations and protein phases variations upon pH cycle**

- **pH cycle to 5.5**

**For reference skim milk**, the colloidal phase was destructured following micellar calcium solubilization during acidification. Upon back alkalization,  $\text{Ca}^{2+}$  reintegrated casein micelles but insufficiently to counteract casein micelles disaggregation. More  $\alpha_{s1}$  and  $\alpha_{s2}$ -caseins monomers were detected in the soluble phase after a pH cycle to 5.5, in relation with casein micelles destructuring.

**For reference soy milks**, the mean volume diameter  $D_{4.3}$  increased due to the acid-induced aggregation and the apparent viscosity increased. Upon alkalization, the ionization state of soy proteins increased again.  $\text{Ca}^{2+}$  equilibrium and  $D_{4.3}$  were reversed. The aggregation was partially or fully reversed for non hydrolysed or hydrolysed soy milks respectively. Upon alkalization,  $\text{Ca}^{2+}$  reintegrated soy proteins.

**For pH cycle to 5.5**, the comparison of milky systems showed similar trends in  $\text{Ca}^{2+}$  variations between skim milk and soy milks, but a major difference of the effect of  $\text{Ca}^{2+}$  variations on the proteic phase. Casein micelles disaggregated upon acidification following MCP solubilization. On the contrary, acidification induced endogenous calcium solubilization contained in soy proteins, involving their regression of ionization and their aggregation ( $D_{4.3}$  increase and  $\eta_{\text{app}}$  increase). Upon alkalization, casein micelles went on to disaggregate despite the reintegration of calcium into the calcium-depleted casein micelles. In soy milk, upon alkalization, the ionization state of soy proteins increased again. Ionized calcium equilibrium and  $D_{4.3}$  were reversed. Yet, apparent viscosity was reversed for hydrolysed soy milk only. Aggregation is partially or fully reversed for non hydrolysed and hydrolysed soy milks respectively.

**For CC-milk**, turbidity increased upon CC-supplementation and would be related to a micellar density increase due to the  $\text{Ca}^{2+}$  association with casein micelles. Simultaneously,  $\alpha_{s1}$  and  $\alpha_{s2}$ -caseins decreased upon CC-supplementation. Then turbidity decreased during acidification, which fitted with the colloidal phase destructuring and the release of  $\alpha_s$ -casein monomers following MCP solubilization. During alkalization, turbidity decreased and then increased again, which fits respectively with casein-based particles destructuring and restructuring. This should reflect reverse interactions of  $\alpha_s$ -casein monomers with colloidal proteins via calcium-interactions.

**With CC-supplementation of soy milks**,  $\text{Ca}^{2+}$  concentration increased. In the meantime, the zeta-potential, the mean volume diameter and the apparent viscosity increased as well. The concomitant variation of these 3 parameters indicated calcium-soy protein interactions of electrostatic nature, which involved aggregates formation. During acidification, calcium bound to proteins or to other anions was released in the soluble phase. A decrease in apparent viscosity was measured but the size of soy proteins aggregates remained almost similar. During alkalization,  $\text{Ca}^{2+}$  was reabsorbed by the protein colloidal phase. Once CC-supplementation, the apparent viscosity increased and no further significant variations were measured on  $D_{4.3}$ . Calcium-induced aggregation in soy milk was irreversible and the following pH cycle had only minor global effect.

#### ➤ pH cycle to 3.5

**For skim milk**, a maximum of turbidity and a maximum of  $\text{Ca}^{2+}$  were measured at about the  $\text{pH}_i$  region during acidification and back neutralization, which fitted with a maximal aggregation. During alkalization, turbidity decreased up to reach a minimum and then increased again. The major difference between reference milk and CC-milk was observed during back neutralization: the minimum of turbidity appeared sooner and was higher for CC-milk. This confirmed the hypothesis of an initial reinforcement of colloidal proteic phase upon CC-supplementation. The rate of restructuring was higher in CC-milk compared to reference milk. Nevertheless, pH cycle effect to 3.5 was not reversed even in CC-milk.

**For soy milks**, all the studied parameters ( $\text{Ca}^{2+}$ ,  $\eta_{\text{app}}$ , and zeta-potential variations) showed two distinct steps during acidification and the reverse events upon alkalization when the  $\text{pH}_i$  region was going through. The maximum of each parameter was reached at the  $\text{pH}_i$  region, where the zeta-potential is null and where the maximum of aggregation occurred. Reverse phenomena occurred upon alkalization.

**With CC-supplementation**, particle size ( $D_{4.3}$ ) increased and was irreversible whatever the soy milk. Calcium-induced soy protein aggregates showed only few variations in size whereas the  $\text{Ca}^{2+}$  concentration, the apparent viscosity and the buffering index varied all over the pH cycle. The variation of calcium density of soy protein aggregates could be expected. To conclude, calcium-induced aggregates were stronger than acid-induced aggregates (higher  $\eta_{\text{app}}$  and higher  $D_{4.3}$ ). Stronger interactions may be involved in the former case.

- **Comparison of buffering index in cow skim milk versus soy milk ( $\text{pH}_{\text{min}} 3.5$ )**

During acidification, the maximum buffering index was measured at approximately pH value of 5.2 for skim milk and was essentially due to MCP solubilization. Yet on back titration, the maximum buffering occurs at pH 6.6 due to the formation of calcium phosphate with the proton release. The buffering index increased with CC supplementation.

The buffering capacity (dB/dpH) was higher in H-soy milk compared to NH-soy milk, in absence or presence of CC-supplementation. According to the whole results, since NH-soy proteins bound more calcium than H-soy proteins and ionized calcium was almost similar in H than in NH-soy milks, we could expect that this was due to a higher proportion of complexed calcium in H-soy milk. This complexed calcium may act more easily and more efficiently than calcium bound to soy proteins in the buffering capacity.

Whatever the soy milk and similarly to skim milk, the buffering capacity was shifted upward with CC supplementation and a peak of buffering capacity, centred on pH 4.5-5.0, appears clearly. Similarly to cow skim milk, the maximum buffering capacity observed in soy milk in the same pH range (4.5-5.0) could be the fact of calcium initially absorbed by the soy protein, aggregated in presence of CC and then solubilized during acidification. Calcium supplementation improved buffering capacity of soy milks. This parameter was maximal in the pH range where the maximum of calcium was solubilized or where the maximum of acid-induced aggregation occurred. With aggregation, more interactions are involved in soy proteins aggregates. Hence these former aggregates are reinforced and consequently more resistant to pH variations.

In the second part of this study, calcium-proteins interactions were thermodynamically characterized using ITC and calcium binding sites were tentatively identified with FTIR spectroscopy. Thermodynamic parameters, such as  $N$ ,  $K$ ,  $\Delta H$ ,  $\Delta S$ ,  $\Delta G$  were determined after fitting the experimental data with a one set of sites model. The calcium-protein interactions were studied in a whole protein sample for skim milk and soy milks. In soy milks, calcium-protein interactions were also studied in soluble protein sample at various temperatures (4, 25 and 50°C) to determine the  $\Delta C_p$  value.

- **Thermodynamic characterization of the calcium-protein interaction**

The thermodynamic profiles of calcium-proteins interactions were similar in whole protein samples of skim milk, NH and H-soy milks and were endothermic. Both milky systems (cow milk or soy milks) were fitted using a one set of sites model (calcium binding sites onto milk/soy proteins equivalent and independent). Whole protein sample of milk could bind up to 8 mg Ca per g of protein at saturation. In comparison, NH/H soy proteins contained in whole protein samples could bind up to 51.7 and 2.2 mg Ca per g of proteins respectively.

The binding could not be supported by solely electrostatic interactions, which otherwise would be strictly exothermic. Nevertheless, the exothermic part of the thermodynamic signal involved by electrostatic phenomenon was completely hidden by the strong endothermic signal coming from the release of water molecules, either from the hydration shell of the calcium ions and/or dehydration of hydrophobic core of milk/soy proteins. Whatever the milky system, binding should rather be described as a counterion exchange between protons and calcium ions, which is at least best energetically neutral with respect to the electrostatic forces involved.

- **Calcium binding sites identification**

In skim milk or soy milks, variations occurred upon CC supplementation in the amid I and amid II bands. The first amide band fitted mainly to the C=O stretching vibrations coupled with the bending vibrations of N-H specific of the amide I function. The amide II region was mainly due to the N-H bending vibration coupled with the C-N stretching modes. These variations were much more important in soy milks than in skim milk, where only a slight decrease was observed. In soy milks, in addition to the amid bands, variations occurred in two other bands, centred at around  $1450\text{ cm}^{-1}$  and  $1400\text{ cm}^{-1}$ . These two former bands fitted respectively with the C-H bending vibrations of proteins and the O-C-O stretching vibrations of carboxylate groups. The decrease of the intensity of the two amid bands and the C-H band could be indirectly related to  $\text{Ca}^{2+}$  interactions onto proteins. Indeed, the calcium ion interactions onto milk or soy proteins could modify the environment of these two amide functions, involving the reinforcement and a higher rigidity of the primary structure of proteins and consequently a decrease of the vibration energy of these two amide bands. The decrease of the intensity of the O-C-O stretching vibrations could be directly related to the  $\text{Ca}^{2+}$  interactions onto carboxylate groups of soy proteins due to the high acid amino-acid content (i.e. glutamic and aspartic acids) in soy proteins.



## PERSPECTIVES

This study was proposed to get a better understanding of the interactions occurring between calcium and proteins in cow skim milk and in soy milk. Indeed the industrial context was to compare both milky systems. Cow skim milk, as a natural vector of calcium, was chosen as a biological model to imitate for further calcium supplementation of soy milks. In order to get useful information, the choice of a complex mixture of protein industrially prepared was made to be closer of real phenomena.

Then, for a better interpretation of the results concerning the calcium equilibrium in cow skim milk or in soy milk, the study of other mineral species than calcium ions, especially citrate or phosphate ions and magnesium ions species could bring interesting information.

To gain in deeper understanding in calcium-protein interactions in milk proteins or soy proteins, some other experiments should be carried out with ITC. Same experiments could be done with purified proteins fractions contained in cow skim milk ( $\alpha_{s1}$ -caseins,  $\alpha_{s2}$ -casein,  $\beta$ -lactoglobulin,  $\alpha$ -lactalbumin...) or in soy milks (7S or 11S soy proteins). Indeed, with such complementary experiments, we could determine the thermodynamic parameters N, K,  $\Delta H$ ,  $\Delta S$  and  $\Delta G$  for each protein and then, distinguish the fractions that are more efficient in binding calcium. Similarly to the study of soluble soy proteins, the same experiments could be carried out on cow skim milk to determine the effect of temperature on the enthalpy measured. Hence the heat capacity change could be determined in the cow skim milk sample as well and a full comparison of the thermodynamic profile of the calcium protein interactions could be achieved.

ITC experiments could be coupled to other techniques than zeta-sizer to obtain more information on the events occurring upon calcium-protein interaction. Similarly to Pochard et al. (1999), conductimetry and turbidity could be used in parallel of ITC experiments. The complexation of  $\text{Ca}^{2+}$  on fully neutralized milk or soy proteins could be monitored by the conductimetric method described by Pochard et al. (1998) and turbidity could be followed to detect the solubility limit of milk or soy protein in presence of calcium. We could also use temperature scanning UV-vis spectroscopy to elucidate the effect of  $\text{Ca}^{2+}$  binding on the protein stability and circular dichroism (CD) spectroscopy to determine the structural changes of milk or soy proteins due to the calcium ion interaction (Saboury et al., 2005). Combining our results obtained in ITC with some structural data would enable calculations quantifying

the change in water-accessible nonpolar surface area. Protein conformational changes upon CC titration could be studied by NMR (Wintrode and Privalov, 1997). Penfold et al. (1998) and Matulis et al. (2000) used electrostatic binding models for calcium binding proteins. The use of such model could quantify the part of electrostatic contribution on the enthalpy signal measured.

In an industrial aim, the efficiency of complexant commonly used in food industry could also be studied with ITC.

Our FTIR approach brought information about variations of the secondary structures occurring in cow skim milk or soy proteins upon calcium supplementation. In order to investigate more in the structural modifications of proteins in interactions with calcium ions, it should be useful to simplify the systems by working on purified proteins fractions. Then the both study of real and simplified systems should be the best way to fully understand the interactions of protein and ions like calcium.

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## VII. List of Publications

### Articles in refereed journals (based on Ph.D. thesis)

**L.S. Canabady-Rochelle**, C. Sanchez, M. Mellema, A. Bot, S. Desobry and S. Banon. Influence of Calcium Salt Supplementation on Calcium Equilibrium in Skim Milk during pH Cycle. *Journal of Dairy Science*, 2007. 90: 2155-2162

**L.S. Canabady-Rochelle**, C. Sanchez, M. Mellema, and S. Banon. Influence of Calcium Salt Supplementation on Calcium Equilibrium in Soy Milk during pH Cycle. *Journal of Agricultural and Food Chemistry*. (in progress).

**L.S. Canabady-Rochelle**, C. Sanchez, M. Mellema, and S. Banon. Thermodynamic characterization of Calcium–soy protein interactions by Isothermal Titration Calorimetry. *Journal of Physical Chemistry B*. (in progress).

**L.S. Canabady-Rochelle**, C. Sanchez, M. Mellema, and S. Banon. Thermodynamic characterization of Calcium–milk protein interactions by Isothermal Titration Calorimetry. *Dairy Science and Technology (formerly Lait)*. (in progress)

### Articles in refereed journals (based on MSc thesis)

R. Tijssen, **L.S. Canabady-Rochelle** and M. Mellema. Gelation Upon Long Storage of Milk Drinks with Carrageenan. *Journal of Dairy Science*, 2007. 90: 2604-2611.

D. Tentcheva, L. Gauthier, S. Jouve, **L. Canabady-Rochelle**, B. Dainat, F. Cousserans, M.E. Colin, B.V. Ball, M. Bergoin. Polymerase Chain Reaction detection of deformed wing virus (DWV) in *Apis Mellifera* and Varoa Destructor. *Apidologie*, 2004. 35: 431-439

### Scientific communications

**L.S. Canabady-Rochelle**, C. Sanchez, M. Mellema, and S. Banon. Thermodynamic characterization of Calcium–milk protein interactions by Isothermal Titration Calorimetry. Oral presentation. *1<sup>st</sup> International Symposium on minerals and dairy products, Octobre 2008, St Malo*.

**L.S. Canabady-Rochelle**, C. Sanchez, M. Mellema, and S. Banon. Calcium-chloride supplemented skim milk subjected to pH cycle ( $\text{pH}_{\text{min}}$ : 3.5). Ionized calcium exchange between soluble and colloidal protein phase. Poster. *1<sup>st</sup> International Symposium on minerals and dairy products, Octobre 2008, St Malo*.

**L.S. Canabady-Rochelle**, C. Sanchez, M. Mellema, and S. Banon. Ionized calcium equilibrium and turbidity variations in calcium supplemented milk: effect of pH cycle. Poster and Proceeding. *International Dairy Federation Conference, Mexico, November 2008*.

**L.S. Canabady-Rochelle**, C. Sanchez, M. Mellema, and S. Banon. Calcium-proteins interactions studied by Isothermal Titration Calorimetry (ITC) – Comparison of milk versus soy proteins. Poster and Proceeding. *International Dairy Federation Conference, Mexico, November 2008*.

**L.S. Canabady-Rochelle**, C. Sanchez, M. Mellema, A. Bot, S. Banon. Relationship between calcium equilibrium and proteic phase behaviour in pH-Cycled and Calcium Supplemented Milk. Article, poster and oral presentation. *Doctoral school symposium 2007*.



## **VIII. Appendix**





**Appendix 1. Lipid composition of Cow's skim milk** (Souci et al., 1994)

Fatty Acids	Unit	Value
Butyric acid*	mg	2.4
Caproic acid*	mg	1.5
Caprylic acid*	µg	900.00
Capric acid*	mg	1.70
Lauric acid*	mg	2.20
Myristic acid*	mg	7.10
Palmitic acid*	mg	17.80
Stearic acid*	mg	6.70
Palmitoleic acid*	mg	2.10
Oleic acid**	mg	17.40
Linoleic acid***	µg	200.00
Linolenic***	µg	100.00

\*Saturated fatty acid

\*\* Mono-Unsaturated Fatty acid

\*\*\* Poly-Unsaturated Fatty Acid

## Appendix 2. Composition of SPI powders

The Solae Company

SUPRO 760 IP Non-GM  
Isolated Soy Protein

Essential Amino Acids	Ref Pattern mg/g Protein	SUPRO 760 IP Non-GM mg/g Protein
Histidine	19	26
Isoleucine	28	49
Leucine	66	82
Lysine	58	63
Total Sulfur AA	25	26
Total Aromatic AA	63	90
Threonine	34	38
Tryptophan	11	14
Valine	35	50

Amino Acids	g AA/100g Product	g AA/100g Protein
Alanine	3.8	4.3
Arginine	6.7	7.6
Aspartic Acid	10.2	11.6
Cysteine	1.1	1.3
Glutamic Acid	16.8	19.1
Glycine	3.7	4.2
Histidine	2.3	2.6
Isoleucine	4.3	4.9
Leucine	7.2	8.2
Lysine	5.6	6.3
Methionine	1.1	1.3
Phenylalanine	4.6	5.2
Proline	4.5	5.1
Serine	4.6	5.2
Threonine	3.4	3.7
Tryptophan	1.2	1.4
Tyrosine	3.3	3.8
Valine	4.4	5.0
Total Sulfur AA	2.3	2.6
Total Aromatic AA	7.9	9.0

Basic Nutrient	Typical Quantity 100g of product
<b>Calories</b>	
From Total Fat	27 kcal
From Sat. Fat	7 kcal
From Unsat. Fat	21 kcal
From Carbohydrate	Less than 4 kcal
From Protein	353 kcal
Total	380 kcal
<b>Protein</b>	
As Is	88.2 g
Moisture Free Basis	92.6 g
Moisture	4.7 g
Ash	4.0 g
Total Fat (Acid Hydrolysis)	3.0 g
Free Fat (Ether Extract)	0.4 g
Saturated Fat	0.8 g
Polyunsaturated Fat	1.8 g
Monounsaturated Fat	0.5 g
Trans Fatty Acid	0 g
<b>Cholesterol</b>	
Sugar Alcohols	Methodology Not Defined
Dietary Fiber	
Soluble Fiber	
Insoluble Fiber	
Total Carbohydrate (by difference)	Less than 1 g
Other Carbohydrate	Less than 1 g
Sugars	Less than 1 g
<b>Minerals</b>	
Calcium	200 mg
Copper	1.4 mg
Iron	16 mg
Phosphorus	800 mg
Potassium	100 mg
Sodium	1200 mg
Zinc	4 mg
<b>Vitamins</b>	
Vitamin A	Less than 15 IU
Vitamin C	Less than 1 mg
Thiamin (B1)	0.2 mg
Riboflavin (B2)	0.1 mg
Biotin	0.03 mg
Niacin	0.3 mg
Pantothenic Acid	0.2 mg
Folacin	200 mcg

## DUPONT

FXP H0219D IP Non-GM  
Isolated Soy Protein

Essential Amino Acids	Ref Pattern mg/g Protein	SUPRO 760 IP Non-GM mg/g Protein
Histidine	19	26
Isoleucine	28	49
Leucine	66	82
Lysine	58	63
Total Sulfur AA	25	26
Total Aromatic AA	63	90
Threonine	34	38
Tryptophan	11	14
Valine	35	50

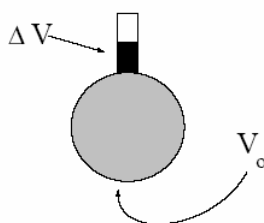
Amino Acids	g AA/100g Product	g AA/100g Protein
Alanine	3.7	4.3
Arginine	6.5	7.6
Aspartic Acid	10.0	11.6
Cysteine	1.1	1.3
Glutamic Acid	16.5	19.1
Glycine	3.6	4.2
Histidine	2.3	2.6
Isoleucine	4.2	4.9
Leucine	7.0	8.2
Lysine	5.4	6.3
Methionine	1.1	1.3
Phenylalanine	4.5	5.2
Proline	4.4	5.1
Serine	4.5	5.2
Threonine	3.3	3.7
Tryptophan	1.1	1.4
Tyrosine	3.3	3.8
Valine	4.3	5.0
Total Sulfur AA	2.2	2.6
Total Aromatic AA	7.7	9.0

Basic Nutrient	Typical Quantity 100g of product
<b>Calories</b>	
From Total Fat	40.5 kcal
From Sat. Fat	7.2 kcal
From Unsat. Fat	TBD kcal
From Carbohydrate	0 kcal
From Protein	344 kcal
Total	385 kcal
<b>Protein</b>	
As Is	86 g
Moisture Free Basis	90.5 g
Moisture	5 g
Ash	4.5 g
Total Fat (Acid Hydrolysis)	4.5 g
Free Fat (Ether Extract)	0.5 g
Saturated Fat	0.8 g
Polyunsaturated Fat	TBD g
Monounsaturated Fat	TBD g
Trans Fatty Acid	Less than 0.5 g
Cholesterol	0 g
<b>Sugar Alcohols</b>	
Dietary Fiber	Methodology Not Defined
Soluble Fiber	
Insoluble Fiber	
Total Carbohydrate (by difference)	0 g
Other Carbohydrate	TBD g
Sugars	0 g
<b>Minerals</b>	
Calcium	200 mg
Copper	TBD mg
Iron	16 mg
Phosphorus	800 mg
Potassium	1300 mg
Sodium	800 mg
Zinc	TBD mg
<b>Vitamins</b>	
Vitamin A	Less than 66 IU
Vitamin C	Less than 0.4 mg
Thiamin (B1)	TBD mg
Riboflavin (B2)	TBD mg
Biotin	TBD mg
Niacin	TBD mg
Pantothenic Acid	TBD mg
Folacin	TBD mcg

## Appendix 3. Equations used for fitting ITC binding data

### I. General Considerations

It will be assumed throughout that the macromolecule  $M$  is in the cell at an initial bulk concentration  $M_t^0$  (moles/liter) before the first injection, and the ligand  $X$  to be injected is initially at zero concentration in the cell. The working volume (cross-hatched area below) of the lollipop-shaped cell is  $V_0$ , the size of the  $i^{\text{th}}$  injection is  $\Delta V_i$  and the total liquid which has been injected at any point during the experiment,  $\Delta V$ , is simply the sum of the individual  $\Delta V_i$  for all injections.



At the beginning of an experiment, both the cell and the long communication tube are filled with macromolecule solution, but it is only that contained within  $V_0$ , that is sensed calorimetrically, because of the total-fill nature of the cell each injection acts to drive liquid out of the working volume and up into the inactive tube as shown by the darkened portion representing  $\Delta V$ . Thus, the concentration of macromolecule in  $V$  changes a small amount with each injection since the total number of moles of macromolecule initially in  $V$  (i.e.  $M_t^0$  times  $V_0$ ) at the beginning of the experiment is later distributed in a larger volume,  $V_0 + \Delta V$ . Since the average bulk concentration of macromolecule in  $\Delta V$  is the mean of the beginning concentration  $M_t^0$  and the present concentration  $M_t$  in the active volume, then the conservation of mass requires that:

$$M_t^0 V_0 = M_t V_0 + \frac{1}{2} (M_t + M_t^0) \Delta V \quad (1)$$

So that

$$M_t = M_t^0 \left( \frac{1 - \frac{\Delta V}{2V_0}}{1 + \frac{\Delta V}{2V_0}} \right) \quad (2)$$

Using similar reasoning, it is easily shown that the actual bulk concentration of ligand in  $V_0$ ,  $X_t$ , is related to the *hypothetical* bulk concentration  $X_t^0$  (assuming that all of the injected ligand remained in  $V_0$ ) as follows.

$$X_t^0 V_0 = X_t V_0 + \frac{1}{2} X_t^0 \Delta V \quad (3)$$

$$X_t = X_t^0 \left( 1 - \frac{\Delta V}{2V_0} \right) \quad (4)$$

The above expressions for  $M_t$  and  $X_t$  are used by Origin to correct for displaced volume effects which occur with each injection.

## II. Single Set of Identical Sites

In the following equations,

$K$  = binding constant,

$n$  = number of sites,

$V_0$  = active cell volume,

$M_t$  and  $[M]$  are bulk and free concentration of macromolecule in  $V_0$ ,

$X_t$  and  $[X]$  are bulk and free concentration of ligand, and

$\Theta$  = fraction of sites occupied by ligand  $X$

$$K = \frac{\Theta}{(1 - \Theta)[X]} \quad (5)$$

$$X_t = [X] + n\Theta M_t \quad (6)$$

Combining equations (5) and (6) above gives

$$\Theta^2 - \Theta \left[ 1 + \frac{X_t}{nM_t} + \frac{1}{nKM_t} \right] + \frac{X_t}{nM_t} = 0 \quad (7)$$

The total heat content  $Q$  of the solution contained in  $V_0$  (determined relative to zero for the unliganded species) at fractional saturation  $\Theta$  is:

$$Q = n\Theta M_t \Delta H V_0 \quad (8)$$

where  $\Delta H$  is the molar heat of ligand binding. Solving the quadratic equation (7) for  $\Theta$  and then substituting this into equation (8) gives:

$$Q = \frac{nM_t \Delta H V_0}{2} \left[ 1 + \frac{X_t}{nM_t} + \frac{1}{nKM_t} - \sqrt{\left( 1 + \frac{X_t}{nM_t} + \frac{1}{nKM_t} \right)^2 - \frac{4X_t}{nM_t}} \right] \quad (9)$$

The value of  $Q$  above can be calculated (for any designated values of  $n$ ,  $K$ , and  $\Delta H$ ) at the end of the  $i^{\text{th}}$  injection and designated  $Q(i)$ . The parameter of interest for comparison with experiment, however, is the change in heat content from the completion of the  $i-1$  injection to completion of the  $i$  injection. The expression for  $Q$  in equation (9) only applies to the liquid contained in volume  $V_0$ . Therefore, after completing an injection, it is obvious that a correction must be made for displaced volume (i.e.,  $\Delta V_i = \text{injection volume}$ ) since some of the liquid in  $V_0$  after the  $i-1$  injection will no longer be in  $V_0$  after the  $i^{\text{th}}$  injection, even though it will contribute to the heat effect (assuming the kinetics of reaction and mixing are fast) before it passes out of working volume  $V_0$ . The liquid in the displaced volume contributes about 50% as much as heat effect as an equivalent volume remaining in  $V_0^a$ . The correct expression then for heat released,  $\Delta Q(i)$  from the  $i^{\text{th}}$  injection, is:

$$\Delta Q(i) = Q(i) + \frac{dV_i}{V_0} \left[ \frac{Q(i) + Q(i-1)}{2} \right] - Q(i-1) \quad (10)$$

The process of fitting experimental data then involves 1) initial guesses (which most often can be made accurately enough by Origin) of  $n$ ,  $K$ , and  $\Delta H$ ; 2) calculation of  $\Delta Q(i)$  for each injection and comparison of these values with the measured heat for the corresponding experimental injection; 3) improvement in the initial values of,  $n$ ,  $K$ , and  $\Delta H$  by standard Marquardt methods; and 4) iteration of the above procedure until no further significant improvement in fit occurs with continued iteration.

### III. Two Sets of Independent Sites

Using the same definition of symbols as above for set 1 and set 2, we have:

$$K_1 = \frac{\Theta_1}{(1 - \Theta_1)[X]} \quad \text{and} \quad K_2 = \frac{\Theta_2}{(1 - \Theta_2)[X]} \quad (11)$$

$$X_t = [X] + M_t (n_1 \Theta_1 + n_2 \Theta_2) \quad (12)$$



Solving equation (11) for  $\Theta_1$  and  $\Theta_2$  and then substituting into equation (12) gives

$$X_t = [X] + \frac{n_1 M_t [X] K_1}{1 + [X] K_1} + \frac{n_2 M_t [X] K_2}{1 + [X] K_2} \quad (13)$$

Clearing equation (13) of fractions and collecting like terms leads to a cubic equation of the form:

$$[X]^3 + p[X]^2 + q[X] + r = 0 \quad (14)$$

where

$$p = \frac{1}{K_1} + \frac{1}{K_2} + (n_1 + n_2)M_t - X_t$$

$$q = \left( \frac{n_1}{K_2} + \frac{n_2}{K_1} \right) M_t - \left( \frac{1}{K_1} + \frac{1}{K_2} \right) X_t + \frac{1}{K_1 K_2}$$

$$r = \frac{-X_t}{K_1 K_2} \quad (15)$$

Equations 14 and 15 can be solved for  $[X]$  either in closed form or (as done in Origin) numerically by using Newton's Method if parameters  $n_1$ ,  $n_2$ ,  $K_1$  and  $K_2$  are assigned. Both  $\Theta_1$  and  $\Theta_2$  may then be obtained from equation 11 above.

As discussed earlier in section II, the heat content after any injection  $i$  is equal to

$$Q = M_t V_0 (n_1 \Theta_1 \Delta H_1 + n_2 \Theta_2 \Delta H_2) \quad (16)$$

After a similar correction for displaced volume, the pertinent calculated heat effect for the  $i$  injection is:

$$\Delta Q(i) = Q(i) + \frac{dV_i}{V_0} \left[ \frac{Q(i) + Q(i-1)}{2} \right] - Q(i-1) \quad (17)$$

which can be used in the Marquardt algorithm to obtain best values for the six fitting parameters.

The first infinitesimal volume element in the  $i$  injection contributes no heat effect since it has already equilibrated at existing concentrations after the  $i-1$  injection. The last volume element of an injection contributes heat effects equal to the liquid remaining in  $V_0$  since its concentrations are equivalent to those in  $V_0$  after the  $i$  injection. Assuming linearity over the small  $\Delta V_i$ , volume increment, then the liquid in the displaced volume is only half as effective in producing heat relative to the liquid in  $V_0$ .

## Appendix 4. The Scatchard plot and the Klotz plot

The Scatchard equation is obtained by transforming the following Langmuir equation:

$$\frac{[AC]}{[A]_t} = \frac{NK[C]}{(K[C]+1)} \quad (\text{Equation 8})$$

where  $[AC]$ , is the amount of calcium bound to protein (M);  $[C]$ , the free  $\text{Ca}^{2+}$  activity (M),  $[A]_t$ , the amount of protein (M);  $N$ , the maximum amount of bound calcium per  $10^5$  g of protein ( $\text{mole.g}^{-1}$ ); and  $K$ , the binding constant for calcium ( $\text{M}^{-1}$ ).

as follows:

$$\frac{[AC]}{[A]_t[C]} = NK - K \frac{[AC]}{[A]_t} \quad (\text{Equation 9})$$

This former equation can be simplified into:

$$\frac{v}{[C]} = K(N - v) \quad (\text{Equation 10})$$

where  $v$  is the average moles of  $\text{Ca}^{2+}$  bound per  $10^5$  g of protein and corresponds to  $[AC]/[A]_t$ .

The representation of  $(\frac{v}{[C]})$  versus  $(v)$  gives the Scatchard plot. For a set of equivalent

binding sites, the plot is a straight line with the intercept on the abscissa given  $N$ , and the slope of the linear Scatchard plot giving  $K$ .

The Klotz plot (Klotz, 1950) is a variant of Scatchard graph, where coordinates change a little, and determine the maximum number ( $n$ ) of moles of calcium bound to  $10^5$  g of proteins:

$$\frac{1}{r} = \frac{1}{K \times n} \times \frac{1}{(\text{Ca}^{2+})} + \frac{1}{n} \quad (\text{Equation 11})$$

where:

$r$ , the number of moles of calcium bound to  $10^5$  g of proteins;

$K$ , the intrinsic binding constant in  $\text{M}^{-1}$ ;

$[\text{Ca}^{2+}]$ , the concentration of free calcium ions in the protein solution in M.

The above equation involves that the plotting of the corresponding curve gives a linear relationship.

AUTORISATION DE SOUTENANCE DE THESE  
DU DOCTORAT DE L'INSTITUT NATIONAL  
POLYTECHNIQUE DE LORRAINE

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VU LES RAPPORTS ETABLIS PAR :

**Madame Sylvie MARCHESSEAU, Maître de Conférences, INRA, Montpellier Sup. Agro, Montpellier**

**Monsieur Frédéric GAUCHERON, Chargé de Recherche, INRA, Rennes**

Le Président de l'Institut National Polytechnique de Lorraine, autorise :

**Madame CANABADY-ROCHELLE Latha-Selvi**

à soutenir devant un jury de l'INSTITUT NATIONAL POLYTECHNIQUE DE LORRAINE,  
une thèse intitulée :

**"Equilibre en calcium dans les systèmes lactés - Etudes des interactions calcium-protéines"**

en vue de l'obtention du titre de :

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Spécialité : « **Procédés biotechnologiques et alimentaires** »

Fait à Vandoeuvre, le 20 mars 2008

Le Président de l'IN.P.L.,

F. LAURENT

