Apports des spectroscopies infrarouge et de fluorescence couplées à la chimiométrie pour la caractérisation de la structure de matrices fromagères et des relations structure-texture

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RESUME

Dans l'industrie laitière, la maîtrise de la qualité en général et de la texture en particulier des produits passera par une meilleure connaissance de leur structure au niveau moléculaire ; ce que ne permettent pas les méthodes classiques utilisées actuellement qui sont souvent destructives.

La première partie de cette thèse porte sur la mise au point de la spectroscopie de fluorescence synchrone et frontale, avec pour modèles d'étude le lait chauffé entre 4°C et 50° ou amené à différents pH allant de 6,8 à 5,1. L'analyse des matrices d'excitation-émission par l'outil chimiométrique PARAFAC a permis de décomposer les matrices de spectres synchrones et de modéliser les spectres d'excitation et d'émission des différents fluorophores présents dans le lait : les signaux des tryptophanes des protéines, de la vitamine A et de la riboflavine ont été identifiés. De plus, l'évolution des propriétés de fluorescence de ces fluorophores lors du chauffage du lait ou de l'acidification a pu être caractérisée et modélisée.

Dans un deuxième temps, l'effet de l'addition des sels (calcium, phosphate, citrate) sur la structure des constituants du lait a été étudié. Ce même chapitre rapporte le suivi des cinétiques de coagulation du lait par la présure et la glucono-delta-lactone à différentes températures aux moyens du test de cisaillement dynamique et des spectroscopies de fluorescence et moyen infrarouge. L'analyse des données spectrales a permis de caractériser sur le plan moléculaire l'impact des enrichissements du lait en minéraux sur la structure des micelles et les différentes étapes de la coagulation et les caractéristiques des coagulums.

Dans la troisième partie, les cinétiques de fusion de plusieurs fromages à pâte pressée cuite ou non (Comté, Raclette) ont été étudiées aux moyens du test de cisaillement dynamique et des spectroscopies de fluorescence et moyen infrarouge. Toutes les techniques mises en œuvre ont permis de déterminer les points de fusion de la matière grasse et les températures de fonte des matrices fromagères. De plus des informations sur l'évolution de la structure moléculaire de la matrice fromagère au cours de la fonte ont été extraites des données spectrales. Enfin, nous avons montré que les techniques spectroscopiques peuvent être utilisées pour authentifier les fromages de type Saint-Nectraire et les classer selon le producteur.

Mots-clés : Lait, minéraux, coagulation, fromage, texture ; spectroscopie moyen infrarouge, spectroscopie de fluorescence synchrone, rhéologie, chimiométrie.

ABSTRACT

In the dairy industry, the control of product quality in general and of texture in particular relies on a better knowledge of their structure at the molecular level; what is not possible using classical methods which are often destructives.

The first part of this thesis focuses on the development of front-face synchronous fluorescence spectroscopy, using as models milk heated between 4 and 50°C or acidified from pH 6.8 till pH 5.1. The analysis of excitation-emission matrices using PARAFAC algorithm allowed to decompose the synchronous spectra matrices and to model the excitation and emission spectra of the different fluorophores found in milk: the signals of protein tryptophans, vitamin A and riboflavin have been identified. Moreover, the changes of fluorescent properties of these fluorophores during milk heating or acidification have been characterized and modelized.

In a second part, the effect of salt (calcium, phosphate, citrate) addition on the structure of milk components has been studied. This chapter reports on the kinetics studies of milk coagulation by rennet or glucono-delta-lactone at different temperatures using dynamic testing rheology and fluorescence and mid-infrared spectroscopies. The analysis of spectral data allowed to characterize at the molecular level the effects of milk fortification with minerals on the micelle structure, as well as the different steps of coagulation and coagulum characteristics.

In the third part, the melting kinetics of several hard (Comté) or semi-hard (Raclette) cheeses have been studied using dynamic testing rheology and fluorescence and mid-infrared spectroscopies. The considered methods allowed to determine melting points of cheese fats and melting temperatures of cheese matrices. In addition, information on the changes of cheese molecular structure during melting has been derived from spectral data. Finally, we have shown the ability of spectroscopic methods to authentify Saint-Nectaire cheeses and to classify them according to cheese producer.

Keywords : Milk, salts, coagulation, cheese, texture, mid infrared spectroscopy, front-face synchronous fluorescence spectroscopy, rheology, chemometrics.

SOMMAIRE

INTRODUCTION GENERALE

Le lait est un élément essentiel dans notre régime alimentaire, qu'il soit sous forme liquide, transformé ou sous forme cachée dans les préparations alimentaires diverses, avec près de 123 kg de produits laitiers consommés par an et par habitant en 2006 (CNIEL, 2007). Sa composition équilibrée en nutriments de base (protéines, lipides, minéraux, glucides) et l'apport qu'il représente en protéines d'excellente qualité en font une source d'alimentation très riche.

Du point de vu physico-chimique, le lait est un produit très complexe. Une connaissance approfondie de sa composition, de la structure de ses constituants et de ses propriétés physiques et chimiques est indispensable à la compréhension des transformations du lait et des bases de la qualité des produits obtenus lors des différents traitements industriels (Amiot et al., 2002). Les progrès dans la connaissance scientifique et technologique des constituants du lait et de leurs propriétés ont permis aux producteurs et aux transformateurs de mieux valoriser l'énorme potentiel nutritionnel et techno-fonctionnel du lait. L'industrie laitière est devenue le secteur d'avant-garde des industries alimentaires par la diversité des moyens technologiques nouveaux mis en œuvre et des produits nouveaux mis en marché.

Sur une production annuelle de près de 23 milliards de litres de lait, environ 3,7 milliards de litres ont été consommés à l'état liquide. Etant un produit fragile et facilement altérable, le lait subit des traitements technologiques pour le stabiliser et le conserver. Mais sans doute, le fromage est l'une des formes de conservation du lait les plus anciennes (Linden & Chamba, 1994). Pâtes molles ou dures, fabriqués à partir du lait de vache, de brebis ou de chèvre cru ou pasteurisé et des conditions de production du lait diverses, les fromages donnent lieu à un ensemble de produits qui présentent des propriétés organoleptiques très variées (Hennequin & Hardy, 1995). La production fromagère atteignait en France 1,7 million de tonnes en 2006 (CNIEL, 2007). Ainsi la France se situe au second rang de la production mondiale de fromages (en volume), après les USA, et au premier rang européen. La catégorie des fromages à pâte molle représente certainement celle où il existe le plus grand nombre de variétés en France. Elle constitue près de 26 % de la production fromagère nationale (438 850 tonnes). Les fromages à pâte pressée cuite et non cuite présentent, aussi, une part non négligeable de variétés et leur production était de 551 269 tonnes en 2006 (CNIEL, 2007). L'Emmental représente la part la plus importante des fromages à pâte pressée cuite, soit 76 %. Avec une consommation annuelle moyenne de 23,1 kg/habitant en 2004

(CNIEL, 2007), les Français se positionnent comme les deuxièmes amateurs de fromages, derrière les Grecs. Le chiffre d'affaire 2004 (CNIEL, 2007) de l'industrie fromagère (hors fromages frais) s'élève à plus de 6,35 milliards d'euros dont 2,1 milliards concernent les fromages à pâtes molles.

Les caractéristiques sensorielles d'un fromage, qui regroupent l'apparence, la texture et la flaveur, sont des critères importants de l'acceptabilité du fromage. Parmi ces caractéristiques, la texture est la composante essentielle de la qualité sensorielle des fromages. Elle peut être évaluée par analyse sensorielle ou par des méthodes d'analyse instrumentales (Creamer & Olson, 1982; Hardy & Scher, 1997; Karoui & Dufour, 2003; Romdhane Karoui et al., 2003; Vassal et al., 1987). Ces différentes méthodes ont permis de montrer que les propriétés de texture des fromages sont déterminées à la fois par les caractéristiques physico-chimiques des laits mis en œuvre et par les paramètres technologiques de fabrication. En effet, des corrélations assez étroites ont été mises en évidence entre l'évolution de certains paramètres physico-chimiques et les modifications des propriétés texturales.

Au fil des années, la consommation et les modes alimentaires des consommateurs évoluent. Le consommateur s'oriente de plus en plus vers la consommation de plats élaborés et prêt à l'emploi. Ce changement a mené les industriels à incorporer le fromage dans les plats cuisinés (Kahyaoglu & Kaya, 2003). En France, on estime que le cinquième du million de tonnes des fromages affinés est utilisé comme ingrédient en cuisine : gratins, pizzas, pâtes, sauces, etc. La part la plus importante de l'utilisation des fromages comme ingrédient est de loin celle de l'Emmental qui représente 70 % (Famelart et al., 2002; Richoux et al., 2001). Malgré son emploi à des fins culinaires, l'appréciation de la qualité des fromages, tel l'Emmental, est souvent réalisée à froid alors que les propriétés fonctionnelles à chaud, tant mécaniques (propriétés de fonte, de coulant, d'étalement à chaud), que visuelles (exsudation d'huile), auxquelles les consommateurs accordent une importance non négligeable, sont difficiles à mesurer en routine du fait de l'absence de méthodes fiables et faciles à mettre en œuvre.

A l'heure actuelle, la maîtrise de la texture des fromages reste une priorité majeure pour les fromagers. La qualité des produits ne dépend pas seulement de leur composition physico-chimique : la distribution de la matière grasse et des protéines dans le produit et les interactions (protéine-protéine, protéine-lipide et protéine-eau) entre les différentes

composantes impliquées dans l'organisation structurale des fromages doivent être considérées, car les propriétés organoleptiques du produit final sont l'expression de cette organisation structurale. C'est pourquoi, les mécanismes d'organisation du grain de caillé, d'égouttage, de fusion des grains de caillé et la structure, phénomènes qui sont encore mal connus, font l'objet de travaux de recherches ces dernières années. Une meilleure compréhension de l'évolution de la structure au cours de la fabrication devrait permettre à terme une meilleure maîtrise de la texture des fromages. Pour appréhender l'effet de la structure du produit sur sa texture, différents niveaux d'observations peuvent être investigués, allant du niveau macroscopique au niveau moléculaire. Dernièrement, un grand nombre de techniques instrumentales non destructives telles que les spectroscopies de fluorescence frontale et infrarouge et le test de cisaillement dynamique ont montré un potentiel intéressant pour la caractérisation respectivement de la structure moléculaire (Dufour et al., 2001; Herbert, 1999; Herbert et al., 1999; Karoui & Dufour, 2003; Karoui, Dufour et al., 2006; R. Karoui et al., 2003; Mazerolles et al., 2001)(Herbert et al., 2000; Dufour et al., 2001; Mazerolles et al., 2001) et de la structure au niveau macroscopique (Famelart et al., 2002; Kulmyrzaev et al., 2008) des fromages.

Par ailleurs, les techniques spectroscopiques présentent un potentiel important pour l'authentification des produits alimentaires qui reste une préoccupation majeure, non seulement des consommateurs mais aussi des producteurs et des distributeurs. Le caractère original ou typique des fromages bénéficiant d'une Appellation d'Origine Contrôlée (AOC) constitue un facteur de préférence qui justifie l'importance accordée aujourd'hui à la promotion et à la défense des signes de qualité.

L'objectif de cette thèse est de développer de nouvelles méthodes rapides et non-destructives pour la caractérisation au niveau moléculaire des produits laitiers et la compréhension des déterminants de la structure et de ses relations avec la texture. Les méthodes basées sur les techniques spectroscopiques telles que les spectroscopies infrarouge et de fluorescence synchrone et frontale ont été utilisées pour l'analyse de la structure au niveau moléculaire des constituants du lait et des fromages, pour comprendre l'évolution de la structure des constituants des fromages au cours de fabrication. Les techniques chimiométriques ont été appliquées sur les jeux de données afin d'extraire l'information pertinente contenue dans les données physico-chimiques, rhéologiques et spectroscopiques et faciliter l'interprétation des résultats.

Après une première partie faisant le point sur l'état de l'art, la deuxième partie de la thèse met en évidence les potentialités de la spectroscopie de fluorescence synchrone couplée à la méthode PARAFAC pour l'étude des changements structuraux des constituants du lait lors du chauffage ou de l'acidification.

Dans une troisième partie ont été étudiés les potentiels des spectroscopies infrarouge et de fluorescence synchrone et frontale couplées aux techniques chimiométriques pour le suivi des changements structuraux des constituants du lait suite à l'ajout de minéraux (calcium, phosphate et citrate) et lors des coagulations acide et présure.

Enfin, la quatrième partie a été consacrée à montrer l'apport des techniques spectroscopiques dans l'étude de la structure des fromages lors de leur fonte (matière grasse et matrice fromagère). La fusion des fromages à pâte pressée (Comté et Raclette) a été suivie par spectroscopies moyen infrarouge et de fluorescence synchrone et la corrélation avec les résultats de rhéologie a été déterminée. Ensuite, les méthodes spectroscopiques (moyen infrarouge et de fluorescence frontale) couplées à la chimiométrie ont été utilisées pour l'authentification et la classification de fromages de type Saint-Nectaire.

CHAPITRE 1 : SYNTHESE BIBLIOGRAPHIQUE

I. Lait

I.1. Composition et caractéristiques

Le lait est un système complexe et hétérogène. Il a la particularité d'être produit chaque jour et d'avoir une composition chimique (Tableau 1) qui varie en fonction de nombreux facteurs zoologiques et d'élevage tels que l'espèce, la race, l'âge, le stade de lactation et le nombre de lactation et l'alimentation (Grappin et al., 2006; Walstra et al., 2005). Le lait correspond à la fois a une solution aqueuse constituée essentiellement de lactose, de protéines sériques, de sels minéraux et de vitamines, à une émulsion de matière grasse sous forme de globules gras (huiles dans l'eau) et à une suspension de micelles constituées de caséines et de sels minéraux (Walstra et al., 2005). Cette composition confère au lait un pH de 6,7, une force ionique d'environ 73 mM et une densité de 1029 $kg.m^{-3}$ (Walstra et al., 2005).

Tableau 1 : Composition moyenne du lait entier de vache (Walstra et al., 2005).

Composants	Concentration moyenne (% p/p)	Gamme de concentrations (% p/p)
Eau	87,1	85,3 - 88,7
Matière grasse	4	2,5 - 5,5
Matières sèches non grasse	8,9	7,9 - 10,0
Lactose	4,6	3,8 - 5,3
Protéines	3,3	2,3 - 4,4
Caséines	2,6	1,7 - 3,5
Protéines sériques	0,6	---
Substances azotées non protéiques	0,06	---
Substances minérales	0,7	0,57 - 0,83
Acides organiques	0,17	0,12 - 0,21
Constituants divers	0,15	----

I.1.1. Les protéines

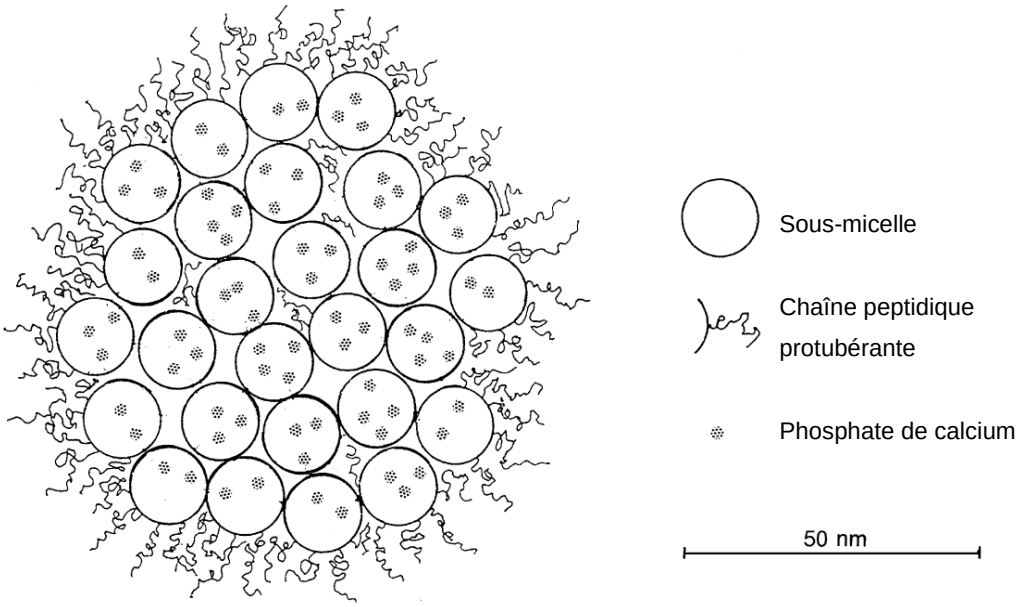
Les protéines du lait sont des éléments essentiels au bon fonctionnement des cellules vivantes et elles constituent une part importante du lait et des produits laitiers. Elles peuvent entre séparées en deux catégories : les caséines insolubles organisées sous forme de micelles à pH 4,6, représentant 80% des protéines du lait et les protéines du lactosérum (20%), solubles à ce pH.

I.1.1.1. Caséines

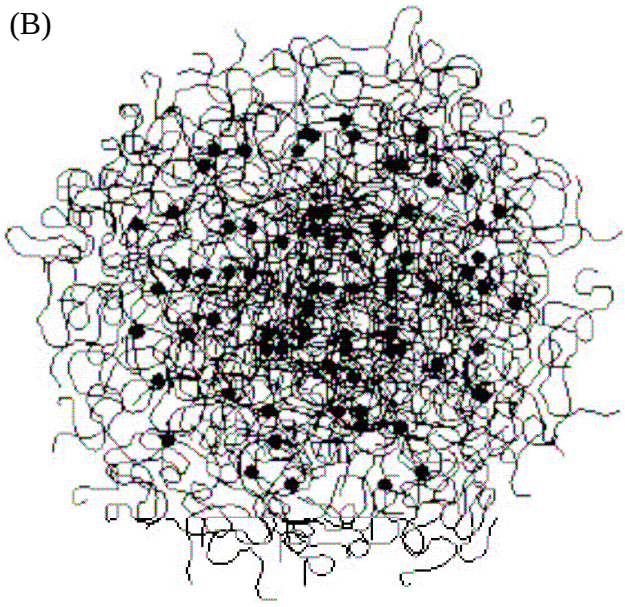
Les caséines ont la particularité d'être des protéines phosphorylées, de lier le calcium, de s'auto-associer pour former des micelles, et de précipiter par acidification rapide à pH 4,6 à 20 °C. La micelle de caséine est constituée de 92% (g/g) de protéines, les caséines α_{s1}/α_{s2} /β/κ dans un rapport 4/1/3,7/1,4 et de 8 % de minéraux dont 90% phosphate de calcium et de 10% d'ions citrate de magnésium (Cayot, 1998). Les micelles de caséine sont de taille et de composition variable. Le nombre moyen de molécules de caséines composant une micelle, d'un diamètre moyen de l'ordre de 150 nm (Schmidt et al., 1973), est de l'ordre de 10^4 (Walstra et al., 2005). L'association de caséines est régie par leurs propriétés (hydrophobie, sensibilité et association au calcium) et par les conditions physico-chimiques du milieu (pH, température, présence d'autre ions comme le citrate, force ionique).

L'organisation de la micelle, la répartition des composants ainsi que leur mode d'association reste encore aujourd'hui du domaine de l'hypothèse. De nombreux travaux ont été menés afin d'élucider le type d'interaction entre les molécules de caséines et leur organisation pour former la micelle. Parmi les nombreux modèles proposés, nous pouvons citer le modèle « cœur-enveloppe » (Parry & Carroll, 1969; Payens, 1966; Waugh & Noble, 1965), le modèle de structure interne (Garnier & Ribadeau Dumas, 1970), le modèle de sous-micelles (Morr, 1967; Ono & Obata, 1989; Schmidt, 1982; Walstra, 1999) (**Figure 1**) et le modèle structure ouverte (Holt & Horne, 1996). De récents travaux (Marchin, 2007; Marchin et al., 2007) ont toutefois écarté le modèle submicillaire au profit du modèle à structure ouverte grâce à des observations en cryo-microscopie à transmission et des analyses en diffusion des rayons X aux petits et ultra-petits angles.

(A)



(B)



- Nanocluster de phosphate de calcium

Figure 1 : Modèle de micelle de caséine (sous-micelle) de (Walstra, 1999) (en haut) et modèle à structure ouverte de (Holt & Horne, 1996) (en bas).

Selon le modèle proposé par (Schmidt, 1980), la micelle résulte d'une association complexe de sous-unités (sous-micelles) non uniformes caractérisées par un cœur hydrophobe formé par la partie apolaire de la caséine. Les parties polaires hydrophiles, notamment les résidus phosphoséryls, seraient localisées à la périphérie. Ces sous-unités s'agrègent entre elles par l'intermédiaire du calcium et du phosphate minéral pour former la micelle (Schmidt, 1980). Les proportions relatives de caséines, notamment la caséine k varient selon les submicelles : celles qui ont une faible teneur en caséine κ sont localisées à l'intérieur de la micelle, alors que celles qui sont riches en ce type de caséine se trouvent à la périphérie.

Selon Ono & Obata (1989), la micelle serait constituée de sous-micelles de caséine β et α_{s1} au cœur et de sous-micelles de caséines α_{s1}, α_{s2} et κ en périphérie. Ils indiquent que les micelles seraient constituées de 2 types de sous-micelles : des sous-micelles F2, à l'extérieure de la micelle, contenant des caséines α_{s1}, α_{s2} et κ et des sous-micelles F3, uniquement au centre de la micelle, contenant des caséines α_{s1} et β.

Dans la structure ouverte, Holt, (2004) et Holt & Horne (1996) suggèrent que le réseau protéique est « cimenté » par des « nanoclusters » de phosphate de calcium. Ces « nanoclusters » correspondent à la forme inorganique des minéraux dans la phase micellaire et leur nature est aujourd'hui discutée.

Horne (2006) a proposé un schéma de croissance du réseau protéique en prenant en compte uniquement un pontage des molécules par les « nanoclusters » et les centres phosphate des caséines (**Figure 2**). Horne a démontré ainsi les limites de ce type d'association. La caséine κ, faute de centre phosphate, ne peut se lier aux « nanoclusters » et, par conséquent, est absente d'un tel modèle d'association. Par ailleurs, ce type d'association laisse supposer une croissance à l'infinie du réseau, ce qui n'est pas le cas. Horne, suggère donc que d'autres types d'interactions interviennent dans la cohésion des molécules de caséine entre elle pour former la micelle.

La grande stabilité des micelles est attribuée principalement à des forces de répulsion électrostatique et stérique. Ces forces sont respectivement dues à une charge potentielle de surface très négative des micelles au pH normal du lait et à une couche d'hydratation résultant en grande partie de la capacité de fixation de l'eau par la partie osidique de la séquence C-terminale de la caséine κ (Mahaut et al., 2000). Tout élément qui modifiera ces facteurs de stabilité entraînera une modification de la dispersion des micelles dans le lait.

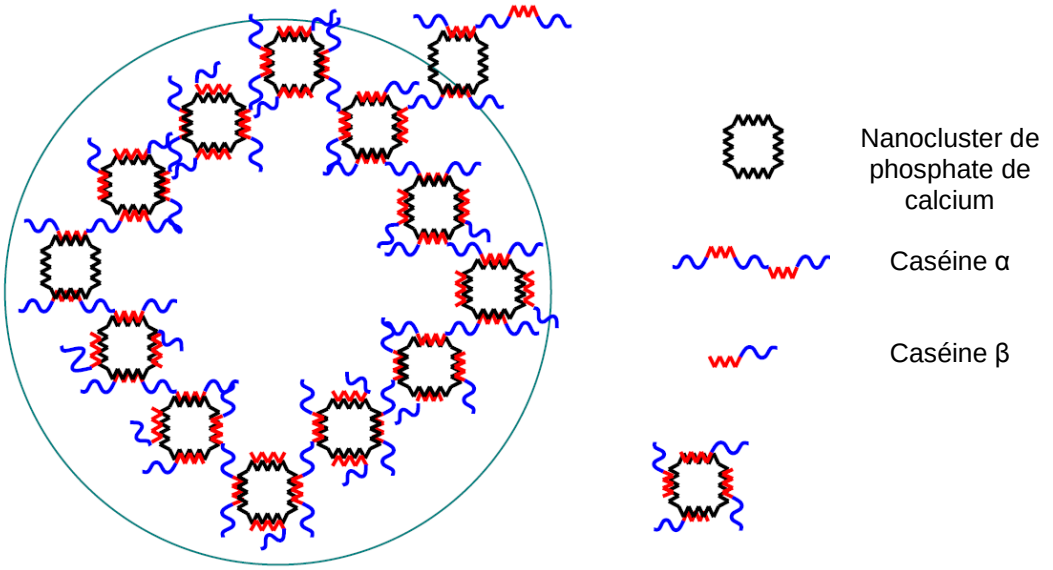


Figure 2 : Croissance du réseau protéique à partir des nonoclustres et des centres phosphate des molécules de caséine (Horne, 2006). Les régions (∿) correspondent aux régions hydrophobes des caséines. Les régions en rouge (∿) s'apparentent aux zones contenant des centres.

La micelle de caséines peut être considérée comme une structure complexe d'un point de vue statique mais aussi d'un point de vue dynamique. Dans des conditions normales, plusieurs constituants sont échangés entre la phase micellaire et la phase aqueuse. C'est le cas des minéraux ou de la caséine β qui peuvent se solubiliser à froid et réintégrer la micelle lorsque la température remonte. Lorsque les conditions physico-chimiques du milieu (température) changent ou lorsque des modifications biologiques (enzymes), chimiques (acidification, addition de minéraux ou de chélatants) ou technologiques (traitement thermique, refroidissement, concentration, séchage, filtration) ont lieu, ces échanges peuvent varier de manière qualitative et quantitative (**Figure 3**). Selon les conditions physico-chimiques et le type de modification, les conséquences sur la micelle sont plus ou moins importantes, réversibles ou non, et peuvent conduire à sa déstabilisation avec des répercussions sur la structure macroscopique du lait.

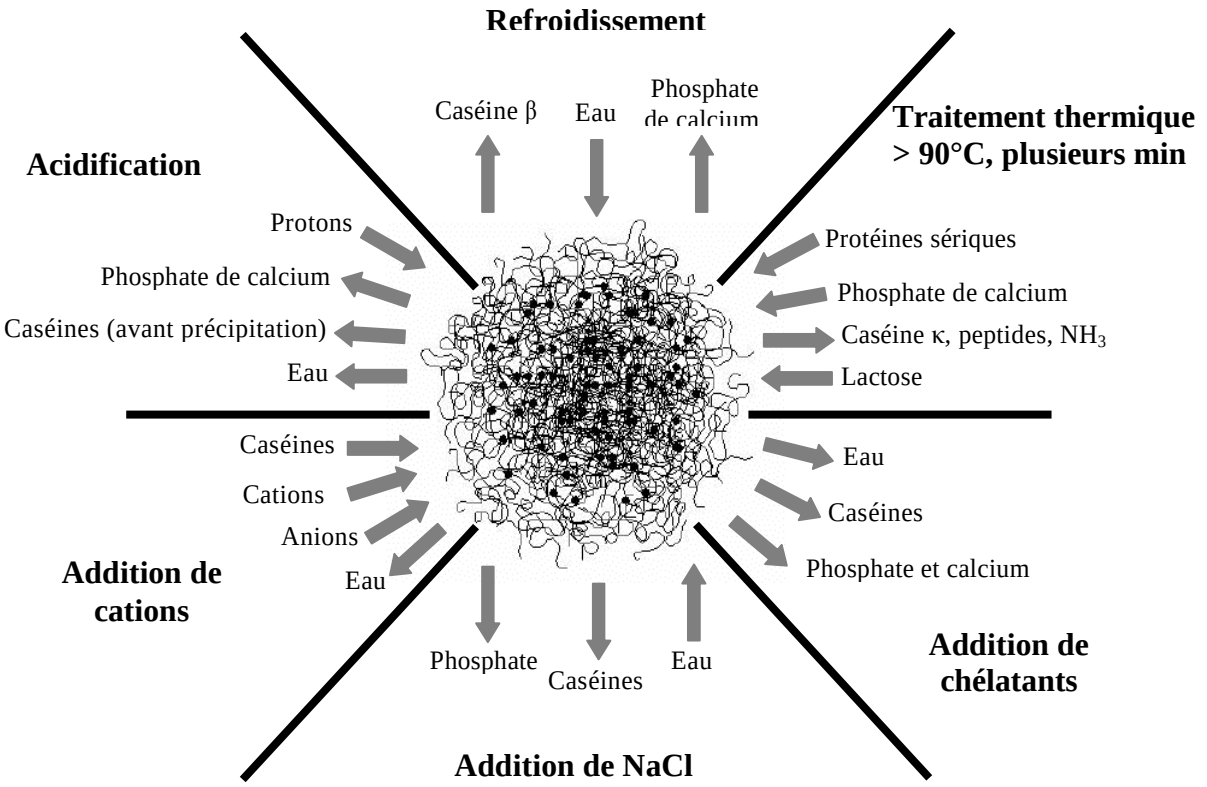


Figure 3 : Schématisation des échanges entre la phase micellaire et la phase solvante lors de modifications physico-chimiques telles que : acidification, refroidissement, traitement thermique > 90°C, addition de chélatants, de cations ou de NaCl (Gaucheron, 2005).

I.1.1.2. Protéines de lactosérum

Les protéines du sérum, qui représentent environ 20 % (p/p) des protéines totales, se trouvent sous forme de solution colloïdale. Les deux principales sont la β- lactoglobuline et l'α-lactalbumine ; les autres protéines du sérum sont les immunoglobulines, la sérum albumine bovine et la lactoferrine. La β-lactoglobuline et l'α-actalbumine sont les plus importantes des protéines du sérum puisqu'elles en représentent environ 55 % et 22 % (p/p), respectivement (Amiot et al., 2002). Par ailleurs, toutes ces protéines sont sensibles au chauffage qui, à plus de 80 °C, provoque leur dénaturation et leurs insolubilisation par agrégation. Ces protéines se différencient des caséines par leur excellente valeur nutritionnelle à cause de leurs teneurs élevées en lysine, tryptophane et plus particulièrement en cystéine.

I.1.2. Matière grasse

Les matières grasses du lait sont sécrétées sous forme de globules gras allant de 0,1 à 20 μm avec une valeur moyenne de 3 à 5 μm (Danthine et al., 2000). Elles se composent principalement de triglycérides, de phospholipides et d'une fraction insaponifiable constituée en grande partie de cholestérol et de β-carotène. La structure de globule gras est hétérogène. Elle est constituée d'un cœur composé de triglycérides à bas point de fusion, liquide à température ambiante et d'une zone corticale qui joue un rôle très important (**Figure 4**). Le globule est formé à la périphérie d'une sorte d'enveloppe contenant premièrement des phospholipides, qui sont hydrophiles et hydrophobes ou lipophiles et qui jouent un rôle d'émulsifiant dans la stabilité du globule gras. Des protéines de membrane viennent compléter la couche externe du globule. Ce sont des lipoprotéines, des enzymes et des agglutinines (Amiot et al., 2002). La membrane du globule gras joue un rôle primordial dans la stabilité des globules gras du lait. Elle s'oppose à la fusion des globules en une masse compacte de matière grasse et leur permet, par sa zone externe hydrophile chargée négativement, de stabiliser l'émulsion (Pougheon & Goursaud, 2001). Cette membrane sépare les triglycérides des autres constituants et les protège de certaines substances présentes, dans le milieu aqueux, en particulier de la lipase fixée sur les micelles de caséines. Le contenu et l'état physique des triglycérides dans les globules gras est un paramètre important dans le domaine de la qualité (texture) des fromages. Les propriétés de fluorescence de la vitamine A située dans le noyau des globules gras et dans la membrane du globule gras peuvent être utilisée pour fournir des informations sur le développement des interactions protéines-lipides pendant la coagulation de lait (Herbert et al., 1999) (Herbert, 1999) et pendant la période d'affinage des fromages (Mazerolles et al., 2001).

I.1.3. Les minéraux

La quantité des minéraux contenus dans le lait après incinération varie de 0,60 à 0,90% (p/p). Les minéraux du lait se trouvent sous deux formes principales : surtout sous forme de sels ionisés et solubles dans le sérum et aussi sous forme micellaire insoluble (**Tableau 2**). Les éléments basiques majeurs comme le calcium, le potassium, le magnésium et le sodium forment des sels avec les constituants acides que sont les protéines, les citrates, les phosphates et les chlorures. En outre, le calcium, le magnésium et les phosphates se trouvent sous forme colloïdale dans les micelles de caséines.

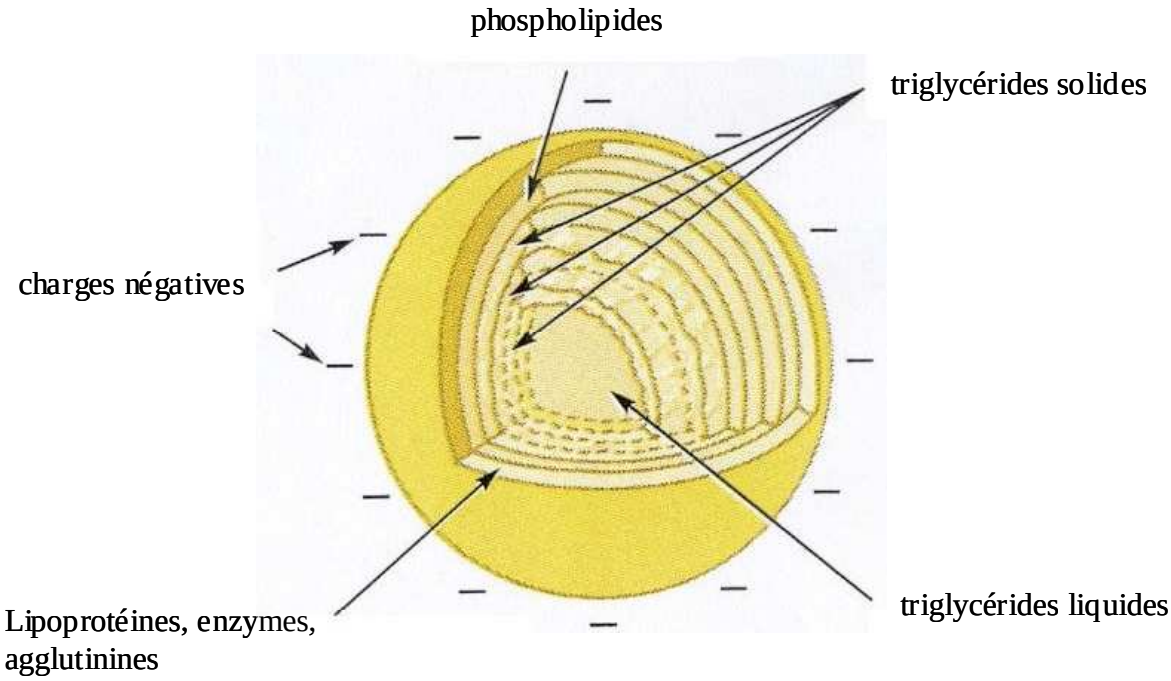


Figure 4 : Structure d'un globule de matière grasse (Amiot et al., 2002)

Tableau 2 : Répartition des principaux minéraux du lait à pH 6,7 d'après (Holt & Jenness, 1984)

Constituants	Concentrations (en mM)			% de soluble
	Totale	Soluble	Micellaire	
Calcium	29,4	9,2	20,2	31
Phosphate inorganique	20,9	11,2	9,7	54
Magnésium	5,1	3,3	1,8	65
Citrate	9,2	8,2	1	89
Sodium	24,2	24,2	0	100
Potassium	34,7	34,7	0	100
Chlorure	30,2	30,2	0	100

Les minéraux du lait se distribuent entre la phase soluble et la phase micellaire. Leurs équilibre et répartitions peuvent être présentés comme dans la **Figure 5**.

Dans cette figure sont représentés les éléments essentiels (calcium, phosphate inorganique, citrate) qui contribuent aux équilibre minéraux. D'autres miniraux comme le magnésium, le sodium, le potassium et le chlorure sont également présents dans le lait en quantité significative, mais ont un rôle moins crucial au niveau de ces équilibres.

Dans cette phase soluble, le sodium, le potassium et le chlorure existent principalement sous forme d'ions libres alors que les éléments calcium, magnésium et citrate sont préférentiellement associés sous forme de sels $CaCit^-$ et $MgCit^-$. Le phosphate est majoritairement sous forme libre (≈ 10 mM) et son état d'ionisation dépend du pH (formes $H_2PO_4^-$ ou HPO_4^{2-}). Il est également associé au calcium pour former du $CaHPO_4$ (0,59 mM), sel très peu soluble.

Dans la phase micellaire, les principaux minéraux sont le phosphate et le calcium, et minoritairement, le citrate et le magnésium. Les différentes formes minérales de la phase soluble et de la phase colloïdale sont en équilibre les unes avec les autres (**Figure 5**), et peuvent évoluer selon les modifications du milieu (pH, température, acidification, addition de minéraux, etc.).

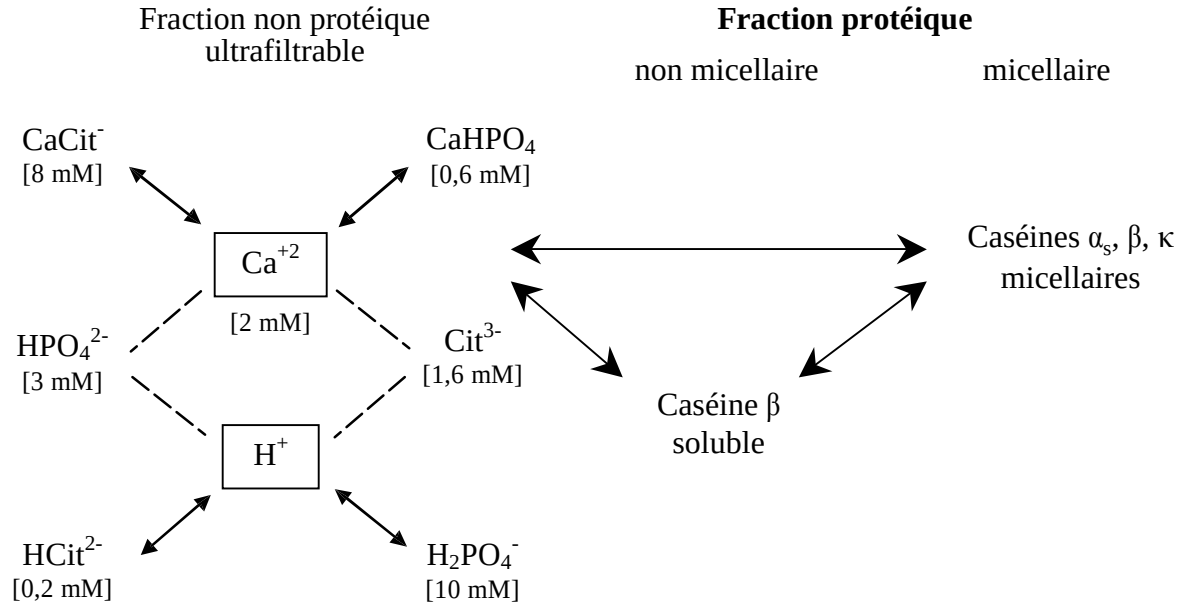


Figure 5 : Les équilibres salins du lait (Brulé, 1981).

I.1.4. La coagulation

La coagulation du lait par voie acide ou par voie enzymatique est étroitement liée à l'organisation structurale et aux modifications physico-chimiques intervenant au niveau de la micelle de caséine. Suivant le mode de coagulation utilisée, les mécanismes de formation du coagulum diffèrent totalement ainsi que les caractéristiques finales des gels.

I.1.4.1. Coagulation par voie acide

La coagulation par voie acide est provoquée par le ferment lactique qui transforme le lactose en acide lactique. L'acidification doit être lente et progressive. En effet, une acidification rapide et brutale (par un acide minéral ou organique) du lait entraîne la formation d'un précipité. Une acidification lente et progressive peut être obtenue par des ferments lactiques ou par une hydrolyse de la glucono-δ-lactone et conduit à la formation d'un gel lisse homogène qui occupe entièrement le volume initial du lait (Brulé et al., 1997). L'abaissement du pH à pour effet de protoner les groupements acides libres des caséines (résidus aspartiques, glutamiques, phosphosériques). Cette protonation provoque une réduction du potentiel de surface conduisant à la formation du gel lactique (Darling & Dickson, 1979), et a pour conséquence de diminuer le pouvoir séquestrant des caséines α et β et d'augmenter la solubilité du phosphate de calcium micellaire (Le Graet & Brulé, 1993). Même une légère acidification modifie suffisamment la structure micellaire pour que les caséines deviennent instable à la chaleur (Amiot et al., 2002). Ainsi, lorsque le pH passe de 6,7 à 5,5, les charges négatives présentes à la surface des micelles de caséines sont neutralisées, ce qui produit une augmentation du diamètre moyen des micelles causée par l'agglomération des petites micelles aux plus grosses. Le diamètre moyen passe de 180 nm à 1300 nm. On observe alors une perte du caractère individuelle des micelles par formation de chaînes de micelles ; toutefois, elles conservent leur forme sphérique. Lorsque l'acidification se poursuit jusqu'à un pH de 5,0, l'agrégation des micelles et même la fusion de certaines d'elles peuvent être observées. L'acidification se poursuit jusqu'au point isoélectrique de la caséine, soit un pH de 4,65. Les micelles perdent complètement leurs structures par dissolution totale du calcium micellaire. Dès lors, las caséines sont dénaturées et perdent leur propriété de suspension colloïdale. Les protéines subissent alors des étirements, peuvent s'enchevêtrer et former un gel.

Le gel formé par acidification est constitué d'amas dispersés de caséines déminéralisées dépourvues de liaisons susceptibles de créer une force de contraction. Ce type

de gel est ferme, extrêmement friable et ne présente qu'un très faible pouvoir de synérèse et donc une aptitude à l'égouttage extrêmement limitée (Brulé et al., 1997).

I.1.4.2. Coagulation par voie enzymatique

Plusieurs enzymes protéolytiques d'origine animale (présure, pepsine), végétales (broméline, ficine, chardon) ou microbiennes (enzymes de certaines moisissures ou de bactéries) ont la propriété de coaguler les caséines du lait. Cependant, en France, seule la présure est utilisée dans l'industrie fromagère (Herbert, 1999). La présure, sécrétée dans le caillette de veau non sevré, est un mélange de chymosine (80%) et de pepsine (20%).

L'addition de la présure au lait provoque sa coagulation par hydrolyse de la caséine κ située en périphérie de la micelle. Cette hydrolyse scinde la liaison peptidique 105-106 et libère le glycomacropeptide qui est la parie hydrophile de la caséine κ chargée négativement et responsable des répulsions électrostatiques. La partie restante de la micelle est la paracaséine qui est plus hydrophobe et peut se lier avec d'autres paracaséines par des liaisons hydrophobes pour créer un coagulum. Cette coagulation commence par une agrégation des petites micelles, puis se complète par l'agrégation des plus grosses micelles et formera le gel de paracaséine (Amiot et al., 2002). Ce type de gel structuré possède une bonne élasticité, une friabilité réduite et un fort pouvoir de contraction, lui conférant ainsi une bonne aptitude à l'égouttage (Ramet, 1997a).

Dans les procédés classiques de fabrication de fromages, la coagulation est toujours obtenue par une l'action conjointe d'une préparation enzymatique coagulante et de l'acidification (Ramet, 1997b). L'importance de chacun de ces deux modes de coagulation induit des propriétés particulières du coagulum qui conditionnent d'aptitude à l'égouttage et les caractéristiques finales du fromage.

II. Fromages à pâtes pressées

Le fromage correspond à une véritable conserve alimentaire, obtenue grâce au jeu croisé de l'élimination plus au moins poussée de l'eau du lait et de la récupération des matières sèches. Selon les paramètres mis en oeuvre au niveau des différentes étapes de transformation du lait en fromage, une grande variété de produits peut être obtenue tel que traduit par (Lenoir et al., 1985)(**Figure 6**).

La catégorie des fromages à pâte pressée désigne un ensemble de fromages très variés dans leurs compositions, leurs formats et leurs aspects extérieurs à croûte sèche ou avec présence de flore microbienne. Les fromages à pâte pressée se divisent en deux sous-familles : les pâtes pressées non cuites et les pâtes pressées cuites.

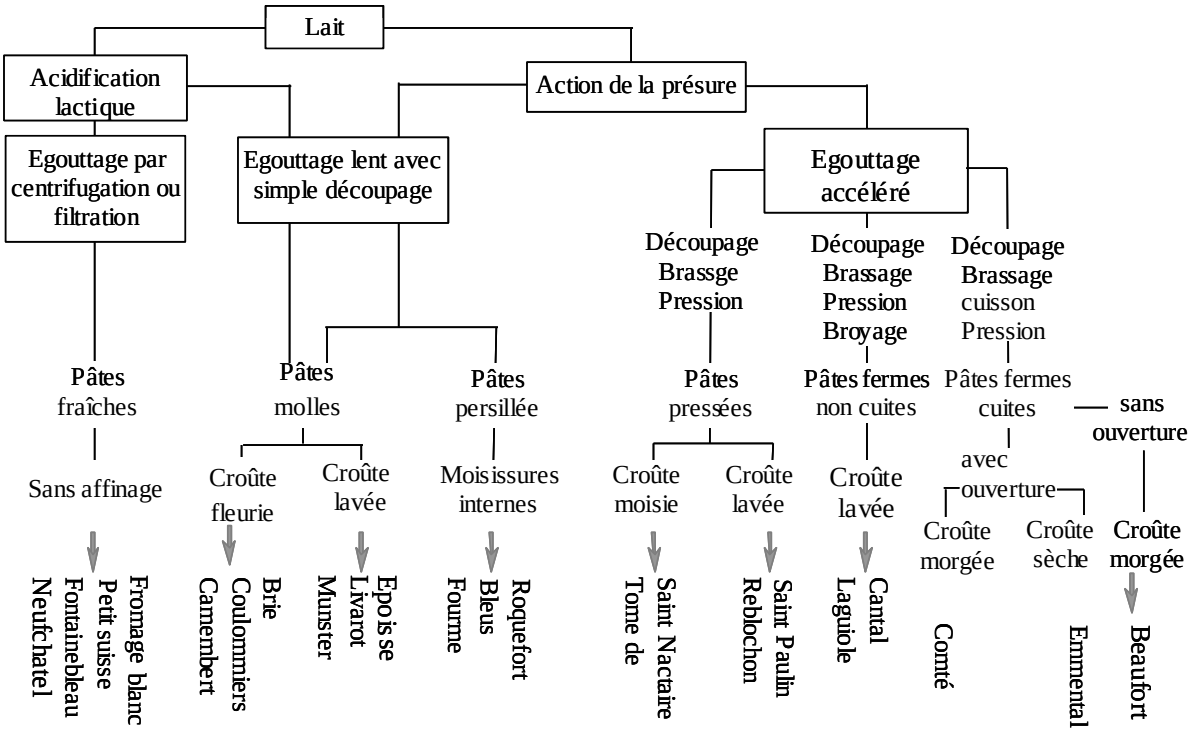


Figure 6 : La diversité des fabrications fromagères (Lenoir et al., 1985).

II.1. Les fromages à pâte pressée non cuites

Les pâtes pressées non cuites ne subissent pas de chauffage ou cuisson lors du brassage en cuve. Leur matière sèche est comprise pour la plupart entre 44 % et 55% ; seul le Cheddar présente un extrait sec plus élevé (63%). Parmi ces fromages, certains sont à croûte sèche tels le Morbier, la Raclette, le Gouda, l'Edam, le Cantal et le Cheddar. D'autre sont affinées avec des flores fongiques comme le Saint-Nectaire et les Tommes ou avec des flores de morge tels que les Saint-Paulin (Ramet, 1997b).

II.2. Les fromages à pâte pressée cuites

Les pâtes pressées cuites subissent un égouttage en cuve plus poussé par cuisson à 52-55 °C pendant 30 à 60 minutes qui permet d'amener l'extrait sec final entre 60 et 63 %. Dans cette catégorie de pâte pressée cuite, on peut distinguer deux groupes de fromage (**Tableau**

3): le groupe de l'Emmental qui se caractérise par des fromages de gros format (65 à 110 kg), à croûte sèche ; la fermentation propionique est développée lors du passage en cave (16 à 18 °C) et les faces sont bombées. Le second groupe est celui du Gruyère où se rangent le Comté, le Beaufort pour la France et le fromage de Jura et de Fribourg pour la Suisse ; ces fromage sont de formats plus réduits (25 à 55 kg) et la fermentation propionique est nulle ou faible (trous de 8 à10 mm) et les faces sont plates et couvertes d'une morge (Ramet, 1997b).

Tableau 3 : Technologie des fromages à pâte pressée cuite (Chamba & Cretin-Maintenaz, 1990).

	Comté	Emmental
Lait utilisé	Lait cru, mélange lait frais et de 12 h, partiellement écrémé. Lait maturé	Lait cru ou thermisé à 63-67 °C pendant 15 à 30 s, partiellement écrémé. Lait maturé
Levains utilisés	Recuite ou ferments sauvages thermophiles, plus rarement ferment sélectionnés. Dominance des lactobacilles, dose environs 2 %	Ferments sauvages ou ferments sélectionnés mésophiles et thermophiles. Dominance des streptocoques, dose 2 à 5%
Coagulation	A 32-33 °C en 30 à 35 min avec recuite ou présure de commerce	A 32-33 °C en 25 min environ avec présure du commerce
Taille des grains	Variable, riz à maïs	Variable, riz à maïs
Chauffage	53 à 56 °C en 35 à 50 min	52 à 55 °C en 35-45 min
Brassage après le feu	40 min à 1 heure	40 min à 1 heure
Temps total de travail en cuve	Normalement 2 h 30 mais de 2 h à 3 h	Normalement 2 h 10, mais de 1h 45 à 2 h 30
Pressage	20 h avec grainage de la croûte par le dernier pressage de la toile sèche, 4 à 5 retournements	Traditionnellement 20 h avec 4 à 5 retournements, de 4 à 20h dans les installations mécanisées avec au maximum un retournement; lissage de la croûte
Salage	Traditionnellement en saumure saturée en cave froide (24 h)	En saumure saturée à 13-15 °C pendant 48 h à 72 h
Affinage	Cave froide 12-14 °C, avec frottage au sel à l'aide d'un chiffon et emmorgeage avec 2 ou 3 retournements hebdomadaires pendant 4 à 6 semaines; cave chaude (HR 90-95 %, 16-18 °C) avec même soins qu'en cave froide pendant 8 semaines environs; cave froide à 12-14 °C avec un lavage de la croûte et un retournement par semaine	Cave froide à 12-14 °C avec lavage et brossage pendant 10 à 20 jours. Cave chaude (HR 75-85 %, 22 à 25 °C) pendant 6 semaines en moyenne; retournements avec lavage et brossage 1 à 2 fois par semaine; cave froide à 10-12 °C jusqu'à l'expédition
Temps d'affinage	4 à 6 mois	2 à 3 mois

I.2. Technologie des fromages à pâtes pressées

Les principaux traits dominants de la technologie des fromages à pâtes pressées sont les suivants :

II.2.1. La coagulation

La coagulation, à caractère enzymatique dominant, est obtenue pour l'emploi de dose élevée en enzyme coagulant (20 à 40 ml/100 l de lait) dans des conditions de température propices à cette activité. Le caractère acide reste limité par l'emploi de lait frais non acide et l'usage de levains lactique à pouvoir acidifiant faible en quantité modérée. Le temps de floculation est court (10 à 30 min), la phase de durcissement est réduite pour éviter la déminéralisation du gel et conserver une bonne aptitude à l'égouttage (Ramet, 1997b).

Les principaux facteurs influençant cette étape sont les conditions de la coagulation (pH et température, quantité d'enzymes coagulant, vitesse d'acidification….), les caractéristiques physico-chimiques du lait (pH, concentration et composition en caséines, concentration en en calcium soluble et en phosphate de calcium colloïdal…) et les traitements technologique préalable sur le lait (refroidissement, chauffage, maturation) (Brulé et al., 1997; Lenoir et al., 1997).

II.2.2. L'égouttage

L'égouttage est un phénomène complexe et ses mécanismes sont encore peu connus. Cependant, il est généralement admis que l'égouttage résulte à la fois d'un processus physique actif appelé la synérèse, qui correspond à la rétraction du gel, et d'un processus passif correspondant à l'aptitude du gel à évacuer le lactosérum occlus, qui dépend de la porosité et de la perméabilité du gel (Walstra et al., 1985). Ce phénomène aboutit à une concentration sélective de certains constituants majeurs du lait (caséines et matière grasse) dans un petit volume, appelé caillé (Ramet, 1997a). L'essentiel du lactosérum est éliminé pendant le travail en cuve par l'application d'un ensemble de traitements physique (tranchage, brassage, chauffage facultatif) et d'une acidification limitée qui améliore la perméabilité du gel. La durée d'égouttage en cuve est comprise en général entre 30 et 90 minutes selon le type de fromage (Ramet, 1997b). Cette phase est suivie par un pressage visant à compacter les grains et expulser une fraction complémentaire, mais relativement faible de lactosérum. L'égouttage permet d'obtenir un taux de matière sèche oscillant entre 44 et 63 % selon le

fromage. En fin d'égouttage, la pâte du fromage présente une bonne cohésion qui autorise la mise en forme de fromages de gros format.

II.2.3. Le salage

Le salage est une étape indispensable dans la fabrication des produits destinés à subir un affinage. Il est réalisé par saupoudrage de sel, immersion en saumure ou par le salage des grains de fromage avant la mise en moule. Le sel ajouté au fromage permet de rehausser la saveur finale. Il complète l'égouttage sous l'effet de la pression osmotique, permet l'arrêt de l'acidification du caillé et prévient une déminéralisation excessive de la pâte. L'ajout du sel permet également le contrôle des bactéries nuisibles ou pathogènes et le développement des micro-organismes utiles à l'affinage (Hardy, 1997; St-Gelais & Tirard-Collet, 2002).

II.2.4. L'affinage

L'affinage est une phase de digestion enzymatique des composants du caillé. Les modifications biochimiques confèrent au caillé des caractères nouveaux que se soit au niveau de la texture ou de la flaveur des fromages. La pâte, à l'origine relativement dure, compacte, sans grande saveur, est modifiée dans sa composition, sa structure et, par suite, dans son aspect, sa consistance et sa couleur. Les principales transformations sont la fermentation du lactose en acide lactique, la dégradation de la matière grasse et l'hydrolyse des protéines (Choisy et al., 1997). Elles ne s'arrêtent pas au stade primaire car les produits formés sont eux même transformés. Les acides aminés peuvent subir la désamination qui est à l'origine de la formation d'ammoniac présent dans toute la pâte provoquant ainsi une augmentation du pH.

L'affinage reste encore un processus complexe et difficile à maîtriser. Il dépend de nombreux facteurs : la nature du substrat (composition chimique et structure physico-chimique du caillé), la variété des agents responsables des transformations (enzymes) ainsi que les conditions de l'affinage (aération, composition de l'atmosphère, température…) (Herbert, 1999).

II.3. Caractérisation des propriétés texturales des fromages

La texture est considérée comme un élément majeur de la qualité dans beaucoup d'aliments. Elle est définie comme l'ensemble des propriétés mécaniques et de structure d'un produit alimentaire perceptible par les mécanorécepteurs, les récepteurs tactiles, et éventuellement visuels et auditifs (AFNOR, 1992). Cette caractéristique est particulièrement

importante pour les fromages. Elle est largement identifiée en tant qu'un des attributs les plus importants pour la détermination de son identité et de sa qualité (Creamer & Olson, 1982; Jack et al., 1993). Dans les fromages à pâte dure et semi-dure, les variables ayant le plus d'influence sur la texture sont respectivement la teneur en protéines, en sel, et en eau, le pH et la teneur en matière grasse (Herbert, 1999).

II.2.5. Méthodes d'évaluation de la texture des fromages

La texture des fromages peut être déterminée par l'analyse sensorielle mais également par des méthodes empiriques et instrumentales. Parmi les méthodes instrumentales, on trouve les méthodes rhéologiques en régime permanent (compression uniaxiale, méthode du profil d'analyse de la texture, méthode de traction, méthode de flexion) et/ou en régime transitoire (méthode de relaxation, méthode de fluage).

II.2.5.1. Analyse sensorielle

L'évaluation sensorielle d'un aliment est basée, d'une part sur le choix des caractéristiques sensorielles à percevoir et, d'autre part, sur la formation et l'entraînement d'un jury de dégustation. Il existe plusieurs méthodes pour évaluer la texture des produits alimentaires (Brandt et al., 1963; Civille & Szczesniak, 1973). Les premières méthodes telles que le profile de la texture ont été développées dans les années 50 et 60. Elles permettaient de décrire et de quantifier une unique dimension sensorielle. Dans les années 70, la méthode QDA (Quantitative Descriptive Analysis) a été développée pour permettre la caractérisation sensorielle des aliments sur plusieurs dimensions (Stone et al., 1974). Cependant, ces méthodes sont très générales pour pouvoir être appliquées à l'étude des fromages. C'est pourquoi, dans un programme européen, une méthode d'évaluation sensorielle de la texture des fromages à pâtes dures et semi-dures à été développée par (Lavanchy et al., 1994). Cette méthode s'appuie sur les perceptions des sens du juge et prend en compte les informations émanant des mécanorécepteurs kinesthésiques, des récepteurs tactiles et des récepteurs visuels. Les descripteurs à évaluer ont été classés dans 4 groupes de caractéristiques :

- mécaniques : élasticité, fermeté, déformabilité, friabilité et adhésivité ;
- de surface : visuelles (ouvertures) et tactiles (degré de rugosité) ;
- géométriques : perceptibilité de la microstructure ;
- autres : plutôt liés à l'organisation de la matière selon des impression buccales, tactiles ou résiduelles (solubilité, impression d'humidité…).

L'analyse sensorielle présente l'inconvénient d'être relativement coûteuse et fastidieuse à mettre en oeuvre. De plus, les jurys d'analyse sensorielle effectuent généralement l'analyse de la texture des fromages à la fin de l'affinage. Un des intérêts de l'analyse sensorielle réside dans le fait qu'elle permet de caractériser la texture, ainsi que le goût et la flaveur des produits.

II.2.5.2. Méthodes d'analyses empiriques

Les caractéristiques texturales des fromages ont souvent été mesurées au moyens de tests simples utilisant des outils très divers simulant de près ou de loin certaines techniques d'appréciation sensorielle et fournissant une évaluation globale de ces propriétés. On peut les classer en cinq catégories : les méthodes de compression, d'extrusion, de cisaillement, de pénétration et de tranchage (Hardy & Scher, 1997). Les paramètres évalués par ces différentes méthodes sont nommées, selon le cas, dureté, fermeté, souplesse, friabilité, consistance, etc.

Les propriétés fonctionnelles des fromages deviennent de plus en plus importantes. Les modes alimentaires des consommateurs sont en évolution permanente et l'on assiste au développement de plats cuisinés utilisant du fromage. Par conséquent, les consommateurs accordent une importance non négligeable aux propriétés fonctionnelles à chaud, tant mécaniques (propriétés de fonte, le coulant, l'étalement à chaud), que visuelles (exsudation d'huile) (Bertola et al., 1996; Guinee & O'Callaghan, 1997; Kindstedt & Rippe, 1990). Ces tests sont fastidieux et ne peuvent être appliqués que par des opérateurs qualifiés. Ils ont été décrits de façon détaillée par (Reparet, 2000).

II.2.5.3. Analyse rhéologique

La rhéologie, science de la déformation de la matière, classe habituellement les effets de l'application d'une contrainte en quatre catégories élémentaires : l'élasticité (déformation réversible instantanément), l'élasticité retardée (déformation réversible, mais dépendant du temps), la viscosité (écoulement dépendant de la force appliquée) et la plasticité (déformation permanente à partir d'un certain seuil de contrainte). Ainsi, lorsqu'on soumet un corps (liquide, pâteux, pulvérulent, etc.) à l'action d'un force dans des conditions données, l'étude de la déformation résultante conduit à l'établissement d'une loi dite de comportement, permettant de caractériser rhéologiquement le produit (Hardy & Scher, 1997).

Les propriétés de texture des fromages évaluées en analyse sensorielle ne peuvent être toutes décrites avec les méthodes rhéologiques. Seules les caractéristiques mécaniques de la

texture peuvent être évaluées. Contrairement à l'analyse sensorielle qui s'effectue généralement après la fin de l'affinage des fromages, les analyse rhéologiques peuvent être réalisées au cours de l'affinage, contribuant à une meilleure connaissance des mécanismes de l'évolution de la texture. Les travaux de (Herbert, 1999; Herbert et al., 2000; Lebecque et al., 2001) ont mis en évidence des corrélations fortes entre des évaluations sensorielles et des mesures instrumentales suggérant que les phénomènes observés aux niveaux macroscopique et moléculaire étaient reliés à la texture des fromages. Les méthodes rhéologiques les plus utilisées pour évaluer la texture des fromages sont les tests de compression uniaxiale, du profil d'analyse de la texture (TPA) et du test de pénétration.

Le test de compression uniaxiale a été utilisé dans de nombreuses études réalisées sur la rhéologie des fromages (Creamer & Olson, 1982; Mpagana & Hardy, 1985, 1986). Pour ce test, un échantillon (généralement de forme cylindrique ou cubique avec des surfaces plates) est comprimé uniaxialement entre deux plateaux parallèles dont l'un avance à une vitesse constante prédéterminée (Van Vliet, 1991). Un capteur enregistre la force développée par l'échantillon durant la déformation. Les paramètres suivants sont calculés : le module d'élasticité (E), la contrainte (σ_F) et la déformation (ε_F) au point de fracture et l'énergie à la fracture (W_F). La **Figure 7** montre une courbe de compression représentant la contrainte en fonction de la déformation.

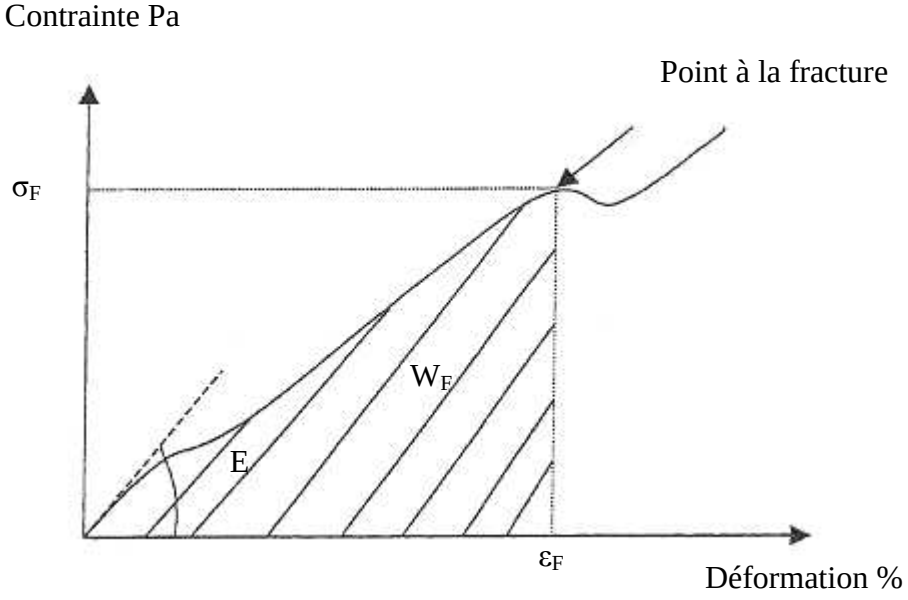


Figure 7 : Allure générale d'une courbe de compression, contrainte en fonction de la déformation, obtenu pour un test de compression uniaxiale (Herbert, 1999).

Le TPA est un test imitatif visant à reproduire de manière partielle le processus de mastication (Hardy & Scher, 1997). Ce test permet d'obtenir une réponse globale du produit vis à vis d'une déformation. Les échantillons, de forme cylindrique, sont comprimés deux fois de suite (test à deux morsures) entre deux plaques d'acier. A partir de la courbe obtenue représentant la force en fonction de la distance (**Figure 8**), la dureté TPA, l'élasticité TPA, l'intensité de la cohésion TPA et l'intensité de l'adhérence TPA sont mesurées. Malgré de nombreux exemple d'application de ce test, la Fédération Internationale Laitière (FIL) déconseille l'utilisation de ce test car la majorité des paramètres rhéologiques est calculée une fois que la fracture a eu lieu (Van Vliet, 1991).

Le test de pénétration consiste à enfoncer une aiguille ou une tige dans l'échantillon à une vitesse constante (Vassal et al., 1987). La force nécessaire à la pénétration est enregistrée. La fermeté est estimée à partir du travail (mesuré en g.cm) effectué pendant l'enfoncement de l'aiguille jusqu'à une profondeur de pénétration fixée.

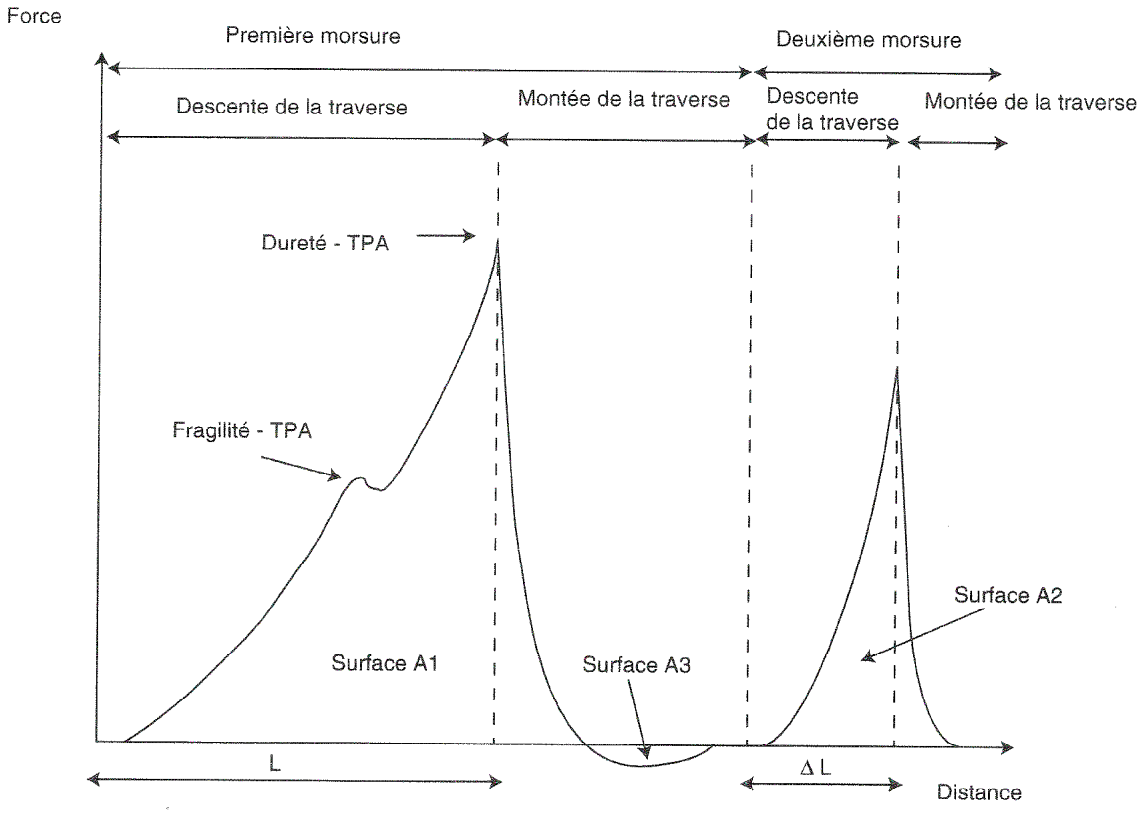


Figure 8 : Allure générale du profil d'analyse de la texture (TPA) (Hardy & Scher, 1997)

A_3 : Intensité de l'adhérence TPA ; A_2/A_1 : Intensité de la cohésion TPA ; AL/L : Elasticité TPA.

Le test de cisaillement dynamique permet d'expliquer les modifications structurales susceptibles d'intervenir dans un échantillon de fromage (Famelart et al., 2002; Ma et al., 1996; Shoemaker et al., 1992). La technique d'oscillation est non destructive et elle permet de mesurer simultanément les comportements visqueux et élastique de l'échantillon. La contrainte (ou la déformation) est appliquée de façon sinusoïdale. Cette méthode peut être mise en application dans la région viscoélastique linéaire. Comme le montre la **Figure 9**, les paramètres rhéologiques (G', G" et tan δ) sont calculés. G' est le module élastique (de stockage) : il sera important pour un matériau de prédominance élastique ou fortement structuré. G", module visqueux (ou dissipatifou de perte) est important pour un échantillon de prédominance visqueuse. Le Tan δ représente l'angle de déphasage de perte où δ est le rapport entre G" et G'. Ce rapport est un indice du caractère viscoélastique de l'échantillon à une température et une fréquence ω données.

Le test de cisaillement dynamique, comparé aux autres méthodes susmentionnées, présente l'avantage d'être rapide et de permettre plusieurs types de balayage (température, en fréquence, en temps…). Le balayage en température peut varier de –10 à 100 °C pour les appareils équipées d'un système à effet Peltier. Cependant, les analyses rhéologiques en régime transitoire et/ou permanent doivent être effectuées à température constante. En outre, ces méthodes présentent l'inconvénient de nécessiter des mesures sur une échelle de temps assez importante, et pouvant provoquer ainsi une déshydratation de l'échantillon, qui pourrait affecter les mesures rhéologiques. Afin surmonter ce type de problème d'évaporation, Herbret a couvert la surface des échantillons du lait par de l'huile de silicone durant les cinétiques de coagulations.

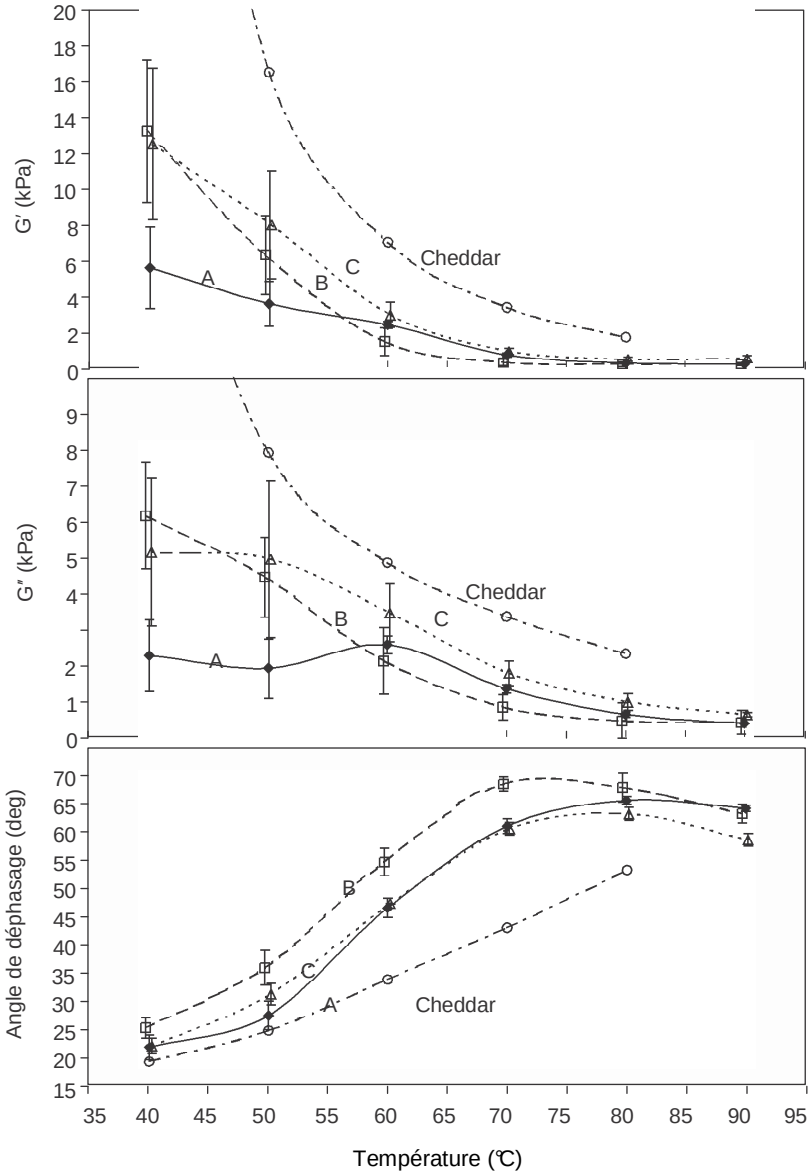


Figure 9 : Évolutions des modules élastiques G' et visqueux G" et de l'angle de déphasage au cours du balayage de température de 40 à 90 °C pour 4 types de fromages (A, B, C et Cheddar) (Famelart et al., 2002)

II.2.5.4. Conclusion

La texture telle qu'elle est perçue par le sens du toucher ou par la bouche lors de la mastication est une combinaison de plusieurs paramètres texturaux élémentaire que le cerveau intègre instantanément. En conséquence, les méthodes précitées ne peuvent, individuellement, couvrir l'ensemble de la définition texturales d'un produit. Il est nécessaire de faire appel à d'autres techniquesplus puissantes, telles que les techniques spectroscopiques, donnant simultanément plusieurs valeur texturales à partir d'un même échantillon.

III. Apports des techniques spectroscopiques couplées à la chimiométrie pour l'étude de la composition et la caractérisation de la structure des fromages

Dans les industries agroalimentaires, une grande partie du contrôle de la qualité des denrées en cours de transformation et des produits finis repose sur leur analyse (bio)chimique. Ces méthodes, considérées de référence, sont en général longues, nécessitent parfois l'utilisation de réactifs onéreux et polluants et ne sont applicables que par des opérateurs qualifiés. De plus ces méthodes (bio)chimiques, ne sont pas assez efficaces pour couvrir la demande croissante. Pour un dosage demandant plusieurs heures par une méthode conventionnelle (comme celui des protéines par la méthode Kjeldahl ou de l'eau par séchage et pesée), la mise en œuvre de méthodes spectroscopique appropriées, telle que la spectroscopie proche infrarouge, rend possible la réalisation de ces dosages en quelques secondes.. La durée prolongée d'une analyse peut ainsi avoir comme conséquence d'augmenter la proportion des produits non conformes. Par ailleurs, les tests empiriques permettant de déterminer les propriétés fonctionnelles des fromages (filant, fondant) sont fastidieux et ne peuvent être appliqués que par des opérateurs qualifiés. De plus, la qualité des produits ne dépend pas seulement de leur composition physico-chimique. La distribution de la matière grasse et des protéines dans le produit et les interactions entre les différentes composantes impliquées dans l'organisation structurale des fromages doivent être considérées (protéine-protéine, protéine-lipide et protéine-eau) : les propriétés organoleptiques du produit final sont l'expression de cette organisation structurale.

Le développement de méthodes d'analyse rapide des aliments repose principalement sur l'exploitation des propriétés physique des substrats en tant que source d'information et la transposition, plus au moins exacte, des méthodes (bio)chimiques manuelles (Bertrand, 2006). Le développement de ces méthodes analytiques (et particulièrement basées sur les techniques spectroscopiques) est intimement lié aux progrès de la chimiométrie.

Des méthodes reposant sur la spectroscopie de fluorescence frontale et la spectroscopie infrarouge ont été développées pour caractériser finement la structure moléculaire des fromages (Dufour et al., 1998; Fagan et al., 2007; Herbert, 1999; Herbert et al., 2000; Herbert et al., 1999; Karoui & Dufour, 2006; Karoui, Dufour et al., 2006; Martín-

del-Campo et al., 2007; Noronha et al., 2008). Ces méthodes sont non destructives, rapides, peu coûteuse et non polluantes.

Selon l'approche de Belton (Belton, 1994), trois étapes de base peuvent être distinguées qui conduisent à une mesure spectrale : l'*excitation*, le *classement de l'information* et sa *détection*. L'étape d'excitation porte sur l'apport contrôlé d'énergie dans l'échantillon. Le classement de l'information en spectroscopie a en effet comme objectif principal de ranger les particules ou les photons en fonction de leur énergie. La courbe de l'intensité en fonction de l'énergie constitue le spectre. L'étape de détection met en œuvre un capteur approprié, souvent spécifique de la méthode spectroscopique.

La spectroscopie de fluorescence présente un intérêt potentiel élevé en tant que méthode d'analyse rapide en agroalimentaire. Cantonnée pendant longtemps à l'étude des solutions diluées, le développement à la fin des années 1960 d'une technique autorisant des mesures de surface a permis l'étude des poudres et des milieux concentrés et turbides. La première application rapportée portait sur la détermination de l'hémoglobine dans un échantillon de sang en l'état (Blumberg et al., 1980). Par la suite, elle a été appliquée à la caractérisation des produits de l'industrie sucrière, à l'appréciation de la teneur en parois végétales des farines et récemment dans la détermination de la structure et à la prédiction de la texture de fromages (Dufour et al., 2001). La spectroscopie infrarouge (proche et moyen) est probablement, par le nombre et la diversité de ses applications analytiques, la méthode physique que l'en rencontre aujourd'hui le plus couramment dans les entreprises et les laboratoires d'analyse pour la caractérisation des produits agroalimentaires (Bertrand, 2006).

III.1. Techniques spectroscopiques

Les techniques de spectroscopie sont des méthodes physiques de caractérisation qui peuvent être une alternative remarquablement efficace aux analyses traditionnelles. Elles peuvent être divisées en deux grandes classes : la spectroscopie photonique qui repose sur l'étude de l'interaction d'une onde électromagnétique avec la lumière, et la spectroscopie des particules. Dans la première classe, on trouve la plupart des méthodes spectroscopiques présentant un intérêt analytique pour le contrôle rapide. La seconde classe est essentiellement représentée par la spectrométrie de masse et les méthodes dérivées, qui portent sur l'étude du rapport masse/charge de molécules ou fragments de molécule ionisés.

Le spectre électromagnétique est généralement divisé comme le montre la **Figure 10** en diverses régions en fonction de la longueur d'onde des radiations : ainsi, on trouve les rayons γ qui sont les plus énergétiques, les rayons X, l'ultraviolet, le visible, l'infrarouge (IR), les micro-ondes et les ondes radio fréquences (Lachenal, 2006). A chaque région on peut associer un type de transition atomique ou moléculaire différente mettant en jeu des énergies différentes.

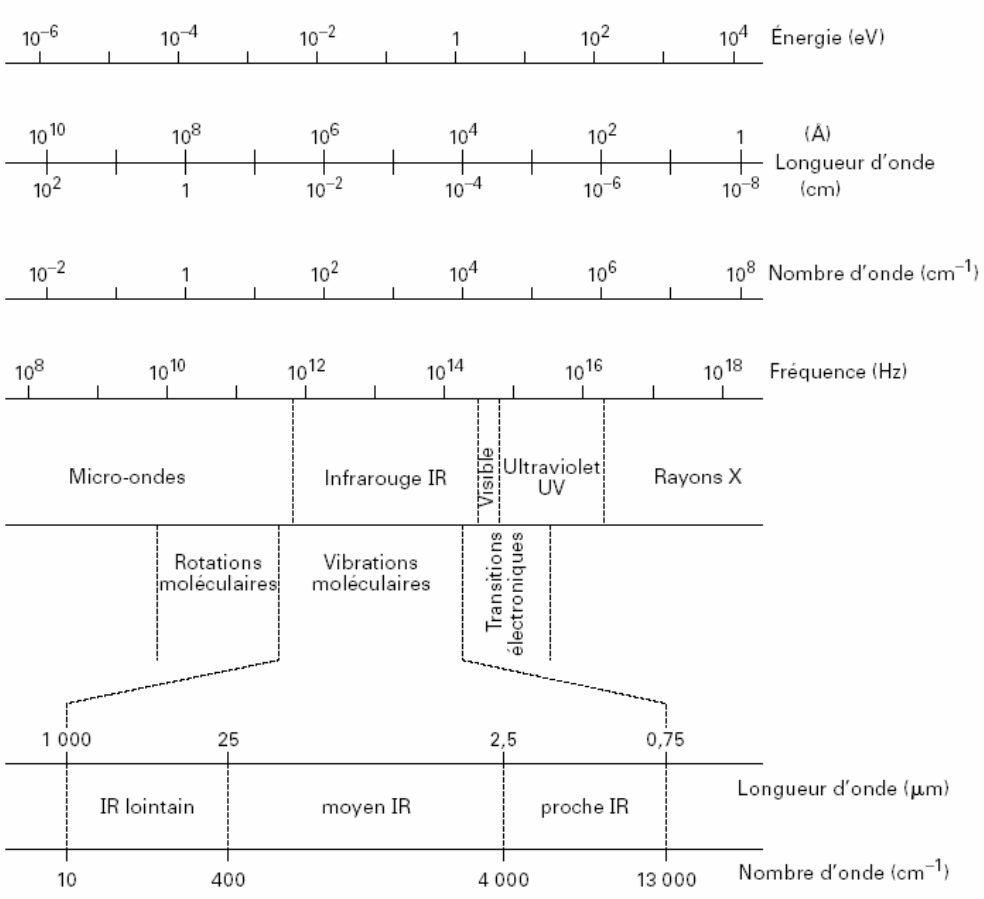


Figure 10 : Domaines spectraux du rayonnement électromagnétique (Dalibart & Servant, 2000)

La radiation électromagnétique a une double nature ondulatoire et quantique. La principale caractéristique ondulatoire du rayonnement est sa fréquence de vibration ν, exprimée en Hertz. La longueur d'onde λ est la distance parcourue pendant un cycle complet. Elle est reliée à la fréquence par l'équation :

$$\lambda = c/\nu$$

avec c la célérité de la lumière (3.108 m.s-1)

L'approche quantique permet la description des interactions énergétiques avec la matière au niveau moléculaire. Une radiation lumineuse se comporte comme si elle était composée de corpuscules appelés photons. Ces photons possèdent la propriété de transporter une quantité d'énergie finie, liée à la fréquence de la radiation. Lorsque la lumière traverse de la matière non-transparente, elle peut être absorbée partiellement ou complètement. Dans le domaine de l'UV et du visible, une autre manifestation de l'interaction lumière-matière est l'absorption de l'énergie lumineuse qui fait passer des électrons d'une molécule d'un état fondamental, stable, à un état de plus haute énergie, instable. Bien entendu, étant instable, cet état ne peut pas vivre longtemps et la molécule retourne à son état fondamental en cédant de l'énergie au milieu, soit sous forme de lumière (luminescence) soit sous forme de chaleur soit sous forme de fluorescence. Pour le domaine de l'IR, les transitions d'énergie observées sont de type vibrationnel, qui peut être définie comme l'étude de l'interaction des ondes électromagnétiques et de la matière sur le domaine des longueurs d'ondes ultraviolet, visible et infrarouge (Banwell, 1983).

La fluorescence est la propriété d'une molécule, d'un atome, ou d'un ensemble d'atome d'émettre un photon suite à l'absorption d'un autre photon. Les énergies mises en jeux sont dans le domaine de l'UV et du visible, le domaine des énergies d'orbitale atomique.

III.1.1. Spectroscopie infrarouge

Le domaine infrarouge est sous divisé en trois catégories selon la fréquence : le proche infrarouge (PIR) est compris entre 750 nm et 2500 nm, entre 2500 nm et 25000 nm se trouve le domaine de l'infrarouge moyen (MIR) et l'infrarouge lointain à des longueurs d'onde supérieures à 25000 nm.

Les combinaisons et les harmoniques sont le cœur de la PIR. Elles se présentent sous forme de larges bandes de faible intensité pouvant se recouvrire partiellement. De nombreux auteurs ont essayé d'attribuer les bandes spectrales du proche infrarouge (Roggo, 2003). Il est difficile de ce fait de donner une affectation précise à chacune d'elle, même après enregistrement des composants à l'état pur (Grappin et al., 2006). L'identification des maxima d'absorption reste difficile car les bandes sont larges, souvent de faibles intensités. De plus les interactions entre les molécules peuvent entraîner des déplacements de longueurs d'onde (Roggo, 2003). D'une manière générale dans les produits laitiers, l'absorptions des

liaisons O-H sont utilisées pour le dosage de l'eau et du lactose, C-H pour le dosage de la matière grasse et N-H pour le dosage des protéines.

La spectroscopie PIR et la chimiométrie ont prouvé leurs utilités dans des domaines variés tels que l'agriculture, les industries alimentaires, pharmaceutiques, chimiques et pétrolières (Dowell et al., 2008; Huang et al., 2008; Roggo et al., 2007; S. Wold & M. Sjöström, 1998; Svante Wold & Michael Sjöström, 1998). Par sa rapidité et par son caractère non destructif, la PIR reste une méthode de choix pour l'analyse des produits en ligne.

De plus, le PIR constitue la technique privilégiée pour l'analyse rapide des produits laitiers solides, semi-solides ou pulvérulents, car il présente l'avantage de pouvoir analyser des échantillons directement en transmittance ou en réflectance, sans qu'il soit nécessaire de procéder à une dissolution de l'échantillon qui pose des problèmes pratiques et diminue la fiabilité des mesures (Grappin et al., 2006).

La partie du spectre infrarouge la plus riche en informations est celle du MIR. Les bandes d'absorptions observées dans le MIR sont principalement associées à des vibrations fondamentales de liaison de valence (ν) de groupes fonctionnels d'une molécule. Les spectres MIR de nombreuses molécules sont d'ores et déjà connus. Les attributions des bandes spectrales dans le MIR sont décrites par (Grappin et al., 2006) (**Tableau 4**).

Dans le domaine de l'agroalimentaire, la spectroscopie MIR a été moins employée pour des mesure en ligne que le PIR. Ceci est expliqué par le fait que l'eau est un constituant majeur de ces produits et contribue fortement au spectre MIR.

Cependant, le développement de la spectroscopie infrarouge à transformée de Fourrier (IRTF) ces dernières années a donné la possibilité d'obtenir des informations intéressantes sur les structures des protéines et des lipides (Casal & Mantsch, 1984). Le développement de la technique de réflexion totale atténuée (RTA) s'est révélée très utile pour l'acquisition des spectres MIR de produits agroalimentaires solides, liquides et pâteux simplement étalés sur une lame cristalline de séléniure de zinc (ZnSe), de silicium (Si) ou de germanium (Ge) (**Figure 11**).

RTA-IRTF : échantillon turbide, concentré ou solide

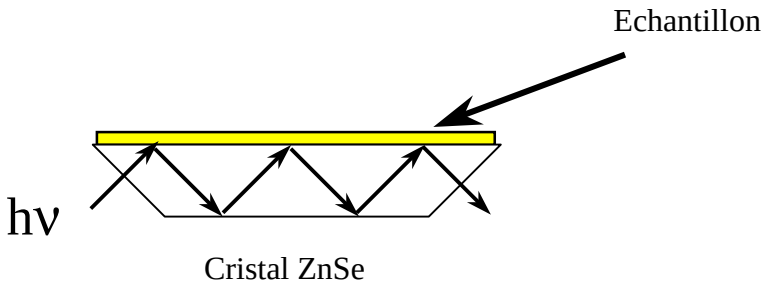


Figure 11 : Dispositifs d'infrarouge IRTF et RTA-IRTF.

La région 3000-900 cm^{-1} du domaine infrarouge est essentiellement utilisés pour l'analyse et le dosage des différents constituants des produits laitiers tels que la matière grasse, les protéines et le lactose.

La région spectrale 3000-2800 cm^{-1} est dominée par les bandes liées aux vibrations (élongation) de valence ν(C-H) des groupements CH, CH_2 et CH_3 des chaînes d'acides gras des lipides (**Tableau 4**).On peut observé deux bandes aux alentour de 2920 et 2845 cm^{-1}. La première résulte de l'élongation asymétrique des deux liaisons CH du groupe méthylène $\nu_{as}CH_2$. La seconde a comme origine l'élongation symétrique du même groupement (ν_aCH_2). Les bandes à 2962 et 2872 cm^{-1} sont attribuable respectivement aux élongations asymétriques $\nu_{as}CH_3$ et symétrique ν_sCH_3. Cette zone spectrale est utilisée pour l'identification et le dosage des acides gras et pour la détermination de l'état physique des triglycérides des globules gras. La région (1700-1500 cm^{-1}) est dominée par les bandes dites amide I vers 1650 cm^{-1} et amide II vers 1550 cm^{-1}, liées aux liaisons peptidiques des protéines. La bande amide I est essentiellement attribuable à une élongation du groupe C=O. La bande amide II caractérise principalement une combinaison hors phase du balancement dans le plan du groupement N-H et de l'élongation C-N. Le balancement dans le plan du groupement C=O ainsi que les élongations C-C et N-C contribuent faiblement à la bande amide II (Dufour & Robert, 2006). La bande amide III qui se situe entre 1200-1400 cm^{-1} est complexe et les chaînes latérales des acides aminés des protéines présentent des contributions dans cette zone. Les modes de vibration associés aux bandes amides I, II et III sont sensibles à la structure secondaire des protéines et ils sont couramment utilisés pour l'étude de conformation des protéines.

Quant à la région spectrale 1200-700 cm^{-1}, elle est plus connue sous le nom d'empreinte digitale : dans cette région, de nombreuses liaisons chimiques absorbent.

Tableau 4. Attribution des bandes spectrales en moyen infrarouge aux principaux composants du lait.

Nombre d'onde en cm^{-1}	Liaison impliquée	Fonction ou groupement	Mode de vibration	Composant du lait
2 955	C-H	$C-H_3$	élongation	matière grasse
2 924	C-H	$C-H_2$	élongation	matière grasse
2 872	C-H	$C-H_3$	élongation	matière grasse
2 854	C-H	$C-H_2$	élongation	matière grasse
1 746	C = O	ester carbonyle	élongation	matière grasse acides
1 651	C = O	amide I	élongation	protéines
1 548	N-H	amide II	élongation	protéines
	C-N		déformation élongation	
1 470-1 446	-CH	CH_2	déformation	matière grasse
	-CH	CH_3	déformation	matière grasse
1 243-1 100	-C(O)-O-	Ester	élongation	matière grasse
	C-O		élongation	matière grasse
1 112-1 050	C-O	alcool I	élongation	lactose
	O-H		déformation	

Lorsque l'on s'intéresse au lait, quatre bandes d'absorption sont retenues pour l'analyse quantitative des principaux constituants (Grappin et al., 2006). Ces bandes correspondent respectivement aux groupements C-N et N-H des liaisons peptidiques qui absorbent à 1 548 cm^{-1} pour le dosage des protéines, au groupement carbonyle C=O des liaisons ester absorbant à 1 746 cm^{-1} et aux groupement C-H des acide gras absorbant à 2 920–2 854 cm^{-1} pour la matière grasse et à la liaison C–O, ainsi qu'au groupement hydroxyle O–H absorbant à 1 041 cm^{-1} pour le dosage du lactose .

Plusieurs travaux ont mis en évidence la potentialité du MIR pour la caractérisation des fromages. McQueen et al. (1995) ont utilisé le moyen infrarouge pour la prédiction de la teneur en protéines, en matière grasse et en matière sèche de 24 échantillons de fromage. (Mazerolles et al., 2001) ont suivi, par le biais du moyen infrarouge les modifications de la

structure des protéines du fromage pendant son affinage. De plus, Karoui et al. (2006) l'ont utilisé pour la détermination des paramètres chimiques des fromages Emmental de production hivernale. Par ailleurs, (Picque et al., 2002) ont montré par spectroscopie moyen infrarouge couplée aux techniques chimiométriques qu'il était possible de discriminer des fromages Emmental provenant de diverses origines géographiques. Enfin, Dufour et al. (2000) ont utilisé les spectres MIR pour l'extraction d'informations sur l'état physique des triglycérides et la structure de la matière grasse des fromages. Les auteurs ont montré que le vecteur propre associé à la seconde variable canonique affiche des bades de signe opposé entre 2847 et 2857 cm^{-1} et à 2914 et 2925 cm^{-1}. Ce profil caractérise le déplacement des bandes méthylène des triglycérides avec l'âge des fromages et indique une cristallisation des triglycérides au cours de l'affinage à basse température des fromages.

III.1.2. Spectroscopie de fluorescence

La fluorescence est le phénomène qui résulte de l'émission de photons par une molécule excitée à l'aide d'une radiation lumineuse dans l'ultraviolet et le visible (Lakowicz, 1983). Lorsqu'un photon rencontre une molécule, un électron absorbe cette énergie et passe du niveau singulet fondamental S_0 à un niveau singulet S_1 (**Figure 12**). L'état excité persiste pendant un temps fini caractéristique de la molécule. Suite à des collisions avec d'autres molécules, une désactivation a lieu et l'électron revient dans le premier niveau vibrationnel S_1. Si la molécule possède suffisamment d'énergie, après un temps court qui est caractéristique de chaque molécule (10^{-9}-10^{-7} secondes), l'électron retourne au niveau S_0 en émettant un photon. Cette émission de photons est appelée fluorescence et le signal enregistré à différentes longueurs d'onde constitue le spectre d'émission de fluorescence. Le photon émis aura donc une énergie moindre que le photon d'excitation : pour une molécule donnée, les longueurs d'onde d'émission seront donc supérieures à celles d'excitation et l'énergie des photons de fluorescence sera plus faible que celle des photons d'excitation. L'ensemble des rayonnements émis lors de la désexcitation par fluorescence constitue le spectre d'émission.

La spectroscopie de fluorescence est une méthode d'analyse sensible, rapide et non-invasive. Elle donne des informations sur la présence des molécules fluorescentes et sur leur environnement dans des échantillons analysés (Alvarez et al., 2008; Blaszczyk & Janoszka, 2008; Dufour et al., 2001; R. Karoui et al., 2003; Lakowicz, 1983). Ainsi, les propriétés de fluorescence des acides aminés aromatiques des protéines (Dufour et al., 1994; Ganzlin et al., 2007; Ladokhin, 2000; Lakowicz, 1983; Longworth, 1971) et des produits fluorescents

résultants de l'oxydation des lipides (Gatellier et al., 2007; Pokorny, 1981; Veberg et al., 2006) ont été utilisés pour étudier la structure des protéines et des lipides, les interactions protéines-protéines et/ou protéines-lipides (Herbert et al., 2000), les interactions protéines-molécules hydrophobes (Dufour et al., 1994).

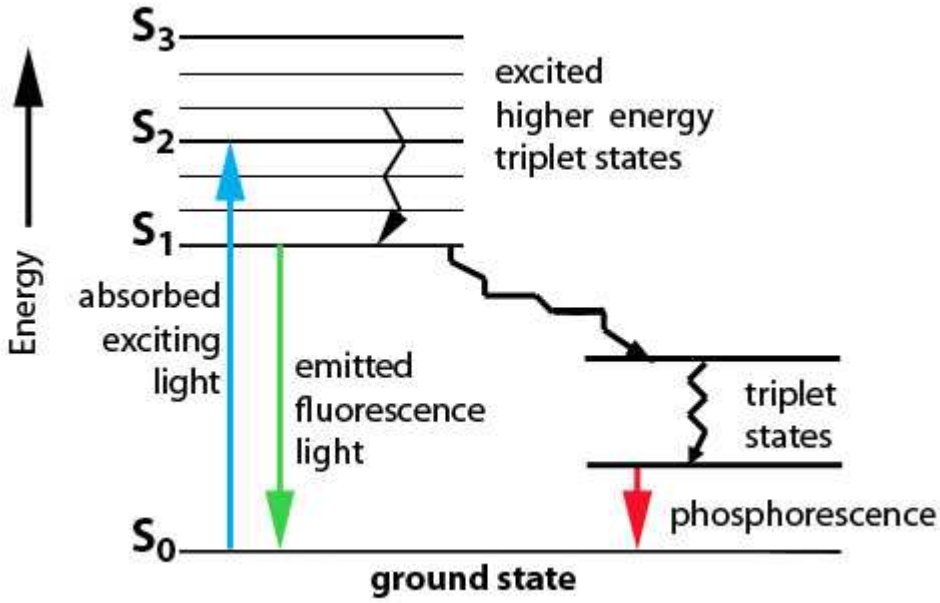


Figure 12 : Désexcitation de molécules et émission de fluorescence ou de phosphorescence

La fluorescence est basée sur des sondes fluorescentes, extrinsèques ou intrinsèques. Les sondes de fluorescence extrinsèques sont ajoutées aux échantillons et sont choisies pour marquer spécifiquement un constituant du produit. Ainsi, le 8-anilino-naphtalène-1-sulphonate (ANS) et le DM-NERF ont été utilisés par (Herbert, 1999) pour marquer les protéines et le suivi du pH au cours de la coagulation du lait, respectivement.

Les fluorophores intrinsèques sont ceux qui sont présents naturellement dans les produits. Ceux-ci incluent les acides aminés aromatiques tels les résidus tryptophane (trp)- les six protéines majeures du lait, α_{s1}, α_{s2}, β et κ caséines, β-lactoglobuline et α-lactalbumine contiennent au moins un résidu de tryptophane (Fox, 1989), un grand nombre de vitamines qui présentent des propriétés de fluorescence comme la vitamine A (Dufour et al., 2001; Herbert et al., 2000) ainsi que certains cofacteurs enzymatiques comme le NADH et la riboflavine.

III.1.2.1. La spectroscopie de fluorescence frontale

La spectroscopie de fluorescence classique, à angle droit (Lakowicz, 1983), ne peut pas être appliquée à l'étude des systèmes turbides tels que les produits laitiers. En effet, la grande majorité des expériences de fluorescence sont réalisée en solutions diluées dont l'absorbance est inférieure à 0,1. Dans le cas où l'absorbance de l'échantillon est élevée, un effet d'écran se produit induisant une diminution de l'intensité de fluorescence par le phénomène d'extinction (quenching) de fluorescence et une distorsion de spectres d'excitation (Genot et al., 1992a). Cependant, La grande difficulté résulte dans l'exploitation des résultats obtenus sur les échantillons dilués de produits alimentaires. Ces résultats ne peuvent pas être extrapolés aux produits de départ; en particulier, lorsque les informations recherchées concernent les changements de conformations dus à la proximité ou aux interactions entre molécules (Genot et al., 1984). Pour contourner ce problème, la méthode de spectroscopie de fluorescence frontale a été développée pour permettre l'investigation de la fluorescence des échantillons de poudres, turbides et concentrés (Genot et al., 1992b) (**Figure 13**). Dans ce cas, seule la surface de l'échantillon est examinée. Les photons émis sont collectés sous un angle de 54° par rapport à l'échantillon afin de minimiser la collection de photons réfléchis.

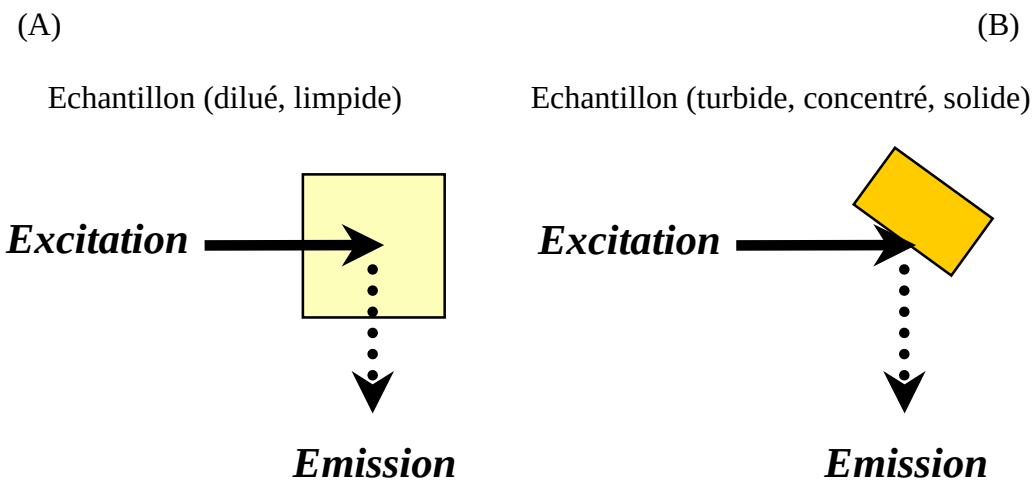


Figure 13 : Dispositifs de fluorescence à angle droit (A) et frontale (B).

L'allure du spectre d'excitation de fluorescence de la vitamine A contenue dans la matière grasse laitière varie fortement avec l'état physique des triglycérides. Il a été montré

que le rapport des intensités de fluorescence à 322 et 295 nm (I.F.322 nm /I.F.295 nm) varie selon l'état solide ou liquide des triglycérides (R. Karoui et al., 2003). La valeur de ce rapport en fonction de la température permet de déterminer le point de fusion de la matière grasse émulsionnée (Dufour et al., 1998). De plus, les travaux de Dufour et Riaublanc (1997) (Dufour & Riaublanc, 1997) ont montré que l'allure des spectres d'excitation de fluorescence de la vitamine A est sensible à l'état de la membrane du globule gras : il est possible de discriminer des laits ayant subi des traitements technologiques (lait natif, chauffé, homogénéisé, chauffé et homogénéisé) altérant partiellement la membrane des globules gras.

Récemment, la riboflavine a été utilisée comme sonde intrinsèque pour déterminer l'état d'oxydation des laits et des produits laitiers (Dowell et al., 2008; Kristensen et al., 2000; Miquel Becker et al., 2003; Wold et al., 2002). La riboflavine joue un rôle important dans le domaine alimentaire en influençant des facteurs de qualité comme la couleur et la valeur nutritionnelle. Cette vitamine est très sensible et instable sous l'action de la lumière et de l'oxygène. Elle se dégrade en composés fluorescents tels le lumichrome qui se traduit par des modifications de l'allure des spectres de fluorescence (Miquel Becker et al., 2003). (Karoui, Mouazen et al., 2006) ont utilisé les spectres de fluorescence de la riboflavine prédire des paramètres chimiques, à la surface et au centre, des fromages à pâte molle.

Malgré son fort potentiel, cette technique n'a connu que dernièrement des utilisations dans le domaine alimentaire. C'est le cas des travaux de (Genot et al., 1992b); (Zandomeneghi, 1999) sur les protéines de blé; ceux de (Dufour & Frencia, 2001) et de (Dufour et al., 2003) dont l'objectif était respectivement de caractériser la qualité des viandes (texture) et la fraîcheur de poissons.

Dans le domaine de l'étude par spectroscopie de fluorescence des fromages, les principales investigations ont essentiellement porté sur les propriétés fluorescentes de trois sondes intrinsèques (tryptophanes, la vitamine A et la riboflavine) mesurées directement sur des échantillons de fromages (Dufour et al., 2001; Herbert, 1999; Herbert et al., 2000; Karoui & Dufour, 2006; Karoui, Dufour et al., 2006; Karoui et al., 2004a, 2004b; R. Karoui et al., 2003; R. Karoui, B. Martin et al., 2005; Karoui et al., 2007; Karoui, Mouazen et al., 2006) et sur les relations entre les mesures instrumentales (fluorescence et infrarouge), les mesures rhéologiques et les données sensorielles (Lebecque et al., 2001).

III.1.2.2. La spectroscopie de fluorescence synchrone

L'idée de la fluorescence synchrone a été suggérée pour la première fois par Lloyd (Lloyd, 1971). Pour cette méthode, la longueur d'onde d'excitation et la longueur d'onde d'émission varient simultanément, tout en conservant entre elles un décalage constant, $\Delta\lambda$ (Peuravuori et al., 2002; Taylor & Patterson, 1987). Pratiquement, on choisit une longueur d'onde d'émission (λ_{em}) de départ supérieure de quelques nanomètre à la longueur d'onde d'excitation (λ_{ex}). Puis les deux monochromateurs se mettent en route et défilent à la même vitesse. On peut jouer sur le rapport des deux bandes passantes, en fixant par exemple une petite bande à l'excitation pour que la structure soit bien résolue et une bande plus importante à l'émission pour augmenter la sensibilité (**Figure 14**).

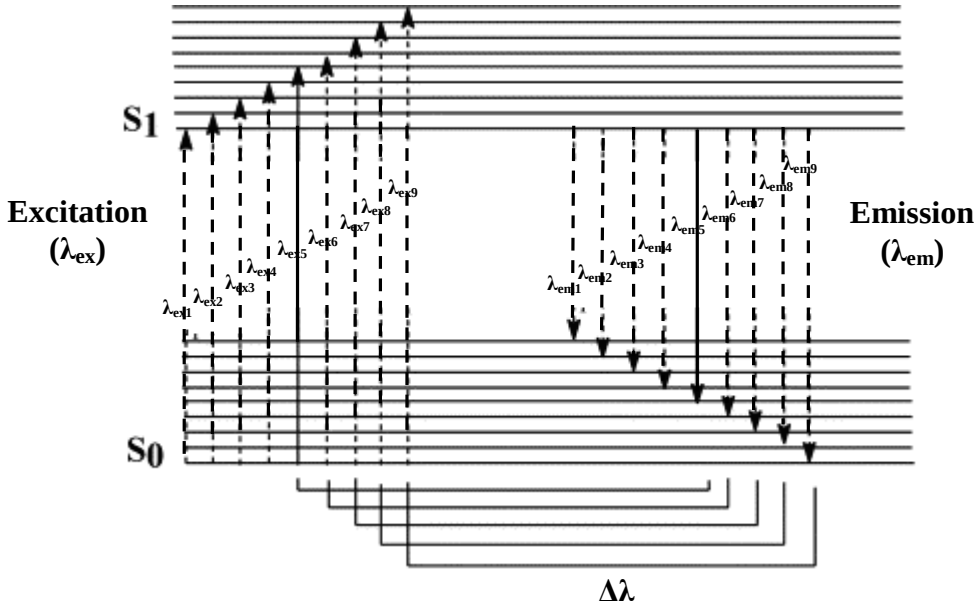


Figure 14 : exemple de diagramme de Jablonski expliquant le balayage de fluorescence synchrone (Patra & Mishra, 2002) : $\Delta\lambda = \lambda_{emi} - \lambda_{exi}$ (i varie de 1 à 9).

Pour mieux comprendre la fluorescence synchrone, on peut se référer au diagramme de Jablonski (**Figure 14**). Une molécule peut être excitée dans toute la plage d'absorption, par exemple, à partir des longueurs d'onde λ_{ex1}, λ_{ex2},…, λ_{ex9} et pourrait donner la fluorescence aux longueurs d'onde λ_{em1}, λ_{em2},…, λ_{em9}. Dans la spectroscopie de fluorescence synchrone,

l'émission à la longueur d'onde λ_{emi} n'est observée que lorsque le monochromateur d'excitation est placé sur la longueur d'onde λ_{exi}.

Au contraire de fluorescence conventionnelle, la spectroscopie de fluorescence synchrone présente l'avantage de pouvoir caractériser plusieurs fluophores à partir d'un seul spectre.

La fluorescence synchrone, très développée dans les domaines de la chimie du pétrole (Cui et al., 2008; Patra & Mishra, 2002), demeure encore très peu utilisée dans le domaine agroalimentaire. Toutefois, (Poulli et al., 2007) ont essayé de discriminer les huiles d'olive, de maïs, de tournesol, de soja, de colza et de noix à partir des spectres de fluorescence synchrone. Les auteurs ont obtenu une bonne discrimination en utilisant un $\Delta\lambda$ de 20 nm. Ils ont également montré que la spectroscopie de fluorescence synchrone peut être utilisée pour la quantification des degrés d'adultération des huiles.

III.2. Chimiométrie

La chimiométrie est la science de l'acquisition, de la validation et du traitement de données dans le domaine de la chimie analytique (Bertrand, 2006). Elle inclut les traitements mathématiques du signal et les méthodes statistiques pour extraire l'information présente dans les données spectrales. Le développement des méthodes analytiques (et particulièrement celles qui sont basées sur les techniques spectroscopiques) est intimement lié au progrès chimiométrique. En effet, l'analyse des collections spectrales présentant de faibles différences nécessite l'emploi de méthodes chimiométriques pour évaluer les données; démarche pratiquée avec succès dans le domaine de la spectroscopie infrarouge depuis de nombreuses années. Le fait d'appliquer ces outils statistiques sur des collections de données renfermant un grand nombre de variables mesurées pour un grand nombre d'échantillons permet d'extraire l'information pertinente et de conclure sur le niveau de signification statistique des faibles différences spectrales observées

Les méthodes chimiométrique (**Figure 15**) peuvent avoir comme objectifs principaux soit la description des données sous forme synthétique, soit la prédiction (ou estimation) d'une ou de plusieurs variables de nouveaux échantillons à partir de données prédictives.

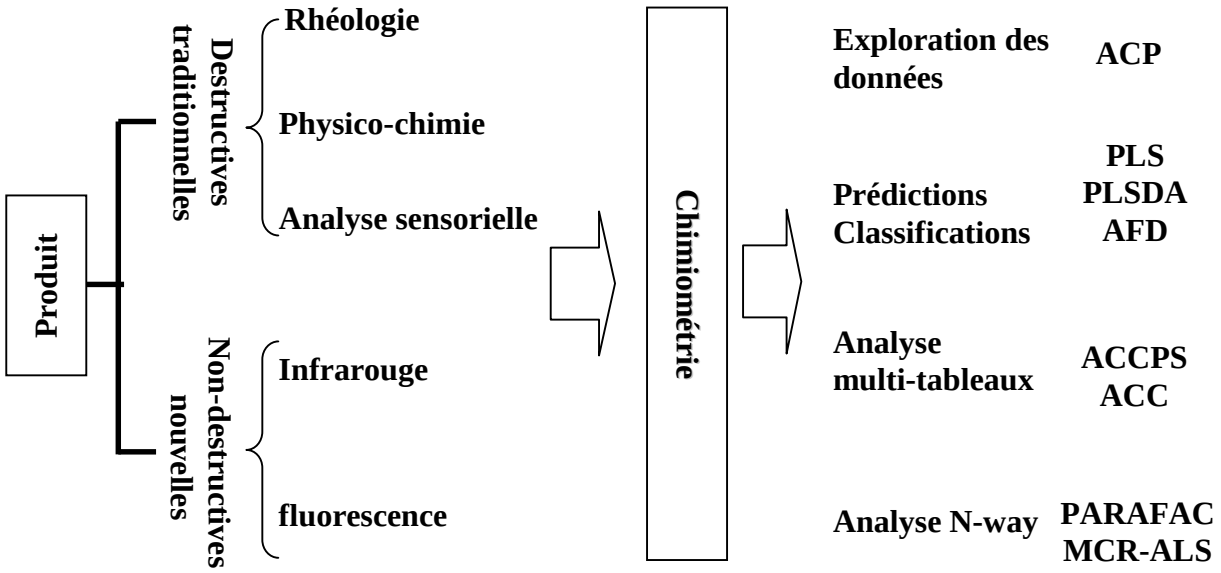


Figure 15 : Evaluation des tableaux de données enregistrés sur des fromages à l'aide des outils chimiométriques.

III.2.1. Pré traitements appliqués aux spectres

Les données spectrales brutes, telles qu'elles sont acquises par un spectromètre, ne revêtent pas obligatoirement la forme la plus adaptée aux traitements chimiométriques ultérieurs. Elles peuvent être entachées de défauts liés à la présence d'un bruit aléatoire ou à des déformations de la ligne de base.

Les prétraitements ont pour objectifs l'amélioration du signal et la condensation des données. Parmi les prétraitements les plus utilisés, on trouve la déviation normale standardisée (SNV) (Barnes et al., 1989; Gendrin et al., 2007), Correction du Signal Orthogonal OSC (Niazi & Goodarzi, 2008), les lissages et les dérivées (Bertrand & Vigneau, 2006).

III.2.2. Méthodes exploratoires

Les méthodes exploratoires ont comme objectif de décrire les données, sans introduire d'éléments de connaissance a priori. On peut ainsi disposer de moyens statistiques permettant de contrôler la validité de la mesure spectrale lors de l'analyse de séries, et s'assurer que les échantillons inconnus à analyser sont bien de même de nature que ceux qui sont servi à la mise au point.

III.2.2.1. Analyse en Composantes Principales (ACP)

Quand le nombre de données devient important, un examen direct est difficile. Avant de commencer une étude quantitative, il faut observer les données spectrales pour apprécier leur structure et détecter, par exemple, la présence d'un spectre aberrant (Roggo, 2003). L'ACP est particulièrement bien adaptée à l'étude exploratoire des données spectrales. Elle calcule de nouvelles variables, appelées composantes principales qui sont des combinaisons linéaires des variables de départ. Ces composantes principales sont mutuellement orthogonales. Puisque l'objectif de l'analyse est la simplification, il faut choisir la dimension de l'espace de représentation en effectuant un compromis entre deux objectifs contradictoires : prendre un espace de faibles dimensions et conserver une variance expliquée maximale. La première composante choisie doit donc être la combinaison linéaire des variables d'origine décrivant la droite d'allongement maximum du nuage. Les autres composantes doivent respecter les règles suivantes :

- être orthogonales aux composantes précédentes.
- respecter la règle de description de l'allongement maximum.

L'ACP projette les données spectrales dans un espace de représentation de faibles dimensions (Danzart, 1990).

Lorsque les données de départ sont des spectres tels les spectres de fluorescence et les spectres infrarouges, les vecteurs propres associés aux composantes principales apparaissent comme analogues à des spectres de produits purs, et les coordonnées factorielles sont analogues à des concentrations (Bertrand et al., 2006).

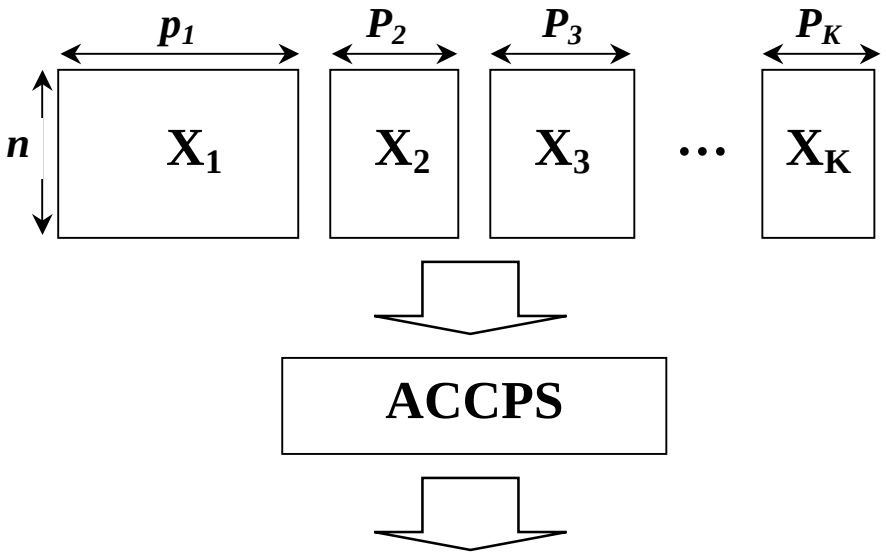
III.2.2.2. Analyse en Composantes Communes et Poids Spécifiques (ACCPS)

Dans de nombreuses situations l'utilisateur dispose de plusieurs tableaux de données pour le même lot d'échantillons. En chimiométrie, des méthodes dites multi-tableaux ont été spécifiquement développées pour analyser simultanément plusieurs tableaux de données. L'analyse conjointe des tableaux de données peut avoir une finalité à caractère descriptive ou prédictive.

En s'inscrivant à l'interface de la chimiométrie et des sciences des aliments, l'objectif est d'étudier dans quelle mesure les méthodes d'analyse multi-tableaux permettent de traiter efficacement l'ensemble des informations collectées par différentes techniques. Cette analyse est très intéressante dans le cas lorsque la nature des variables mesurées est différente d'une

table de données à l'autre, les mesures réalisées pouvant ainsi provenir de domaines d'expertise différents (spectroscopique, chimique, physique…). L'ACCPS a pour objectif de décrire plusieurs tableaux de données collectées sur les mêmes échantillons, en prenant en compte le maximum de variance présentée par chacune d'entre elles. Ella a été initialement développée pour analyser des données provenant de mesures d'analyse sensorielle (Qannari et al., 2000).

L'hypothèse de travail justifiant l'emploi de l'ACCPS est l'existence de structures communes aux différents tableaux de données. Cette méthode permet de déterminer un espace de représentation commun à tous les tableaux de données (**Figure 16**). Chaque tableau sera doté d'un poids qui représente sa contribution à chacune des dimensions de l'espace commun de représentation. Dans cet espace, les liens entre variables des différents tableaux peuvent donc être déclinés dimension par dimension en fonction des relations initialement présentes dans l'ensemble du jeu de données (Bertrand et al., 2006).



Λ_k : poids spécifiques

Q : Composantes communes

E : résidu

Figure 16 : structure de l'analyse en composantes communes et poids spécifiques (ACCPS).

(Mazerolles et al., 2002) ont utilisé l'ACCPS pour caractériser l'affinage de fromages à pâte pressée en utilisant simultanément les données de spectroscopie infrarouge et de fluorescence. Cette démarche a permis de d'interpréter les modifications présentées par les fromages au cours de l'affinage à la lueur de ces deux techniques spectroscopiques. Dans les travaux de (Karoui et al., 2004a), l'ACCPS réalisée sur les jeux de données des spectres de fluorescence des tryptophanes et de la vitamine A a permis la discrimination des fromages Emmental en fonction du producteur et de la marque (nationale, distributeur et premier prix).

III.2.2.3. PARAllel FACtor analysis (PARAFAC)

L'utilisateur peut être intéressé par l'analyse de plusieurs tableaux de données lorsque les spectres sont enregistrés pour les mêmes produits dans des conditions différentes de pH, température, etc. C'est également le cas lorsque les spectres sont enregistrés au cours du temps, de façon de suivre l'évolution d'un phénomène ou d'une réaction chimique. Des méthodes d'analyse d'un nombre n (avec 2 >2) de tableaux doivent alors être mises en œuvre (Bro, 1997). Il existe des méthodes descriptives (PARAFAC, MCR-ALS, méthode de Tucker) aussi bien que prédictives (N-PLS) (Lorho et al., 2006).Dans notre cas, nous nous sommes intéressés à la méthode PARAFAC qui est une méthode d'analyse de plusieurs cubes de données développées dans le domaine de la psychométrie par Harshmann (1970) et Carroll & Chang (1970). Cette méthode trouve de nombreux développement en chimiométrie et a reçu un nombre significatif d'application dans le domaine analytique. On considère à titre d'illustrartion (**Figure 17**), un cube de taille *n x p x m*. Ce cube correspond, par exemple, aux valeurs de fluorescence, pour *p* longueurs d'ondes mesurées sur *n* individus dans *m* conditions différentes. Le modèle de PRAFAC consiste en une décomposition tri-linéaire de ce tableau de données selon les trois modes suivants : individus, intensités de fluorescence et conditions d'acquisition. L'algorithme de décomposition du cube de données est itératif et il repose sur l'algorithme des moindres carrés alternés qui permet de minimiser la somme des carrés des résidus. Le nombre de dimensions n'est pas aisé à déterminer a priori mais l'examen de la décroissance de la somme des carrés des résidus peut aider à faire ce choix. Les algorithmes les plus courants permettent d'imposer des contraintes particulières aux valeurs obtenues telles que l'orthogonalité ou la positivité en fonction des objectifs poursuivis ou des données traitées. Bro (1997) a présenté une description des principaux algorithmes d'estimation.

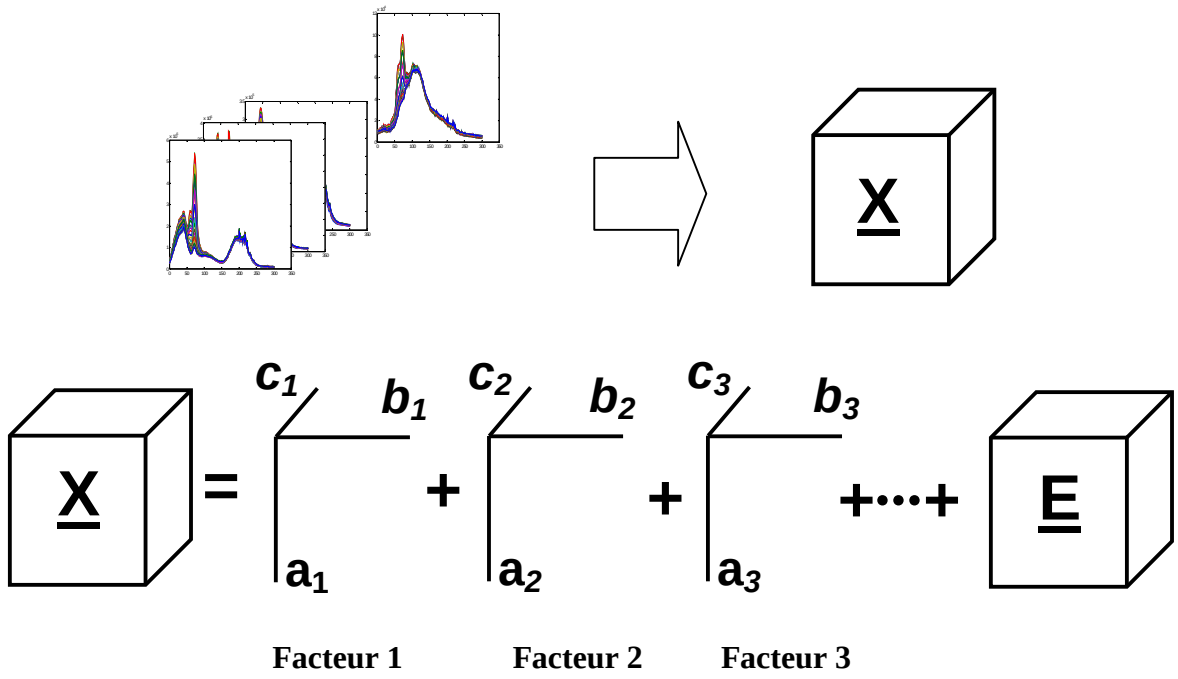


Figure 17 : Illustration de la décomposition de PARAFAC d'un cube de données de 3 dimensions. Les données sont décomposées à des facteurs *i* ayant des modes a_i, b_i et c_i.

III.2.3. Méthodes prédictives

Les méthodes prédictives (régression et discrimination) utilisées pour construire une équation d'étalonnage à partir de données spectrales sont nombreuses. Elles peuvent être classées en méthodes de régression et méthodes de discrimination.

III.2.3.1. Partial Least Squares PLS (moindre carrés partiels)

La régression PLS est actuellement la méthode la plus connue et la plus utilisée dans de nombreux domaines (Vigneau et al., 2006). Elle a été introduite par Wold (1966) et a fait l'objet de nombreuses adaptations et développement.

Comme pour l'ACP, la PLS est basée sur la construction de facteurs à partir des données spectrales initiales. Le but de cette méthode est de réduire la quantité de données et d'éviter ainsi les problèmes de surentraînement sans éliminer les informations utiles. Les composantes ou variables de la nouvelle base vectorielle, dites variables latentes, sont des combinaisons linéaires des variables dans l'ancienne base. Mais la différence avec l'ACP est

que la PLS construit ses facteurs en tenant compte de la corrélation entre les variables prédictives X et les variables prédites Y (Agnar, 1988; Wold et al., 2001). La condensation des données se fait donc suivant les directions les plus pertinentes en terme de prédiction des variables Y (Geladi & Kowalski, 1986). La régression sur la matrice des variables latentes est utilisée pour construire l'équation de prédiction.

Il existe de nombreuses versions de l'algorithme de régression PLS. Elles diffèrent au niveau des normalisations et des calculs intermédiaires, mais elles aboutissent toutes à la même régression. Parmi ces versions, on distingue deux algorithmes, l'algorithme NIPALS initialement proposé par Sjöström et al. (1983) et l'algorithme SIMPLS initialisé par De Jong (1993).

Il existe une distinction dans l'application de la méthode de régression PLS. Il faut séparer le cas où il y a une seule variable Y à prédire de celui où il y en a plusieurs. Dans le premier cas, on parle de régression PLS univariée (PLS1) et dans le second cas de régression PLS multivariée (PLS2)(Vigneau et al., 2006).

III.2.3.2. Analyse Factorielle Discriminante (AFD)

L'AFD est l'une des méthodes de discrimination, appelées « d'apprentissage supervisé », qui ont pour objet de mettre en relation une variable qualitative indiquant l'appartenance des individus à des groupes et un ensemble de variables quantitatives (Vigneau et al., 2006).

Les deux objectifs de l'AFD sont de séparer au mieux les groupes d'individus identifiés au préalable à l'aide des variables quantitatives et d'affecter de nouvelles observations aux groupes dont elles sont les plus proches. L'approche de cette méthode consiste à choisir les axes factoriels qui séparent au mieux les centres de gravité des groupes et de projeter des points correspondant aux observations d'un groupe donné de telle sorte que ces projections soient les plus concentrées possible autour de leur centre de gravité afin d'éviter le recouvrement des groupes après projection.

Les facteurs discriminants, qui seront des combinaisons linéaires des variables d'origine, doivent restituer au mieux l'inertie du nuage de points formé par les centres de gravité des groupes (Lebart et al., 1977). Les facteurs discriminants sont calculés de telle façon que :

- la variance intragroupe soit minimale ;
- la variance intergroupe soit maximale.

Typiquement, on fait appel en analyse discriminante à un jeu de données d'apprentissage (étalonnage) qui sert de référence pour construire la règle d'affectation, auquel est adjoint un jeu de validation qui sert à vérifier la pertinence du modèle de discrimination (Vigneau et al., 2006).

Dans le cas de données spectroscopiques, les variables spectrales étant très redondantes, l'inversion de la matrice de variance-covariance, nécessaire pour définir les fonctions linéaires discriminantes, est généralement impossible.

Une solution consiste à remplacer, dans l'AFD, les données spectrales par les composantes principales, résultantes de l'application de l'ACP sur les données spectroscopiques, associées à des valeurs propres non nulles (Devaux et al., 1988). Mais l'utilisation de composantes principales comme variables de départ pour l'AFD pose le problème choix du nombre de composantes à introduire dans le modèle. En choisissant trop peu de composantes, il y a un risque de ne pas prendre en compte des informations spectrales utiles à la discrimination. A l'inverse, en considérant trop de composantes, les dernières seront source d'instabilité dans le modèle. Une solution peut être de choisir les composantes par une procédure pas à pas (Devaux et al., 1988).

III.2.3.3. Analyse discriminante PLS (PLSDA)

L'analyse discriminante PLS est une méthode de classification supervisée. Elle est maintenant très couramment utilisée dans le domaine de l'agroalimentaire (Downey, 2006).

L'objectif de la méthode est, comme pour l'AFD, de séparer le mieux des groupes d'individus. Son principe fondamental repose sur la création de nouvelles variables Y, de dimension $n \times k$, formée par les indicateurs des groupes où n et k représentent le nombre d'individus et de groupes, respectivement, et d'appliquer la régression PLS2 sur ces nouvelles variables. Considérons, l'individu i appartenant au groupe k. La ligne i de Y est un vecteur dont tous les éléments valent 0, à l'exception de l'élément en position k, qui prend la valeur 1. Comme dans le cas de l'ACP et la PLS, les composantes discriminantes de la PLSDA peuvent être représentées sous forme de cartes factorielles. Le nombre de variables latentes choisies

pour construire le modèle influencera directement celui-ci. Ce choix du nombre de variables latentes résulte d'un compromis entre stabilité et précision, entre complexité du modèle et les problèmes de sur-interprétation des données. Un modèle établi avec un faible nombre de dimensions risque de donner une erreur résiduelle assez élevée, mais le modèle sera fiable. A l'opposé, avec un trop grand nombre de dimensions, le modèle sera plus précis mais reposera sur des informations associées à des phénomènes de faible intensité. Un pourcentage de bonne classification peut être calculé, permettant ainsi d'évaluer la bonne prédiction de l'appartenance d'un individu à un groupe qualitatif.

CHAPITRE 2

POTENTIEL DE LA SPECTROSCOPIE DE FLUORESCENCE SYNCHRONE COUPLEE A LA METHODE PARAFAC POUR L'ETUDE DES CHANGEMENTS STRUCTURAUX DU LAIT LORS DU CHAUFFAGE OU DE L'ACIDIFICATION

Ce chapitre a fait l'objet d'une publication :

- Boubellouta, T. & Dufour, E (2008) Effects of Mild Heating and Acidification on the Molecular Structure of Milk Components as Investigated by Synchronous Front-Face Fluorescence Spectroscopy Coupled with Parallel Factor Analysis, *Applied Spectroscopy*, 62 (5) 490-496.

I. Introduction

La composition du lait, d'un point de vue quantitatif, peut être affectée par des facteurs génétiques (race, individu), physiologiques (état sanitaire de l'animal, âge, stade et nombre de lactation), zootechniques (alimentation, exercice, conditions de traite) et environnementaux (saison, région, climat, exposition à la lumière, altitude) (Karoui et al., 2005; Walstra et al., 2005). Cette variabilité inhérente à tout produit agricole peut avoir des répercussions sur la qualité des produits.

Dans un souci de qualité, l'industrie laitière française effectue plusieurs analyses sur les produits qu'elle traite. Pour les laits qui vont être stérilisés UHT, un aspect essentiel de la qualité est la stabilité du lait cru au traitement thermique qui est estimée à l'aide des tests à la chaleur, à l'éthanol et au phosphate. Ces tests sont éffectués sur les laits crus au moment du dépotage afin de prévoir leur stabilité aux traitements thermiques, ou sur les laits après traitement UHT afin de prédire l'évolution de leur stabilité au stockage.

Pour répondre à ces questions, nous avons conduits deux études en collaboration avec Isabelle GAUCHER et Frédéric GAUCHERON de l'UMR STLO de l'INRA Rennes (Articles en Annexes 1 et 2). La première avait pour objectif de déterminer les facteurs influençant la réponse de chaque test et les relations entre les trois tests. La deuxième étude a portée sur des effets saison, région laitière, traitement de stérilisation et conditions de stockage sur les caractéristiques physico-chimiques du lait et du lait UHT.

II. Effet du chauffage et de l'acidification du lait cru suivi par spectroscopie de fluorescence synchrone couplée a la chimiométrie

Parmi les productions agricoles, le lait a la particularité d'être hétérogène et d'avoir une composition complexe. Par ces propriétés, il est forme un système thermo-dynamiquement instable et constitue en outre un milieu fermentescible.

Du point de vu physico-chimique, le lait est un produit très complexe. Une connaissance approfondie de sa composition, de sa structure et des propriétés physiques et chimiques est indispensable à la compréhension des transformations du lait et des produits obtenus lors des différents traitements industriels (Amiot et al., 2002).

La structure de globule gras du lait est hétérogène. Elle est constituée d'un cœur composé de triglycérides à bas point de fusion, liquide à température ambiante et d'une zone corticale qui joue un rôle très important. Les protéines présentes à l'état soluble dans la phase aqueuse ont des caractéristiques particulières. En effet, toutes ces protéines (hormis les protéoses-peptones) sont sensibles au chauffage qui, à plus de 80 °C, provoque leur dénaturation et leur insolubilisation par agrégation. La structure des protéines, qu'elles soient présentes dans le lactosérum ou sous forme de micelles, peut être modifiée selon les traitements utilisés en transformations alimentaires. Ces traitements affectent les différentes liaisons chimiques, essentiellement non-covalentes, qui maintiennent cette structure en place. Ils provoquent ainsi des changements plus ou moins importants et il en résulte des modifications de leurs propriétés de solution ou micellaire.

L'acidification du lait touche particulièrement les caséines. Même une légère acidification modifie suffisamment la structure micellaire pour que les caséines deviennent instables à la chaleur. Par ailleurs, le chauffage du lait change l'état physique des triglycérides des globules gras.

La spectroscopie de fluorescence offre plusieurs avantages pour la caractérisation de la structure des interactions et des réactions moléculaires dans les produits alimentaires. Les spectres de fluorescence donnent des informations sur la présence des molécules fluorescentes et sur leur environnement dans les échantillons. Dans le lait, il existe plusieurs fluorophores

intrinsèques qui sont extrêmement sensibles à leur environnement. Ceux-ci incluent les acides aminés aromatiques tels les résidus tryptophane, tyrosine et phénylalanine des protéines, un grand nombre de vitamines qui présentent des propriétés de fluorescence comme la vitamine A, ainsi que certains cofacteurs enzymatiques comme le NADH et la riboflavine.

Les résidus des tryptophanes qui sont enfouis dans le cœur de la protéine ont des propriétés fluorescentes différentes de celles des résidus qui se trouvent exposés au solvant aqueux. Leur sensibilité à l'environnement permet de caractériser les changements conformationnels, comme ceux attribuables à la dénaturation thermique et les interactions des protéines avec d'autres molécules.

La vitamine A située dans le noyau et la membrane du globule gras peut être utilisée pour fournir des informations sur l'état physique des triglycérides et le développement des interactions protéines-lipides pendant la coagulation de lait (Herbert et al., 1999) et pendant la période d'affinage des fromages.

Dans la spectroscopie de fluorescence frontale, un spectre d'excitation ou d'émission donne, en général, des informations sur la présence d'une molécule fluorescente et sur son environnement alors qu'il existe plusieurs molécules fluorescentes dans le lait. Un spectre doit donc être enregistré individuellement pour chaque sonde fluorescente. La spectroscopie de fluorescence synchrone présente l'avantage de pouvoir enregistrer les signaux de plusieurs fluorophores simultanément à partir d'une seule acquisition. Les spectres synchrones sont plus riches en information par rapport aux spectres d'excitation et d'émission. Cette méthode est utilisée dans les domaines de la chimie et pétrolier, mais elle reste encore peu employée dans le domaine agroalimentaire.

L'objectif de cette étude est de suivre par spectroscopie de fluorescence synchrone couplée à la méthode PARAFAC l'évolution de structures des composants du lait et leurs interactions lors du chauffage et de l'acidification. La méthode, PARAFAC permet de décomposer des cubes de données spectrales enregistrées à des différentes conditions et donc d'identifier les spectres d'excitation et d'émission des fluorophores présents dans les cubes de données.

CHAPITRE 3

CHANGEMENTS STRUCTURAUX DES CONSTITUANTS DU LAIT SUITE A L'AJOUT DE MINERAUX OU LORS DE LA COAGULATION

Ce chapitre a fait l'objet de trois publications :

- Boubellouta, T., Galtier, V. & Dufour, E. (2008) Effects of added minerals (calcium, phosphate and citrate) on the molecular structure of skim-milk as investigated by mid-infrared and synchronous fluorescence spectroscopies coupled with chemometrics. (*Applied Spectroscopy*) (soumis);

- Boubellouta, T., Galtier, V. & Dufour, E. (2008) Structural changes of milk components during coagulation as studied by spectroscopic methods. 1 – Acid-induced coagulation of milk investigated at different temperatures, *Journal of Agricultural and Food Chemistry*, (soumis) ;

- Boubellouta, T., Galtier, V. & Dufour, E. (2008) Structural changes of milk components during coagulation as studied by spectroscopic methods. 2 – Rennet-induced coagulation of milk investigated at different temperatures : Comparison with acid-induced milk coagulation, *Journal of Agricultural and Food Chemistry* (soumis).

I. Introduction

Le lait est un aliment produit chaque jour et livré aux entreprises pour être transformé en une grande diversité de produits que sont les laits de consommation, les produits frais fermentés, le beurre, les fromages et les produits secs. Lorsque le lait est transformé en produits laitiers, il va subir des traitements physiques (chauffage, homogénéisation, séparation de phase) et des transformations biochimiques (actions enzymatiques, fermentations) qui vont entraîner des modifications plus au moins importantes de ses constituants.

Le lait étant un produit fragile et facilement altérable, des traitements technologiques, notamment thermiques, sont utilisés pour le stabiliser. Ainsi, 93 % des laits liquides consommés ont subi une stérilisation à Ultra Haute Température (UHT). Ce traitement consiste en une stérilisation (environ 140°C) pendant un temps très court (quelques secondes), suivi d'un conditionnement aseptique. Le lait peut ainsi être conservé pendant 3 mois à température ambiante dans un emballage fermé.

II. Effets de l'enrichissement en minéraux sur les structures des composants du lait

Le marché des produits laitiers enrichis en minéraux est important. Pour apporter des minéraux supplémentaires, le lait et les produits laitiers sont donc des vecteurs intéressants car ils sont consommés par une grande partie de la population des pays industrialisés. Les enrichissements en minéraux de produits laitiers répondent aux exigences et motivations variées qui peuvent être :

- une amélioration des propriétés technologiques et/ou fonctionnelles ;
- une amélioration des propriétés nutritionnelles ;
- une amélioration de la stabilité thermique des laits ;
- une modifications des propriétés organoleptiques ;
- une sélections des micro-organismes et activités enzymatiques potentiellement présents dans ces produits ;
- une amélioration de l'image du produit en lui associant une connotation santé.

De façon générale, dans des liquides laitiers contenants des micelles de caséines (cas du lait, des rétentats, etc.) ou ne contenant pas de micelles de caséines (cas des différents types de lactosérums et caséinates, perméats, etc.), les ions monovalents (sodium, potassium, chlorure) sont présents en *quasi* totalité dans la phase aqueuse alors que les ions à valence multiple (calcium, magnésium, phosphate et citrate) sont en parie liés aux protéines. Cette répartition minérale entre phase protéique et phase aqueuse est soumise aux lois des équilibres chimiques et varie significativement selon les conditions physicochimiques et les traitements technologiques (thermiques principalement).

L'équilibre ionique est très important dans la stabilité de la suspension colloïdale des caséines et dans la stabilité des protéines du lactosérum. Puisque les micelles de caséines sont chargées négativement au pH du lait (6,6 – 6,8), l'ajout d'ions positifs bivalents comme Ca^{2+} ou Mg^{2+} peut provoquer la formation de ponts phosphates de calcium entre les micelles et favorise leur agrégation. Par contre, l'addition de sels contenant des phosphate ou des citrate favoriserait la stabilité du lait lors des traitement thermiques puisque ces ions négatifs ont la possibilité de séquestrer le Ca^{2+} et le Mg^{2+}, ce qui limite l'association des différentes protéines (Amiot et al., 2002).

L'addition des sels modifie l'environnement ionique et les équilibres minéraux, et par conséquents la physico-chimie du système enrichi. La nature et la concentration du sel ajouté, qui dépendent de la cible à atteindre, ont évidemment une influence sur ces altérations. En plus des effets spécifiques de chaque ion ajouté, l'ajout de sels augmente la force ionique. Cette augmentation de force ionique réduit le coefficient d'activité et augmente la solubilité du phosphate, et du citrate de calcium (Gaucheron et al., 2004).

Le but cette étude est d'étudier les changements induits de la structure des constituants et des équilibres minéraux du lait par l'addition de sels (calcium, phosphate et citrate) aux moyens des spectroscopies infrarouge et de fluorescence. Le recours aux techniques d'analyse chimiométriques, telles que l'ACP et l'ACCPS, utilisées pour facilite cette investigation, a permis de mieux comprendre les changements structuraux au niveau moléculaire suite à l'addition des différents sels.

III. Suivi des cinétiques de coagulation acide et présure du lait par spectroscopies moyen infrarouge et de fluorescence synchrone et frontale : corrélation avec les données rhéologiques

La texture des fromages est un paramètre important pour l'appréciation de leurs qualités. Elle dépend à la fois de la composition physico-chimique des laits de départ mis en œuvre, des paramètres technologiques de fabrication mais également de la structure physique des produits qui en résulte (Dufour et al., 2001; Herbert, 1999; Herbert et al., 2000). Par conséquent, afin de mieux maîtriser la texture des produits laitiers, il apparaît indispensable de mieux connaître l'évolution de leur structure au cours de différentes phases de fabrication. De plus, la coagulation du lait, première étape dans la production de la plupart des produits laitiers, est très importante dans le développement de la texture des produits. La coagulation par voie acide ou par voie enzymatique a des répercussions très différentes sur l'organisation des micelles de caséines. En fromagerie, la coagulation du lait résulte le plus souvent de l'action combinée d'un enzyme et de l'acidification ; la proportion de l'une et de l'autre variant d'un fromage à l'autre.

Pour appréhender l'effet de la structure du produit sur la texture, différents niveaux d'observations peuvent être investigués, allant du niveau macroscopique au niveau moléculaire. Actuellement, très peu de techniques permettent d'étudier de manière non destructive les structures moléculaire et macroscopique. Cependant, les spectroscopies infrarouge et de fluorescence synchrone et frontale et le test de cisaillement dynamique ont montré un potentiel intéressant pour la caractérisation, respectivement, des structures moléculaire (Dufour et al., 2001; Herbert et al., 2000) et macroscopique (Famelart et al., 2002; Reparet & Noël, 2003) des fromages. Les objectifs de cette étude étaient, d'une part, de caractériser finement les différences de structures moléculaire et macroscopique en fonction de type et des conditions de coagulation, et, d'autre part, d'analyser les relations existantes entre les paramètres physico-chimiques, la structure au niveau moléculaire et les propriétés rhéologiques. La finalité de ces travaux est d'avoir une meilleure connaissance des composants de la structure moléculaire qui sont à l'origine des variations de texture observés sur les produits finals.

Ces méthodes d'analyse structurale doivent permettre la caractérisation des changements structuraux au cours de la coagulation. Pour investiguer le potentiel de ces méthodes, nous avons choisi de travailler sur les deux types de coagulation : coagulation induite par acidification et par emprésurage. En effet, ces deux types de coagulation sont connus pour générer différentes structures et textures (Herbert, 1999).

Dans notre étude, les systèmes de coagulation ont étés réalisés à partir de lait de vache récolté à la ferme du lycée agricole Louis Pasteur. Le lait a été écrémé par centrifugation à 30 °C pour éviter la présence de crème à la surface des échantillons au cours des cinétiques. Le système de coagulation acide utilisant la glucono-δ-lactone à 17,5 g/L (GDL, Sigma-Aldrich Chemie GmbH, Heidenheim, Allemagne). Le système de coagulation induit par la présure a été préparé par addition de présure (COOPER, Melun, France), contenant 50 mg/L de chymosine, à une concentration de 40 µg/L dans le lait.

Les cinétiques de coagulation ont été suivies pendant 300 minutes et réalisées à 30 et à 40 °C. Il est noté que les conditions expérimentales choisies dans cette étude sont assez éloignées des conditions de coagulation utilisées en fromagerie. En effet, elles ont été sélectionnées pour obtenir des coagulations sans synérèses.

Les données spectroscopiques et rhéologiques ont été enregistrés durant toute la durée de coagulation (5 heures). Les spectres moyen infrarouge ont été enregistrés entre 3000-900 cm^{-1} tandis que les spectres de fluorescence synchrone ont été enregistrés dans la gamme spectrale 250-500 nm avec un $\Delta\lambda$ = 80 nm. Le test de cisaillement dynamique effectué en mode balayage dans le temps s'utilise pour déterminer les variations des différents moduli structurels au cours de la coagulation. Durant les expérimentations, la surface de l'échantillon a été couverte par un film plastique pour les acquisitions spectrales et par de l'huile de silicone pour les mesures rhéologiques.

L'application de l'ACP aux données spectroscopiques et de rhéologie a permis d'étudier l'évolution de la structure de coagulums au niveau moléculaire. Les spectres infrarouge et de fluorescence synchrone sont sensibles aux modifications de la structure des constituants du laits et aux interactions au cours de la coagulation telles que les interactions protéine-protéine et protéine-lipides.

Les résultats et les discussions de ces études sont présentés dans les articles qui suivent.

CHAPITRE 4

APPORT DES TECHNIQUES SPECTROSCOPIQUES POUR L'ETUDE DE LA STRUCTURE DES FROMAGES LORS DE LA FONTE ET POUR L'AUTHENTIFICATION DE FROMAGES AOC

Ce chapitre a fait l'objet de deux publications :

- Boubellouta, T. & Dufour, E. (2008) Investigation of cheese-matrix rheology and structure during melting using dynamic testing rheology and mid infrared and synchronous front-face fluorescence spectroscopies coupled with chemometrics, *International Dairy Journal* (soumis) ;

- Boubellouta, T. Lebecque, A. & Dufour, E. (2008) Utilisation of attenuated total reflectance MIR and front-face fluorescence spectroscopies coupled to chemometrics for the identification of Saint-Nectaire cheeses varying by manufacturing conditions, *Le Lait* (soumis).

I. Introduction

L'objectif de cette étude était de tester les potentialités des spectroscopies infrarouge et de fluorescence (classique et synchrone) couplées à la chimiométrie à (1) suivre l'évolution de la structure d'un fromage à pâte pressée cuite (Comté) et d'un fromage à pâte pressée non cuite (Raclette) au cours du chauffage (fusion de la matière grasse et de matrice fromagère) et (2) à authentifier de fromages de type Saint-Nectaire (AOC).

II. Suivi de la fusion des fromages a pâte pressée par spectroscopies moyen infrarouge et de fluorescence synchrone et frontale. Corrélation avec les résultats rhéologiques

La consommation et les modes alimentaires des consommateurs évoluent au fil des années. Le consommateur s'oriente de plus en plus vers la consommation de plats élaborés et prêt à l'emploi. Ce changement a mené les industriels à incorporer le fromage dans les plats cuisinés (Kahyaoglu & Kaya, 2003). En France, on estime que le cinquième du million de tonnes des fromages affinés est utilisé comme ingrédient en cuisine : gratins, pizzas, pâtes, sauces, etc.

La texture des fromages dépend à la fois de la composition physico-chimique des laits de départ mais aussi des procédés de fabrication (Dufour et al., 2001; Herbert, 1999; Herbert et al., 2000; R. Karoui et al., 2003). Les modifications de texture sont reliées à la fois à des changements de l'état physique et physico-chimique des fromages au cours de l'affinage. Le développement de nouveaux produits présentant de nouvelles propriétés fonctionnelles repose sur une meilleure connaissance de la structure de la matrice fromagère.

Pour mesurer les propriétés fonctionnelles de ces produits ou les prévoir, des méthodes de caractérisation rapides et fiables sont nécessaires. En effet, ces propriétés sont le plus souvent évaluées à l'aide des tests empiriques imitant certaines techniques d'appréciations sensorielles et fournissant une évaluation globale de ces propriétés. Actuellement, très peu de techniques permettent d'étudier de manière non destructive les propriétés rhéologiques à chaud des fromages. Les travaux de Karoui et al. (2003) laissent entrevoir la possibilité de prédire les caractéristiques rhéologiques à chaud des fromages à pâte pressée à partir des données de spectroscopie de fluorescence.

L'objectif de ce travail était d'évaluer le potentiel de spectroscopie infrarouge et de fluorescence synchrone couplée à la chimiométrie pour déterminer les propriétés rhéologiques à chaud d'un types de fromage à pâte pressée cuite (Comté) et un type de fromage à pâte pressée non cuite (Raclette). Nous avons donc voulez caractériser la structure de la matière grasse et de la matrice fromagère au cours du chauffage par les techniques spectroscopiques et

d'étudier les structures aux niveaux moléculaire et macroscopique afin de mettre en évidence les corrélations entre ces deux niveaux d'observation.

Dans cette optique, les analyses chimiométriques peuvent être utilisées pour faciliter l'exploitation de la grande quantité des données et de trouver les corrélations entre ces deux domaines de mesure en tenant compte de l'ensemble de variables de chaque domaine étudié. L'ACCPS est une méthode descriptive multi-tableaux qui permet de mettre en évidence les propriétés, exprimées dans chaque domaine, qui peuvent être utilisées conjointement pour caractériser globalement les échantillons étudiés De plus les vecteurs propres associés aux composantes principales ou aux composantes communes nous ont permis une interprétation au niveau moléculaire des données spectrales.

III. Authentification de fromages par spectroscopies moyen infrarouge et de fluorescence frontale couplées a la chimiométrie. Application a un fromage AOC – le Saint Nectaire

Les fromages se fondent sur la notion de terroir pour affirmer leur typicité et leur spécificité. Les caractéristiques de ces fromages dépendent fortement des procédés de fabrications. Mais elles sont aussi fonction des conditions de production du lait et cette relation avec la qualité du produit fini est particulièrement importante à étudier s'agissant de fromages bénéficiant d'une AOC (Agabriel et al., 2001; Coulon et al., 1996). Ces fromages de qualité sont marqués par les conditions locales dans lesquels ils ont été élaborés et procurant une forte valeur ajoutée. La production de ces fromages est caractérisée par des pratiques et des systèmes de productions liés à un terroir, une culture et une histoire locale. Si les produits sont suffisamment spécifiques, leurs propriétés peuvent alors servir à déterminer leur origine, autorisant ainsi une certification du produit. Outre, le rôle important de la certification pour le commerce de ces produits, elle permet souvent de générer une plus value bénéficiant aux différents acteurs de la filière.

Les relations entre les caractéristiques de fromages et leur territoire d'origine avaient jusqu'à présent fait l'objet de peu de travaux de recherche. Les travaux récents ouvrent les perspectives d'application importantes pour la définition et le contrôle des fromages bénéficiant d'Appellation d'Origine Protégée (AOP) et d'Indication Géographique Protégée (IGP).

De nombreuses méthodes de mesure (chimiques, physiques, biochimiques, rhéologiques, spectroscopiques et sensorielles) permettent de différencier les fromages en fonction des conditions locales de production du lait, telles la nature des fourrages (pâturage, foin), la composition botanique des prairies et la localisation géographique des sites de production (Karoui, Bosset et al., 2005; Karoui et al., 2004a; Karoui et al., 2007). Ces méthodes utilisées pour l'authentification des produits sont destructives, et généralement longues à mettre en oeuvre. Pouvoir donner une information rapide sur l'identité et la nature du produit nécessite le développement et la mise en oeuvre de méthodes analytiques qui, en quelque sorte, révèlent leur empreinte digitale.

Actuellement, les recherches s'orientent vers la mise au point de techniques d'authentification rapides et non destructives. Les techniques spectroscopiques telles que la spectroscopie infrarouge et la spectroscopie de fluorescence frontale semblent prometteuses. La mise en oeuvre de ces méthodes analytiques pour la détermination de l'origine géographique des fromages répond aussi à une forte demande sociale. En effet, le consommateur n'attend plus seulement d'un produit qu'il soit bon et sain et qu'il lui apporte des éléments nutritifs. Il s'interroge sur l'alimentation donnée aux animaux et sur les conditions dans lesquelles le produit a été élaboré et sur l'origine géographique du produit. La spectroscopie infrarouge et de fluorescence frontale ont été utilisée pour la reconnaissance de l'origine géographique de laits, de fromages à pâte pressée cuite du Jura et de fromages Gruyère AOC et L'Etivaz AOC (Karoui, Bosset et al., 2005; Karoui et al., 2007). La spectroscopie de fluorescence dont le potentiel d'application est le plus prometteur du fait de son faible coût et sa facilité d'utilisation. Elle a également permis de discriminer des laits ayant subi des traitements technologiques différents (lait cru, chauffé, homogénéisé, chauffé et homogénéisé) (Dufour & Riaublanc, 1997). Cette vitamine est très sensible et instable puisque sous l'action de la lumière et de l'oxygène, elle se dégrade en composés fluorescents tels le lumichrome qui se traduit par des modifications de l'allure des spectres de fluorescence (Miquel Becker et al., 2003). A cette liste de composés on peut ajouter nombre de composés des fromages dont les propriétés de fluorescence sont moins bien connues mais néanmoins réelles comme certains terpènes, des acides gras insaturés conjugués tel l'acide linoléique conjugué (ALC), des composés phénoliques et des hydrocarbures aromatiques, polycycliques ou non, qui peuvent être trouvés en concentrations très faibles dans les produits alimentaires (Bosset et al., 1997).

Les objectifs de cette étude étaient, d'une part, de caractériser finement les différences de structures moléculaire et macroscopique des fromages de type Saint-Sectaire et, d'autre part, d'analyser les relations existantes entre les paramètres physico-chimiques, la structure au niveau moléculaire et les propriétés rhéologiques, afin d'avoir une meilleure connaissance des composants de la structure moléculaire qui sont à l'origine des variations de texture observés sur ces fromages et qui permettent leur authentification. Afin d'extraire l'information des données de physico-chimie, de rhéologie et de spectroscopies infrarouge et de fluorescence frontale, nous avons mis en oeuvre plusieurs techniques chimiométriques telles que l'ACP et l'AFD.

Les résultats de cette étude présentés dans l'article qui suit ont permis de montrer que les techniques spectroscopiques, de part leur rapidité et leur simplicité sont des techniques non-destructive qui présente un fort potentiel pour discriminer les fromages de type Saint-Nectaire. Les techniques spectroscopiques couplées avec les méthodes chimiométriques ont permis une très bonne classification des fromages par rapport aux données de rhéologie et physico-chimiques.

CONCLUSION ET PERSPECTIVES

Malgré les progrès récents réalisés dans la connaissance de la structure des constituants des produits laitiers et de leur comportement techno-fonctionnel, il subsiste de nombreux points d'interrogation qui sont autant de verrous technologiques au développement de nouveaux produits et à une diversification des utilisations des produits laitiers. De plus, d'autres facteurs tels que l'évolution des procédées, des marchés et des habitudes alimentaires modifient les stratégies d'utilisation de ces produits alimentaires.

L'optimisation des propriétés organoleptiques des produits alimentaires, en général, et des produits laitiers, en particulier, reste un enjeu majeur dans les industries agro-alimentaires. La bonne connaissance des constituants des matières premières est indispensable, mais pas suffisante pour prédire les propriétés organoleptiques des produits : la somme des propriétés physico-chimique des constituants ne permet pas à elle seule de prédire ou de modéliser la structure et les propriétés organoleptiques de l'aliment. Ces propriétés organoleptiques sont les composantes les plus déterminantes pour l'acceptation du produit de la part du consommateur. Par conséquence, l'objectif des industriels consiste souvent à développer des structures qui donnent aux produits les qualités organoleptiques recherchées par le consommateur. Or, en science des aliments, l'étude des bases de la structure moléculaire de la matrice fromagère et des relations structure-texture est encore peu développée.

Pour appréhender l'effet de la structure du produit sur la texture, différents niveaux d'observations peuvent être investigués, allant du niveau macroscopique au niveau moléculaire. Ainsi, une meilleure connaissance des relations structure - texture est primordiale pour l'optimisation de la texture de ces produits. Une connaissance approfondie du comportement des différents constituants de la matrice fromagère et de leurs interactions lors de la coagulation, du chauffage (fromage à pâte pressée) et/ou de l'affinage est indispensable pour maîtriser la qualité finale du fromage.

Parmi les objectifs de cette thèse figuraient l'évaluation du potentiel de la spectroscopie de fluorescence synchrone et frontale couplée à la méthode PARAFAC pour l'étude de la structure des constituants du lait au cours du chauffage et de l'acidification. La

décomposition du cube de données spectrales par PARAFAC a permis l'attribution des différentes bandes des spectres de fluorescence synchrone du lait aux différentes sondes intrinsèques (tryptophane, vitamine A et riboflavine). En effet, les profils spectraux calculés par cette méthode chimiométrique étaient similaires aux spectres de fluorescence (excitation et émission) spécifiques de ces fluorophores. De plus nous avons montré que les spectres de fluorescence synchrone de la vitamine A sont particulièrement bien adaptés à la détermination de l'état physique de la matière grasse du lait au cours du chauffage du lait.

Les changements structuraux des constituants du lait suite à l'ajout de minéraux ou lors de la coagulation ont été étudiés par les méthodes spectroscopiques infrarouge et de fluorescence synchrone. L'approche a montré que les changements induits par l'ajout de sels (calcium, phosphate et citrate) peuvent être suivis par ces méthodes spectroscopiques. Notre travail s'est porté ensuite sur l'étude du développement du réseau de caséines au cours de la coagulation du lait par voie acide et par la présure aux moyens de nouvelles méthodes rapides et non destructives d'analyse de la structure au niveau moléculaire. L'évolution de la structure moléculaire au cours des cinétiques de coagulations acide et présure du lait a été suivie par spectroscopie infrarouge et de fluorescence synchrone. Les spectres de fluorescence synchrone et infrarouge ont permis de suivre les différentes étapes de la coagulation du lait : les spectres synchrones correspondant aux fluorescences des sondes intrinsèques (vitamine A, tryptophane et riboflavine) et les spectres infrarouges de la région amide I et amide II (1700 – 1500 cm^{-1}) et de la région des empreintes (1500-900 cm^{-1}) ont été enregistrés dans deux conditions différentes de température (30 et 40 °C). L'application des méthodes chimiométriques sur les données spectrales a permis de suivre les différentes étapes de la coagulation et de déterminer les temps de coagulation selon la température et pour chacun des systèmes. Les résultats obtenus étaient corrélés avec ceux obtenus par le test de cisaillement dynamique. De plus, cette approche a permis de caractériser finement les différents systèmes de coagulation en fonction du coagulant et de la température.

Un autre objectif de cette thèse était d'évaluer le potentiel des spectroscopies infrarouge et de fluorescence synchrone et du test de cisaillement dynamique couplés aux méthodes chimiométrique pour déterminer les propriétés rhéologiques à chaud d'un fromage à pâte pressée cuite (Comté) et d'un fromage à pâte pressée non cuite (Raclette). Les méthodes chimiométriques appliqués d'une part aux spectres infrarouge et de fluorescence synchrone et d'autre part aux paramètres rhéologiques (G', G'', tan δ, η*) ont permis de suivre

les transitions de la phase solide à la phase liquide des fromages. Les températures de fonte de la matière grasse et de fusion des deux fromages, calculées à partir des données spectrales, étaient similaires à celles obtenues par la méthode de référence (rhéologie). Les deux techniques spectroscopiques apparaissent comme des méthodes prometteuses compte tenu de la bonne discrimination des échantillons de fromages, ainsi que de la facilité et la rapidité de la mise en oeuvre de ces mesures. Les résultats obtenus indiquent que les spectres infrarouge et de fluorescence synchrone enregistrés sur un fromage sont spécifiques de celui-ci. Les propriétés de fluorescence synchrone de la vitamine A et des tryptophanes ainsi que les régions des lipides (3000-2800 cm^{-1}) et des protéines (1700-1500 cm^{-1}) en moyen infrarouge apparaissent particulièrement bien adaptées à la caractérisation des fromages, et à la détermination de l'état de la matière grasse et à la description des caractéristiques de la matrice fromagère.

Enfin notre étude a porté sur le potentiel des méthodes spectroscopiques pour authentifier et discriminer différents fromages de type Saint-Nectaire. Les résultats obtenus indiquent clairement le fort potentiel des spectroscopies infrarouge et de fluorescence synchrone à discriminer les fromages selon le fabriquant : les pourcentages de bonnes prédictions étaient de 100%. Cette étude, avec d'autres, montre qu'il est donc possible sur la base des spectres infrarouge et de fluorescence synchrone de déterminer l'origine du fromage.

Les développements en matière de méthodes analytiques ouvrent aujourd'hui la voie vers une approche globale et intégrée qui, grâce aux méthodes chimiométriques, permet de prendre en compte l'ensemble de l'information fournie par les différentes techniques (spectroscopiques, rhéologiques, physico-chimiques). Notre travail a conduit à valider de nouvelles techniques permettant de mieux comprendre le changement de la structure moléculaire des constituants du lait au cours du chauffage, de la coagulation et suite à l'enrichissement en minéraux. Nos travaux ont également montré l'intérêt de l'utilisation des techniques chimiométriques pour faciliter l'exploitation de grandes bases de données. En effet, seule une approche globale s'intéressant aux différents niveaux d'organisation de la matrice fromagère est à même de donner les clés qui permettront de modéliser la structure de la matrice fromagère et de préciser les relations entre structure et texture. C'est là un élément incontournable pour qui envisage de maîtriser la texture des fromages et de développer de nouveaux produits aux propriétés recherchées.

Cette thèse ouvre de nouvelles perspectives dans le domaine de la recherche en science des aliments en général et en technologie fromagère en particulier. A partir des données spectrales, il serait possible de mettre en place des modèles mathématiques pour prédire à un stade précoce (après salage) la texture des fromages en fin d'affinage. Cette nouvelle approche permettra également de mettre au point des tests rapides pour mesurer les propriétés fonctionnelles (le fondant, le filant) et de texture des fromages qui pourrait remplacer avantageusement les tests empiriques actuellement mis en oeuvre. De plus, les produits alimentaires en général et les fromages en particulier étant des systèmes hétérogènes, il apparaît important d'appréhender la microstructure et l'organisation spatiale des principaux constituants de la matrice fromagère : l'imagerie multi-spectrale, la microscopie confocale à balayage laser et de la micro-spectroscopie dans le MIR sont adaptées à la réalisation de cartographies des produits. Ces techniques d'imagerie et de micro-spectroscopie de fluorescence et dans le MIR permettraient d'appréhender cette hétérogénéité spatiale (au niveau du grain de caillé ou à la jonction des grains de caillé) qui intervient dans la définition des propriétés rhéologiques des fromages.

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ANNEXES

I. Annexe 1

II. Annexe 2

Queries for apls-62-05-17

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Effects of Mild Heating and Acidification on the Molecular Structure of Milk Components as Investigated by Synchronous Front-Face Fluorescence Spectroscopy Coupled with Parallel Factor Analysis

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This paper reports the potential of synchronous front-face fluorescence spectroscopy in the characterization at the molecular level of milk changes during mild heating from 4 to 50 °C and acidification in the pH range of 6.8 to 5.1. Synchronous fluorescence spectra were collected in the 250–550 nm excitation wavelength range using offsets of 20, 40, 60, 80, 100, 120, 140, 160, 180, 200, and 240 nm between excitation and emission monochromators. The potential of parallel factor (PARAFAC) analysis in the decomposition of the whole synchronous fluorescence data set into the contribution of each of the fluorescent compounds present in milk has been investigated for heating and acidification data sets. Models were fitted from 1 to 7 components. Considering the core consistency values, PARAFAC models with three components have been considered. The first three components explained 94.43% and 94.13% of the total variance for heating and acidification data sets, respectively. The loading profiles of the first and second components derived from PARAFAC analysis performed on heating and acidification data sets corresponded quite well with the characteristics of tryptophan and vitamin A fluorescence spectra, respectively. The third component corresponded to the riboflavin fluorescence spectrum. Considering the heating experiment, the profile of the concentration mode for the second component showed large variations according to the temperature, which were assigned to the melting of triglycerides between 4 and 50 °C. For the acidification experiment, drastic changes in the concentration modes of the three components were observed for pH below 5.6, in agreement with structural changes in casein micelles.

Index Headings: **Milk; Heating; Acidification; Synchronous front-face fluorescence spectroscopy; Fluorescence; Parallel factor analysis; PARAFAC; Molecular structure.**

INTRODUCTION

Fluorescence spectroscopy is a rapid and sensitive method for quantifying a fluorescent component and characterizing its molecular environment in all sorts of biological samples. This technique offers several inherent advantages for the characterization of molecular interactions and reactions. First, it is 100 to 1000 times more sensitive than other spectrophotometric techniques. Second, fluorescent compounds are extremely sensitive to their environment. For example, tryptophan residues that are buried in the hydrophobic interior of a protein have different fluorescent properties than residues that are on a hydrophilic surface. This environmental sensitivity enables the characterization of conformational changes such as those attributable to the thermal, solvent, or surface denaturation of proteins, as well as the interactions of proteins with ligands.

The techniques used in conventional fluorescence spectroscopy allow the recording of excitation spectra or emission spectra. The idea of synchronous fluorescence spectroscopy (SFS) was first suggested by Lloyd.[1] In SFS the excitation wavelength, λ_{ex}, and the emission wavelength, λ_{em}, are scanned synchronously with a constant wavelength interval, $\Delta\lambda = \lambda_{em} - \lambda_{ex}$. For well-defined absorption and quantum yield maxima, the optimum value of the offset $\Delta\lambda$ is set by the difference in wavelength of the emission and excitation maxima, which is known as the Stoke's shift.[2] SFS makes it possible to narrow the spectral band and simplify the emission spectrum. This technique has been used to overcome the overlapping of complex mixtures of molecules with chemical similarity, such as different poly aromatic hydrocarbons (PAHs). Thus, several publications describe SFS as a useful analytical tool for simultaneous determination of multicomponent PAHs in mixtures.[3–7] In addition, SFS presents two interesting advantages from our point of view: firstly, it allows the consideration of the whole fluorescence landscape, i.e., spectra recorded at different offsets, and, secondly, a synchronous fluorescence spectrum retains information related to several fluorophores, compared to a classical emission spectrum, which is mainly specific to a sole fluorophore.

The application of autofluorescence in analysis of food has increased during the last decade, probably due to the increased use of chemometrics. Food products retain numerous molecules, several of them being fluorescent, resulting in complex spectra despite the fact that fluorescence spectroscopy is generally recognized as being relatively selective. The most valuable way to evaluate the data is to use chemometric tools to extract qualitative or quantitative information from the spectra. These methods encompass descriptive techniques such as principal component analysis (PCA),[8] common components, and specific weights analysis (CCSWA)[9] and predictive techniques such as partial least squares regression (PLSR)[10] and factorial discriminant analysis (FDA).[8] New chemometric tools such as parallel factor analysis (PARAFAC) and multivariate curve resolution–alternating least squares (MCR-ALS)[11–13] make it possible to handle fluorescence landscapes while keeping the two-dimensional structure of each measurement. These techniques are known as multiway chemometric techniques, and in the case of fluorescence signals, a three-way (sample × excitation × offset) data analysis is considered. Multiway analysis allows the use of the original and true structure of the data, which can stabilize the decomposition of the data and increase the interpretability.

Milk contains intrinsic fluorophores such as vitamin A and tryptophan residues present in fats and proteins, respectively, which give typical excitation and emission spectra. These spectra provide specific information on the characteristics of fats and proteins and on the environment of the fluorescent

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probes in food samples, allowing the investigation of structural changes.[14] For example, the melting temperature of fat in cheese has been determined from vitamin A fluorescence spectra recorded at different temperatures.[15] Dairy products also contain intrinsic fluorophores such as tyrosine and phenylalanine in proteins, vitamins A and B2 (riboflavin), NADH, derivatives of pyridoxal and chlorophyll, some nucleotides, and numerous other compounds that can be found at a low or very low concentration in food.[16]

In dairy research, fluorescence spectroscopy coupled with chemometrics has previously been considered to monitor structural changes in milk proteins or fats and their physicochemical environment during milk heating,[17,18] milk coagulation,[19] and cheese manufacture and ripeninig.[20–23]

The aim of this paper is (1) to investigate the potential of synchronous fluorescence spectroscopy in the characterization of milk during mild heating and acidification, (2) to show the potential of PARAFAC in the decomposition of the synchronous fluorescence data set into the original fluorescence characteristics of intrinsic fluorophores contained in milk, and (3) to retrieve information from the whole synchronous fluorescence spectra coupled with PARAFAC analysis on the structural changes at the molecular level of milk components during mild heating and acidification.

MATERIALS AND METHODS

Milk Samples. Bovine milk samples were collected from the tank of a local farm and a given milk sample was used in the course of the day.

Fluorescence Spectroscopy. Fluorescence spectra were recorded using a FluoroMax-2 spectrofluorimeter (Spex-Jobin Yvon, Longjumeau, France) mounted with a front-surface cuvette holder, and the incidence angle of the excitation radiation was set at 56° to ensure that reflected light, scattered radiation, and depolarization phenomena were minimized. The spectrofluorimeter was equipped with a thermostatically controlled cuvette holder and the temperature was controlled by a Haake temperature controller (Haake, Champlan, France).

Excitation and Emission Spectra of Protein Tryptophan Residues, Vitamin A, and Riboflavin. The excitation and emission fluorescence spectra of tryptophan, vitamin A, and riboflavin (vitamin B2) were recorded on milk at 20 °C. The emission spectrum (305–400 nm) of tryptophan residues was recorded with the excitation wavelength set at 290 nm and the excitation spectrum (250–310 nm) was recorded with the emission wavelength set at 340 nm.[17] The excitation spectrum (250–350 nm) of vitamin A was recorded with the emission wavelength set at 410 nm and the emission spectrum (350–500 nm) was recorded with the excitation wavelength set at 322 nm.[17] The excitation (300–500 nm) and emission (480–600 nm) spectra of riboflavin were recorded following emission and excitation wavelengths set at 520 nm and at 380 nm, respectively.[24]

Synchronous Fluorescence Spectra. Synchronous fluorescence spectra were collected in the 250–550 nm excitation wavelength range using offsets of 20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 220, and 240 nm between excitation and emission monochromators. For the mild heating experiment, the synchronous spectra of milk samples placed in a quartz cuvette were recorded at 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, and 50 °C. Considering the acidification experiment, the synchronous spectra of milk samples placed in a quartz cuvette thermostatically controlled at 20 °C were recorded for pH values of 6.8, 6.6, 6.4, 6.2, 6.0, 5.8, 5.6, 5.4, 5.2, and 5.1. The pH values of the milk samples were adjusted to the desired pH using 1 M HCl.

Spectra were recorded in triplicate for each condition using different samples.

Chemometrics..

Principal Component Analysis. Principal component analysis was applied to the synchronous spectra in order to investigate differences in the spectra. This statistical multivariate treatment made it possible to draw similarity maps of the samples.[17,19,22]

Parallel Factor Analysis. Parallel factor analysis is a chemometric decomposition method and is a generalization of principal component analysis (PCA) to higher-order arrays.[12,25]

We have used the PARAFAC Toolbox[11] (available at http://www.models.kvl.dk). PARAFAC decomposes an N-order array ($N \geq 3$) into a sum of the outer products of N loading components.[26] The number of PARAFAC components necessary to reconstruct the data is an important parameter.[26] Several methods can be used to determine this parameter.[27,28] In this study, the core consistency diagnostic (CORCONDIA) has been used.[29] The core consistency diagnostic is often used to assess the model deviation of ideal multi-linearity and guide the choice of the number of components to consider.[11] When the core consistency drops from a high value (above about 60%) to a low value (below about 50%), it indicates that an appropriate number of components has been attained.[30] In addition, non-negativity has been applied to build the components of the models. Imposing non-negativity constraints on decomposition model parameters of fluorescence three-way spectral data is common practice since both the spectral intensities and fluorophore concentrations are known to be positive.[31,32]

Chemometric analyses were performed in MATLAB (The Mathworks Inc., Natic, MA).

RESULTS AND DISCUSSION

Synchronous Spectra Recorded During Mild Heating of Milk. The synchronous scan collects fluorescent emission only from the wavelengths where the absorption and emission bands of a species overlap by the specified wavelength interval, $\Delta\lambda$.[30]

As fluorescence spectra were recorded in triplicate for different $\Delta\lambda$ (in the range 20–240 nm, with 20 nm intervals) and different temperatures (4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, and 50 °C), a total of 432 synchronous fluorescence spectra were recorded. The synchronous fluorescent landscape for milk at 4 °C is shown in Fig. 1. The synchronous fluorescence data were constructed in such a way that the x-axis represents the synchronous excitation wavelength and the y-axis represents the wavelength interval ($\Delta\lambda$), while the z-axis is plotted by linking points of equal fluorescent intensity.

Figure 2 presents milk synchronous spectra recorded at 4 °C and obtained with different offsets ($\Delta\lambda = 40$, 80, and 180 nm). Depending on the offset values, bands appeared and disappeared in the spectra. The milk synchronous spectrum recorded at 4 °C for $\Delta\lambda = 40$ nm exhibited a sharp intense band at 290 nm (emission at 330 nm), a smaller band at 320 nm (emission at 360 nm), and a broader band centered at 460 nm (emission at 500 nm). The sharp intense band observed at 290 nm (emission at 330 nm) can be attributed to tryptophan residues of

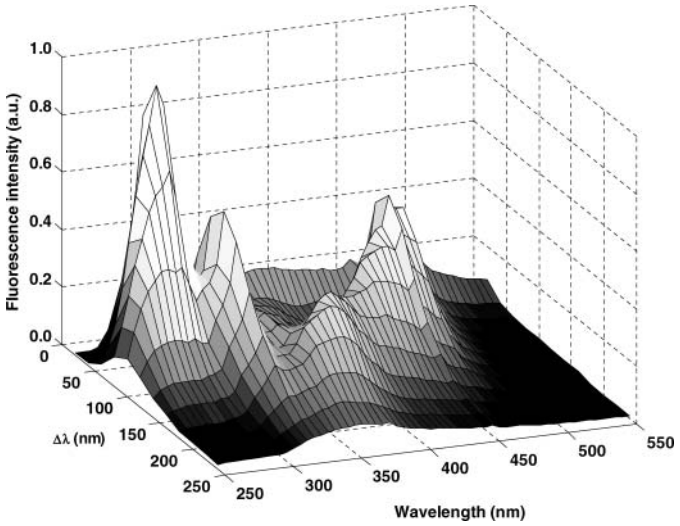

FIG. 1. Three-dimensional plot of the synchronous fluorescent landscape for milk at 4 °C.

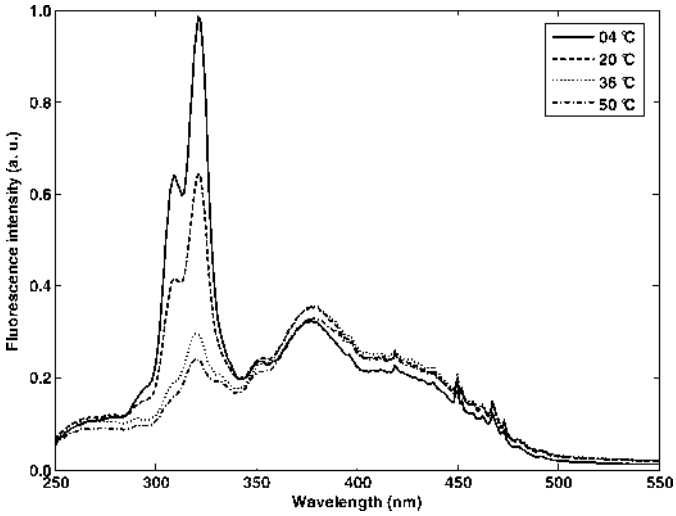

FIG. 3. Milk synchronous fluorescence spectra ($\Delta\lambda = 140$ nm) recorded at different temperatures: 4°C (—), 20°C (- - -), 36°C (····), and 50 °C (– · –).

proteins.[17,19,22,33,34] An increase of $\Delta\lambda$ to 80 nm drastically changed the synchronous spectrum of milk at 4 °C. The intensity of the band at 290 nm remains in the same range, whereas the highest intensity is now observed for the band at 320 nm (emission at 400 nm). In addition, a shoulder appeared at 310 nm. The band centered at 320 nm and the shoulder at 310 nm are similar to the ones found in the vitamin A excitation spectrum recorded for milk.[17] This spectrum also showed a broad band centered at 460 nm (emission at 540 nm) and a weak band at 360 nm (emission at 440 nm). For $\Delta\lambda =$ 180 nm, the band at 320 nm (emission at 500 nm) exhibited again the highest intensity and the shoulder at 310 nm was still present in the milk synchronous spectrum. This spectrum also showed a large band centered at 360 nm (emission at 540 nm) and a shoulder at 350 nm. These bands may be attributed to riboflavin.[24]

The results show that the influence of $\Delta\lambda$ can be substantial on the shape, location, and signal intensity of fluorescence peaks.[35] It appears from this study on milk that SFS makes it possible to narrow the spectral bands and, compared to excitation or emission spectra, to have information on the fluorescent properties of several intrinsic fluorophores on a given spectrum. Indeed, bands corresponding to tryptophan, vitamin A, and riboflavin were observed on the synchronous spectrum for $\Delta\lambda = 80$ nm.

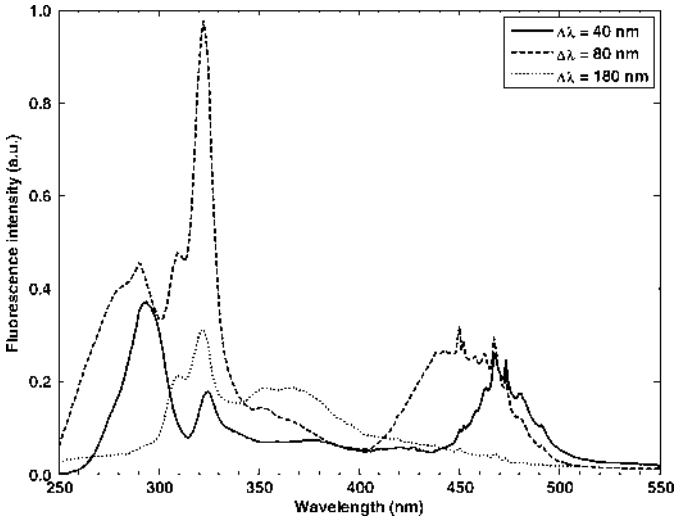

FIG. 2. Milk synchronous fluorescence spectra recorded at 4 °C for different $\Delta\lambda = 40$ nm (—), 80 nm (- - -), and 180 nm (····).

Figure 3 shows synchronous spectra for $\Delta\lambda = 140$ nm recorded at 4, 20, 36, and 50 °C. The shapes of the spectra were quite the same except in the 300–350 nm region. In this region, the intensity of the band centered at 320 nm was reduced by 80% when the temperature was increased from 4 °C to 50 °C. Such changes were reported for vitamin A excitation spectra (270–350 nm) recorded on milk[36] and cheeses[15] at different temperatures. These authors showed that the changes in the shape of the vitamin A excitation spectra were related to the melting of triglycerides in fat globules during the increase of temperature from 4 °C to 50 °C, allowing the determination of the melting temperature. Indeed, it is well known that changes in the solvent properties (such as viscosity) of a fluorescent probe drastically modify its quantum yield.[37]

Synchronous Spectra Recorded During Acidification of Milk. As fluorescence spectra were recorded in triplicate for different $\Delta\lambda$ (in the range 20–240 nm, with 20 nm intervals) and different pH values (6.8, 6.6, 6.4, 6.2, 6.0, 5.8, 5.6, 5.4, 5.2, and 5.1), a total of 360 synchronous fluorescence spectra were recorded. Figure 4 presents milk synchronous spectra recorded at 20 °C and obtained at different pH values (6.8, 6.2, 5.6, and 5.1) for $\Delta\lambda = 60$ nm. As observed in the previous section, the milk synchronous spectrum recorded at pH 6.8 for $\Delta\lambda = 60$ nm exhibited four bands, i.e., a sharp, intense band at 290 nm (emission at 350 nm), a band at 320 nm (emission at 380 nm), a smaller band at 380 (emission at 420 nm), and a broader and noisy band centered at 460 nm (emission at 520 nm). Based on the literature,[17,19,22,24] these fluorescence bands were ascribed to tryptophan residues of proteins, vitamin A, and riboflavin, respectively. Changes in the fluorescence intensity in the synchronous spectra were observed as a function of pH (Fig. 4).

Principal component analysis was applied to the synchronous spectra recorded at the investigated pH values for $\Delta\lambda = 60$ nm and $\Delta\lambda = 180$ nm to check the ability of synchronous

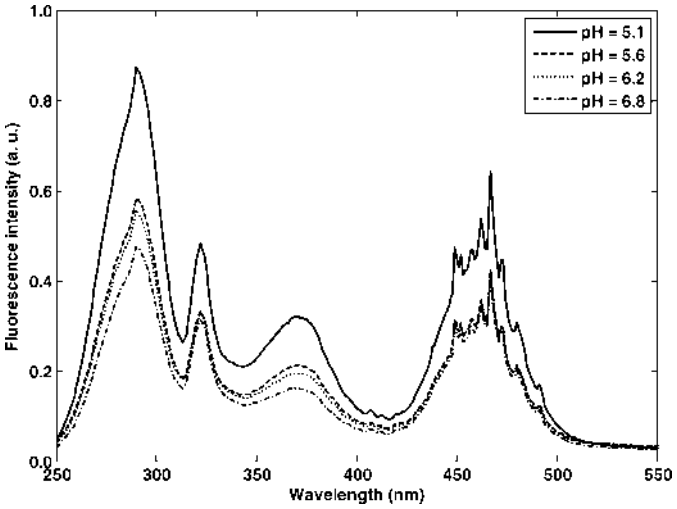

FIG. 4. Milk synchronous fluorescence spectra ($\Delta\lambda = 60$ nm; temperature = 20 °C) recorded at different pH values: 5.1 (—), 5.6 (- - -), 6.2 (····), and 6.8 (– · –).

spectra to (1) discriminate the milk samples as a function of pH and (2) retrieve various information for different offset values. Figure 5 shows the PCA similarities maps defined by the principal components 1 and 2 for synchronous spectra with $\Delta\lambda = 60$ nm (Fig. 5A) and $\Delta\lambda = 180$ nm (Fig. 5B). In both cases,

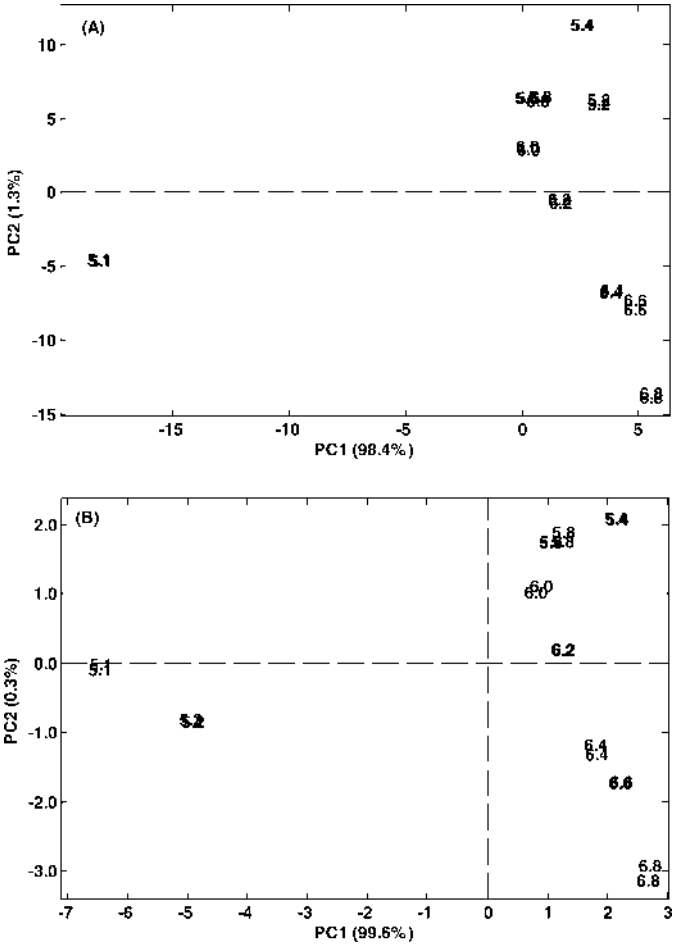

FIG. 5. PCA similarity maps defined by PC1 and PC2 for the acidified milk synchronous spectra recorded with (**A**) $\Delta\lambda = 60$ nm and (**B**) $\Delta\lambda = 180$ nm.

TABLE I. Core consistency values and the explained variance obtained with PARAFAC models derived from synchronous spectra recorded on mild-heated milk samples.

	Number of components of PARAFAC models						
	1	2	3	4	5	6	7
Core consistency (%)	100.00	99.93	81.56	14.97	0.03	0.80	0.30
Explained variance (%)	80.37	89.42	94.43	98.31	98.97	99.63	99.74

the first two principal components (PCs) accounted for more than 99.7% of the total variance, with a predominance of component 1 (98.4% ($\Delta\lambda = 60$ nm) and 99.6% ($\Delta\lambda = 180$ nm)). As a discrimination of the pH 5.1 sample from all the others was observed according to component 1 for the data set with offset $\Delta\lambda = 60$ nm, a slightly different trend appeared for the data set with offset $\Delta\lambda = 180$ nm. All the other samples were discriminated as a function of pH according to component 2, i.e., the milk sample at pH 6.8 had a negative score whereas a positive score was observed for the milk sample at pH 5.4. These results showed that the synchronous spectra retained information related to the molecular structure of milk components allowing the discrimination of samples as a function of pH. More interestingly, the differences observed in the two similarity maps (Figs. 5A and 5B) indicate that synchronous spectra recorded at two different offsets contain complementary molecular information regarding the investigated system.

Calculation of the Parallel Factor Analysis Models for Heating and Acidification Data Sets. The objective of PARAFAC is to resolve the synchronous fluorescence signal into the contribution of each of the fluorescent compounds present in the set of milk spectra, i.e., to estimate the profiles of fluorophores directly from the synchronous fluorescent landscape measurements (432 and 360 spectra for heating and acidification experiments, respectively).

In order to investigate how many components are needed, different models were fitted by using a number of components varying from 1 to 7. Tables I and II present the core consistency values and the explained variance obtained with the different models for heating and acidification data sets. Regarding milk samples submitted to heat, the obtained results indicated that three components were suitable, since a core consistency superior to 81.56% and an explained variance amounting to 94.43% were observed. Upon adding the fourth component, the core consistency decreased to 14.97% and this model explained 98.31% of the total variance (Table I), suggesting that the model with four components was unstable and over-fitted. Similar results were also obtained from the acidification experiment (Table II). Indeed, the fit values indicated that a model with three components was also suitable,

TABLE II. Core consistency values and the explained variance obtained with PARAFAC models derived from synchronous spectra recorded on acidified milk samples.

	Number of components of PARAFAC models						
	1	2	3	4	5	6	7
Core consistency (%)	100.00	99.03	81.31	14.11	0.03	0.66	0.31
Explained variance (%)	79.13	88.05	94.13	97.81	98.25	99.56	99.71

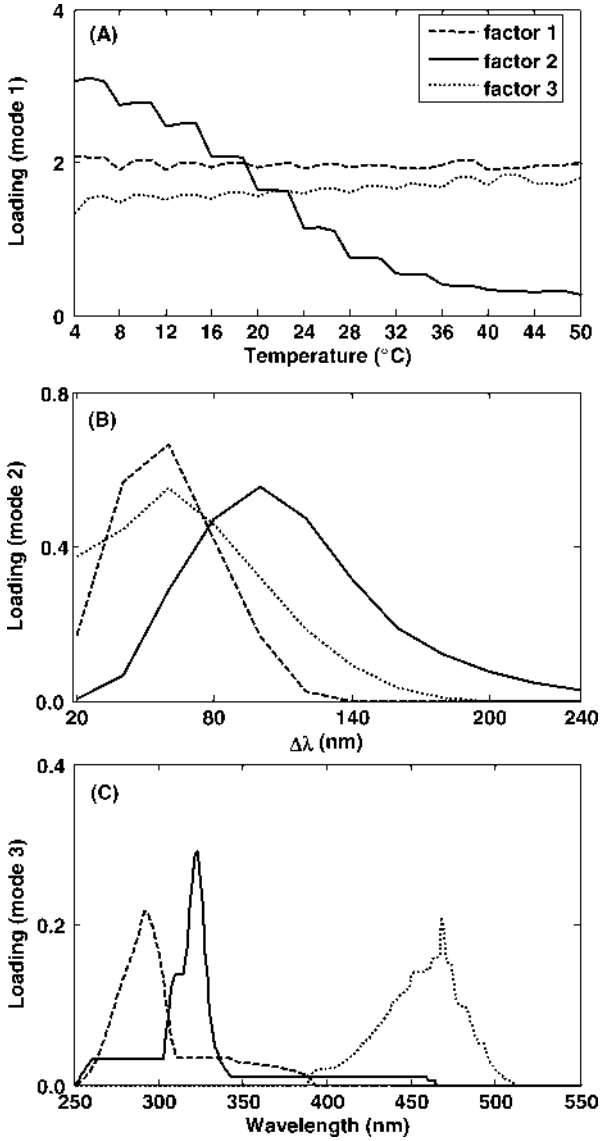

FIG. 6. Three-component non-negativity constraint model derived from the mild heated milk synchronous fluorescence spectra. (**A**) Concentration mode, (**B**) $\Delta\lambda$ profiles, and (**C**) loading profiles. Legend: component 1 (- - -), component 2 (—), and component 3 (····).

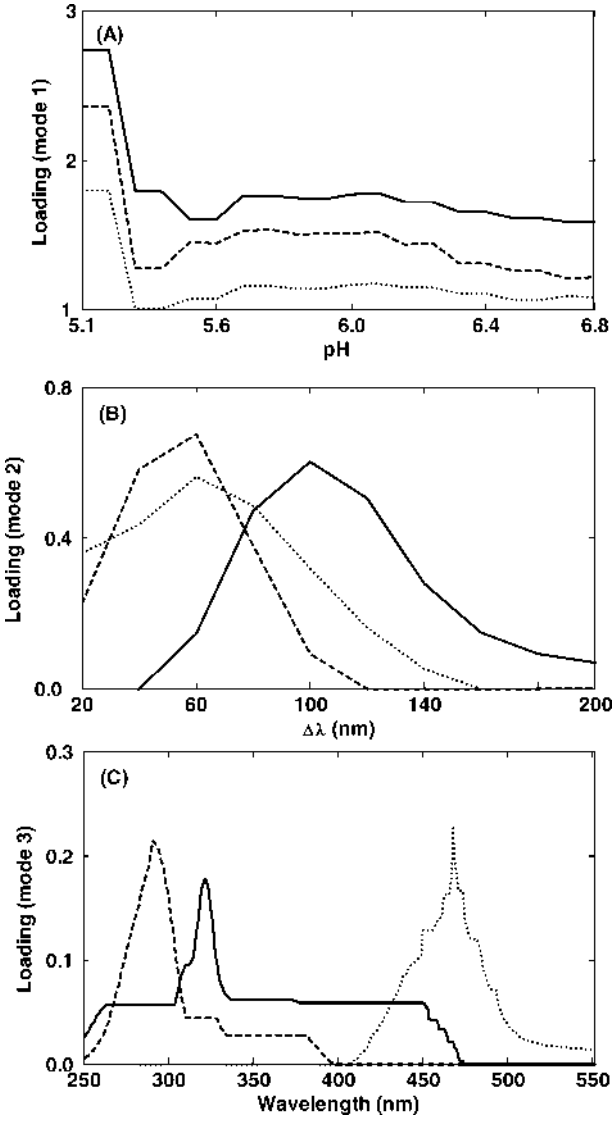

FIG. 7. Three-component non-negativity constraint model derived from the acidified milk synchronous fluorescence spectra. (**A**) Concentration mode, (**B**) $\Delta\lambda$ profiles, and (**C**) loading profiles. Legend: component 1 (—), component 2 (—), and component 3 (····).

exhibiting a core consistency superior to 81.31% and an explained variance amounting to 94.13%.

In the following paragraphs, PARAFAC models with three components will be considered and discussed, indicating that three PARAFAC components could be identified in milk samples during both heating and acidification. These three components looked similar, even identical, for the heating and acidification experiments performed on milk samples. These similar results obtained using PARAFAC on heating and acidification data sets agreed with the stability of the models and their validity.[7,26]

The loading profiles (Figs. 6C and 7C) of the first and second components performed on the two data sets corresponded quite well to the characteristics of tryptophan and vitamin A fluorescence spectra, respectively. Indeed, the shapes of spectra obtained with PARAFAC were similar to the excitation spectra of tryptophan and vitamin A spectra (Fig. 8), respectively. In addition, the optimal $\Delta\lambda$ of 50 nm and 100 nm for the first and second components (Figs. 6B and 7B, Table III) agreed well with the emission maxima of tryptophan residues and vitamin A (Fig. 8), respectively.

Considering the loading profiles (Figs. 6C and 7C), the third component with optimal $\Delta\lambda$ of 60 nm (Figs. 6B and 7B) corresponded to riboflavin fluorescence spectra (Fig. 8B, Table III). Indeed, the excitation spectrum (Fig. 8B) of riboflavin in milk was characterized by a maximum located at 448 nm, whereas the emission maximum was observed at 520 nm as shown in Fig. 8B. The obtained values derived from the fluorescence spectra were similar to the ones found with the PARAFAC model (Table III), strengthening the hypothesis that the third component corresponded to riboflavin compound.

The loading in sample mode (Figs. 6A and 7A) represents the concentration mode for each fluorophore corresponding to the two experiments. But the so-called "concentration mode" could also reveal structural changes in the environment of the considered fluorophores. The loading values are arranged in such a way that the changes of the properties of fluorophores

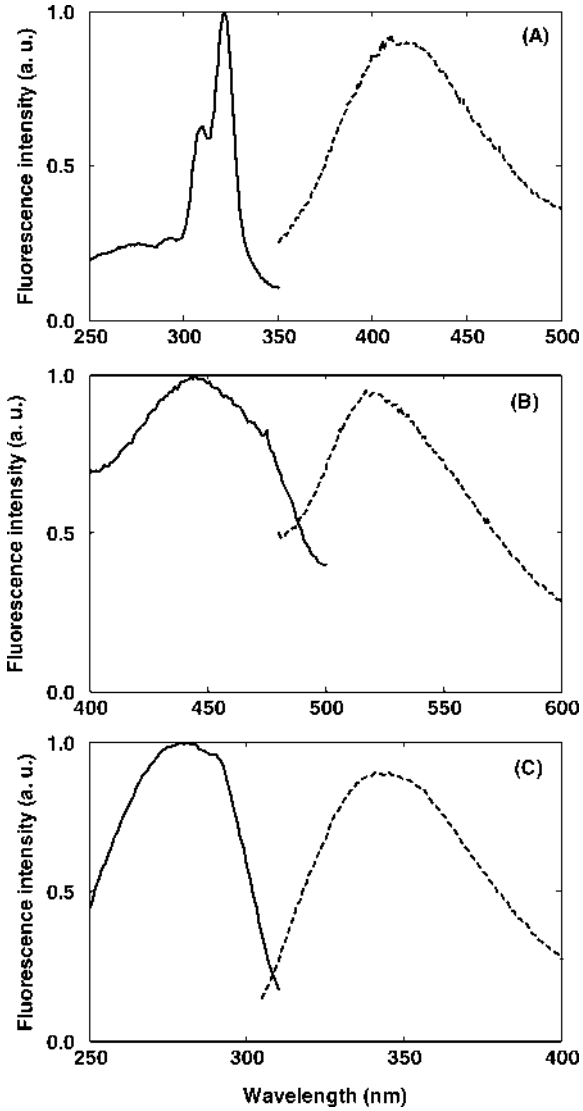

FIG. 8. Excitation (—) and emission (- - -) spectra of (**A**) vitamin A, (**B**) riboflavin, and (**C**) tryptophan recorded on milk (see Materials and Methods section for experimental conditions).

can easily be caught throughout the heating temperature or the acidification. Considering the heating experiment, the profiles of the estimated concentrations for the first and third components showed negligible variations according to the temperature. This might be explained by the mild heat treatment (4 to 50 °C) applied to the milk, which did not allow significant denaturation of proteins or riboflavin. Regarding the loading of component 2, corresponding to vitamin A, a significant decrease was observed when milk samples were submitted to heating. In this temperature range, the triglycerides were mostly the sole milk component whose structure was modified. This could reflect some changes in vitamin A fluorescence properties related to the melting of triglycerides in milk fat globules. This is in agreement with previous findings reporting that the shape of the vitamin A excitation spectrum is correlated with the physical state of the triglycerides in the fat globule.[36] In addition, the shape of the vitamin A excitation spectra of milk fat in water emulsion stabilized by β-lactoglobulin[15,36] and of cheeses[15] were found to show changes at the molecular level (protein–protein, protein–lipid interactions, etc.) when the temperature varied between 4 °C and 50 °C, allowing the melting point of the triglycerides to be derived.

TABLE III. Emission and excitation maxima of the three components of the PARAFAC models derived from synchronous spectra recorded on mild heated and acidified milk samples.

	Component		
	1	2	3
λ_{max} excitation (nm)	292	323	460
λ_{max} emission (nm)	342	423	520

Considering the acidification experiment, the concentration modes for the three components showed variations according to pH (Fig. 7A). Two main regions might be distinguished: the first region, from pH 6.8 to pH 5.5, showing a slow increase in the loadings for the three components, and a later one from pH 5.5 to pH 5.1, which is characterized by a slight decrease followed by a sharp increase in the loadings for the three components. The minima of the loadings for the three components were not observed at the same pH, suggesting that the environments of these fluorophores were not altered in the same way in this pH range and they might characterize different molecular environments. The structural changes of casein micelles, the organization of which is pH dependent, were extensively investigated by physical, chemical, and spectroscopic methods. Considering pH ranging between pH 6.8 and pH 5.6, the changes of the loading values of component 1 corresponding to protein tryptophan residues may characterize the decrease of casein micelles voluminosity.[38,39] It has been demonstrated that, for pH below 5.6, the voluminosity of the casein micelles increased with a maximum at about pH 5.3, correlating with the observed minimum for the loading values of component 1. During this step, a partial micellar disintegration induced by the solubilization of micellar calcium phosphate takes place, which causes a looseness of the micelles. The next step, from pH 5.3 to pH 5.1, corresponding to the sharp increase of the component 1 loading values, is marked by the formation of reversible aggregates of casein micelles.[38–40] The results showed that the changes of the concentration mode of component 1 corresponding to protein tryptophan residues was associated with modifications of micelle structure induced by acidification.

The variation of the loading values of component 2, corresponding to vitamin A, in the 5.5 to 5.1 pH range may result from the interactions between casein micelles and fat globules as previously described by Dufour et al.[41] Less is known about the effect of acidification on the fluorescence properties of riboflavin in milk: more experiments are needed to have a better understanding of the variation of the concentration mode of component 3 corresponding to riboflavin, as a function of pH.

CONCLUSION

This exploratory study on milk demonstrates the potential of synchronous front-face fluorescence and chemometrics applied to the analysis of dairy products. Recorded synchronous fluorescence spectra reveal information at the molecular level about milk heated at various temperatures or milk acidification. PARAFAC provided the same mathematical decomposition for

both data sets. The synchronous fluorescent signal from the milk samples is suggested to derive from tryptophan, vitamin A, and riboflavin. In addition, this analytical method provides a simultaneous determination of the fluorescence level (i.e., loading in sample concentration) of all these compounds. From the three components, only the second one attributed to vitamin A showed a change in the fluorescence signal during milk heating, corresponding to the melting of triglycerides. This investigation underlines the potential of synchronous fluorescence spectroscopy in combination with chemometrics as a nondestructive innovative method that can be applied to dairy products for monitoring quality during manufacturing and storage, and perhaps in the development of new fast quantitative analyses of vitamins exhibiting intrinsic fluorescence. The usefulness of SFS is not limited to the simultaneous determination of several components in mixture, as usually reported in the literature. But this analytical method also has the potential to address questions related to the structure of fats and, potentially, proteins, as well as to fat–protein interactions, in milk and milk products.

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Effects of added minerals (calcium, phosphate and citrate) on the molecular structure of skim-milk as investigated by mid-infrared and synchronous fluorescence spectroscopies coupled with chemometrics

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ABSTRACT

Minerals play an important role in the structure and stability of casein micelles: minerals and caseins in milk are in dynamic equilibrium. Front-face synchronous fluorescence and mid-infrared spectra in combination with multivariate statistical analysis have been used to investigate, at a molecular level, the effects of added minerals (calcium, phosphate and citrate) on mineral and casein equilibria, the distribution of calcium phosphate between the soluble and insoluble phases and the micellar structure. Synchronous fluorescence spectra were recorded in the 250–500 nm excitation wavelength range using offset of 80 nm between excitation and emission monochromators for skim-milk samples fortified with 0, 3, 6 and 9 mM of calcium, phosphate or citrate, at 30 °C and 4 °C. Regarding mid infrared spectroscopy, the region located between 1700–1500 cm^{-1} corresponding to the amide I and II bands, and the 1500– 900 cm^{-1} region called the fingerprint region were considered for the characterization of the fortified skim-milk samples at the two considered temperatures. Principal component analysis was applied to the collections of fluorescence and infrared spectral data of the two systems to optimize their description. The results show that the phenomena induced by the addition of phosphate were different from the ones observed following the addition of calcium or citrate, a calcium-chelating agent. Finally, common components and specific weights analysis was applied to infrared spectra and fluorescence data collected on fortified skim-milk samples. This analyse enabled the relationship between the different data tables to be established.

Keywords: milk, minerals, calcium, phosphate, citrate, mid-infrared, front-face synchronous fluorescence, chemometrics.

INTRODUCTION

The mineral fraction (about 8 – 9 g/L) of milk contains cations (calcium, magnesium, sodium and potassium) and anions (inorganic phosphate, citrate and chloride). These ions play an important role in the structure and stability of casein micelles of milk (Gaucheron, 2004; Holt, 1997; Walstra and Jenness, 1984). Milk comprises two phases between which minerals are partitioned. Monovalent ions such as sodium, potassium and chloride are mostly present in the soluble phase, whereas calcium and phosphate are much more present in the colloidal phase. As reported in the literature, the physical chemistry of the calcium phosphate salts is complex, and these salts can have many compositions (different Ca/P ratio) and exist in different forms (amorphous or crystallized). Moreover, in casein micelles, micellar calcium phosphate is partly associated to the colloidal inorganic phosphate and partly bound to casein by the intermediate phosphoserine residues (Gaucheron, 2005). A good understanding of the dynamic and interactions with proteins of milk ions is important for fundamental research , as well as for the development of dairy products. It is well known that minerals play an important role in the structure and stability of casein micelles (Walstra, 1990). The minerals and caseins in milk are in dynamic equilibrium; small alterations in the distribution of calcium phosphate between the soluble and insoluble phases can lead to important effects on micellar stability.

The addition of divalent cations induces important modifications in the salt distribution between aqueous and micellar phases. Thus, after the addition of 10 mM calcium chloride to milk, about 80% of this cation was associated to casein micelles (Philippe et al, 2003). In parallel, displacements of inorganic phosphate and citrate from the diffusible fraction to he micellar structure were observed. Consequently, casein micelle structure was strongly altered, exhibiting lower zeta potentials and hydrations (Philippe et al, 2003). The impact of calcium chelating agents, such as citrate, on some properties of milk has been investigated (Udabage et al, 2000). The calcium-chelating agents disrupt the casein micelles by reducing calcium concentration and colloidal calcium phosphate content, causing casein micelle dissociation.

Mid-Infrared (MIR) and fluorescence spectroscopies have become increasingly important for a wide variety of analytical applications in biology and chemistry. This is due to great technical advances in both instrumentation and data analysis tools in the past two decades. Coupled with chemometric tools such as principal component analysis (PCA), principal component regression (PCR), factorial discriminant analysis (FDA) and Partial Least Squares

(PLS) regression (Armanta et al., 2007; Roggo et al., 2007; Qannari et al., 2000), information on composition, physicochemical properties and molecular structure of food systems can be extracted from fluorescence and infrared spectra in an accurate, high sensitive and rapid manner (Karoui and de Baerdemaeker, 2007; Mazerolles et al, 2002).

Fluorescence spectroscopy is a rapid and sensitive method for quantifying a fluorescent component and characterising its molecular environment in all sorts of biological samples. This environmental sensitivity enables to characterise conformational changes such as those attributable to the thermal, solvent or surface denaturation of proteins, as well as the interactions of proteins with ligands.

While the techniques used in conventional fluorescence spectroscopy allow to record excitation spectra or emission spectra, the excitation wavelength - λex, and the emission wavelength - λem, are scanned synchronously with a constant wavelength interval, $\Delta\lambda = \lambda em - \lambda ex$ in synchronous fluorescence spectroscopy (SFS) (Lloyd, 1971). For well defined absorption and quantum yield maxima, the optimum value of the offset $\Delta\lambda$ is set by the difference in wavelength of the emission and excitation maxima which is known as Stoke's shift (Peuravuori et al., 2002). SFS makes it possible to narrowing of the spectral band and simplify the emission spectrum. In addition, SFS presents an interesting advantage from our point of view: a synchronous fluorescence spectrum retains information related to several fluorophores, compared to a classical emission spectrum that is mainly specific of a sole fluorophore.

Milk contains intrinsic fluorophores such as vitamin A and tryptophan residues present in fats and proteins, respectively, which give typical excitation and emission spectra. These spectra provide specific information on the characteristics of fats and proteins and on the environment of the fluorescent probes in food samples allowing to investigate structural changes (Mazerolles et al., 2002). For example, the melting temperature of fat in cheese has been determined from vitamin A fluorescence spectra recorded at different temperatures (Karoui et al., 2003).

MIR represents the spectrum of the absorption of all the chemical bonds having an infrared activity between 4000 and 400 cm^{-1}. The acyl-chain is mainly responsible for the absorption observed between 3000 and 2800 cm^{-1}, whereas the peptidic bound C-NH is mainly responsible of the absorption occurring between 1700 and 1500 cm^{-1}. As water also strongly

absorbs in the Amide I region, the changes of pattern of the casein-bound water with structural changes of the micelles alter the shape of the spectra in this region. Finally, the 1500 and 900 cm^{-1} region is known to exhibit bands attributed to peptidic bonds and phosphate.

In the present work, front-face synchronous fluorescence and mid-infrared spectra in combination with multivariate statistical analysis are used to investigate, at a molecular level, the effects of added minerals (calcium, phosphate and citrate) on mineral and casein equilibria, the distribution of calcium phosphate between the soluble and insoluble phases and the micellar structure.

MATERIAL and METHODS

- Milk samples

Bovine milk samples were collected from the tank of a local farm and a given milk sample was used in the course of the day. The milk was skimmed by centrifugation at 30 °C using a Elecrem centrifuge (Electrem, France).

- Addition of salts to milk

Trisodium citrate ($C_6H_5Na_3O_7*2H_2O$, VWRInternational SAS, Fontenay sous Bois, France), calcium ($CaCl_2$, Merck KGaA, Darmstadt, Germany) and phosphate ($Na_2HPO_4*2H_2O$, VWR International SAS, Fontenay sous Bois, France) were separately added to milk at various concentrations (0, 3, 6 and 9 mM) by slow addition with continuous stirring for 1 hour (Ozcan-Yilsay et al., 2007). The experiments were performed at two different temperatures, i.e., 4 and 30 °C.

- Mid-infrared spectra

Infrared spectra were recorded between 3500 and 900 cm^{-1} at a resolution of 4 cm^{-1} on a Varian 3100 FT-IR Excalibur Series Fourier-transform spectrometer (Varian Inc., Palo Alto, USA) mounted with a thermostated ATR accessory equipped with a grip. The ATR cell is 6 reflections and was made of a horizontal ZnSe crystal which presented an incidence angle of 45°. The temperature was controlled by a Julabo temperature controller (Julabo, Germany).

Enriched milk samples were placed on the crystal for analyis. In order to improve the signal to noise ratio, 32 scans were co-added for each spectrum. Before each measurement, the spectrum of the ZnSe crystal was recorded in the conditions described above and used as

background. Base line and ATR corrections were applied to the spectra using Varian Software (Resolution Pro 4.0).

The regions of the mid infrared spectra located between 1700 and 1500 cm^{-1} (protein region) and 1500 and 900 cm^{-1} (fingerprint region) have been considered in this study.

For each milk sample, the spectra were recorded at 4 and 30 °C in triplicate.

- Synchronous fluorescence spectra

Synchronous fluorescence spectra were recorded using a FluoroMax-2 spectrofluorimeter (Spex-Jobin Yvon, Longjumeau, France) mounted with a front-surface cuvette-holder and the incidence angle of the excitation radiation was set at 56° to ensure that reflected light, scattered radiation, and depolarisation phenomena were minimized. The spectrofluorimeter was equipped with a thermostatically controlled cuvette-holder and the temperature was controlled by a Haake temperature controller (Haake, Champlan, France).

Synchronous fluorescence spectra were collected in the 250–500 nm excitation wavelength range using offset of 80 nm between excitation and emission monochromators (Boubellouta & Dufour, 2008). For the experiments, the synchronous spectra of milk samples (2 cm x 1 cm x 0.3 cm) placed in quartz cuvette were recorded at 4 and 30 °C. For each item, three spectra were recorded using different milk samples.

Chemometrics **:**

- *Principal Component Analysis (PCA)*

PCA is a multidimensional data treatment which provides a synthetic description of large data sets. When applied to spectral data, PCA allows similarity maps of the samples to be drawn and spectral patterns obtained (Bertrand et al., 1987). PCA was applied to the synchronous spectra in order to investigate differences in the spectra. This statistical multivariate treatment made it possible to draw similarity maps of the samples and to get spectral patterns (Jollife, 1986).

- *Common Components and Specific Weight Analysis (CCSWA)*

CCSWA was developed within a sensory framework as a tool to analyze several data sets (Qannari et al., 2000). The objective of CCSWA is to describe several data tables observed on the same *n* samples by recovering the maximum inertia (total variance) of each of them. It was subsequently applied in chemometrics for combining different kinds of measurements made on food products (Mazerolles et al., 2002; Pram Nielsen et al., 2001). The rationale behind this method is the existence of a common structure to the data tables. Therefore, the

method determines a common space of representation for all the data sets. Each table is allowed having a specific weight (or salience) associated with each dimension of this common space (Mazerolles et al., 2006) .
Chemometric analyses were performed in MATLAB (The Mathworks Inc., Natic, MA, USA) using “Saisir” package available at the address: http://easy-chemometrics.fr.

RESULTS and DISCUSSION

It is well known that minerals play an important role in the structure and stability of casein micelles (Walstra, 1990): minerals and caseins in milk are in dynamic equilibrium. The minerals such as calcium, citrate and phosphate are present in different forms in milk (Holt, 1997). For a total calcium concentration of 32 mM, 22 mM are in the colloidal state and 10 mM are diffusible. Only 2 mM of this diffusible calcium are free ionic calcium. The remainder is essentially complexed with citrate, phosphate, caseins and whey proteins. In the colloidal state, calcium can be complexed with phosphoester, carboxyl groups of micellar caseins or with colloidal phosphate and citrate associated with casein micelles. Moreover, the addition of citrate, a calcium chelating agent, to milk induces solubilization of colloidal calcium phosphate leading to the partial dissociation of caseins from casein micelles (Udabage et al, 2000).
It has been shown that the changes in mineral equilibria in milk alter the structure of casein micelles. As a result of altered structure of casein micelles, decreases of hydration and charges, as well as increases of whiteness, turbidity and hydrophobicity, has been reported (Philippe et al, 2003).
This study was aimed at investigating milk structural evolution at a molecular level following mineral addition using front-face synchronous fluorescence and mid infrared spectroscopies. More precisely, we focused on changes of protein environment. This approach requires the ability to discriminate between different structural changes induced by the addition of calcium, phosphate or citrate.

Structural changes as studied by synchronous fluorescence spectroscopy. Effects of temperature

Figure 1A presents synchronous spectra ($\Delta\lambda$ = 80 nm) recorded at 30 °C of skim-milk and skim milk added with 9 mM calcium, phosphate or citrate. The skim-milk synchronous

spectrum recorded at 30 °C exhibited an intense band at 287 nm (emission at 367 nm), a band at 370 nm (emission at 450 nm) and a broad band centered at 450 nm (emission at 530 nm). The intense band observed at 287 nm (emission at 367 nm) can be attributed to tryptophan residues of proteins (Boubellouta and Dufour, 2008). The broad bands centered at 370 nm and 460 nm may be attributed to NADH and riboflavin (Karoui and Dufour, 2006), respectively. The addition of minerals as different effects on the fluorescence intensity of the different bands. Whereas the addition of calcium to skim milk induced an increase of fluorescence intensities at 287, 370 and 450 nm, phosphate and citrate additions resulted in a decrease of the fluorescence intensities of the 3 major bands. It suggested that the addition of 9 mM calcium, phosphate or citrate induced different changes in the environment of the investigated fluorophores (protein tryptophan residues, NADH, riboflavin). Considering riboflavin, it is generally bound to proteins. Riboflavin binding protein - a monomeric, two-domain protein - has been originally purified from hens' egg white (Wasylewski, 2000). In plasma, it has been shown that riboflavin is bound to proteins, predominantly albumin, but also to immunoglobulins: this vitamin may bind to a large number of proteins. Like others fluorophores, the fluorescence emission of riboflavin is highly sensitive to its local environment, and it can be used as an indicator group for protein conformation and interaction changes in cheese matrices during ripening since it interacts with proteins (Karoui and Dufour, 2006).

PCA was applied to the set of spectra recorded for the different minerals and the three concentrations at 30 °C in order to obtain additional structural information. This method is well suited to optimize the description of data collection by extracting the most useful data and rejecting redundant data. Figure 2A shows a PCA similarity map defined by the principal components 1 and 2 for synchronous fluorescence spectral data. The first two principal components accounted for 97.0% of the total variability with a predominance of component 1 (93.5%). The map showed that the spectra recorded on skim milk exhibited coordinates close to the origin. Interestingly, the principal component 1 separated mainly the spectra recorded on samples added with calcium (negative scores) from the ones recorded on samples added with citrate (positive scores). In addition, principal component 1 discriminated skim milk samples added with 3, 6 and 9 mM calcium, the latest one exhibiting the highest negative score. A similar trend, but in the other direction compared to the origin, was observed for milk samples added with citrate: the sample with 9 mM citrate exhibiting the highest positive score. Considering the principal component 2, a discrimination of the spectra recorded on

milk samples with added phosphate was observed: the spectrum of the milk with 3 mM phosphate had a positive score, whereas the ones for 6 and 9 mM phosphate had negative values. These trends observed on the map defined by principal components 1 and 2 indicated that 1) considering the orthogonality of principal components, the addition of phosphate to milk had a different effect on spectra than calcium and citrate, and 2) two phenomena were observed following the addition of phosphate to milk: the first one was observed for concentrations below 3 mM phosphate and the second one for concentrations above this value.

The spectral patterns associated with principal components 1 and 2, providing the most discriminant wavelengths of the spectral data set recorded at 30 °C, are presented Figure 2B. Considering spectral pattern 1, the most discriminant wavelengths were 285, 370 and 450 nm. They corresponded to the maxima of the bands observed on the synchronous spectra. In fact the spectral pattern was the mean spectrum of the spectra in the data base. The spectral pattern associated with PC 2 exhibited a contrast between a negative peak at 271 nm and a shoulder at 300 nm and a positive peak at 463 nm. It suggested that the addition of 6 and 9 mM phosphate to skim milk induced a broadening of the band of tryptophan residues of proteins, whereas the addition of 3mM phosphate was characterized by a sharpening of this band. The positive band at 463 indicated changes in the environment of riboflavin.

It has been shown by Philippe et al (2003) that Ca supplementation induces association of Ca ions with phosphate and citrate initially present in milk. Consequently, inorganic phosphate and citrate ion concentrations decrease in the aqueous phase. Moreover, these authors showed that calcium absorbs on casein micelles for milks fortified with this mineral. Due to mineral equilibrium modifications, Philippe et al (2003) showed that casein micelles undergo physico-chemical changes such as decreases of hydration and charges and increases of whiteness, turbidity and hydrophobicity. As shown above, synchronous fluorescence spectroscopy was able to detect these changes in the casein micelle properties.

Structural changes as studied by mid-infrared spectroscopy (1700–1500 cm^{-1} region). Effects of temperature

Most of the spectral information useful for the analysis of the spectra is located in: (1) the 3000–2800 cm^{-1} range corresponding to C-H stretching, (2) the region located between 1700–

1500 cm^{-1} corresponding to the amide I and II bands, and (3) the 1500–900 cm^{-1} region called the fingerprint region.

The 3000–2800 cm^{-1} spectral region corresponds to the C–H bond of methyl and methylene groups of fatty acids. As skim-milk was considered in this study, only very weak bands were observed in this region. The 1700–1500 cm^{-1} region was characterised by the presence of bands related to peptides and proteins. Thus, these bands contain some information on the proteins and on the interaction of these proteins with other components, such as ions, water and other proteins. But this region may also exhibit contribution of several lateral chains of amino acids and of carboxylic group of molecules such as citrate. Contribution to the amide I band, which is used to investigate the secondary structure of proteins, can be observed at about 1638 cm^{-1} (Figure 1B). The absorption band with a maximum at about 1553 cm^{-1} is generally assigned to the amide II vibrations. Only weak differences were observed between the spectrum of raw milk and milk samples added with 9 mM minerals.

PCA was applied to the Amide I&II spectral data recorded at 30 °C and 4 °C in order to get a deeper insight into structural changes induced by mineral addition. Considering the results of PCA performed on spectral data recorded at 30 °C, the first two principal components took into account 93.1% of the total inertia. Figure 3A shows the similarity map defined by principal components 1 and 2. The first principal component discriminated raw-milk, milk plus calcium and milk plus phosphate samples exhibiting positive scores from milk plus citrate samples with negative scores: higher was the citrate concentration, more negative was the score. The spectral pattern corresponding to the principal component 1 is given Figure 3B and revealed a sole broad peak centered at 1580 cm^{-1}. It corresponded to the absorbance of citrate.

Considering the second principal component, raw milk and milk fortified with 3 mM phosphate had the lowest score, then were observed the samples with 6 mM phosphate, and, finally, milks plus 9 mM phosphate were close to the origin. Indeed, Le Ray et al (1998) showed that addition of 2-8 mM phosphate to micellar casein or milk does not induced of calcium ions from the micellar phase to the aqueous one. The authors suggested that micellar calcium has an higher affinity for micellar phosphate than for added phosphate. However, Le Ray et al (1998) observed that the added phosphate was partly bound to the micellar phase for the highest concentrations of added phosphate, inducing micelle structural changes. Indeed,

the mid infrared spectra in the 1700–1500 cm^{-1} region showed changes in the spectra for 6 and 9 mM added phosphate: we concluded that the structure of casein micelles was modified for these high concentrations of added phosphate. It is in agreement with the results derived from the evaluation of fluorescence spectra (see above) and of mid infrared spectra in the 1500–900 cm^{-1} region (see below).

This conclusion was strengthened by the results obtained at 4°C. The analysis by PCA of the spectra recorded in the 1700–1500 cm^{-1} region on milk samples at 4°C showed that, according to principal component 3, milk fortified with 6 mM phosphate had the highest score, then were observed close to the origin the raw milk samples, and, finally, milks plus 3 and 9 mM phosphate showed positive scores (Figure 3). As it may be hypothesized, lowering the temperature modifies the binding of phosphate to casein micelles: the mineral equilibria are displaced from the micellar phase to the aqueous one (Gaucheron, 2004). It is in agreement with the results derived from the evaluation of fluorescence spectra and of mid infrared spectra in the 1500–900 cm^{-1} region recorded on milk samples at 4°C.

Regarding the second principal component, the spectral pattern (Figure 3B) exhibited negative peaks at 1514, 1539, 1653 and 1678 cm^{-1}. This spectral pattern was more complex and as a consequence more difficult to interpret. This pattern indicated that that changes occurred in the secondary structure of casein micelles following the addition of phosphate and calcium. It is in agreement with Le Ray et al (1998) and Philippe et al. (2003) that reported binding of phosphate and calcium to casein micelles following supplementation of milk with these minerals.

Structural changes as studied by mid-infrared spectroscopy (1500–900 cm^{-1} region). Effects of temperature

The 1500–900 cm^{-1} region is called the fingerprint region and numerous chemical bonds are absorbing in this region (Figure 1C). Considering the spectra recorded for skim milk and fortified milks, differences were observed for bands located at 1250, 1150 1067 and 1040 cm^{-1}. The bands observed in the 1000–1100 cm^{-1} region could be related to P=O stretching (Bellamy, 1975).

As phosphate absorbs in the fingerprint region, Figure 4 presents the spectra recorded on skim milk with 0, 3, 6 and 9 mM. It was supposed that the intensity of bands related to phosphate absorbance should have increase with increasing concentration of phosphate. But the spectra of skim milk with 0 or 3 mM phosphate were superimposed in the 1500–900 cm^{-1} region. On the other hand, spectra of milk fortified with 6 and 9 mM phosphate exhibited an increase of absorbance at 1250 and 1150 cm^{-1}, but a decrease at 1067 and 1040 cm^{-1}. As described above for fluorescence and mid infrared (1700–1500 cm^{-1} region) data recorded at 30 °C, two phenomena were observed following the addition of phosphate to milk: the first one was observed for concentrations below 3 mM phosphate and the second one for concentrations above this value.

PCA was applied to 1500–900 cm^{-1} spectral data recorded at 30 °C and 4 °C in order to get a deeper insight into structural changes induced by mineral additions. Considering the results of PCA performed on spectral data recorded at 30 °C, the first two principal components took into account 97.5% of the total inertia. Figure 5A shows the similarity map defined by principal components 1 and 2: whereas spectra of skim milks fortified with 0, 3, 6 and 9 mM phosphate were well discriminated according to PC1, the milk samples fortified with calcium and citrate were grouped altogether in right top corner of the map. Regarding milk samples fortitifed with phosphate (Figure 5A), it appeared that samples with 3 mM phosphate had negative scores, whereas samples plus 6 and 9 mM phosphate had positive scores. There was no linear relationship between the concentration of added phosphate and the score of the samples according to PC1. Again two phenomena were observed following the addition of phosphate to milk: the first one was observed for concentrations below 3 mM phosphate and the second one for concentrations above this value.

The spectral patterns (Figure 5B) associated to the principal components 1 and 2 exhibited negative peaks at 1076 (PC 2), 1100 (PC 1), 1150 (PC1) and 1250 (PC1) cm^{-1} in the 1500-900 cm^{-1} region. As principal component mainly discriminated samples fortified with phosphate, it can be concluded that bands at 1100, 1150 and 1250 cm^{-1} were related to phosphate absorbance.

CCSWA analyse of the relationships and differences between the data tables collected during acid- and rennet-induced coagulations

The Common Components and Specific Weights Analysis (CCSWA) method was applied on the 3 data sets containing the synchronous fluorescence and the infrared spectra (Amide I&II and fingerprint regions) data recorded on the milk samples at 30 °C. The two first dimensions gave different saliences for spectroscopies data sets: the first dimension (D1) expressed 92.0 and 39.1 % of the inertia of the fluorescence data and infrared data corresponding to Amide I&II spectral region, respectively, and a smaller part (13.7 %) of the inertia of the fingerprint region 1). On the contrary, the second dimension (D2) expressed 88.2% of the inertia of the fingerprint region and part of the inertia of the infrared data (22.4 and 2.0 % for Amide I&II region and fluorescence spectral data, respectively) (Table 1).

Considering the similarity maps defined by the common components D1 and D2, a discrimination of milk samples supplemented with citrate exhibiting positive scores from milk samples fortified with calcium with negative scores was observed according to the common component D1 (Figure 6A). The spectral pattern in fingerprint region associated with the common component D1 (Figure 6B) showed positive strong bands at 1078 and 1393 cm^{-1} that, according to Figure 6A, are associated with citrate absorbance. In addition, the common component D2 discriminated the different milk samples fortified with phosphate: it appeared that samples with 3 mM phosphate had the lowest scores, samples plus 6 mM phosphate had intermediate negative scores and samples plus 9 mM phosphate were close to the origin. The discrimination of milk samples supplemented with phosphate according to common component D2 was related to phosphate concentrations. Considering the spectral pattern (Figure 6B) associated with the principal component D2, the milk samples fortified with 3 phosphate exhibited higher absorbances at 1111 (weak), 1152 (strong), 1250 (strong) and 1379 cm^{-1} (weak) thank milk samples supplemented with 6 and 9 mM phosphate.

CCSWA performed on the 3 data tables allowed to manage in an efficient way the whole information collected. It summed up the major part of the information on two dimensions (D1 and D2).

CONCLUSION

It is generally assumed that the changes in mineral equilibria in milk alter the structure of casein micelles and the properties of milk. However, few techniques allow the investigation at a molecular level of turbid and complex products such as milk. Front-face synchronous fluorescence spectroscopy and mid infrared spectroscopy, two non-invasive techniques, in combination with chemometric methods make it possible to study the structure of milk components and mineral equilibria at a molecular level. Indeed, the fluorescence properties of tryptophan residues are modified by the changes in micelle structure and protein interactions induced by mineral supplementation. This study shows that infrared spectroscopy is useful for characterizing the changes in micelle structure following the addition of calcium, citrate or phosphate.

Using these spectroscopic methods coupled to chemometrics, it has been possible to demonstrate that the phenomena induced by the addition of phosphate were different from the ones observed following the addition of calcium or citrate, a calcium-chelating agent. Fluorescence and infrared data are spectra that allow us to derive information on the molecular structure and interactions of milk.

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Table 1: Saliences for the common components 1 to 4 of CCSW analysis performed on the spectroscopic data recorded on milk samples at 30 °C.

	Components			
Tables	D1	D2	D3	D4
SFS	0.920	0.020	0.033	0.006
MIR 1700-1500 cm^{-1}	0.391	0.224	0.224	0.125
MIR 1500-900 cm^{-1}	0.027	0.882	0.058	0.008

Legends of the figures

Figure 1: (A) Synchronous fluorescence spectra (250 and 500 nm; $\Delta\lambda$ = 80 nm), and (B) 1700-1500 cm^{-1} and (C) 1500-900 cm^{-1} mid infrared spectra recorded at 30 °C for skim milk (—) and skim milks supplemented with 9 mM calcium (---), phosphate (···) or citrate (-·-).

Figure 2: PCA similarity maps defined by PC1 and PC2 (A) for synchronous fluorescence spectra recorded at 30 °C and spectral patterns (B) corresponding to the principal components 1 (—) and 2 (---).

Figure 3: PCA similarity map defined by PC1 and PC2 (A) for 1700-1500 cm^{-1} mid-infrared spectra recorded at 30 °C and spectral patterns (B) corresponding to the principal components 1 (—) and 2 (---).

Figure 4: Mid-infrared spectra recorded between 1500-900 cm^{-1} at 30 °C for skim milk with 0 (—), 3 (---), 6 (···) and 9 mM (-·-) phosphate.

Figure 5: PCA similarity map defined by PC1 and PC2 (A) for 1500-900 cm^{-1} mid-infrared spectra recorded at 30 °C and spectral patterns (B) corresponding to the principal components 1 (—) and 2 (---).

Figure 6: CCSWA similarity maps defined by the common components D1 and D2 (A) for data recorded at 30 °C and spectral patterns (B) associated with the common component D1 (—) and D2 (---) (1500-900 cm^{-1} mid infrared spectra).

Figure 1 : Boubellouta et al

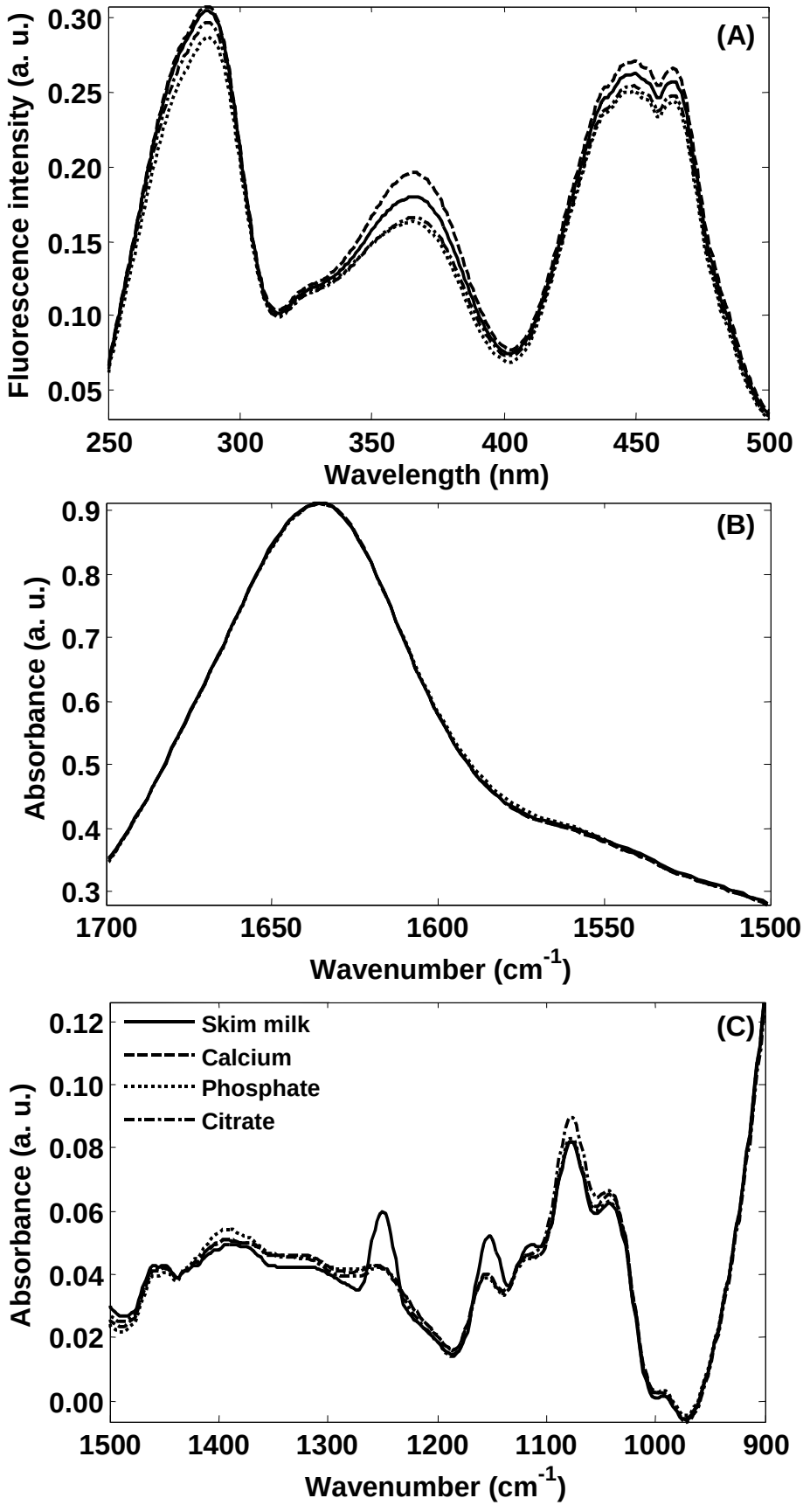


Figure 2 : Boubellouta et al

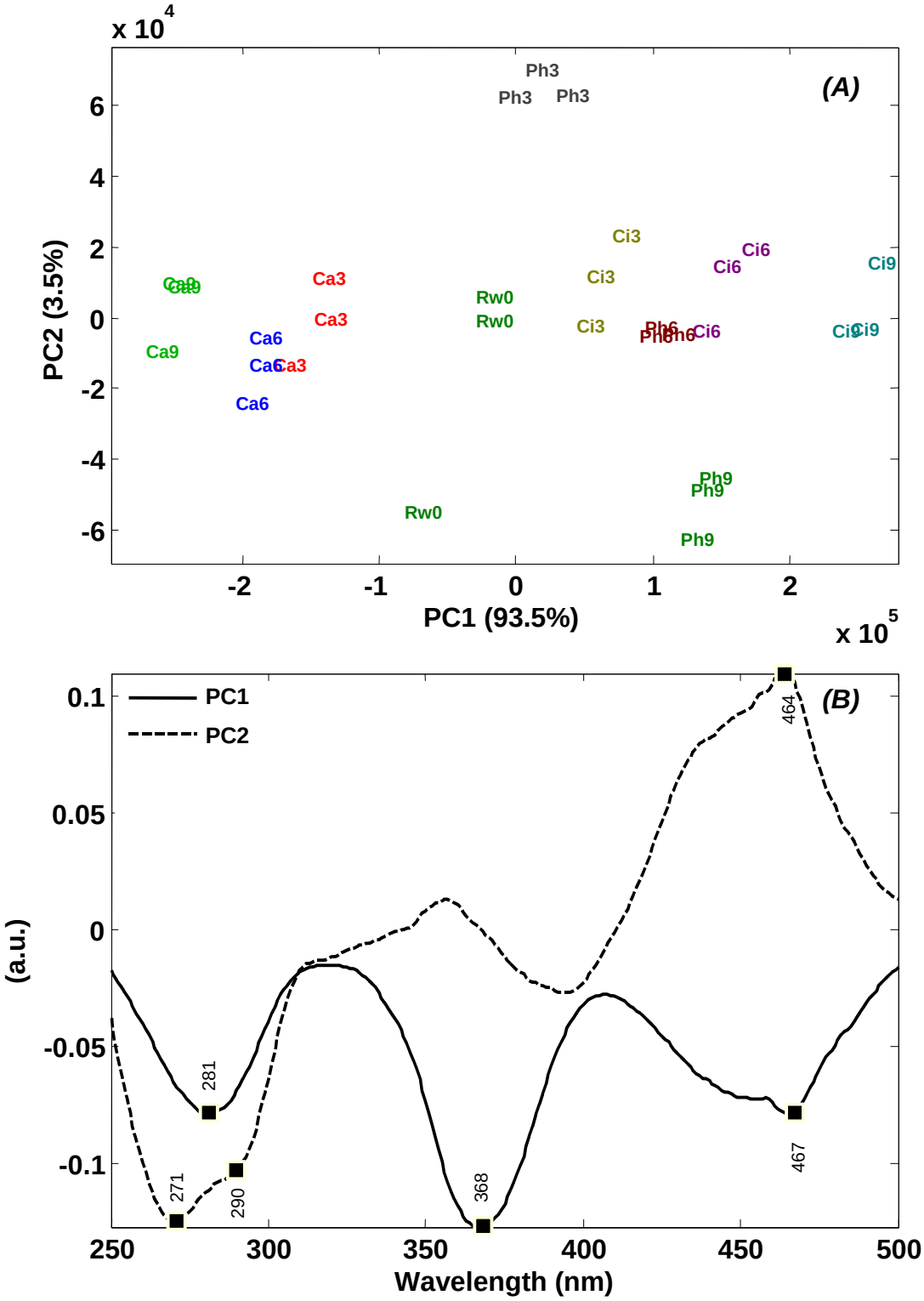
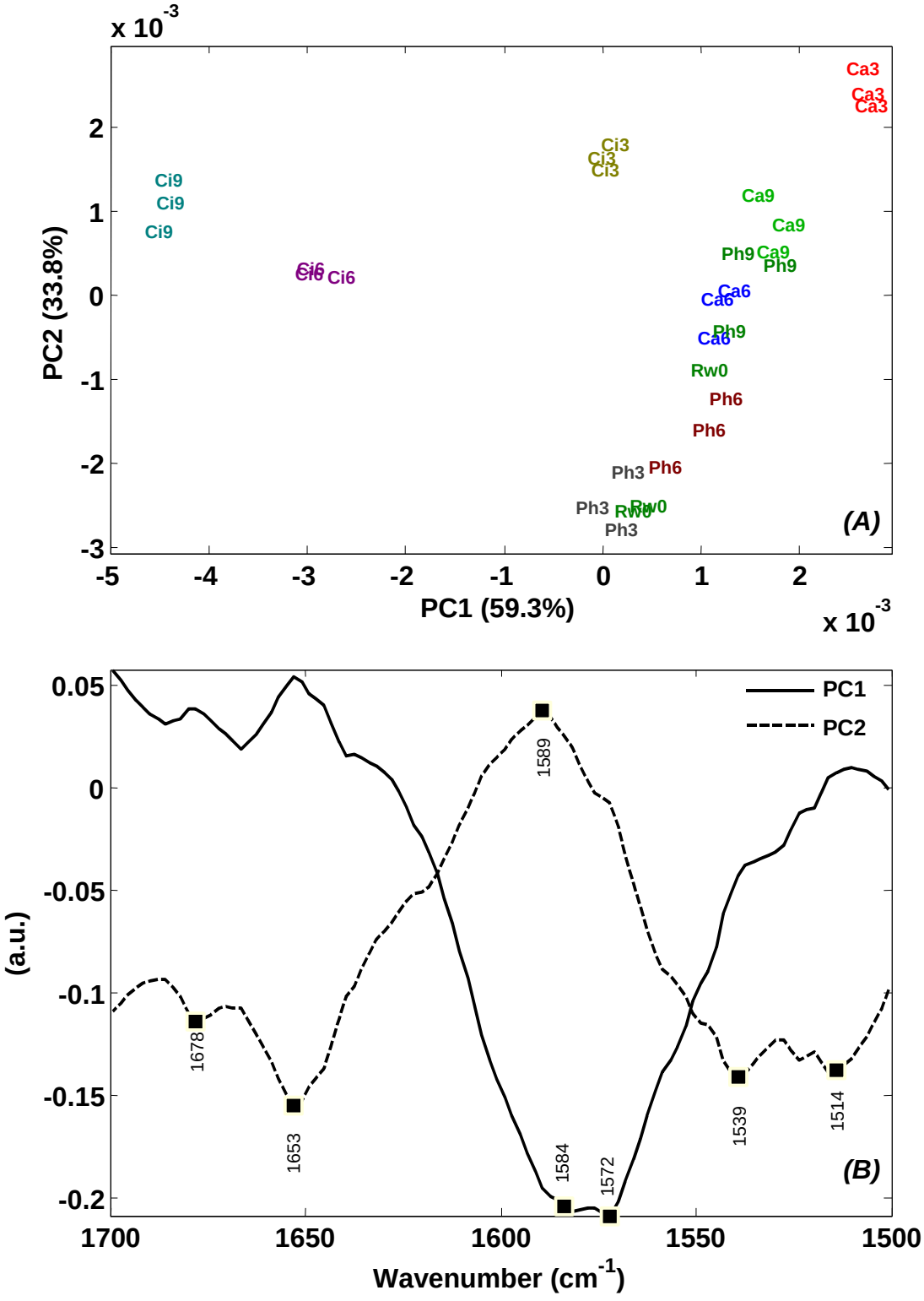


Figure 3 : Boubellouta et al



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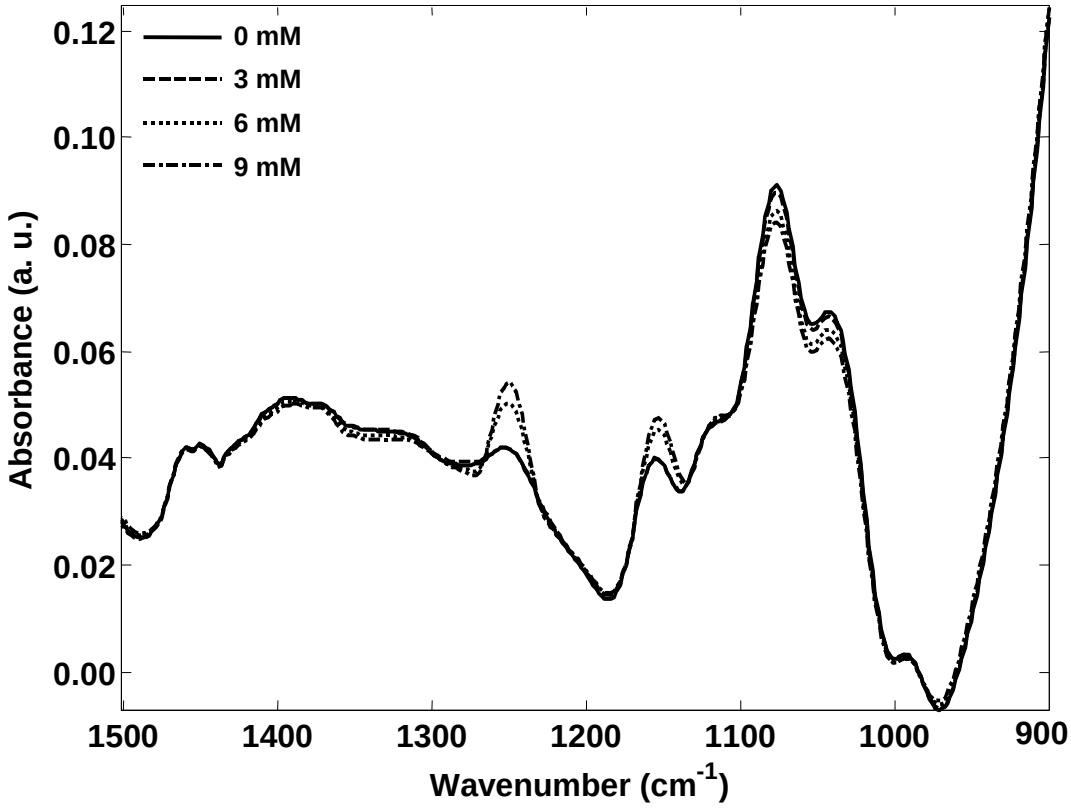

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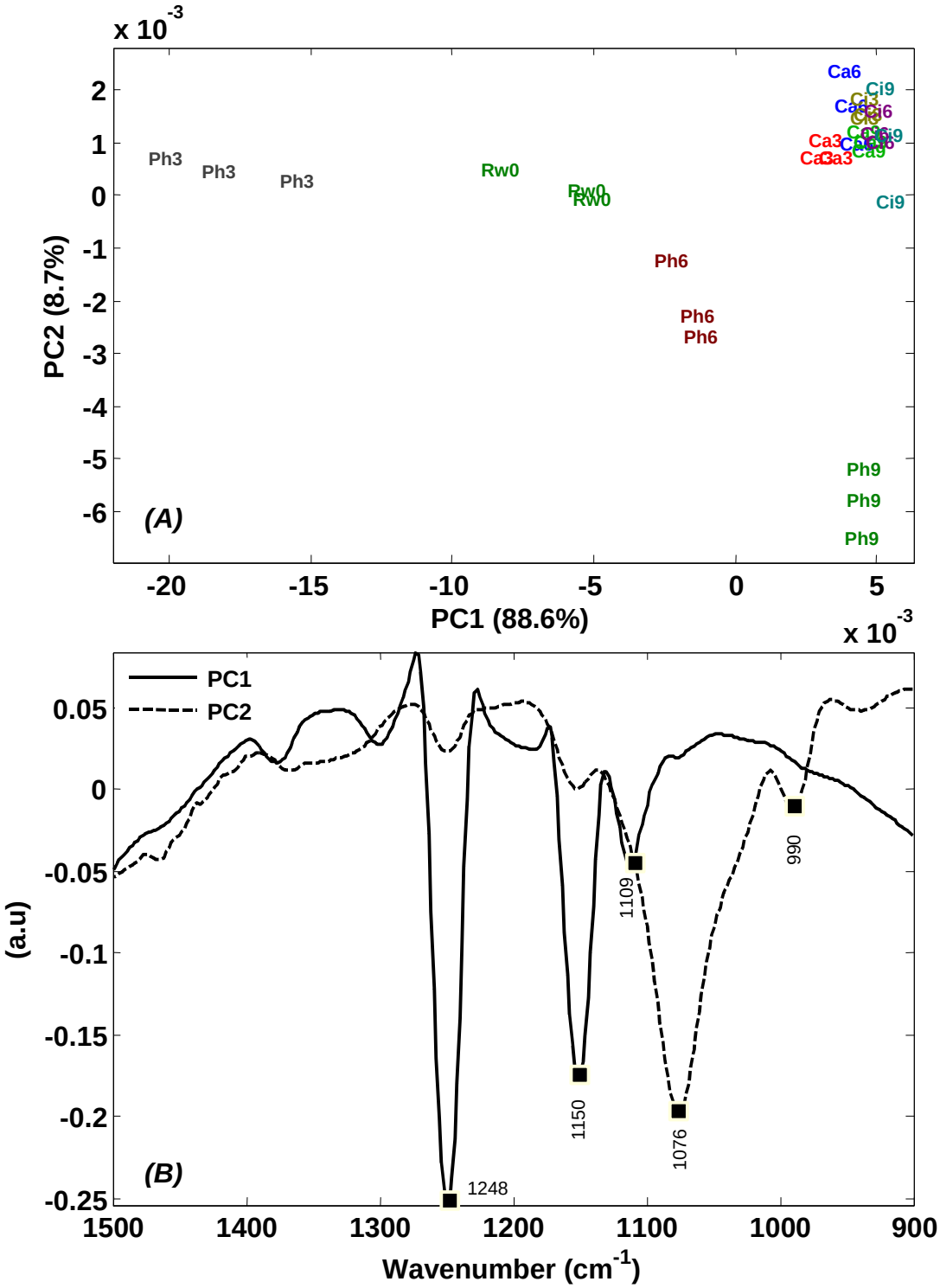
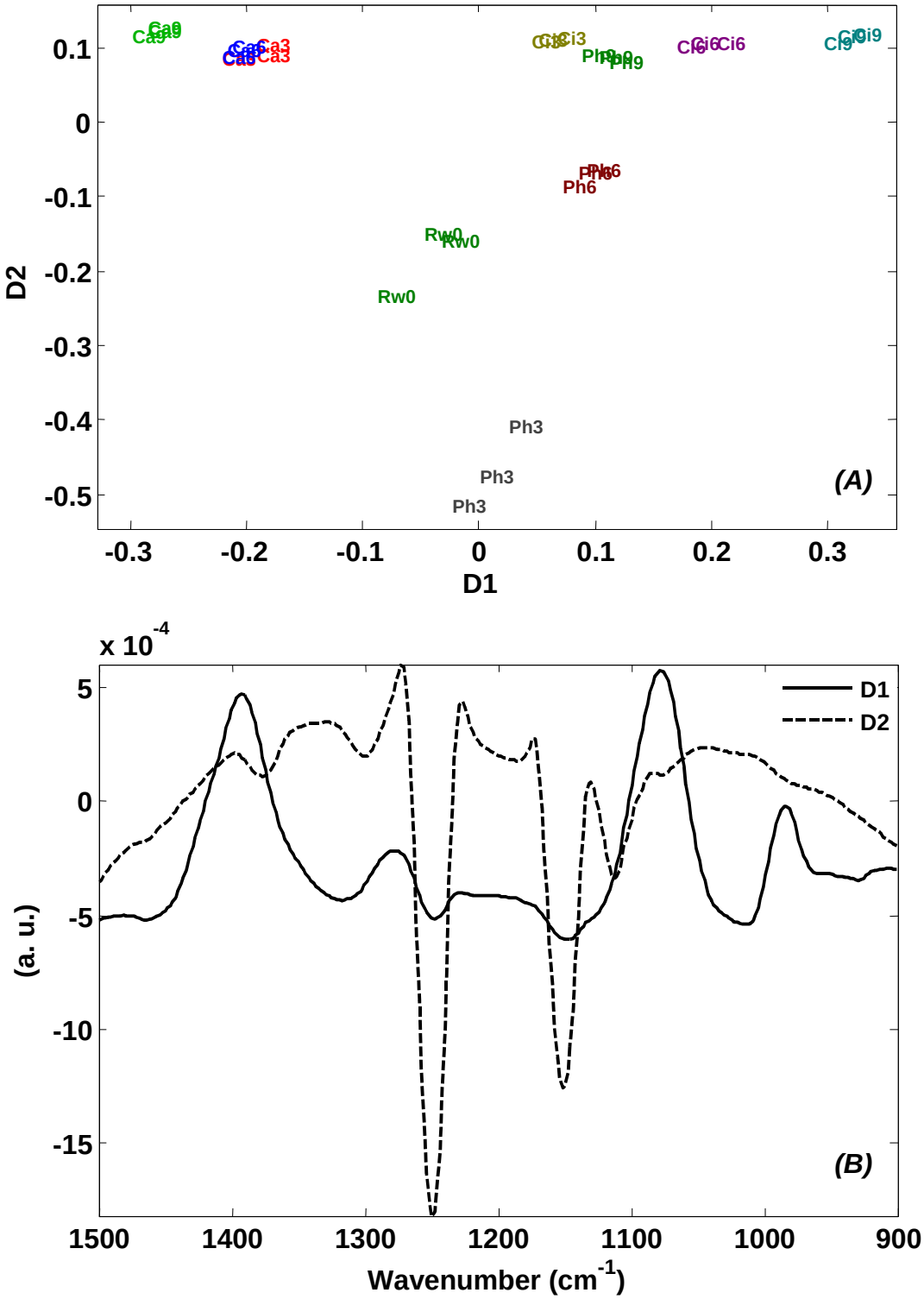


Figure 6 : Boubellouta et al



Structural changes of milk components during coagulation as studied by spectroscopic methods. 1 – Acid-induced coagulation of milk investigated at different temperatures.

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ABSTRACT

Milk coagulation is the primary step in the development of the texture of most dairy products. Front-face synchronous fluorescence and mid infrared spectroscopies have been used to investigate structure evolution, at a molecular level, during milk coagulation. This study was performed using skim milk at two different temperatures (30°C and 40°C) added with glucono-δ-lactone to generate gels exhibiting different textures and structures. Synchronous fluorescence spectra were recorded in the 250–500 nm excitation wavelength range using offset of 80 nm between excitation and emission monochromators for each system during the 300 min coagulation kinetics. Regarding mid infrared spectroscopy, the region located between 1700–1500 cm^{-1} corresponding to the amide I and II bands, and the 1500– 900 cm^{-1} region called the fingerprint region were considered for the characterization of milk coagulation kinetics. Principal component analysis was applied to the collections of fluorescence and infrared spectral data of the two systems to optimize their description. The results show that synchronous fluorescence and infrared data allowed the detection of the structural changes in casein micelles during coagulation and the discrimination of the different dynamics of the two systems. The rapid and non-invasive methods developed in this study allowed the investigation of network structure development and molecular interactions during milk coagulation.

Keywords: Milk, acidification, coagulation, molecular structure, synchronous front-face fluorescence, mid-infrared spectroscopy, chemometrics.

INTRODUCTION

Milk coagulation is the primary step in the production of most dairy products. Coagulation can be induced by acid, rennet, or both. In the case of the acid-induced gelation, upon lowering the pH of milk, colloidal calcium phosphate is solubilised from casein (CN) micelles, micellar disintegration takes place and the CN associate to form a gel (1, 2). The major driving force for protein-protein interactions during the acid coagulation of CN micelle are hydrophobic interactions (3). However, electrostatic and hydrogen bonds contribute to the specificity and stability of the interactions and, as a consequence, to specific structures. The kinetics of coagulation, influenced by temperature, and the structural aspects of protein-protein and protein-fat globule interactions determine the rheology properties of gels and thus their syneresis behaviour, as well as the texture of the final product. Water binding to the caseins plays also a very important role in influencing the physical and functional properties of these proteins in food systems (4). Several factors determine the special nature of water binding to the caseins: the hydrophobicity of the caseins, a loose packing density (or high voluminosity), and the extent of phosphate hydration in the caseins.

Mid-Infrared (MIR) and fluorescence spectroscopies have become increasingly important for a wide variety of analytical applications in biology and chemistry. This is due to great technical advances in both instrumentation and data analysis tools in the past two decades. Coupled with chemometric tools such as Principal Component Analysis (PCA), Principal Component Regression (PCR), Factorial Discriminant Analysis (FDA) and Partial Least Squares (PLS) regression (5,6,7), information on composition, physicochemical properties and molecular structure of food systems can be extracted from fluorescence and infrared spectra in an accurate, high sensitive and rapid manner (8,9).

Fluorescence spectroscopy is a rapid and sensitive method for quantifying a fluorescent component and characterising its molecular environment in all sorts of biological samples. This environmental sensitivity enables to characterise conformational changes such as those attributable to the thermal, solvent or surface denaturation of proteins, as well as the interactions of proteins with ligands.

While the techniques used in conventional fluorescence spectroscopy allow to record excitation spectra or emission spectra, the excitation wavelength - λex, and the emission wavelength - λem, are scanned synchronously with a constant wavelength interval, $\Delta\lambda = \lambda em - \lambda ex$ in synchronous fluorescence spectroscopy (SFS) (10). For well defined absorption and quantum yield maxima, the optimum value of the offset $\Delta\lambda$ is set by the difference in

wavelength of the emission and excitation maxima which is known as Stoke's shift (11). SFS makes it possible to narrowing of the spectral band and simplify the emission spectrum. In addition, SFS presents an interesting advantage from our point of view: a synchronous fluorescence spectrum retains information related to several fluorophores, compared to a classical emission spectrum that is mainly specific of a sole fluorophore.

Milk contains intrinsic fluorophores such as vitamin A and tryptophan residues present in fats and proteins, respectively, which give typical excitation and emission spectra. These spectra provide specific information on the characteristics of fats and proteins and on the environment of the fluorescent probes in food samples allowing to investigate structural changes (8). For example, the melting temperature of fat in cheese has been determined from vitamin A fluorescence spectra recorded at different temperatures (12).

MIR represents the spectrum of the absorption of all the chemical bonds having an infrared activity between 4000 and 400 cm^{-1}. The acyl-chain is mainly responsible for the absorption observed between 3000 and 2800 cm^{-1}, whereas the peptidic bound C-NH is mainly responsible of the absorption occurring between 1700 and 1500 cm^{-1}. As water also strongly absorbs in the Amide I region, the changes of pattern of the casein-bound water with pH-driven structural changes of the micelles alter the shape of the spectra in this region. Indeed, it has been reported in the literature that the presence of molecule such as sucrose (13) or NaCl (14) in water modifies the shape of the MIR spectrum of water: the shape of water band at about 1650 cm^{-1} is sharpened and its intensity increases in the presence of NaCl. Thus, care must be exercised not to overinterpret these observed spectral changes in the Amide I region in terms of variation of the secondary structure of caseins, but rather in terms of hydration changes (15). Finally, the 1500 and 900 cm^{-1} region is known to exhibit bands attributed to peptidic bonds and phosphate.

In the present work, front-face synchronous fluorescence and mid-infrared spectra in combination with multivariate statistical analysis are used to investigate, at a molecular level, structure evolution during milk acid-induced coagulation processes at two temperatures. Moreover, spectral data recorded during the gelation kinetics of milk coagulation processes are compared to rheology measurements.

MATERIAL and METHODS

- *Milk samples*

Bovine milk samples were collected from the tank of a local farm and a given milk sample was used in the course of the day. The milk was skimmed by centrifugation at 30 C using a Electrem centrifuge (Electrem, France).

- *Coagulation system*

Milk coagulation was performed by progressive acidification with glucono-δ-lactone (GDL, Sigma-Aldrich Chemie GmbH, Heidenheim, Germany) added at the concentration of 1.75 g-L^{-1}. The coagulation kinetics were realised at 30 and at 40 °C. The pH was measured every 5 min until 300 min using a pH meter HQ30d (Hach Company, USA). For each temperature, three kinetics were recorded.

- Mid-infrared spectra

Infrared spectra were recorded between 4000 and 900 cm^{-1} at a resolution of 4 cm^{-1} on a Varian 3100 FT-IR Excalibur Series Fourier-transform spectrometer (Varian Inc., Palo Alto, USA) mounted with a thermostated ATR accessory equipped with a grip. The ATR cell is 6 reflections and was made of a horizontal ZnSe crystal which presented an incidence angle of 45°. The temperature was controlled by a Julabo temperature controller (Julabo, Germany).

Milk samples added with GDL were placed on the crystal. In order to improve the signal to noise ratio, 32 scans were co-added for each spectrum. For each kinetic, the spectra were recorded at 30 and 40 °C in triplicate. Before each measurement, the spectrum of the ZnSe crystal was recorded and used as background. Base line and ATR corrections were applied to the spectra using Varian Software (Resolution Pro 4.0).

The regions of the mid infrared spectra located between 1700 and 1500 cm^{-1} (protein region) and 1500 and 900 cm^{-1} (fingerprint region) have been considered in this study.

- Synchronous fluorescence spectra

Synchronous fluorescence spectra were recorded using a FluoroMax-2 spectrofluorimeter (Spex-Jobin Yvon, Longjumeau, France) mounted with a front-surface cuvette-holder and the incidence angle of the excitation radiation was set at 56° to ensure that reflected light, scattered radiation, and depolarisation phenomena were minimized. The spectrofluorimeter was equipped with a thermostatically controlled cuvette-holder and the temperature was controlled by a Haake temperature controller (Haake, Champlan, France).

Synchronous fluorescence spectra were collected in the 250–500 nm excitation wavelength range using offset of 80 nm between excitation and emission monochromators (16). For the experiments, the synchronous spectra of milk samples plus GDL placed in quartz cuvette (2 cm x 1 cm x 1 cm) were recorded at 30 and 40 °C. For each temperature, three time kinetics were performed using different samples.

- Dynamic oscillatory experiments

Milk samples added with GDL were placed on cylinder disks (2 mm thick and 20 mm diameter). The dynamic oscillatory experiments were performed with a rheometer (CP 20, TA Instrument, Guyancourt, France) with plate geometry of 20 mm diameter. Oscillation experiments were performed in the linear viscoelastic region by applying a constant force of 0.1 N and a constant frequency of 1 Hz. All the experiments were carried out at temperatures of 30 and 40 °C by applying a Peltier plate that provided very accurate and rapid temperature control. The data obtained included the two components of shear modulus G*, i.e., the elastic component G' (storage modulus) and the viscous component G" (loss modulus). The complex viscosity (η*) and tan δ were also measured. Data were recorded every 5 min until 300 min. For each temperature, three kinetics were recorded.

Chemometrics **:**

- *Principal Component Analysis (PCA)*

PCA is a multidimensional data treatment which provides a synthetic description of large data sets. When applied to spectral data, PCA allows similarity maps of the samples to be drawn and spectral patterns obtained (17,18). PCA was applied to the spectra in order to investigate differences in the spectra.

RESULTS AND DISCUSSION

This study was aimed at investigating milk structural evolution at a molecular level using front-face synchronous fluorescence and mid infrared spectroscopies. More precisely, we focused on changes of protein environment during the milk coagulation process. This approach requires the ability to discriminate between different dynamic and structural

changes. This study was conducted using acid-induced milk coagulation processes at different temperatures known to yield different structures and textures (19). The physico-chemical properties of the 2 systems (30 and 40°C) were characterized in a preliminary phase.

Physico-chemical properties of the two systems

pH evolution

Figure 1 shows changes in the pH of the two systems. For the two temperatures, pH varied from 6.70 to 4.25 after 300 min of kinetic, but the pH drop during the first step of the kinetic was faster at 40°C than at 30°C.

Rheological properties

The evolutions of the elastic modulus (G') recorded *versus* time during the GDL-induced coagulation phase of the milks at 30 and 40°C are shown in Figure 2. All the samples showed a pre-gel stage characterised by a G' modulus close to the 0 Pa value. The method chosen to evaluate the gel point consisted in a linear extrapolation of the rapidly rising elastic modulus G' to the intercept with the time axis. The change in the G' modulus over the given time span was very different for the two coagulation procedures. At 30°C, the GDL system had a gelation time of 102±6.3 min and, then, the G' modulus increased till 300 min. At 40°C, the gelation time was at 57±2.9 min and G' modulus increased rapidly till 100 min followed by a slower increase up to 300 min. The strength of the gel obtained at 40°C after 300 min is lower (28.45 ± 0.36 Pa) than the one (37.80 ± 0.43 Pa) at 30°C.

Network structure formation as studied by synchronous fluorescence spectroscopy. Effects of temperature on the kinetics

Figure 3 presents skim-milk synchronous spectra ($\Delta\lambda$ = 80 nm) recorded at 30 °C during the coagulation kinetic for 5, 50, 105 and 300 min. The skim-milk synchronous spectrum recorded at 30 °C exhibited an intense band at 290 nm (emission at 370 nm), a small shoulder at 320 nm (emission at 400 nm), a band at 370 nm (emission at 450 nm) and a broad band centered at 460 nm (emission at 540 nm). The intense band observed at 290 nm (emission at 370 nm) can be attributed to tryptophan residues of proteins (16). The weak shoulder centred at 320 nm has the same location to the one found in the vitamin-A excitation spectrum recorded on full-fat milk (16): it is well known that skim milk retains minute amounts of fat exhibiting vitamin A. This hypothesis is also confirmed by the weak mid-infrared absorption

bands observed between 3000 and 2800 cm^{-1} (data not-shown). The broad bands centered at 370 nm and 460 nm may be attributed to NADH and riboflavin (20), respectively. During the acidification kinetic, a decrease of the fluorescence intensity was observed at 290 nm and 460 nm, whereas the intensity of the band at 370 nm was increasing. Considering riboflavin, it is generally bound to proteins. Riboflavin binding protein - a monomeric, two-domain protein - has been originally purified from hens' egg white (21). In plasma, it has been shown that riboflavin is bound to proteins, predominantly albumin, but also to immunoglobulins: this vitamin may bind to a large number of proteins. Like others fluorophores, the fluorescence emission of riboflavin is highly sensitive to its local environment, and it can be used as an indicator group for protein conformation and interaction changes in cheese matrices during ripening since it interacts with proteins (22).

A weak shift toward higher wavelengths of the band at 290 nm was observed (Figure 3): it agreed with the changes in protein interactions, leading to a more hydrophilic environment for tryptophanyl residues during the time span of the reaction. In addition, the band centred at 370 nm is broadening during the acidification kinetic at 30°C.

Similar spectra were observed during the acidification kinetic performed at 40°C (data not shown).

PCA was applied to the set of spectra recorded during acid-induced coagulation at 30°C in order to obtain additional structural informations. This method is well suited to optimize the description of data collection by extracting the most useful data and rejecting redundant data. Figure 4a shows a PCA similarity map defined by the principal components 1 and 2 for synchronous fluorescence spectral data of the acidification kinetic at 30°C. The first two principal components accounted for 98.6% of the total variability with a predominance of component 1 (91.7%). A separation of spectra was observed as a function of time according to component 1. The spectra recorded between 5 and 50 min had negative scores according to PC1 and were separated mainly on PC2 and reaching a minimum for 50 min, whereas spectra recorded between 50 and 105 min were separated principally on axis 2 with a maximum for 105 min. The spectrum recorded at 50 min corresponded to a pH = 5.3 and the spectra obtained between 50 and 80 min were only discriminated according to PC2. After 80 min kinetic, the spectra started to be discriminated according to PC1, despite a predominance of PC2. Finally, spectra recorded between 105 (gelation point) and 300 min were mainly separated according to PC1. Considering PCA similarity map defined by the principal components 1 and 3 (Figure 4b), it appeared that PC 3 (0.7%) discriminated the spectra

recorded before gelation point, whereas PC1 separated the spectra recorded after milk gelation point. These results show that acidification and gelation of milk induce different modifications in the fluorescence properties of milk intrinsic fluorophores. However, considering the data variance explained by principal components 1 and 3, it appeared that gelation modified milk fluorescence properties more dramatically than acidification.

Regarding the results (data no shown) of PCA applied to the spectral data recorded at 40°C, the spectra obtained between 5 and 85 min were discriminated according to PC 2 (7.3%), whereas spectra recorded between 85 and 300 min were principally separated on PC 1 (92.2%). The discrimination according to PC2 of spectra recorded between 5 and 85 min showed in fact two phases with the inflexion point corresponding to the gelation time (55 min).

The spectral patterns associated with principal components 1, 2 and 3, providing the most discriminant wavelengths of the spectral data set recorded at 30°C, are presented Figure 5. Considering spectral pattern 1, the most discriminant wavelengths were 281, 371 and 450 nm. The contrast between negative peaks at 281 and 450 nm and a positive one at 371 nm indicated that, during the time course of the coagulation process, the fluorescence intensities at 281 and 450 nm decreased, whereas the fluorescence intensity at 371 nm increased for all the spectra discriminated according to PC 1. The spectral pattern associated with PC 2 exhibited a contrast between two positive peaks at 260 and 300 nm and two negative ones at 322 and 450 nm. It indicated a fluorescence transfer (23) between tryptophanyl residues of caseins and vitamin A located in the membrane of residual fat globules present in skim milk. This is in agreement with an interaction of caseins with fat-globule membrane during the formation of the protein network since PC 2 discriminated spectra recorded between 5 and 105 min. The spectral pattern associated with PC 3 showed an opposition between a negative peak at 281 nm and a positive one at 301 nm suggesting a shift to higher wavelengths of the peak centered at 290 nm during the first 80 min of the kinetic at 30°C. This spectral pattern was also characterized by a negative peak at 322 nm and a positive one at 482 indicating changes in the environment of vitamin A and riboflavin, respectively.

Changes in the fluorescence intensities at 281 nm as a function of pH for kinetics recorded at 30°C and 40°C are shown in Figure 6. Considering fluorescence intensity at 30 °C, six regions may be distinguished: a first phase from pH 6.19 to pH 5.52, a second domain characterized by a plateau, a third one between pH 5.31 and pH 5.10 showing an increase of the fluorescence followed by a decrease for pH from 5.10 till 4.91, a fifth phase characterized

by a plateau for pH ranging between 4.91 and 4.79 and finally a sharp decrease of fluorescence for the sixth phase from pH 4.79 to pH 4.38. Changes in the fluorescence intensities at 281 nm as a function of pH for kinetic recorded at 40°C showed similar trends than the one observed at 30°C, with most of the differences in the pH region ranging between 6.2 and 5.0.

These modifications in the fluorescence properties of milk proteins can be paralleled with the well known effects of pH on micelle structure and milk gelation (1,24,25,26,27,28). Indeed, the alteration of micelle structure during milk acidification involves two main steps. The first step from pH 6.7 to pH 5.3-5.1 is characterized by the solubilisation of micellar calcium phosphate (23, 6, 11). Between pH 5.8 and pH 5.3-5.1, solubilisation of micellar calcium became faster and at pH 5.3-5.1, practically all the micellar inorganic phosphate was transferred to the serum phase. Whereas about 14-17% of the calcium was still present in the micelles (25,27,28). In this study, the end of the first phase corresponds to pH 5.1. It has also been reported that the release of calcium phosphate on lowering the pH involved the dissociation either of β-CN only from micelles into the serum phase (24, 25) or of all CN - αs1-, αs2-, β-, and κ-CN, from the micelles (26,27). However, CN dissociation is temperature-dependent and, at 30°C, only less 10 % of total CN dissociated (26). Moreover, this first phase was characterized by a diminution in micelle voluminosity (24, 25, 27) from pH 6.7 to pH 6.0-5.8 and by an increase in the voluminosity of CN micelle with a maximum at pH 5.4-5.2 which is attributed to the swelling of the micelles and the dissociation of CN (24, 25). During this first step, a partial micellar disintegration took place, which caused a looseness in the micelle. The second stage from pH 5.2 to pH 4.45 is marked mainly by the reabsorption of CN and the aggregation of micelles involving the formation of new particles completely different in structure and composition from the original micelles (1, 27). These changes induced a large decrease in the voluminosity of CN micelle, with a minimum at pH 4.6 (24, 25) and, from pH 4.6, the voluminosity increases again (24), leading to the formation of the final network of the acid milk gel (1, 27).

The change in fluorescence intensity at 281 nm paralleled the changes of CN micelle structure induced by lowering of the pH. It appears that the change in the fluorescence intensity at 281 nm reflects pH-induced physico-chemical changes of CN micelles and, in particular, structural changes in the micelles. These changes induced specific modifications in the shape of the synchronous fluorescence spectra and the PCA maps defined by the PC 1 and 2 and PC

1 and 3 described the modifications of protein structure and interactions during milk coagulation.

Network structure formation as studied by infrared spectroscopy. Effects of temperature on the kinetics

Most of the spectral information useful for the analysis of the mid-infrared spectra is located in: (1) the 3000–2800 cm^{-1} range corresponding to C-H stretching, (2) the region located between 1700–1500 cm^{-1} corresponding to the amide I and II bands, and (3) the 1500– 900 cm^{-1} region called the fingerprint region.

The 3000–2800 cm^{-1} spectral region corresponds to the C–H band of methyl and methylene groups of fatty acids. As skim-milk was considered in this study, only very weak bands were observed in this region. The 1700–1500 cm^{-1} region was characterised by the presence of bands related to peptides and proteins. Thus, these bands contain some information on the proteins and on the interaction of these proteins with other components, such as ions, water and other proteins. Contribution to the amide I band, which is used to investigate the secondary structure of proteins, can be observed at about 1638 cm^{-1} (data no shown). The absorption band with a maximum at about 1553 cm^{-1} is generally assigned to the amide II vibrations. The 1500–900 cm^{-1} region is called the fingerprint region and numerous chemical bonds are absorbing in this region (Figure 7). Considering the recorded spectra, the important differences were observed at the strong absorbing bands located at 1100 and 1000 cm^{-1}. These bands could be related to P=O stretching (29). Indeed, this author reported that PO^{2-} and PO^{3-} compounds present bands in the 1323–1092 cm^{-1} and 1140 and 1065 cm^{-1} regions, respectively. Additional smaller bands were also observed at about 1240, 1400 and 1450 cm^{-1}.

In order to obtain a more detailed description of spectral variations during kinetics, a second derivative was applied to the infrared spectra. Then, PCA was realized to the amide I and II derivative spectral data recorded at 30°C and 40°C in order to get a deeper insight into casein conformational changes during gelation. Considering the results of PCA performed on spectral data recorded at 30°C, the first two principal components took into account 99.3% of the total inertia. Figure 8A shows the similarity map defined by principal components 1 and 2. As a discrimination of the spectra recorded between 5 and 50 min was observed along the second principal component, the first principal component discriminated the sample recorded between 50 and 300 min. Furthermore, the similarity map defined by principal components 1 and 3 (Figure 8B) showed an interesting pattern. The spectra recorded before gelation (5 to

105 min) had negative scores according to component 1 and were separated according to principal components 1 and 3, whereas spectra recorded after milk gelation (110 to 300 min) had positive scores according to principal component 1 and were essentially separated on principal component 3. It was noticed that mid-infrared spectra recorded at 30°C in the Amide I & II region allowed to characterize three phenomena during the gelation kinetic. The spectral pattern corresponding to the principal component 1 is given Figure 9 and revealed characteristic wavenumbers at 1650, 1624 and 1545 cm^{-1}. It roughly corresponded to mean spectrum of proteins in this spectral region. Regarding the third principal component, the spectral pattern (Figure 9) exhibited a major positive peak at 1618 cm^{-1} and a minor one at 1530 cm^{-1}, as well as a large negative peak at 1652 cm^{-1} and a small one at 1575 cm^{-1}. As addressed in the introduction section, we suggest that the opposite peaks at 1652 and 1618 cm^{-1} depicted the changes of casein-bound water during the kinetics, rather than the transformation of α-helix into intermolecular β-sheets. It is well known that water also strongly absorbs in the amide I region and the changes of pattern of the CN-bound water with pH-driven structural changes of the micelles alter the shape of the spectra in this region. Indeed, it has been reported in the literature that the presence of molecule such as sucrose (13) or NaCl (14) in water modifies the shape of the MIR spectrum of water: the shape of water band at about 1650 cm^{-1} is sharpened and its intensity increases in the presence of NaCl.

Changes in the absorbances at 1618 cm^{-1} as a function of pH for kinetics recorded at 30°C and 40°C are shown in Figure 10. Considering the kinetic performed at 30°C, the absorbances showed a minimum for pH ranging between 5.4 and 5.2 (corresponding the inflexion point observed at 50 min in Figure 8A) which may be related with an increase of micelle voluminosity, and as a consequence of micelle hydration. It has been reported that a maximum in the CN micelle voluminosity is at pH 5.4-5.2 which is attributed to the swelling of the micelles and the dissociation of CN (24, 25). Then a second stage occurring from pH 5.2 to pH 4.45 is marked mainly by the reabsorption of CN and the aggregation of micelles involving the formation of new particles completely different in structure and composition from the original micelles (1, 27). These changes induced a large decrease in the voluminosity of CN micelle which may be associated with the increase of the absorption at 1618 cm^{-1}.

The acidification kinetic of milk performed at 40°C showed different trends as shown in Figure 10, i.e., there was no absorbance minimum at 1618 cm^{-1} for pH 5.25 as observed at 30°C. We concluded that temperatures of 30°C and 40°C have different effects on the changes in micelle structure, as well as micelle hydration: the maximum in the CN micelle voluminosity characterized at pH 5.4-5.2, which is attributed to the swelling of the micelles

and the dissociation of CN (24, 25), was only observed at 30°C.

PCA was applied to 1500–900 cm^{-1} spectral data recorded at 30°C and 40°C in order to get a deeper insight into phosphate-state changes during acidification. Considering the results of PCA performed on spectral data recorded at 30°C, the first two principal components took into account 97.9% of the total inertia. Figure 11A shows the similarity map defined by principal components 1 and 2 and a discrimination of the spectra recorded between 5 and 60 min was observed along the second principal component. The first principal component discriminated the sample recorded between 60 and 300 min with a minimum for 105 min corresponding to the gelation time. Furthermore, the similarity map defined by principal components 1 and 3 (Figure 11B) showed an interesting pattern: the third principal component discriminated spectra recorded between 105 and 300 min.

Regarding the principal components 1, 2 and 3, the spectral patterns (data no shown) exhibited major positive peaks at 1010 (PC1), 1060 (PC1), 1063 (PCs2 and 3) and 1110 (PC3) cm^{-1} and large negative peaks at 1003 (PC3), 1030 (PCs 2 and 3) and 1100 (PCs 1 and 2) cm^{-1} in the 1150–1000 cm^{-1} region.

Changes in the absorbances at 1063 cm^{-1} as a function of pH for kinetics recorded at 30°C and 40°C are shown in Figure 12. It appears that the change in the absorbances at 1063 cm^{-1} reflects pH-induced phosphate dissolution. This phosphate dissolution modifies physico-chemical characteristics of CN micelles and, in particular, induced structural changes in the micelles leading to coagulation.

Considering the absorbances at 1063 cm^{-1} at 30 °C, five regions may be distinguished: a first phase from pH 6.19 to pH 5.60, a second domain characterized by a sharper decrease till pH 5.25, a plateau between pH 5.25 and pH 5.05 followed by a increase of the absorbances for pH from 5.05 till 4.85, and finally a plateau for pH ranging between 4.85 and 4.35. Changes in the absorbances at 1063 cm^{-1} as a function of pH for kinetics recorded at 40°C showed similar trends than the one observed at 30°C, with most of the differences in the pH region ranging between 5.0 and 3.8. These data also showed that the state of phosphate was different at 40°C compared to 30°C for pH ranging between 5.0 and 3.8. It is well known that acid-induced milk coagulation processes at different temperatures yield different structures and textures (19).

CONCLUSIONS

It is common knowledge that the process used to coagulate milk has broad effects on the texture of the final product. Rheological methods are useful for characterizing the texture of milk products and cheeses. This method made it possible to show that the coagulation of milk by GDL at different temperatures investigated in this study showed different rheology properties. It is generally assumed that the properties of gels at a macroscopic level are related to their molecular structure. However, few techniques allow the investigation at a molecular level of turbid and complex products such as milk. Front-face synchronous fluorescence spectroscopy and mid infrared spectroscopy, two non-invasive techniques, in combination with chemometric methods make it possible to study the coagulation of milk at a molecular level. Indeed, the fluorescence properties of tryptophan residues are modified by the changes in micelle structure and protein interactions induced by milk acidification. This study shows that infrared spectroscopy is useful for characterizing the changes in micelle structure/hydration and in phosphate dissolution during milk gelation.

Using these spectroscopic methods, it has been possible to demonstrate that gels exhibiting different rheology properties have different structures at the molecular level. In addition, it was possible to follow the different steps of the gelation processes.

Spectroscopic methods such as fluorescence and mid infrared spectroscopy combined with chemometric tools have the potential to evaluate structure at the molecular level. Fluorescence and infrared data are spectra that allow us to derive information on the molecular structure and interactions of the dairy-product matrix. Moreover, it is also suggested that the phenomena observed at molecular (fluorescence, infrared) and macroscopic (rheology) levels are related to the texture of dairy products.

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Legends of the figures

Figure 1: Evolution of the pH during the milk coagulation kinetics at 30°C (○) and 40°C (●).

Figure 2: Evolution of the storage modulus (G') over a period of 300 min during the coagulation of milk at 30°C (○) and 40°C (●).

Figure 3: Synchronous fluorescence spectra recorded between 250 and 500 nm ($\Delta\lambda$ = 80 nm) during the coagulation kinetic at 30°C for 5, 50, 105 and 300 min.

Figure 4: PCA similarity map defined by PC1 and PC2 (A) and PC1 and PC3 (B) for synchronous fluorescence spectra recorded at 30°C.

Figure 5: Spectral patterns corresponding to the principal components 1 (—), 2 (---) and 3 (····) for the synchronous fluorescence spectra recorded at 30°C.

Figure 6: Evolution of the fluorescence intensity at 281 nm recorded at at 30°C (○) and 40°C (●).as a function of pH.

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Figure 8: PCA similarity map defined by PC1 and PC2 (A) and PC1 and PC3 (B) for 1700-1500 cm^{-1} mid-infrared spectra recorded at 30°C

Figure 9: Spectral patterns corresponding to the principal components 1 (—), 2 (---) and 3 (····) for 1700-1500 cm^{-1} mid-infrared spectra recorded at 30°C.

Figure 10: Evolution of the absorbance at 1618cm^{-1} for mid-infrared spectra recorded at 30°C (○) and 40°C (●) as a function of pH.

Figure 11: PCA similarity map defined by PC1 and PC2 (A) and PC1 and PC3 (B) for 1500-900 cm^{-1} mid-infrared spectra recorded at 30°C.

Figure 12: Evolution of the absorbance at 1063 cm^{-1} for mid-infrared spectra recorded at 30°C (○) and 40°C (●) as a function of pH.

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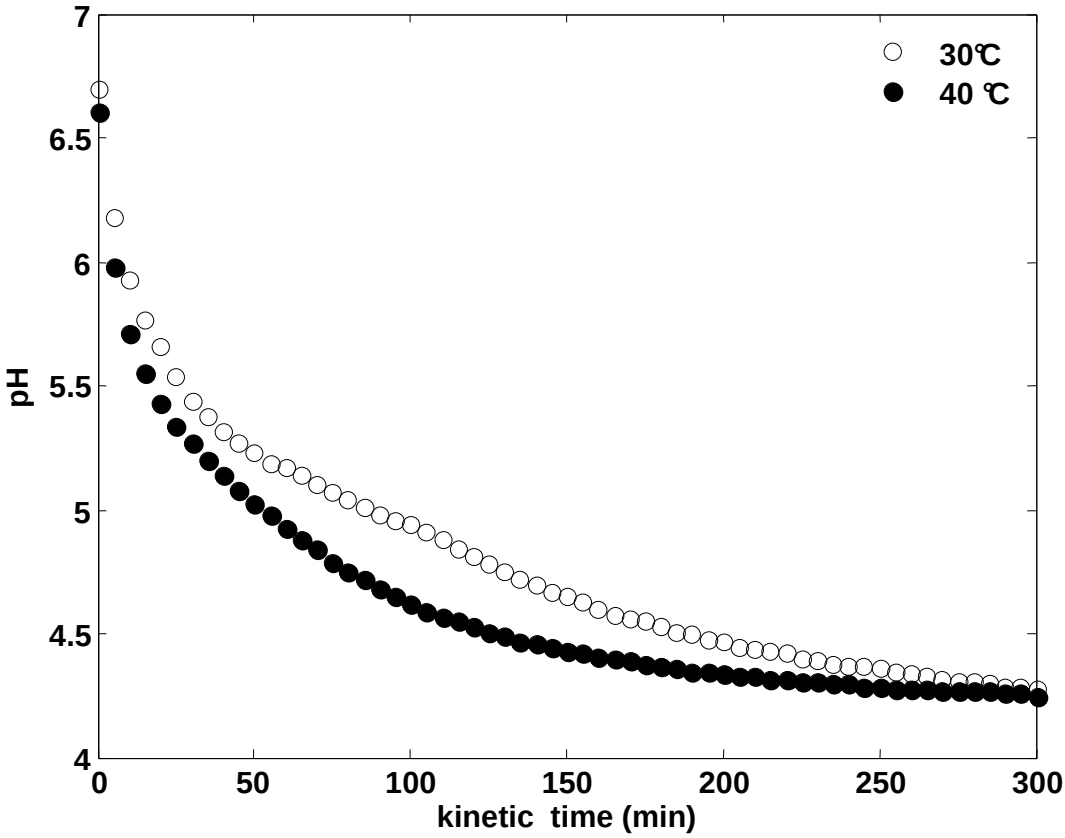

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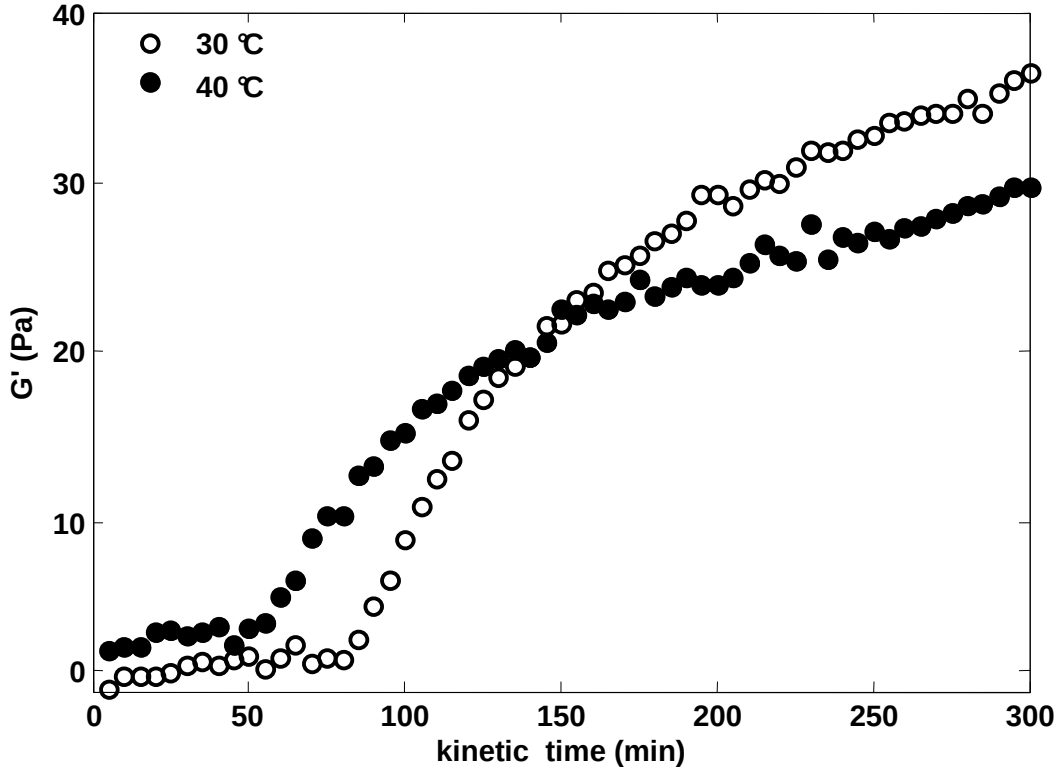

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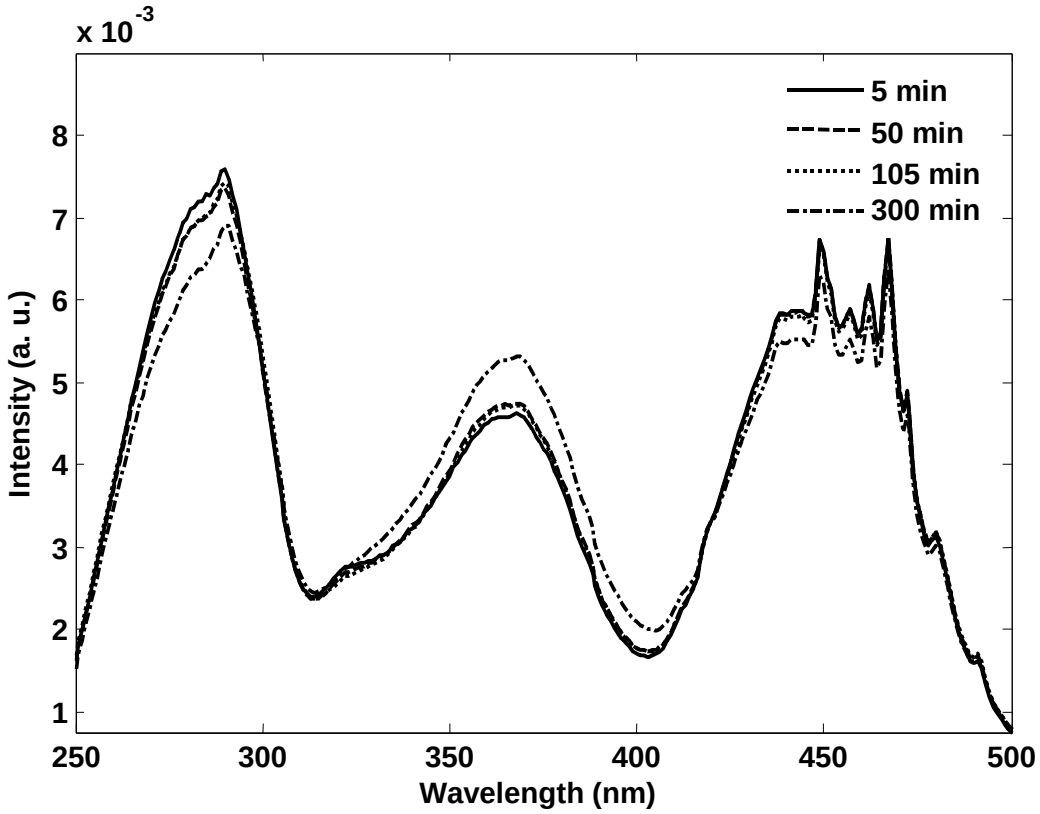

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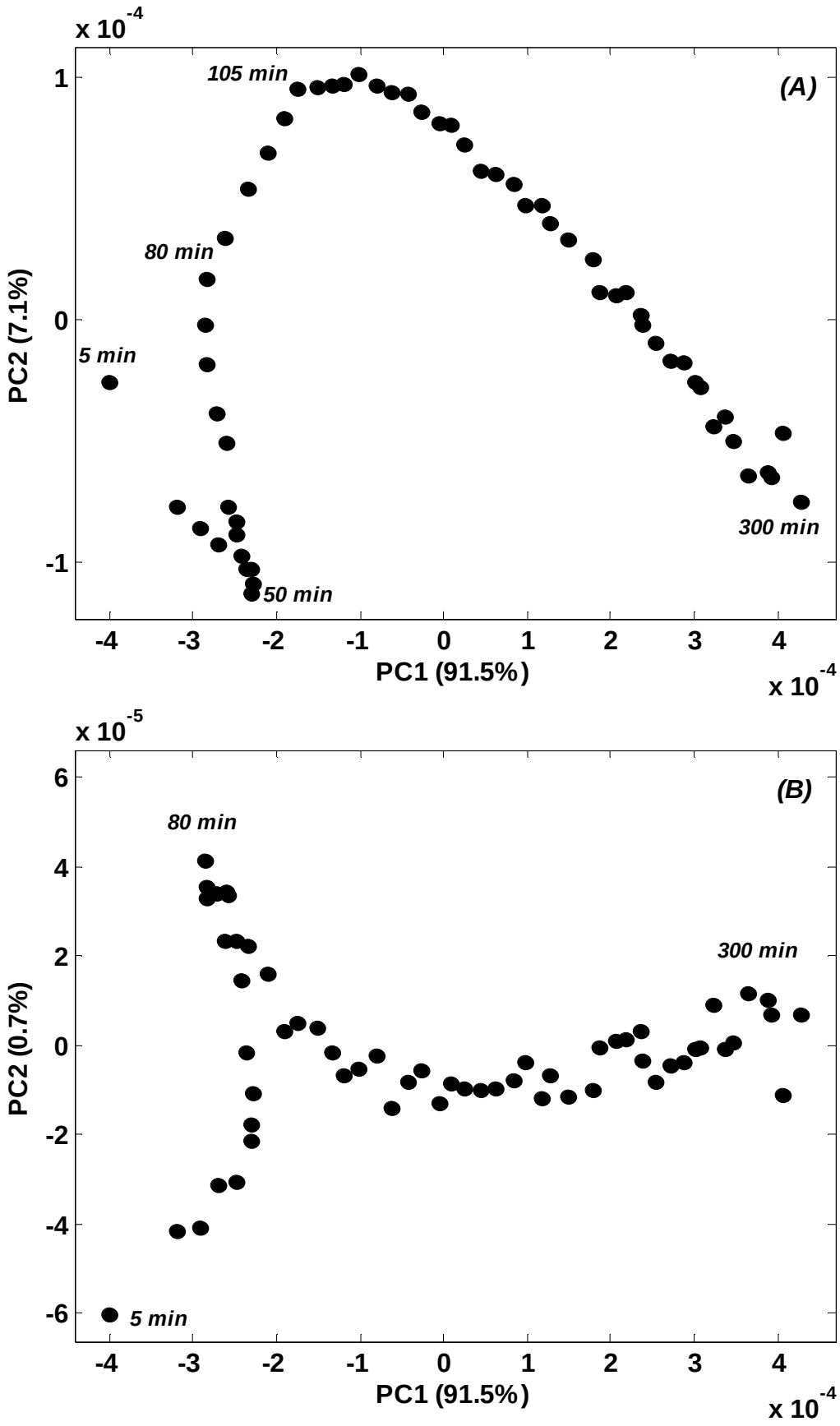

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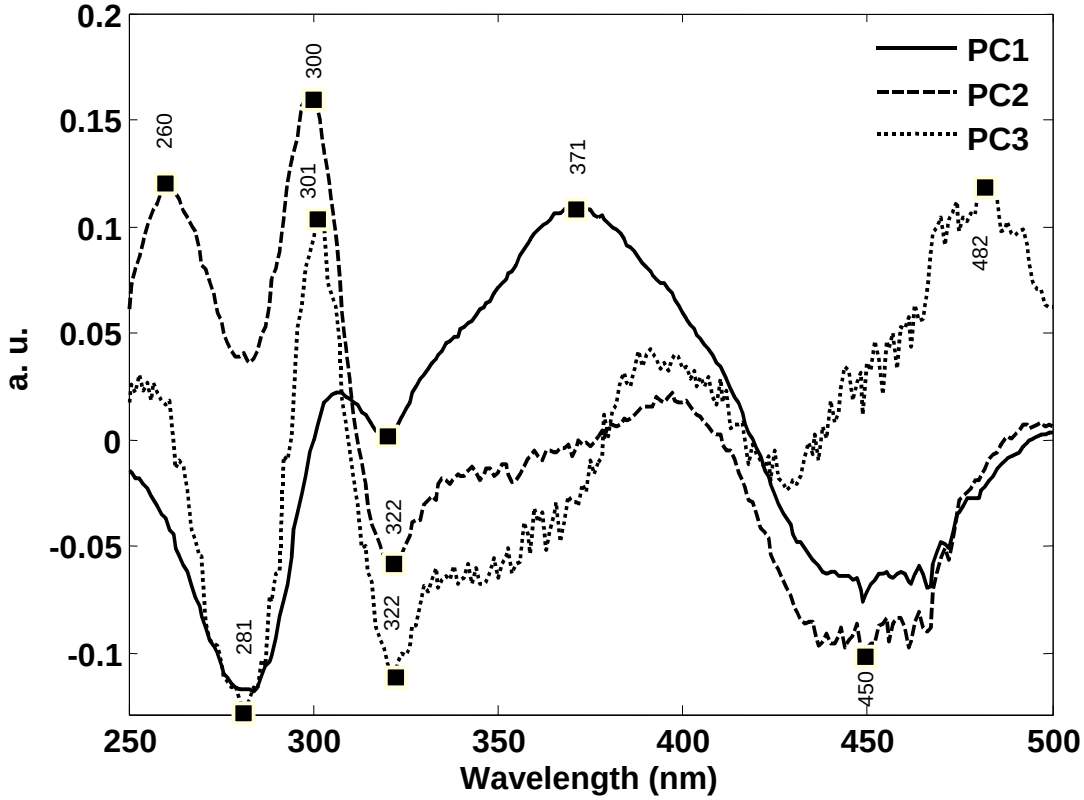

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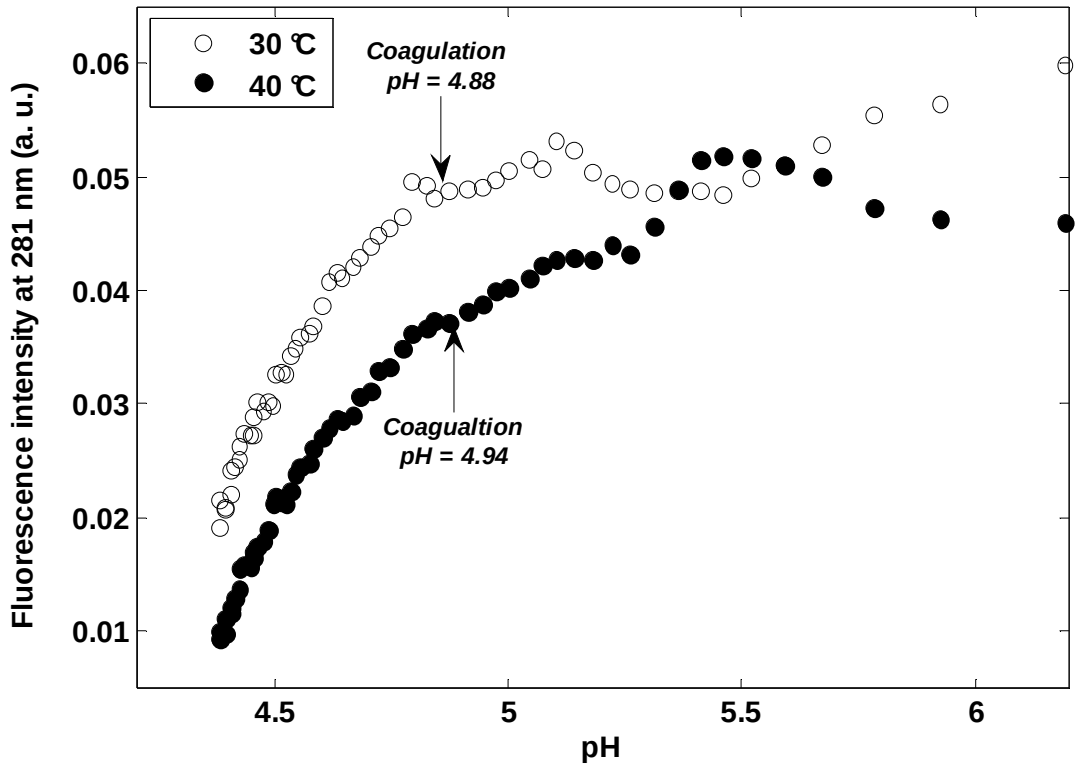

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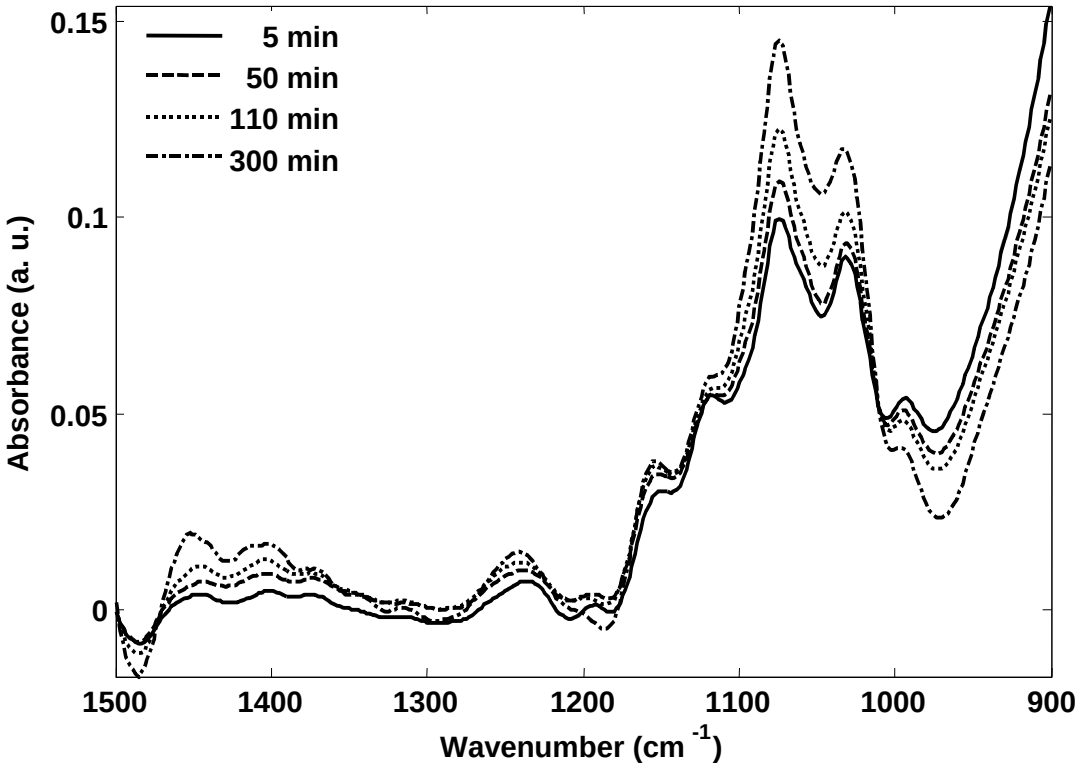

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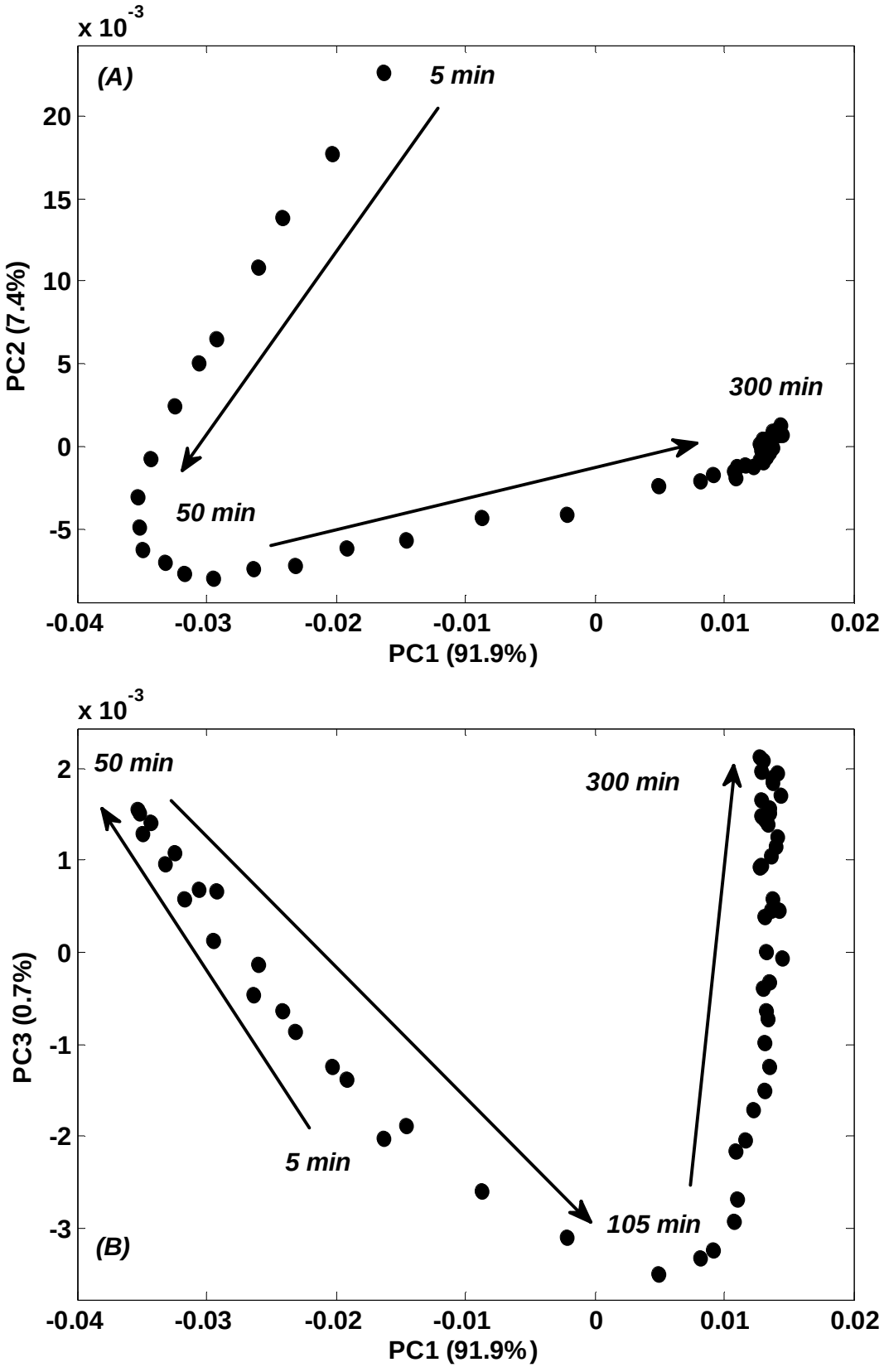

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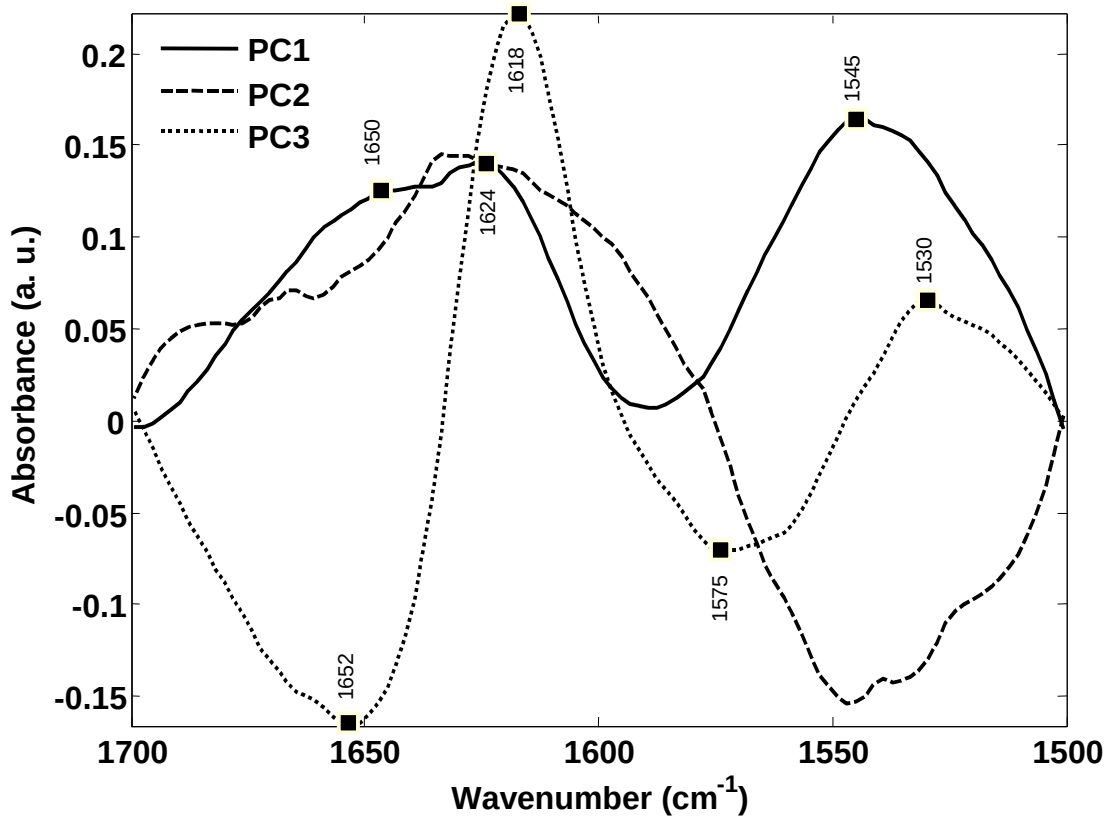

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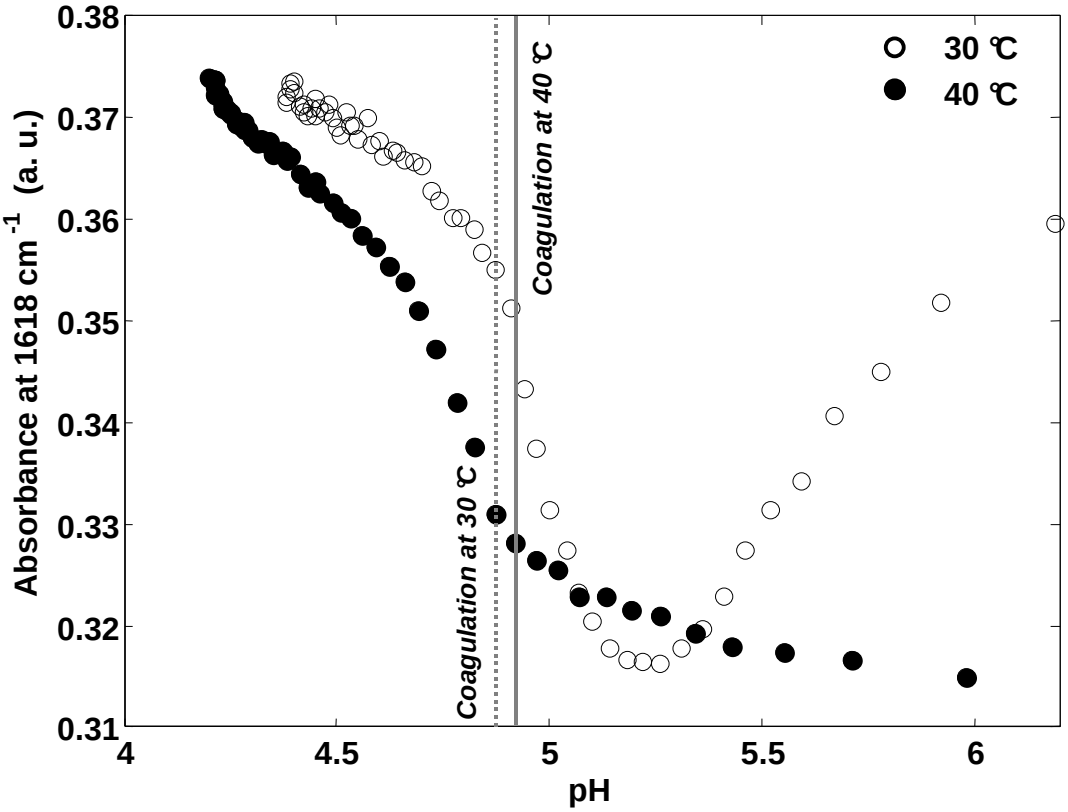

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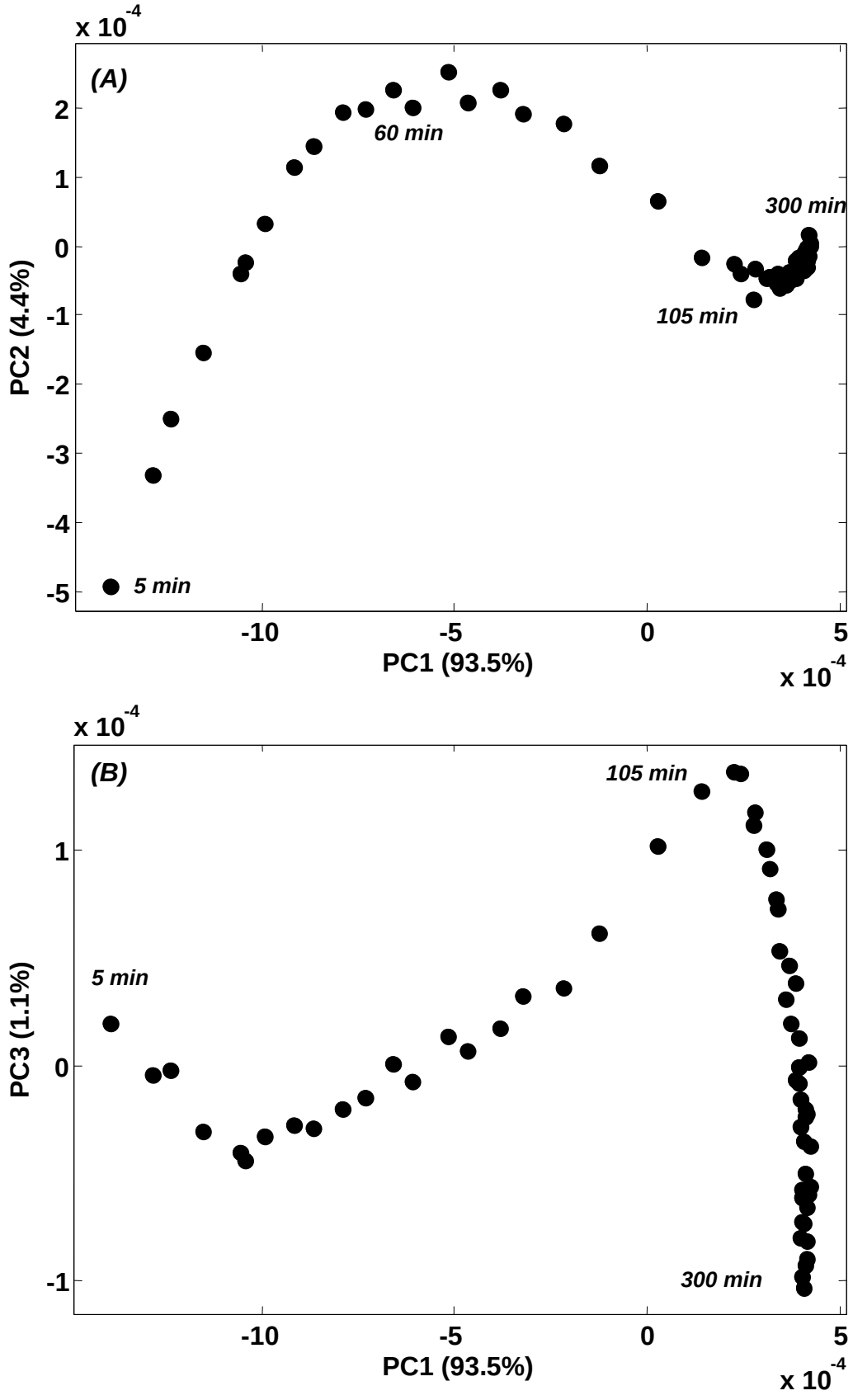

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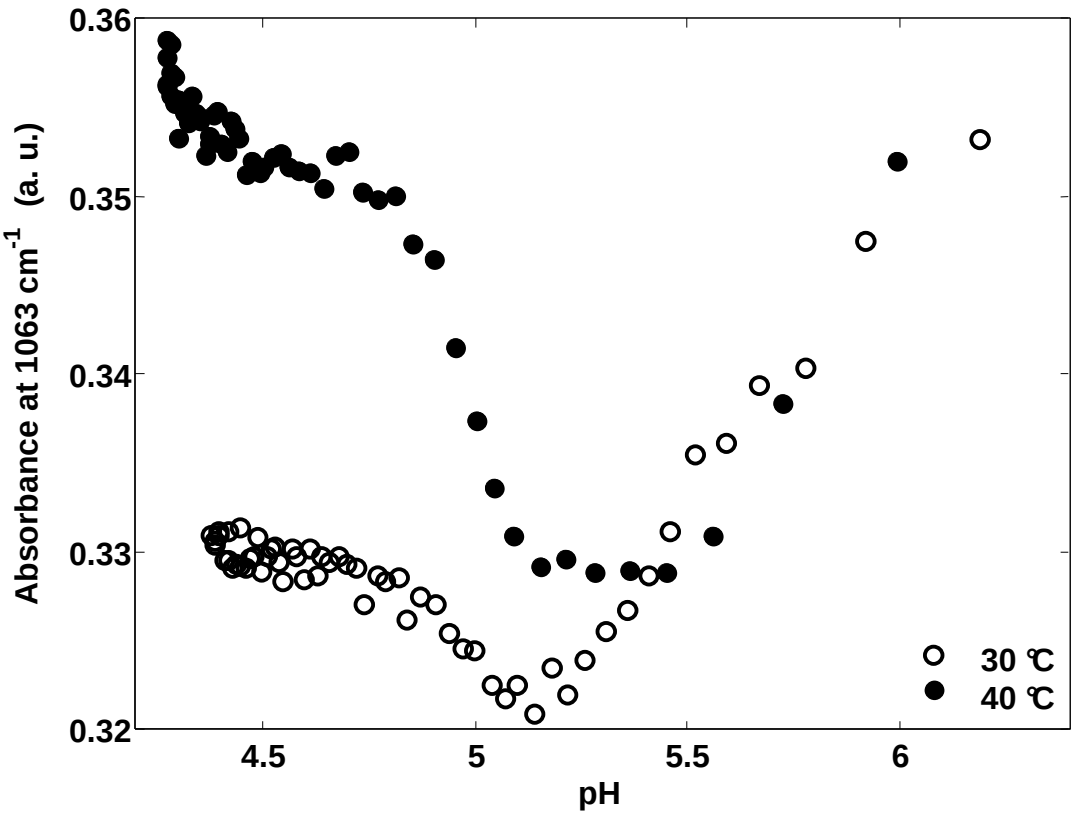

Structural changes of milk components during coagulation as studied by spectroscopic methods. 2 – Rennet-induced coagulation of milk investigated at different temperatures.
Comparison with acid-induced milk coagulation

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ABSTRACT

Milk coagulation is the primary step in the development of the texture of most dairy products. However, there is a lack of techniques to study the kinetic of the network development at a molecular level. Front-face synchronous fluorescence and mid infrared spectroscopies have been used to investigate structure evolution, at a molecular level, during milk coagulation. This study was performed using skim milk at two different temperatures (30°C and 40°C) added with rennet to generate gels exhibiting different textures and structures. Synchronous fluorescence spectra were recorded in the 250–500 nm excitation wavelength range using offset of 80 nm between excitation and emission monochromators for each system during the 300 min coagulation kinetics. Regarding mid infrared spectroscopy, the region located between 1700–1500 cm^{-1} corresponding to the amide I and II bands, and the 1500– 900 cm^{-1} region called the fingerprint region were considered for the characterization of milk coagulation kinetics. Principal component analysis was applied to the collections of fluorescence and infrared spectral data of the two systems to optimize their description. The results show that synchronous fluorescence and infrared data allowed the detection of the structural changes in casein micelles during coagulation and the discrimination of the different dynamics of the two systems. The rapid and non-invasive methods developed in this study allowed the investigation of network structure development and molecular interactions during milk coagulation. Finally, common components and specific weights analysis was applied to the rheology, infrared spectra and fluorescence data collected during acid- and rennet-induced coagulation kinetics of milk. This analyse enabled the relationship between the different data tables to be established.

Keywords: Milk, rennet, coagulation, molecular structure, synchronous front-face fluorescence, mid-infrared spectroscopy, chemometrics.

INTRODUCTION

Milk coagulation is the primary step in the production of most dairy products. Coagulation can be induced by acid, rennet, or both. Rennet coagulation is characterized by three phases. The first of these is an enzymatic phase which involves the cleavage of the surface κ-casein in the CN micelles to para-κ-CN and soluble caseinomacropeptides. The resulting para-CN micelles spontaneously aggregate in a second phase of the process before a rearrangement step takes place (1, 2). The major driving force for protein-protein interactions during the rennet coagulation of CN micelle are hydrophobic interactions (3). However, electrostatic and hydrogen bonds contribute to the specificity and stability of the interactions and, as a consequence, to specific structures. The kinetics of coagulation, influenced by temperature, and the structural aspects of protein-protein and protein-fat globule interactions determine the rheology properties of gels and thus their syneresis behaviour, as well as the texture of the final product. Water binding to the caseins plays also a very important role in influencing the physical and functional properties of these proteins in food systems (4). Several factors determine the special nature of water binding to the caseins: the hydrophobicity of the caseins, a loose packing density (or high voluminosity), and the extent of phosphate hydration in the caseins.

Mid-Infrared (MIR) and fluorescence spectroscopies have become increasingly important for a wide variety of analytical applications in biology and chemistry. This is due to great technical advances in both instrumentation and data analysis tools in the past two decades. Coupled with chemometric tools such as principal component analysis (PCA), principal component regression (PCR), factorial discriminant analysis (FDA) and Partial Least Squares (PLS) regression (5,6,7), information on composition, physicochemical properties and molecular structure of food systems can be extracted from fluorescence and infrared spectra in an accurate, high sensitive and rapid manner (8,9).

Fluorescence spectroscopy is a rapid and sensitive method for quantifying a fluorescent component and characterising its molecular environment in all sorts of biological samples. This environmental sensitivity enables to characterise conformational changes such as those attributable to the thermal, solvent or surface denaturation of proteins, as well as the interactions of proteins with ligands.

While the techniques used in conventional fluorescence spectroscopy allow to record excitation spectra or emission spectra, the excitation wavelength - λex, and the emission

wavelength - λem, are scanned synchronously with a constant wavelength interval, Δλ = λem – λex in synchronous fluorescence spectroscopy (SFS) (10). For well defined absorption and quantum yield maxima, the optimum value of the offset Δλ is set by the difference in wavelength of the emission and excitation maxima which is known as Stoke's shift (11). SFS makes it possible to narrowing of the spectral band and simplify the emission spectrum. In addition, SFS presents an interesting advantage from our point of view: a synchronous fluorescence spectrum retains information related to several fluorophores, compared to a classical emission spectrum that is mainly specific of a sole fluorophore.

Milk contains intrinsic fluorophores such as vitamin A and tryptophan residues present in fats and proteins, respectively, which give typical excitation and emission spectra. These spectra provide specific information on the characteristics of fats and proteins and on the environment of the fluorescent probes in food samples allowing to investigate structural changes (8). For example, the melting temperature of fat in cheese has been determined from vitamin A fluorescence spectra recorded at different temperatures (12).

MIR represents the spectrum of the absorption of all the chemical bonds having an infrared activity between 4000 and 400 cm^{-1}. The acyl-chain is mainly responsible for the absorption observed between 3000 and 2800 cm^{-1}, whereas the peptidic bound C-NH is mainly responsible of the absorption occurring between 1700 and 1500 cm^{-1}. As water also strongly absorbs in the Amide I region, the changes of pattern of the casein-bound water with structural changes of the micelles alter the shape of the spectra in this region. Indeed, it has been reported in the literature that the presence of molecule such as sucrose (13) or NaCl (14) in water modifies the shape of the MIR spectrum of water: the shape of water band at about 1650 cm^{-1} is sharpened and its intensity increases in the presence of NaCl. Thus, care must be exercised not to overinterpret these observed spectral changes in the Amide I region in terms of variation of the secondary structure of caseins, but rather in terms of hydration changes (15). Finally, the 1500 and 900 cm^{-1} region is known to exhibit bands attributed to peptidic bonds and phosphate.

In the present work, front-face synchronous fluorescence and mid-infrared spectra in combination with multivariate statistical analysis are used to investigate, at a molecular level, structure evolution during rennet-induced milk coagulation processes at two temperatures. Moreover, spectral data recorded during the gelation kinetics of milk coagulation processes are compared to rheology measurements. Finally, common components and specific weights analysis (CCSWA) was applied to the rheology, infrared spectra and fluorescence data

collected during acid- and rennet-induced coagulation kinetics of milk. This analyse enabled the relationship and differences between the different data tables to be established.

MATERIAL and METHODS

- *Milk samples*

Bovine milk samples were collected from the tank of a local farm and a given milk sample was used in the course of the day. The milk was skimmed by centrifugation at 30 °C using a Electrem centrifuge (Electrem, France).

- *Coagulation system*

The coagulation kinetics were realised at 30 and at 40 °C by adding 40 µl/L commercial rennet (COOPER, Melun, France) containing 50 mg/L of chymosin.. The coagulation kinetics were realised at 30 and at 40 °C. The pH was measured during the kinetics using a pH meter HQ30d (Hach Company, USA).

- Mid-infrared spectra

Infrared spectra were recorded between 4000 and 900 cm^{-1} at a resolution of 4 cm^{-1} on a Varian 3100 FT-IR Excalibur Series Fourier-transform spectrometer (Varian Inc., Palo Alto, USA) mounted with a thermostated ATR accessory equipped with a grip. The ATR cell is 6 reflections and was made of a horizontal ZnSe crystal which presented an incidence angle of 45°. The temperature was controlled by a Julabo temperature controller (Julabo, Germany).

Milk samples added with rennet were placed on the crystal. In order to improve the signal to noise ratio, 32 scans were co-added for each spectrum. For each kinetic, the spectra were recorded at 30 and 40 °C in triplicate. Before each measurement, the spectrum of the ZnSe crystal was recorded and used as background. Base line and ATR corrections were applied to the spectra using Varian Software (Resolution Pro 4.0).

The regions of the mid infrared spectra located between 1700 and 1500 cm^{-1} (protein region) and 1500 and 900 cm^{-1} (fingerprint region) have been considered in this study.

- Synchronous fluorescence spectra

Synchronous fluorescence spectra were recorded using a FluoroMax-2 spectrofluorimeter (Spex-Jobin Yvon, Longjumeau, France) mounted with a front-surface cuvette-holder and the incidence angle of the excitation radiation was set at 56° to ensure that reflected light,

scattered radiation, and depolarisation phenomena were minimized. The spectrofluorimeter was equipped with a thermostatically controlled cuvette-holder and the temperature was controlled by a Haake temperature controller (Haake, Champlan, France).

Synchronous fluorescence spectra were collected in the 250–500 nm excitation wavelength range using offset of 80 nm between excitation and emission monochromators (16). For the experiments, the synchronous spectra of a milk sample added with rennet placed in quartz cuvette (2 cm x 1 cm x 0.3 cm) were recorded at 30 and 40 °C. For each temperature, three time kinetics were performed using different samples.

- Dynamic oscillatory experiments

Milk samples added with rennet were placed on cylinder disks (2 mm thick and 20 mm diameter). The dynamic oscillatory experiments were performed with a rheometer (CP 20, TA Instrument, Guyancourt, France) with plate geometry of 20 mm diameter. Oscillation experiments were performed in the linear viscoelastic region by applying a constant force of 0.1 N and a constant frequency of 1 Hz. All the experiments were carried out at temperatures of 30 and 40 °C by applying a Peltier plate that provided very accurate and rapid temperature control. The data obtained included the two components of shear modulus G*, i.e., the elastic component G' (storage modulus) and the viscous component G" (loss modulus). The complex viscosity (η*) and tan δ were also measured. Data were recorded every 5 min until 300 min. For each renneted milk kinetics, three curves were recorded using different milk samples.

Chemometrics **:**

- *Principal Component Analysis (PCA)*

PCA is a multidimensional data treatment which provides a synthetic description of large data sets. When applied to spectral data, PCA allows similarity maps of the samples to be drawn and spectral patterns obtained (17). PCA was applied to the spectra in order to investigate differences in the spectra. This statistical multivariate treatment made it possible to draw similarity maps of the samples and to get spectral patterns (18).

- Common Components and Specific Weights Analysis

The objective of common components and specific weights analysis (CCSWA) is to describe several data tables observed for the same n samples taking into account the maximum of inertia (total variance) of each of them. This method consists in determining a common space of representation for all the data sets, each table having a specific weight (or salience) associated with each dimension of this common space. CCSWA was applied within the sensory framework and more precisely in sensory profiling analysis where judges assess a set of products using several sensory attributes. This chemometric method deals with the co-inertia, which allows to describe the overall information, collected and takes into account the relation between the different data tables (19). Similarity maps and patterns can be drawn. CCSWA has been used to 1) describe in a simple and synthetic manner the overall information collected, 2) extract and use the relevant information related to the structural changes of the studied component, 3) provide tools for a molecular interpretation of the results and 4) investigate the relations between structures and rheology properties. CCSWA was carried out using MATLAB (Version 6, Release 12).

RESULTS AND DISCUSSION

This study was aimed at investigating milk structural evolution at a molecular level using front-face synchronous fluorescence and mid infrared spectroscopies. More precisely, we focused on changes of protein environment during the milk coagulation process. This approach requires the ability to discriminate between different dynamic and structural changes. This study was conducted using rennet-induced milk coagulation processes at different temperatures known to yield different structures and textures (20). The physico-chemical properties of the 2 systems (30 and 40°C) were characterized in a preliminary phase.

The pH was measured during the experiments and it was shown that only minute changes occurred during the time course of the kinetics.

Rheological properties of the two systems

The evolutions of the elastic modulus (G') recorded *versus* time during the rennet-induced

coagulation phase of the milks at 30 and 40°C are shown in Figure 1. All the samples showed a pre-gel stage characterised by a G' modulus close to the 0 Pa value. The method chosen to evaluate the gel point consisted in a linear extrapolation of the rapidly rising elastic modulus G' to the intercept with the time axis. The change in the G' modulus over the given time span was very different for the two coagulation procedures. At 30°C, the renet system had a gelation time of 122 ± 15 min and, then, the G' modulus increased till 300 min. At 40°C, the gelation time was at 97 ± 10 min and G' modulus increased rapidly till 170 min followed by a slower increase up to 300 min. The strength of the gel obtained at 40°C after 300 min was higher (80 ± 12 Pa) than the one (49 ± 08 Pa) at 30°C.

Network structure formation as studied by synchronous fluorescence spectroscopy. Effects of temperature on the kinetics

Figure 2 presents milk synchronous spectra ($\Delta\lambda$ = 80 nm) recorded at 30 °C and recorded during the coagulation kinetic for 5, 1200, 240 and 300 min. The skim-milk synchronous spectrum recorded at 30 °C for $\Delta\lambda$ = 80 nm exhibited an intense band at 290 nm (emission at 370 nm), a band at 360 nm (emission at 440 nm) and a broad band centered at 450 nm (emission at 530 nm). The intense band observed at 290 nm (emission at 370 nm) can be attributed to tryptophan residues of proteins (16). The broad bands centered at 360 nm and 450 nm may be attributed to NADH and riboflavin (21), respectively. During the renneting kinetic, a decrease of the fluorescence intensity was observed at 290 nm and 450 nm, whereas the intensity of the band at 360 nm was increasing. Considering riboflavin, it is generally bound to proteins. Riboflavin binding protein - a monomeric, two-domain protein - has been originally purified from hens' egg white (22). In plasma, it has been shown that riboflavin is bound to proteins, predominantly albumin, but also to immunoglobulins: this vitamin may bind to a large number of proteins. Like others fluorophores, the fluorescence emission of riboflavin is highly sensitive to its local environment, and it can be used as an indicator group for protein conformation and interaction changes in cheese matrices during ripening since it interacts with proteins (23).

A weak shift toward higher wavelengths of the band at 290 nm was observed (Figure 2): it agreed with the changes in protein interactions, leading to a more hydrophilic environment for tryptophanyl residues during the time span of the reaction. In addition, the band centered at 360 nm is broadening during the renneting kinetic at 30°C.

Similar spectra were observed during the acidification kinetic performed at 40°C (data not shown).

PCA was applied to the set of spectra recorded during rennet-induced coagulation at 30°C in order to obtain additional structural information. This method is well suited to optimize the description of data collection by extracting the most useful data and rejecting redundant data. Figure 3A shows a PCA similarity map defined by the principal components 1 and 2 for synchronous fluorescence spectral data of the renneting kinetic at 30°C. The first two principal components accounted for 99.2% of the total variability with a predominance of component 1 (97%). A separation of spectra was observed as a function of time according to component 1. The spectra recorded between 5 and 120 min had negative scores according to PC1 and were separated on PC2 and reaching a minimum for 120 min (gelation point), whereas spectra recorded between 120 and 300 min were principally separated on axis 1 with a maximum for 240 min.

As the first part of the kinetic (5 to 120 min) involved the cleavage of the surface κ-casein in the CN micelles to para-κ-CN and soluble caseinomacropeptides, the second phase (120 to 300 min) corresponded to the aggregation of the resulting para-CN micelles (1, 2). This phase also showed different parts, i.e., a main one between 120 and 240 min and an other one between 240 and 300 min (figure 3A), that may be related to the structural rearrangements of para-CN micelles in the gel.

Considering the results (Figure 3B) of PCA applied to the spectral data recorded at 40°C, the spectra obtained between 5 and 95 min were discriminated according to PC 2 (5.2%), whereas spectra recorded between 95 and 300 min were separated principally on PC 1 (92.8%). The discrimination according to PC1 of spectra recorded between 95 and 300 min showed in fact two phases with a maximum corresponding to 220 min.

The spectral patterns associated with principal components 1 and 2 providing the most discriminant wavelengths of the spectral data set recorded at 30°C, are presented Figure 4A. Considering spectral pattern 1, the most discriminant wavelengths were 281, 370 and 450 nm. The contrast between negatives peaks at 281 and 450 nm and a positive one at 370 nm indicated that, during the time course of the coagulation process, the fluorescence intensities at 281 and 450 nm decreased, whereas the fluorescence intensity at 370 nm increased for all the spectra discriminated according to PC 1. The spectral pattern associated with PC 2 exhibited a contrast between two positive peaks at 321 and 436 nm and two negative ones at

264 and 297 nm. These bands provided valuable structural information about the changes that occurred in the milk components during renneting. The spectral pattern associated with principal component 2 was characterized by the opposition of bands at 297 and 321 nm. It indicated that fluorescence intensity at 321 nm decreased between 5 and 120 min, whereas it increased at 297 nm during the same time. Interestingly, the fluorescence intensity at 321 nm increased again after 120 min kinetic reaching a maximum for 240 min (Figures 2 and 3A). It has been shown that fluorescence excitation at about 320 nm originated from vitamin A (24) located in the residual fat globules present in skim milk. The changes in the shape of the synchronous fluorescence spectra in the 250-330 nm region are related to modifications in the environment of tryptophan residues (bands at 264 and 297 nm) and of vitamin A (band at 321 nm). Focusing on the region located between 250 and 300 nm, i.e., the tryprophan residues region, the opposition between two minima (bands at 264 and 297 nm) and a maximum at 280 nm indicated a weak broadening of the excitation band centered at 280 nm from 5 to 120 min (see also Figure 2). These changes in casein tryptophan fluorescence were in agreement with the cleavage by rennet of the surface κ-casein in the CN micelles and their structure changes. The second part of the kinetic (120 to 240 min) was characterized by a sharpening of the band centered at 280 nm as shown Figures 2 and 4A: this phenomena was associated with the development of the casein network. This second spectral pattern was also characterized by a negative peak at 395 nm and a positive one at 436 indicating changes in the environment of NADH and riboflavin during the coagulation kinetic, respectively.

Changes in the fluorescence intensities at 281 nm as a function of renneting time recorded at 30°C are shown in Figure 4B. Considering fluorescence intensity at 281 nm, four regions may be distinguished: a first phase from 5 to 110 min, a second domain characterized by a sharp decrease till 135 min, a third one between 110 and 255 min showing a slower decrease of the fluorescence and finally a plateau for the fourth phase from 255 to 300 min.

These modifications in the fluorescence properties of milk proteins can be paralleled with the well known rennet-induces coagulation process, i.e., effects of rennet on κ-casein in the CN micelles, aggregation of the resulting para-CN micelles and structural rearrangements of para-CN micelles in the gel (1, 2).

The profile of the changes in the fluorescence intensities at 297 nm as a function of renneting time is different: a plateau was observed from 0 to 70 min, then an increase of the intensity

took place till 120 min followed by a decrease up to 200 min and finally the intensity reached a plateau (200 to 300 min).

Network structure formation as studied by infrared spectroscopy. Effects of temperature on the kinetics

Most of the spectral information useful for the analysis of the spectra is located in: (1) the 3000–2800 cm^{-1} range corresponding to C-H stretching, (2) the region located between 1700–1500 cm^{-1} corresponding to the amide I and II bands, and (3) the 1500–900 cm^{-1} region called the fingerprint region.

The 3000–2800 cm^{-1} spectral region corresponds to the C–H bond of methyl and methylene groups of fatty acids. As skim-milk was considered in this study, only very weak bands were observed in this region. The 1700–1500 cm^{-1} region was characterised by the presence of bands related to peptides and proteins. Thus, these bands contain some information on the proteins and on the interaction of these proteins with other components, such as ions, water and other proteins. Contribution to the amide I band, which is used to investigate the secondary structure of proteins, can be observed at about 1638 cm^{-1} (data no shown). The absorption band with a maximum at about 1553 cm^{-1} is generally assigned to the amide II vibrations. The 1500–900 cm^{-1} region is called the fingerprint region and numerous chemical bonds are absorbing in this region (Figure 5). Considering the recorded spectra, the weak differences were observed all along the wavenumber range investigated. The bands observed in the 1000–1100 cm^{-1} region could be related to P=O stretching (25). Additional smaller bands were also observed at about 1240, 1400 and 1450 cm^{-1}.

PCA was applied to the Amide I&II spectral data recorded at 30°C and 40°C in order to get a deeper insight into casein conformational changes during gelation. Considering the results of PCA performed on spectral data recorded at 30°C, the first two principal components took into account 100% of the total inertia. Figure 6A shows the similarity map defined by principal components 1 and 2. As a discrimination of the spectra recorded between 5 and 120 min was observed along the second principal component, the first principal component discriminated the sample recorded between 120 and 300 min. The spectral pattern corresponding to the principal component 1 is given Figure 6B and revealed characteristic wavenumbers at 1643 and 1547 cm^{-1}. It roughly corresponded to mean spectrum of proteins in this spectral region suggesting that the observed changes in the spectra were only related to

absorbance difference over the whole spectrum. Regarding the second principal component, the spectral pattern (Figure 6B) exhibited positive peaks at 1689, 1676 and 1597 cm^{-1}, as well as large negative peaks at 1539, 1532 and 1511 cm^{-1}. This spectral pattern was more complex and as a consequence more difficult to interpret. If we consider the Amide I region, the spectral pattern related to PC2 exhibited an opposition between two positive bands located at 1689 and 1597 cm^{-1} and an other band with a minimum at 1647 cm^{-1}. This pattern indicated that the width of the spectra in the Amide I region was larger for the ones located on the positive side of the PCA map than those on the negative side (26).

In the introduction section, we reported that water band at about 1650 cm^{-1} is sharpened and its intensity increases in the presence of NaCl (15). The observed changes in the spectra recorded during rennet-induced coagulation of milk may be thus interpreted as changes of casein-bound water during the kinetics, rather than the transformation of α-helix into intermolecular β-sheets.

PCA was applied to 1500–900 cm^{-1} spectral data recorded at 30°C and 40°C in order to get a deeper insight into phosphate-state changes during renneting. Considering the results of PCA performed on spectral data recorded at 30°C, the first two principal components took into account 98.4% of the total inertia. Figure 7A shows the similarity map defined by principal components 1 and 2 and a bell-shaped curve was observed. The top of the bell-shaped curve corresponded to 120 min, the gelation point. Regarding the principal components 1 and 2, the spectral patterns (Figure 7B) exhibited major positive peaks at 974 (PCs 1 and 2), 1060 (PCs 1 and 2), 1229 (PCs 1 and 2), 1389 (PC1), 1445 (PC2) and 1450 (PC1) cm^{-1} and negative peaks at 1009 (PCs 1 and 2), 1132 (PCs 1 and 2), 1256 (PCs 1 and 2), 1410 (PC1), 1414 (PC2), 1450 (PC2) and 1455 (PC1) cm^{-1} in the 1500-900 cm^{-1} region.

Analyse using CCSWA of the relationships and differences between the different data tables collected during acid- and rennet-induced coagulations

The Common Components and Specific Weights Analysis (CCSWA) method was applied on the 4 data sets containing the synchronous fluorescence, the infrared spectra (Amide I&II and fingerprint regions) and the rheology data of the acid- and rennet-induced coagulation kinetics. The two first dimensions gave different saliences for spectroscopies and rheology data sets: the first dimension (D1) expressed 89.4, 67.7 and 73.3 % of the inertia of the

rheology, infrared data corresponding to Amide I&II and fingerprint spectral region bands, respectively, and a tiny part (6.3 %) of the inertia of the fluorescence data (Table 1). On the contrary, the second dimension (D2) expressed 87.6 % of the inertia of the fluorescence data and part of the inertia of the infrared data (21.2, and 16.5% for Amide I&II and fingerprint spectral regions, respectively) and a tiny part (5.5%) of the inertia of the rheology data (Table 1).

Considering the similarity maps, the plane defined by the components D1 and D2 allowed to discriminated the types of coagulation (acid/rennet) and the data collected between the gelation points and 300 min (Figure 8A), respectively.

Considering the plane defined by the components D2 and D3, the data of acid coagulation kinetic recorded between 5 and 110 min had negative scores according to D2 and were separated mainly on D3, whereas data of acid-induced coagulation kinetic recorded between 110 and 300 min were separated principally on axis D2 (Figure 8B). The plane defined by the components D2 and D3 discriminated the data in a similar way to the plane defined by components PC1 and PC2 of the PCA run on the synchronous fluorescence spectra recorded during the acid-induced coagulation kinetic of milk (see manuscript on acid-induced gelation). The third dimension (D3) expressed 2.2, 9.8 and 7.9 % of the inertia of fluorescence data, and infrared data corresponding to Amide I&II and fingerprint spectral regions, respectively, and a tiny part (0.2 %) of the inertia of the rheology data (Table 1). In addition, data recorded during rennet-induced coagulation kinetic were quite exclusively discriminated according to D2 (Figure 8B).

The examination of the spectral pattern in synchronous fluorescence spectra associated with the common component D2 (Figure 9) showed similarity with principal components 1 (Figure 3 of this manuscript and Figure 5 of the manuscript on acid-induced coagulation of milk) of the PCAs performed on synchronous fluorescence spectra recorded during acid- and rennet-induced coagulation kinetics at 30°C. Similar conclusions were derived for the spectral pattern associated with the common component D3 (Figure 9) and principal components 2 (Figure 3 of this manuscript and Figure 5 of the manuscript on acid-induced coagulation of milk) of the PCAs performed on synchronous fluorescence spectra recorded during acid- and rennet-induced coagulation kinetics at 30°C. It appeared that the most discriminant wavelengths and wavenumbers in the spectra recorded after the coagulation points were similar whatever the coagulation type, i.e., acid or rennet: as shown in Figure 8, these spectra were discriminated according to common component D2. But the spectra recorded during

acid- and rennet-induced coagulation kinetics at 30°C showed differences since they are discriminated according to common component D1.

CCSWA performed on the 4 data tables allowed to manage in an efficient way the whole information collected. It summed up the major part of the information on three dimensions (D1, D2 and D3). In addition, each of the spectral data sets provided information on the different phases taking place during coagulations.

CONCLUSIONS

It is common knowledge that the process used to coagulate milk has broad effects on the texture of the final product. Rheological methods are useful for characterizing the texture of milk products and cheeses. This method made it possible to show that the coagulation of milk by glucono-delta-lactone and rennet at different temperatures investigated in this study showed different rheology properties. It is generally assumed that the properties of gels at a macroscopic level are related to their molecular structure. However, few techniques allow the investigation at a molecular level of turbid and complex products such as milk. Front-face synchronous fluorescence spectroscopy and mid infrared spectroscopy, two non-invasive techniques, in combination with chemometric methods make it possible to study the coagulation of milk at a molecular level. Indeed, the fluorescence properties of tryptophan residues are modified by the changes in micelle structure and protein interactions induced by milk acidification and renneting. This study shows that infrared spectroscopy is useful for characterizing the changes in micelle structure before gelation of milk and micelle aggregation during casein network development.

Using these spectroscopic methods, it has been possible to demonstrate that gels exhibiting different rheology properties have different structures at the molecular level. In addition, it was possible to follow the different steps of the gelation processes.

Spectroscopic methods such as fluorescence and mid infrared spectroscopy combined with chemometric tools have the potential to evaluate structure at the molecular level. Fluorescence and infrared data are spectra that allow us to derive information on the molecular structure and interactions of the dairy-product matrix. Moreover, it is also suggested that the phenomena observed at molecular (fluorescence, infrared) and macroscopic (rheology) levels are related to the texture of dairy products.

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Table 1: Saliences for the common components 1 to 4 of CCSW analysis performed on the acid- and rennet-induced coagulation kinetic data recorded at 30°C.

	D1 (69.9%)	D2 (27.7%)	D3 (2.2%)	D4 (0.1%)
Rheoloy	0.894	0.055	0.002	0.003
SFS	0.063	0.876	0.022	0.026
MIR (1700 1500 cm^{-1})	0.677	0.212	0.098	0.002
MIR (1500 900 cm^{-1})	0.733	0.165	0.079	0.003

Legends of the figures

Figure 1: Evolution of the storage modulus (G') over a period of 300 min during the coagulation of milk at 30°C (○) and 40°C (●).

Figure 2: Synchronous fluorescence spectra recorded between 250 and 500 nm ($\Delta\lambda$ = 80 nm) during the coagulation kinetic at 30°C for 5, 120, 240 and 300 min.

Figure 3: PCA similarity maps defined by PC1 and PC2 for synchronous fluorescence spectra recorded at 30°C (A) and 40°C (B).

Figure 4: (A) Spectral patterns corresponding to the principal components 1 (—) and 2 (---) for the PCA performed on synchronous fluorescence spectra recorded at 30°C and (B) evolution of the fluorescence intensity at 281 nm recorded at 30°C (○).

Figure 5: Mid-infrared spectra recorded between 1500-900 cm^{-1} during the coagulation kinetic at 30°C for 5, 120 and 300 min.

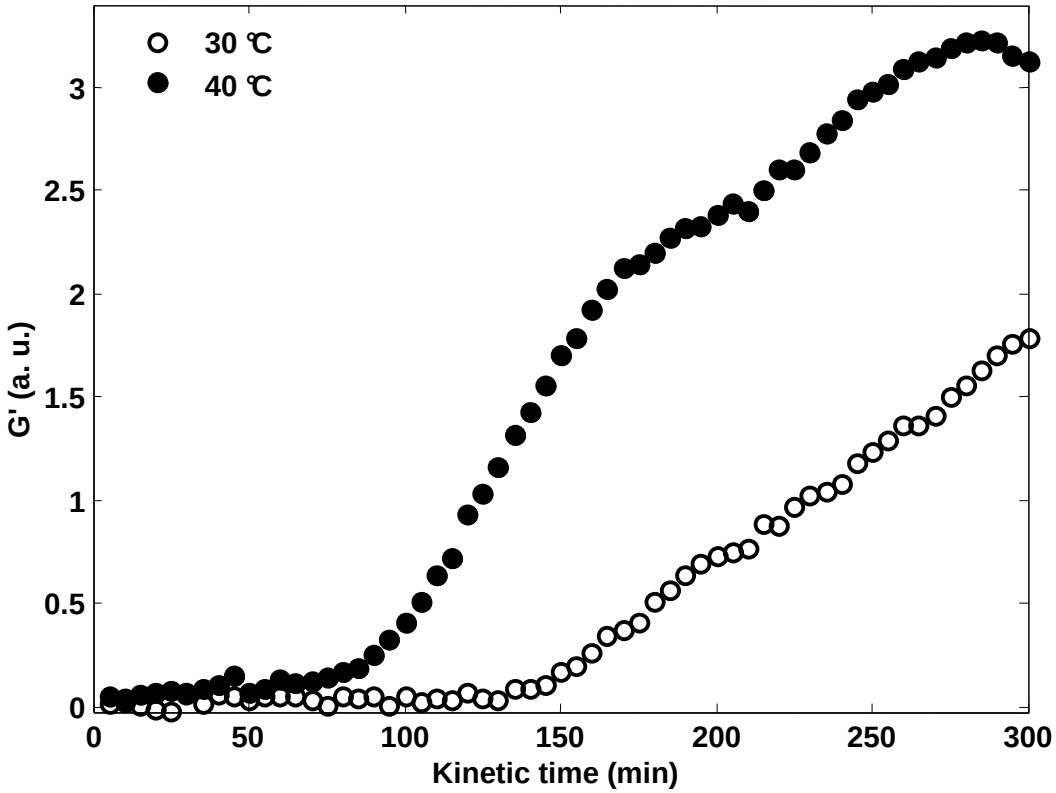
Figure 6: PCA similarity map defined by PC1 and PC2 (A) for 1700-1500 cm^{-1} mid-infrared spectra recorded at 30°C and spectral patterns (B) corresponding to the principal components 1 (—) and 2 (---).

Figure 7: PCA similarity map defined by PC1 and PC2 (A) for 1500-900 cm^{-1} mid-infrared spectra recorded at 30°C and spectral patterns (B) corresponding to the principal components 1 (—) and 2 (---).

Figure 8: CCSWA similarity maps defined by the common components D1 and D2 (A) and D2 and D3 (B) for the acid- and rennet-induced coagulation kinetic data recorded at 30°C.

Figure 9: Spectral pattern associated with the common component D2 (—) and D3 (---) (fluorescence data sets recorded at 30°C).

Figure 1 : Boubellouta et al.



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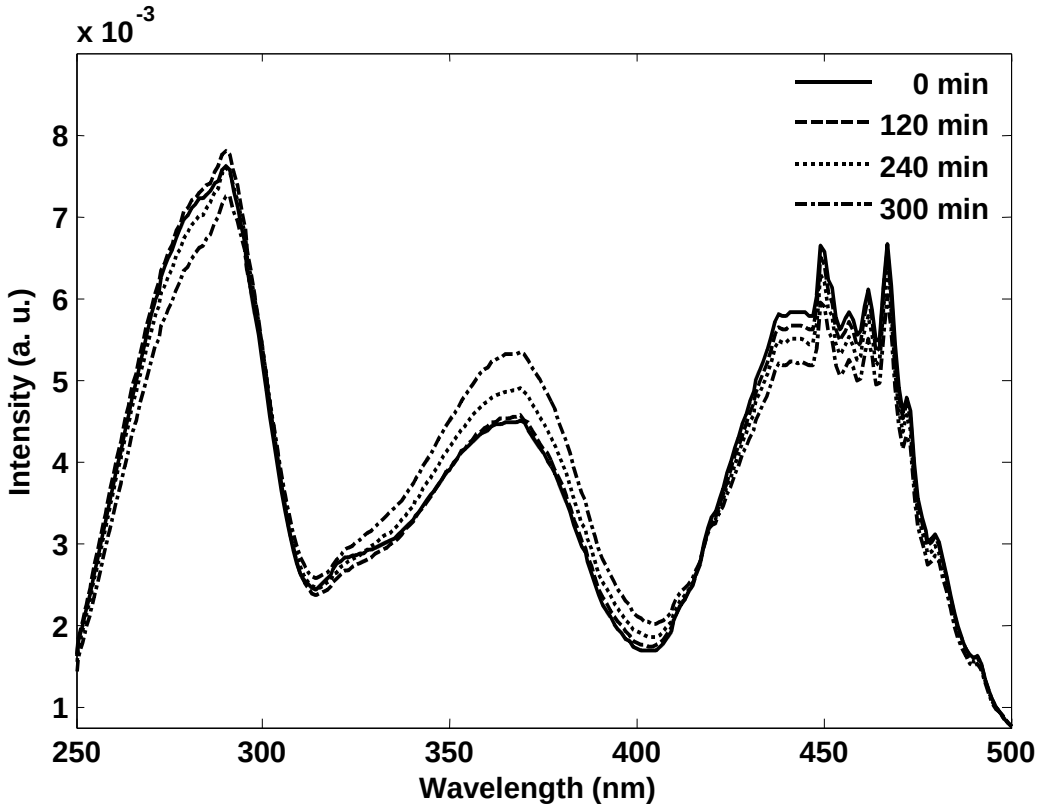
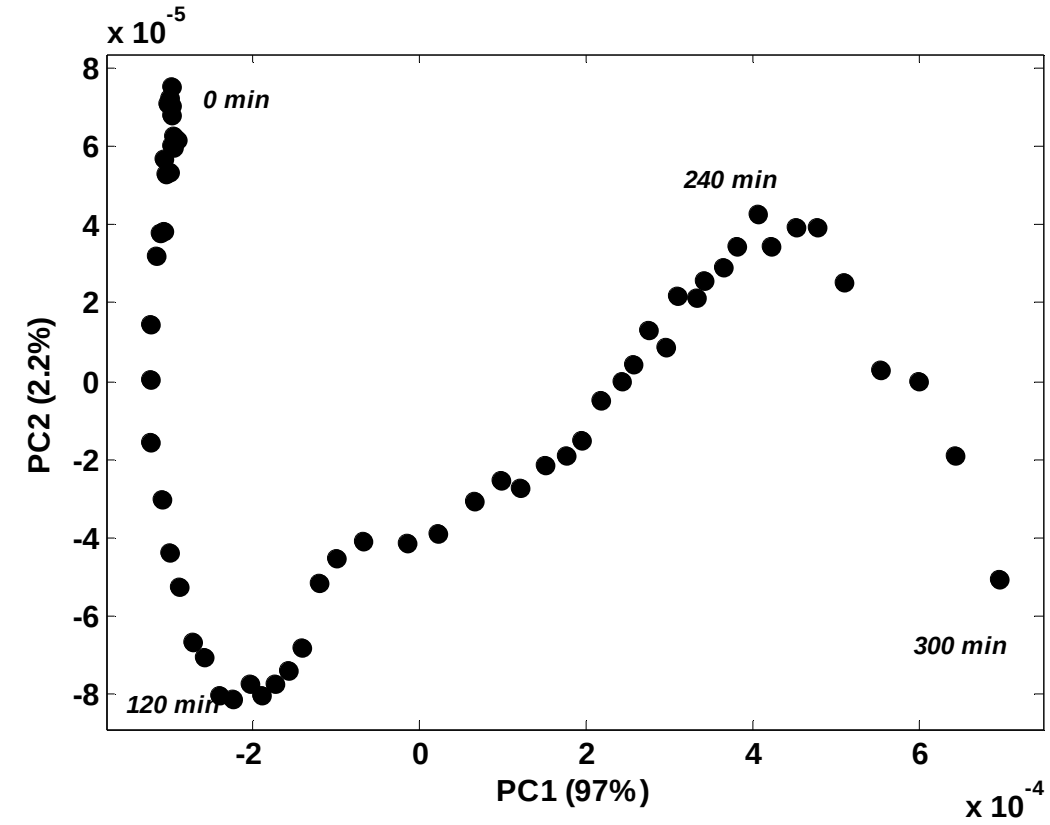


Figure 3 : Boubellouta et al.

(A)



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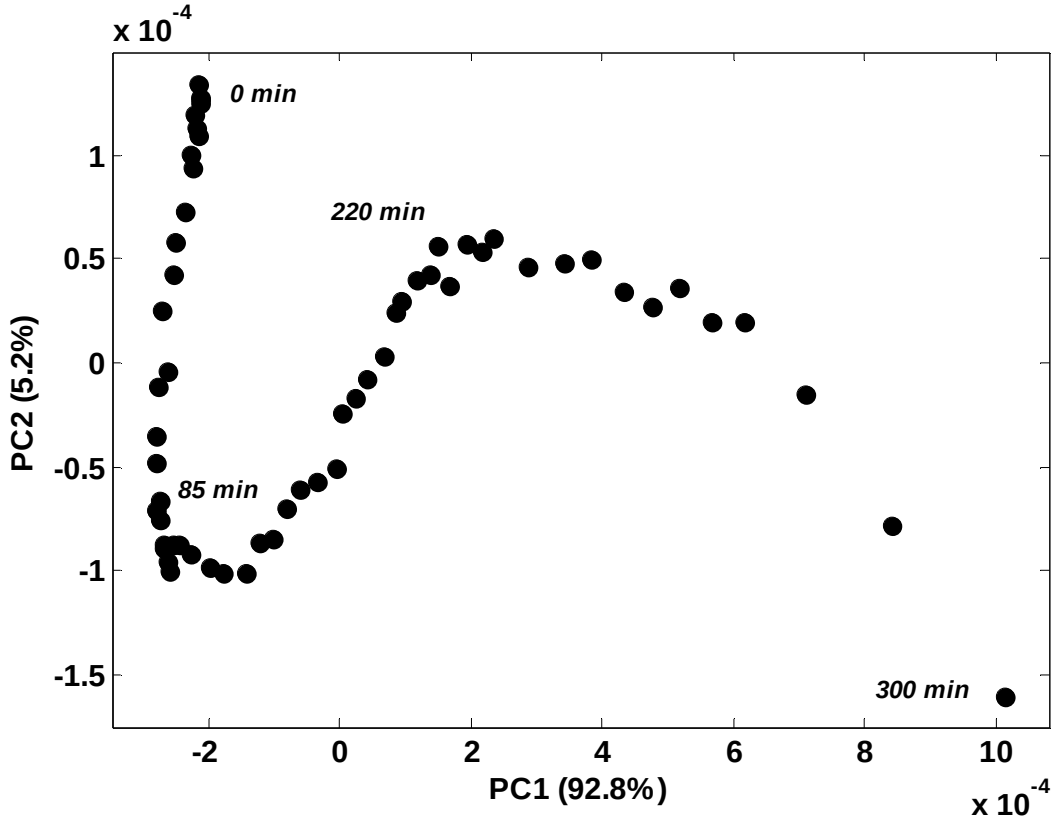

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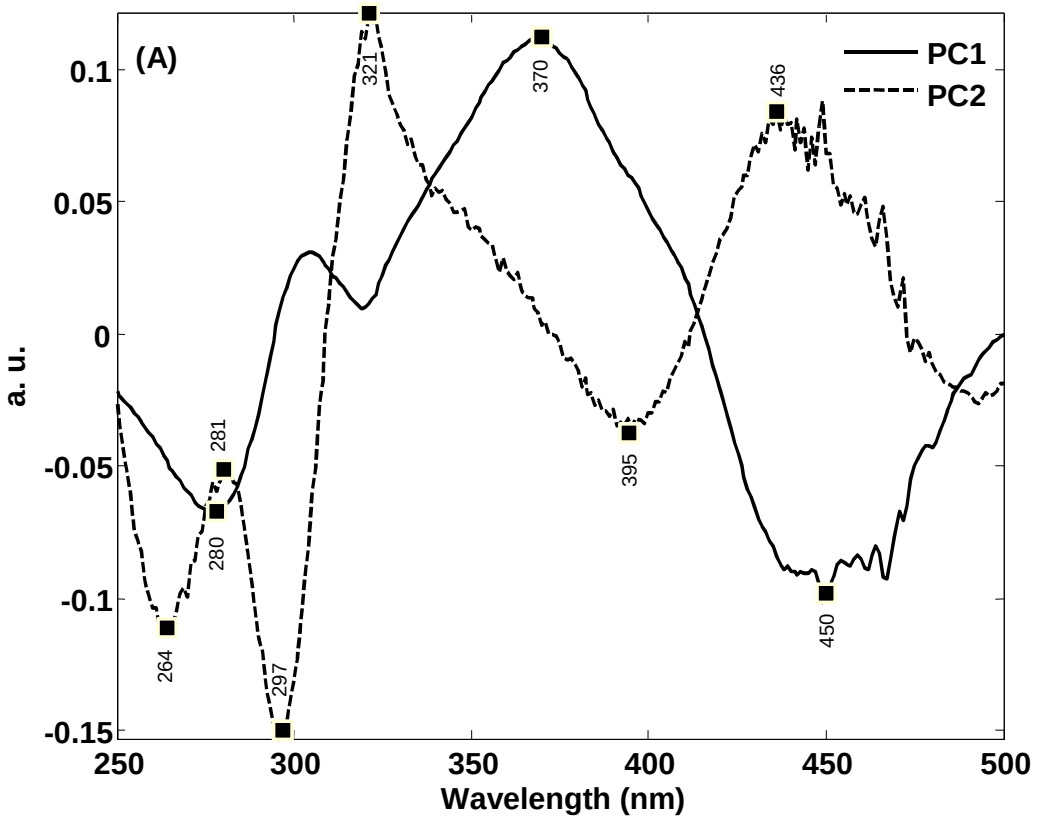

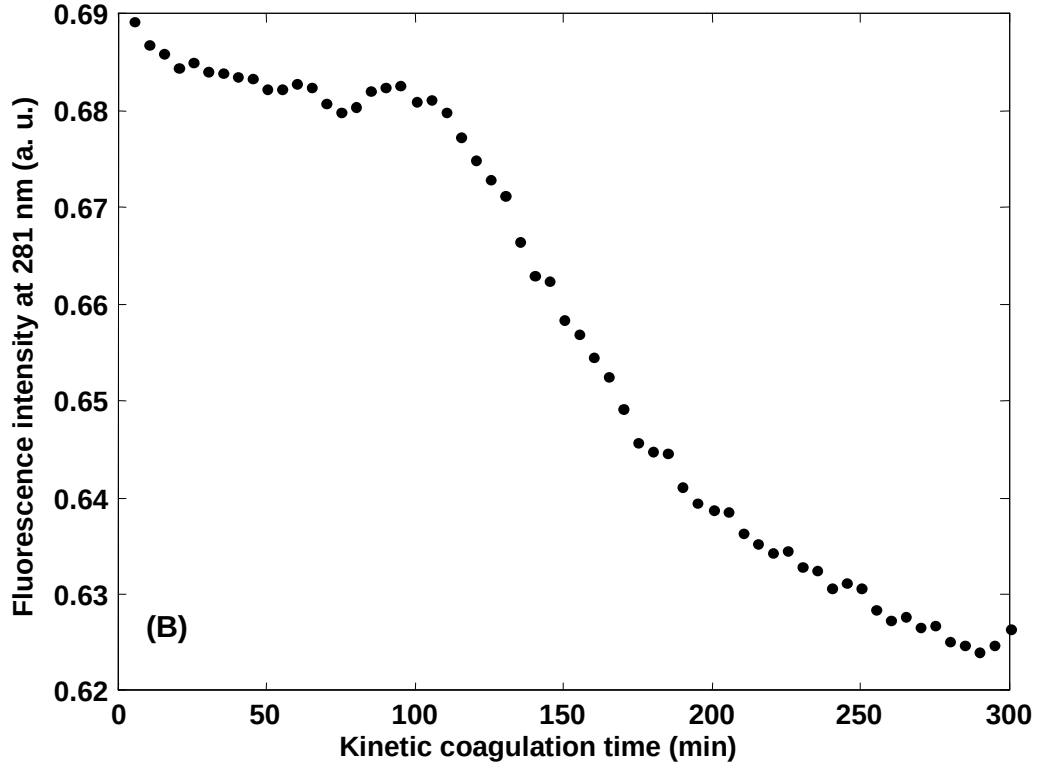

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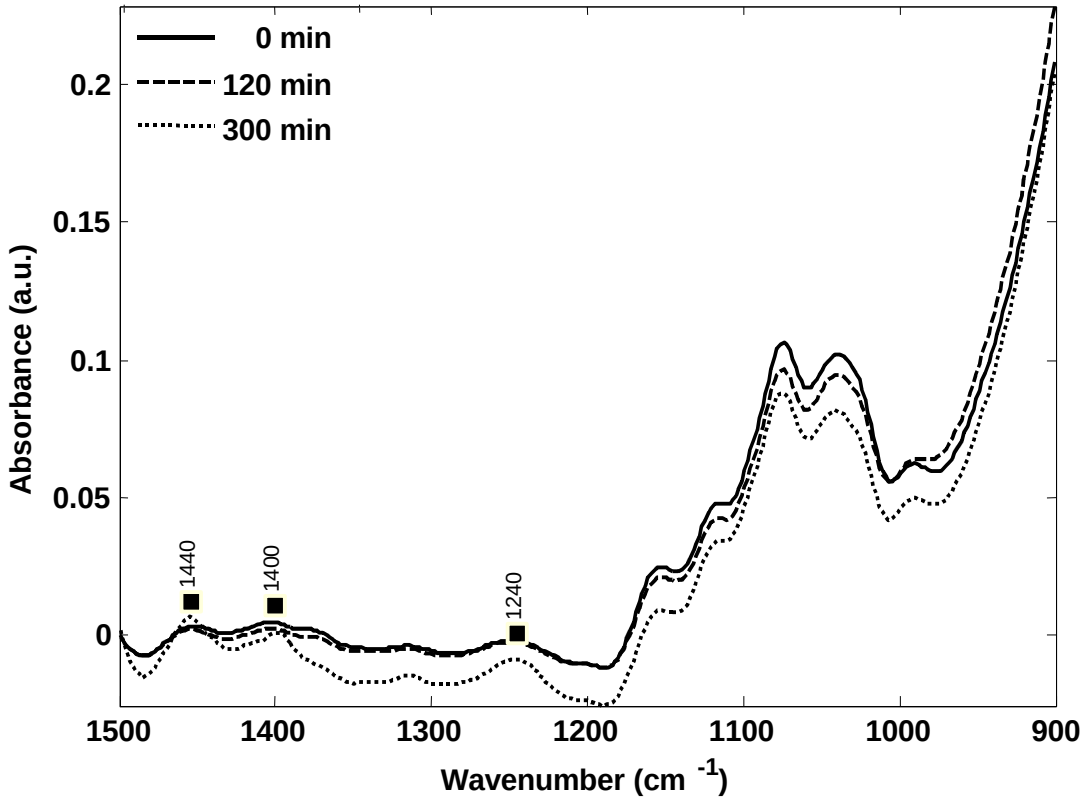

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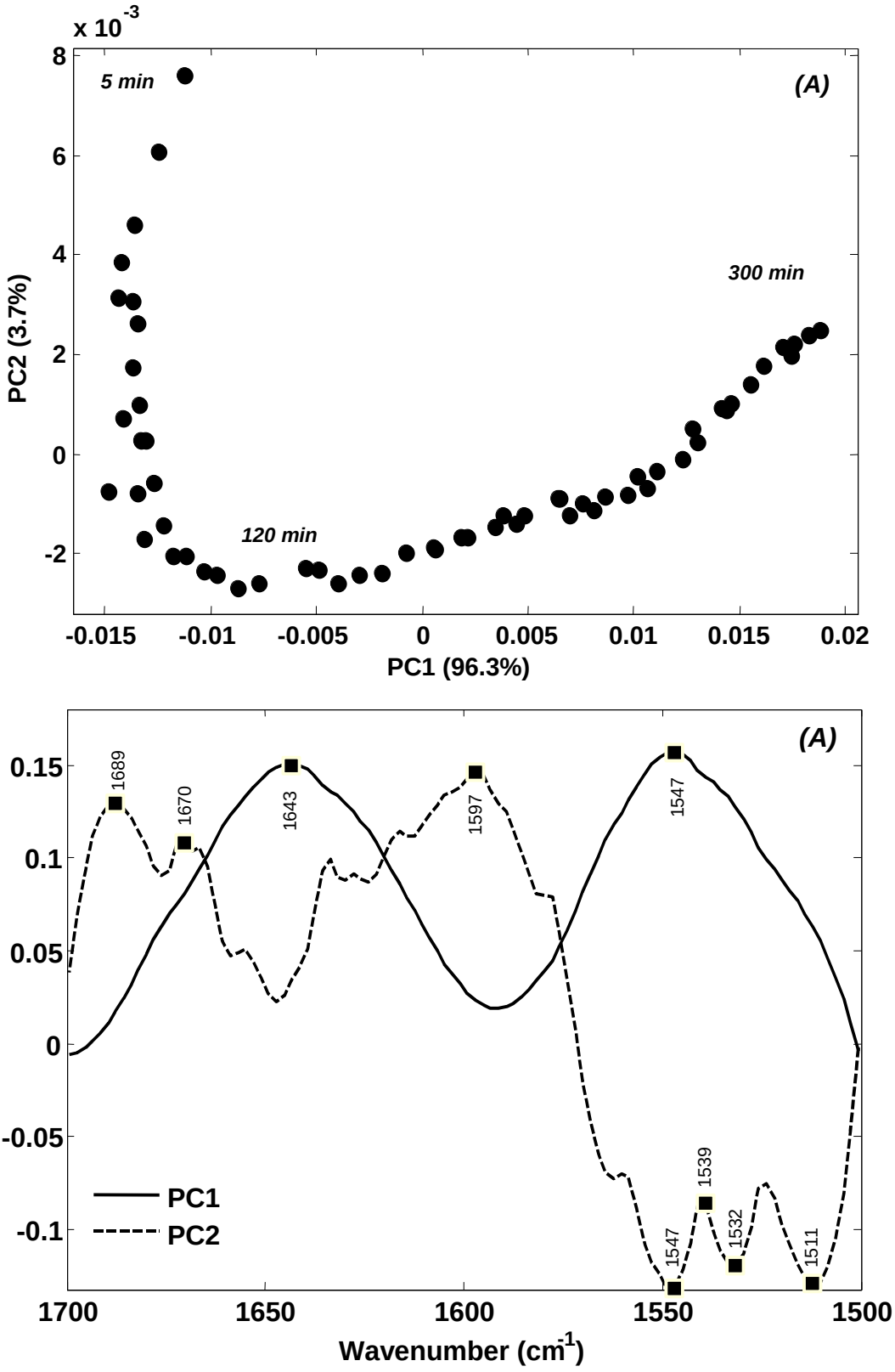

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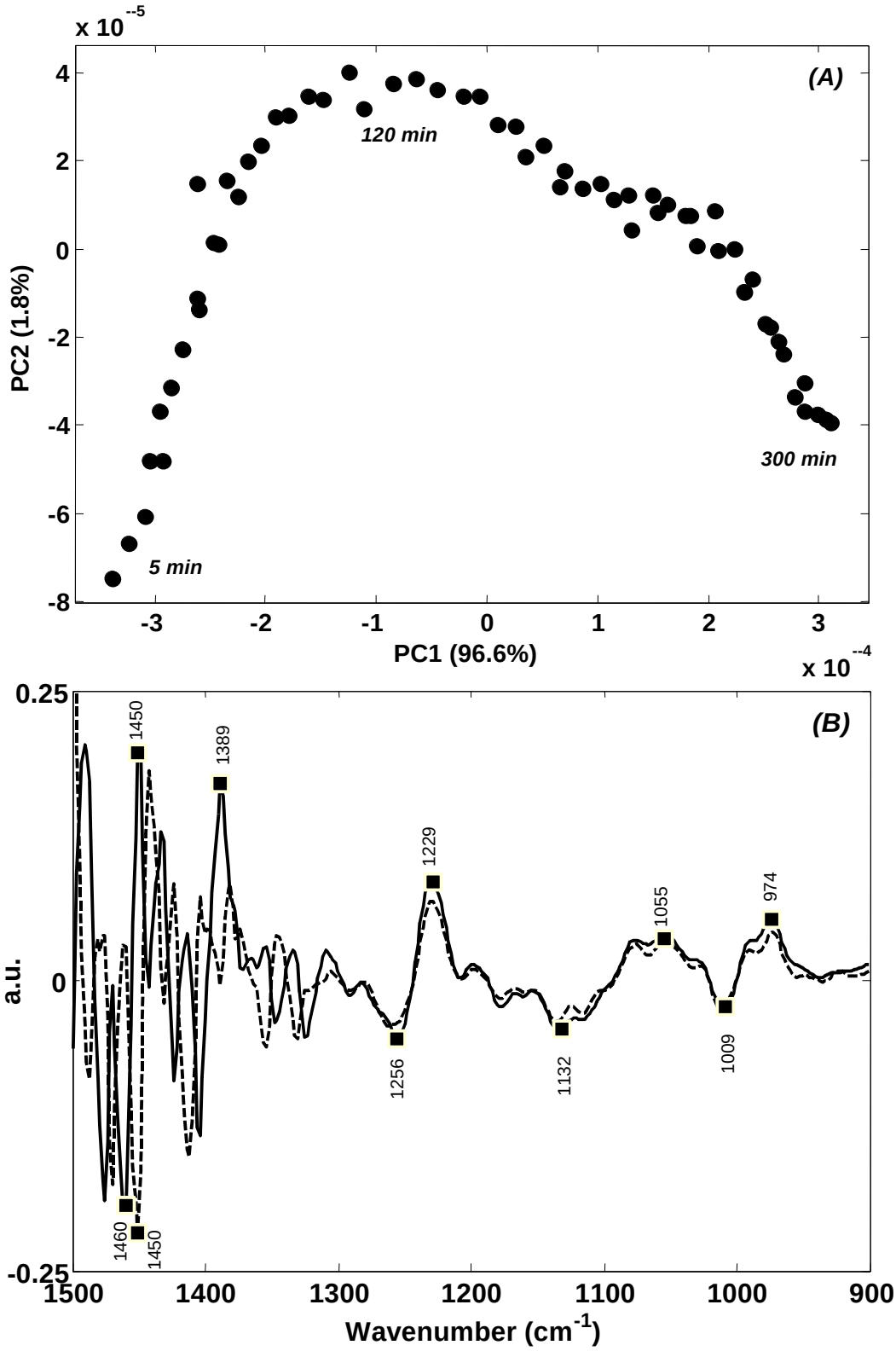

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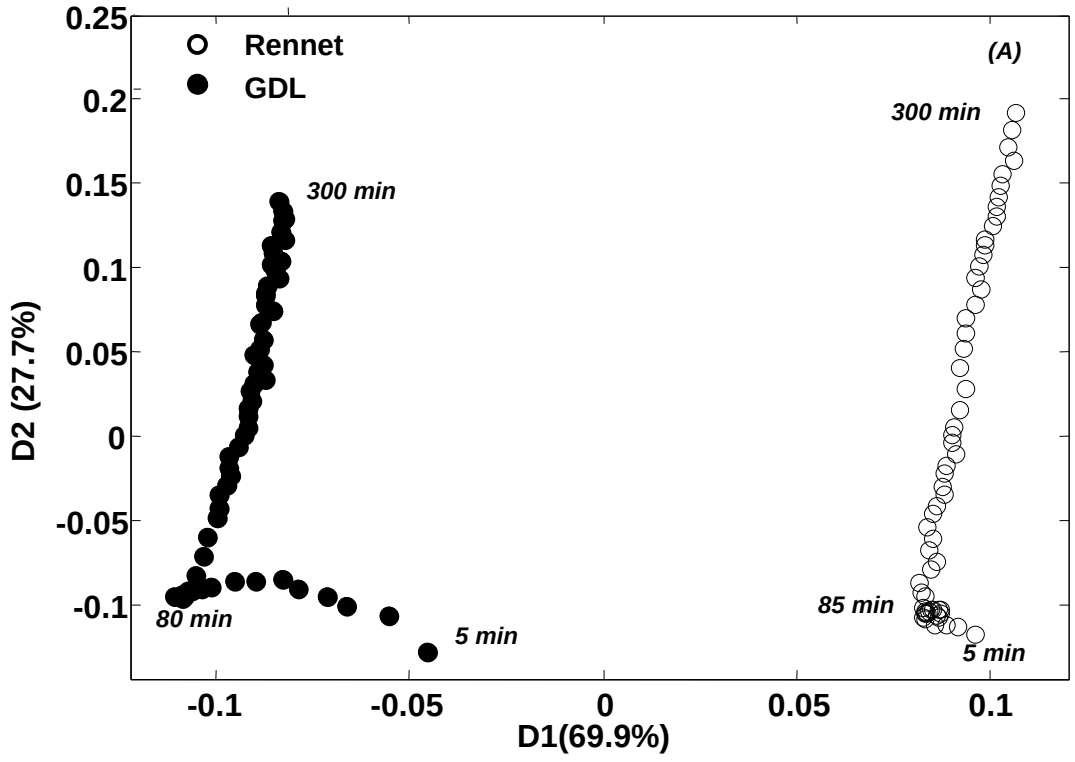

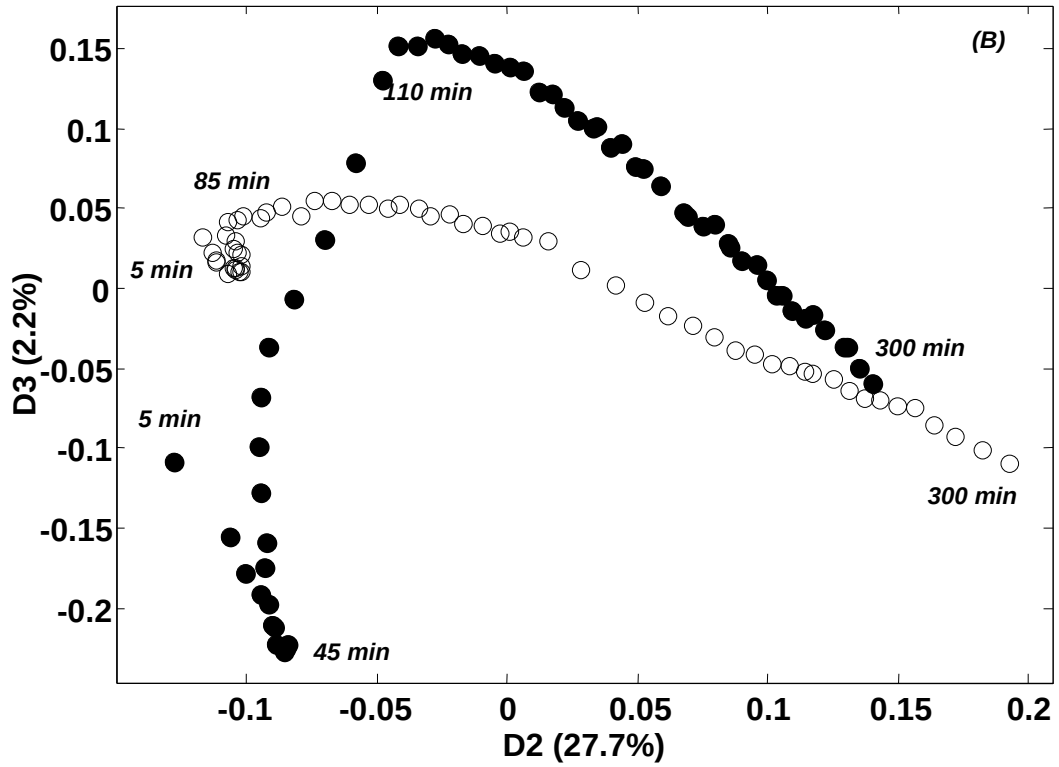

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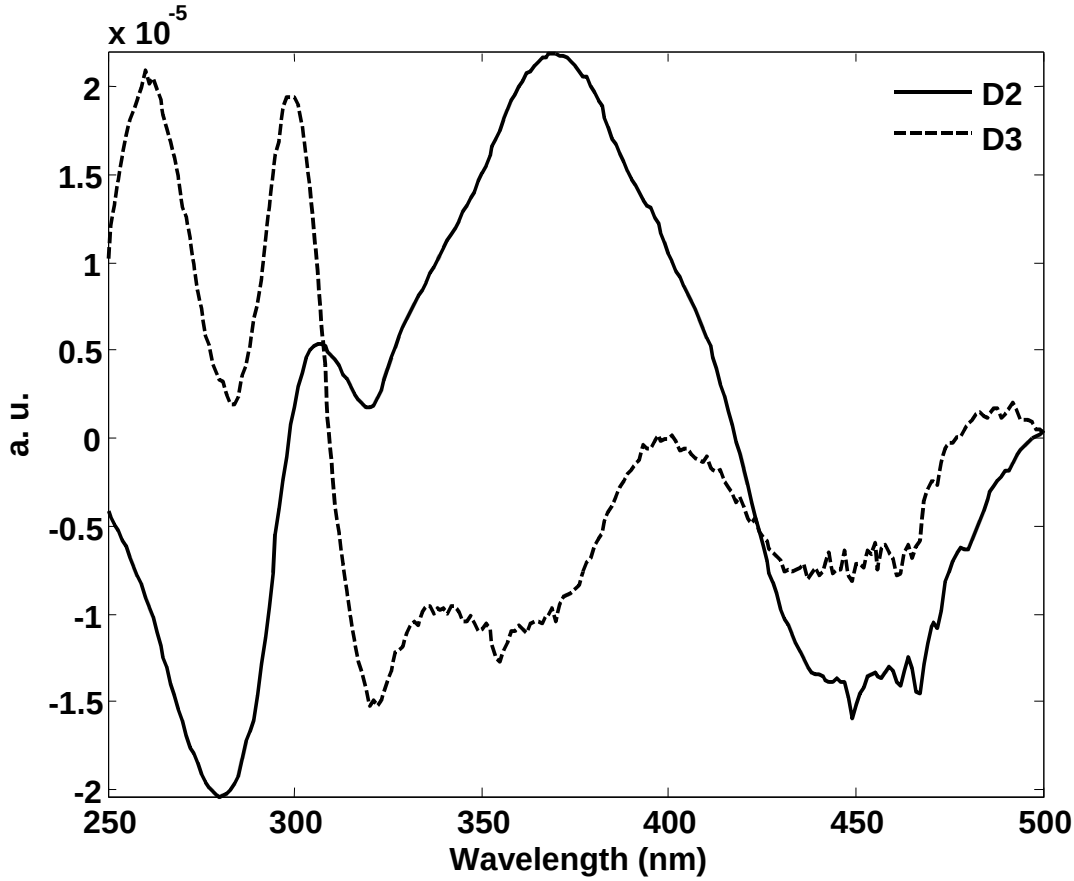

Utilisation of attenuated total reflectance MIR and front-face fluorescence spectroscopies coupled to chemometrics for the identification of Saint-Nectaire cheeses varying by manufacturing conditions

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ABSTRACT

Twelve samples of 3 types of Saint-Nectaire (SNF, SNM, SND) and Savaron cheeses from 12 different producers were studied using attenuated total reflectance MIR and front-face fluorescence spectroscopies, dynamic testing rheology and physico-chemical analysis. Fluorescence spectra (tryptophan residues, vitamin A and riboflavin) and MIR (3000-2800 (fat region), 1700-1500 (protein region) and 1500-900 cm^{-1} (fingerprint region)) spectra were recorded on cheese samples. The potential of the data tables was investigated for determining the production conditions of the different cheeses. The results of factorial discriminant analysis (FDA) performed on the fluorescence and mid-infrared spectra showed a good discrimination of the cheese groups. Considering validation data sets, the best classifications (100%) were achieved from the mid-infrared and fluorescence spectra, while only 91.7% and 72.2% of correct classification were obtained by applying FDA to rheology and physico chemical data, respectively. These results showed that spectroscopic techniques may provide useful fingerprints and allow the identification of investigated cheeses according to manufacturing conditions. Simple and rapid spectroscopic methods offer a promising approach to the authentication of cheeses.

Keywords: Saint-Nectaire cheese, authentication, mid-infrared, front-face fluorescence, chemometrics.

RESUME :

Douze échantillons de trois groupes de fromages Saint Nectaire (SNF, SNM, SND) et Savaron provenant de 12 producteurs ont été analysés par les spectroscopies moyen infra-rouge et de fluorescence, le cisaillement dynamique et les techniques physico-chimiques.
Les spectres de fluorescence (résidus tryptophanes, vitamine A, riboflavine) et moyen infrarouge (3000-2800 (région de la matière grasse), 1700-1500 (région des protéines) et 1500-900 cm^{-1} (région empreinte)) ont été enregistrés sur les échantillons. La possibilité de prédire les conditions de production des fromages à partir des données a été étudiée. Les résultats des analyses factorielles discriminantes (AFD) réalisées sur les spectres de fluorescence et infrarouge montrent une bonne discrimination des 4 groupes de fromages. Si l'on considère les jeux de données de validation, les meilleures classification (100%) ont été obtenues à partir des spectres de fluorescence et infra-rouge, alors que seulement 91,7% et 72,2% de bonnes classifications ont été obtenues pour les AFD réalisées respectivement sur les données rhéologiques et physico-chimiques. Les résultats montrent que les techniques spectroscopiques peuvent générer des empreintes spectrales utiles et permettent d'identifier les fromages étudiés selon leurs conditions de production. Les méthodes spectroscopiques simples et rapides sont des méthodes prometteuses pour authentifier les fromages.

Mots-clés : fromage Saint Nectaire, authentification, moyen infra-rouge, fluorescence frontale, chimiométrie.

INTRODUCTION

Cheese properties play a key role in consumer acceptance [1,8,22,45]. Nowadays, objective and authentic food information is a major concern of many consumers, and it is gaining importance [40,42]. Cheeses with origin identification are generally high-priced and bring in a higher benefit to the producers than ordinary cheeses. For consumers with an extensive choice of food commodities, authenticity is a guarantee of safety and eating quality.

The quality of milk plays a very important role in the production of all types of cheeses, affecting both cheese yield and characteristics of the cheese [44]. In regions with high production costs, agriculture needs to produce food of good quality. The products can be labelled according to the specific conditions which characterise their origin and the processing technology [6]. These regions can be designed for products presenting Protected Designation of Origin (PDO) or Protected Geographical Indication (PGI).

The rheological characterisation of cheeses is important as a means of determining body and texture for quality and identity as a function of composition, processing techniques and storage conditions. It is generally assumed that at room temperature and for a given manufacturing process, milk proteins contribute to firmness and milk fats provide smoothness to cheese: the higher the fat content, the softer the cheese [35].

In recent decades, spectroscopic techniques have been used more and more in the agricultural and food industries. More recently, it has become increasingly clear that the application of spectroscopic methods to food analysis can alleviate important problems in the processing and distribution of food and food products.

Spectroscopic techniques are fast, relatively low-cost and provide a great deal of information with only one test [16,24]. They are considered to be sensitive, non-destructive, rapid, environmentally friendly and non-invasive, which make these methods suitable for on-line or at-line process control. In addition, these analytical tools require limited sample preparation. Spectroscopic methods for measurements of food quality include ultraviolet and visual absorption, fluorescence emission, near-infrared and mid-infrared absorption, Raman scattering, nuclear magnetic resonance and microwave absorption. These spectroscopic techniques are based on different regions of the electromagnetic spectrum and different

physical principles resulting in different sensing capabilities. The methods, however, share the ability to provide rapid multivariate information on the sample being monitored, which in turn makes it possible to simultaneously determine several quality parameters [3].

Mid-infrared spectroscopy is a method used for milk and dairy product analysis [4,17,33,39]. Indeed, each chemical substance (apart from some salts and very simple chemical compounds) has its own distinctive spectrum. Substances occurring in a very low concentration (below 0.1%) are difficult to determine, as the noise of the method might be encountered in such a case.

Fluorescence spectroscopy offers several inherent advantages for the characterisation of molecular interactions and reactions. It is about 1000 times more sensitive than absorption spectroscopy. This spectroscopic technique is more specific and more sensitive than normal infrared technique, allowing measurements in the ppm range. In addition, fluorescent compounds are extremely sensitive to their environment. For example, tryptophan residues that are buried in the hydrophobic interior of a protein have different fluorescent properties than residues that are in a hydrophilic environment [36]. This environmental sensitivity enables the characterisation of conformational changes such as those attributable to the thermal, solvent or surface denaturation of proteins, as well as the interactions of proteins with other food components [11,18,19,21]. It has been shown that front-face fluorescence spectroscopy can discriminate milk samples subjected to heat treatment from those subjected to homogenisation [16], as well as raw milk cheeses from pasteurized milk cheeses [32]. It can also be used to monitor structural changes in milk coagulation [14,21] and cheese manufacture. Front-face spectroscopy has been utilised to determine the geographic origin of milk [31] and Emmental cheeses from different European geographic origins, manufactured during winter and summer periods [25,27,28].

Traditional analytical strategies to uncover adulteration and guarantee quality have relied on wet chemistry to determine the amounts of identified markers in a material [13]. This approach suffers from a number of disadvantages, namely, the ever-increasing range of analytes which may be included in any test procedure. For all these reasons, there is a continuing demand for new, rapid and relatively cheaper methods for direct quality measurement. The overall objective of the present investigation was to study the reliability

and accuracy of mid-infrared and fluorescence spectroscopies coupled with chemometric methods for identifying Saint-Nectaire cheeses manufactured in different conditions.

MATERIAL and METHODS

- Cheese samples

Three different types of Saint-Nectaire cheeses were considered in this study. The first group comprised Saint-Nectaire cheeses manufactured with raw milk and ripened in farm workshops (SNF), the second was formed by cheeses manufactured in farm workshop and repined in dairy plant (SNM) and the third group was made by Saint-Nectaire cheeses manufactured using pasteurized milk and repined in dairy plants (SND). A fourth group, i.e; Savaron cheeses (SAV), was also considered in this study. The manufacturing process of Savaron cheeses is similar to Saint-Nectaire one, but SAV are not PDO cheeses. Each group comprised four cheeses purchased from different producers. A total of 12 cheeses were considered for this study. Cheeses ranges in age from 4 to 8 weeks reflecting the time/age of cheese normally retailed. The temperatures of ripening were between 8 and 12 °C and between 8 and 10 °C for Saint-Nectaire and SAV cheeses, respectively.

Slices were cut off in the middle of the cheese height 20 mm from the rind for mid-infrared, fluorescence, rheology and physico-chemical analysis.

- Physico-chemical analysis

The determination of pH, dry matter, calcium, fat content, total nitrogen and water-soluble nitrogen as protein content for the 12 cheeses was described by [7]. All the analyses were done in triplicate.

- Rheology (dynamic oscillatory experiments)

Cheeses were sliced into thin disks (2 mm thick and 20 mm diameter) with a cheese slicer and the sliced samples were placed into plastic bags to prevent dehydration and stored at 20 °C until analysis. The dynamic oscillatory experiments were performed with a rheometer (CP 20, TA Instrument, Guyancourt, France) with plate geometry of 20 mm diameter. Oscillation experiments were performed by applying force at a constant frequency of 1Hz and the temperature was fixed at 20 °C. The temperature of the lower plate of the measuring system was maintained by applying a Peltier plate that provided very accurate and rapid temperature

control. All the experiments were carried out at force ramp ranging between 0.02 and 2 N corresponding to the linear viscoelastic region.

Data were collected and rheology parameters were calculated using TA instrument software programme. The recorded data included the two components of shear modulus G*, i.e., the elastic component G' (storage modulus) and the viscous component G'' (loss modulus). Tan δ and strain were also measured.

- Mid-infrared spectra

Infrared spectra were recorded between 3000 and 900 cm^{-1} at a resolution of 4 cm^{-1} on a Varian 3100 FT-IR Excalibur Series Fourier-transform spectrometer (Varian Inc., Palo Alto, USA) mounted with a thermostated ATR accessory equipped with a grip. The ATR cell is 6 reflections and was made of a horizontal ZnSe crystal which presented an incidence angle of 45°. The temperature was controlled by a Julabo temperature controller (Julabo, Germany).
Slices of cheeses were deposed on the crystal, a pressure on the grip ensuring a good contact between the two elements. In order to improve the signal to noise ratio, 32 scans were co-added for each spectrum. For each cheese sample, the spectra were recorded at 20°C in triplicate using different samples. Before each measurement, the spectrum of the ZnSe crystal was recorded in the conditions described above and used as background. Base line and ATR corrections were applied to the spectra using Varian Software (Resolution Pro 4.0).
The regions of the mid infrared spectra located between 3000 and 2800 cm^{-1} (fat region), 1700 and 1500 cm^{-1} (protein region) and 1500 and 900 cm^{-1} (fingerprint region) have been considered in this study. A second derivative was applied to the mid-infrared spectra [15]

- Fluorescence spectra

Fluorescence spectra were recorded using a FluoroMax-2 spectrofluorimeter (Spex-Jobin Yvon, Longjumeau, France) mounted with a front-surface cuvette-holder and the incidence angle of the excitation radiation was set at 56° to ensure that reflected light, scattered radiation, and depolarisation phenomena were minimized. The spectrofluorimeter was equipped with a thermostatically controlled cuvette-holder and the temperature was controlled at 20 °C by a Haake temperature controller (Haake, Champlan, France).

The emission spectra of tryptophan residues (305–400nm) and riboflavin (400–640 nm) were recorded with the excitation wavelength set at 290 and 380 nm, respectively [16,26]. The

excitation spectra of vitamin A (250–350 nm) were scanned with the emission wavelength set at 410 nm [16]. For each cheese, three spectra were recorded on different samples.

- *Chemometrics* **:**

- *Principal Component Analysis (PCA)*

PCA is a multidimensional data treatment which provides a synthetic description of large data sets. When applied to spectral data, PCA allows similarity maps of the samples to be drawn and spectral patterns obtained [5]. PCA was applied to the spectra in order to investigate differences in the spectra. This statistical multivariate treatment made it possible to draw similarity maps of the samples and to get spectral patterns [23].

- *Factorial Discriminant Analysis (FDA)*

FDA is a multivariate method that allows the testing of hypotheses [37]. It belongs to the field of decisional statistics, and is based on the comparison of multidimensional intra-group variances to inter-group variances. These methods can show the presence of certain relationships between a qualitative explained criterion and a group of quantitative explanatory characters, and they allow one to describe these latter relationships [4]. The introduction of a qualitative variable within a population allows the division of this population into different groups, with each individual assigned to one group. Discrimination of the groups consists of maximising the variance between their centres of gravity: one can then clarify the properties that distinguish the different groups. If the individual is close to the centre of gravity of its group, it is correctly classified. In the case where the distance to the centre of gravity of its group is superior to that to the centre of gravity of another group, the individual is incorrectly classified and it will be reassigned to this other group. In this study, full cross validation (leave-one-out) was applied as validation method [38].
Chemometric analyses were performed in MATLAB (The Mathworks Inc., Natic, MA, USA) using “Saisir” package available at the address: http://easy-chemometrics.fr.

RESULTS and DISCUSSION

- Physico-chemical characterisation of cheeses

The results for pH, fat, fat in dry matter, total nitrogen, water-soluble nitrogen and calcium determinations for the 12 cheeses are reported in Table 1. On the one hand, SND-cheese fat contents were lower than SNF, SNM and SAV, while the SAV cheeses presented the lowest

water-soluble nitrogen contents. On the other hand, no significant difference was observed for the pHs of the four cheeses. The SNF cheeses had higher dry matters than other cheeses and the SND cheeses had the lowest ones. This can be explained by the differences related to the milk composition, manufacturing process and ripening parameters, which have an effect on the nature and the intensity of proteolysis [10,34].

PCA, a descriptive method, was applied to the normed physico-chemical data. The purpose of this technique is to obtain an overview of all the information in the data set. The PCA results showed that the first two principal components took into account 72.5% of the total variance with a large predominance of principal component 1 (44.3%), and no clear discrimination of the samples as a function of cheese groups (data no shown) was observed.

The aim of FDA is different. It is to predict the membership of an individual to a qualitative group defined as a preliminary. The method requires the development of prediction models for samples that have to be assigned to qualitative groups (SNF, SND, SNM and SAV) such as manufacturing conditions in this study. The results of FDA applied to the normalized data showed that the first two discriminant factors took into account 44.4% and 42.3% of the total variance, respectively. The map defined by discriminant factors 1 and 2 showed that the data were not well suited for the discrimination of the 4 cheese groups (data no shown): the physico-chemical data allowed 72.2% of good classification (Table 2).

- *Characterization of cheese texture by dynamic oscillatory measurements*

Factors such as milk composition, cheese matrix structure, pH, levels of salt and moisture, and changes in protein breakdown markedly affect the viscoelastic behaviour of cheeses [46]. Rheology measurements were carried out in order to investigate cheese characteristics and to investigate their potential to discriminate between the four groups of cheeses.

For the 4 groups, G', G" (Table 1) and η^* (data not shown) decreased, but tan δ increased (data not shown) when the force increased. The SND and SNM cheeses had the highest values for G’ and G’’. In contrast, SAV and SNF cheeses exhibited the lowest values for G’ and G’’. These differences in the values of these rheology parameters could be attributed to the differences in dry matter and fat content in the cheeses.

As the rheology data of the four cheeses exhibited slight differences, univariate analysis was not really appropriate to statistically analyse the data sets. Multivariate statistical techniques such as PCA and FDA make it possible to extract information related to the rheological differences in cheeses. The ability of the rheology data to differentiate between the four cheeses was investigated by applying FDA on the first 10 PCs of the PCA performed on the matrix gathering the rheology data for the four groups of cheeses.

A discrimination of SAV and SND cheeses from SNF and SNM cheeses was essentially observed according to discriminant factor 1 which accounted for 44.2 % of the total variance (Fig. 3). Considering discriminant factor 1, SND cheeses were observed on the right, whereas SNF and SNM cheeses were located on the left. A good classification was observed for 94.5% and 91.7% of calibration (data no shown) and validation samples (Table 2), respectively.

- Discrimination of cheeses from their mid-infrared spectra

The mid-infrared spectrum of a cheese contains information for compounds whose concentrations are higher than 0.1%. Most of the spectral information used for the discriminant analysis is located in: (1) the 3000–2800 cm^{-1} range corresponding to C-H stretching, (2) the region located between 1700–1500 cm^{-1} corresponding to the amide I and II bands, and (3) the 1500– 900 cm^{-1} region called the fingerprint region.

The 3000–2800 cm^{-1} spectral region corresponds to the C–H bond of methyl and methylene groups of fatty acids. This region was dominated by two strong bands located at 2919 cm^{-1} and 2851 cm^{-1} which have been assigned to methylene anti-symmetric and symmetric stretching modes [9], respectively (Fig. 1a). The weaker bands, located at 2955 cm^{-1} and 2870 cm^{-1}, were assigned to the anti-symmetric and symmetric stretching modes of the terminal methyl groups.

SNF cheeses presented the highest intensity at 2921 and 2851 cm^{-1}, while SAV cheeses presented the lowest ones. In addition, the spectra showed different shapes according to the type of cheese. Furthermore, a slight shift to higher wavelengths of the CH_2 stretching mode was observed for SAV cheeses. Differences in the shape of the 3000– 2800 cm^{-1} spectral region could be due to differences in both the nature, concentration and physical state of fatty acids [9,15]. Indeed, Collomb et al. (2002) [12] reported that milks produced in highlands had

smaller amounts of saturated short- and medium-chain fatty acids and more poly-unsaturated fatty acids than those collected from lowlands. Moreover, Dufour et al. (2000) [15] reported that the structure of triglycerides acyl chains in fat globules was modified during cheeses ripening and the shift of the methylene bands in the infrared region agreed with partial crystallization of triglycerides from 21 to 81 days of storage.

The 1700–1500 cm^{-1} region was characterised by the presence of bands related to peptides and proteins. Thus, these bands contain some information on the proteins and on the interaction of these proteins with other components, such as ions, water and other proteins. Fig. 1b shows the shapes of the spectra of the investigated cheeses. Contribution to the amide I band, which is used to investigate the secondary structure of proteins, can be observed at about 1638 cm^{-1} for SNF, SNM and SND cheeses and at about 1639 cm^{-1} for SAV cheeses. The absorption band with a maximum at about 1545 cm^{-1} is generally assigned to the amide II vibrations (Fig. 1b).

The 1500–900 cm^{-1} region is called the fingerprint region and numerous chemical bonds are absorbing in this region (data no shown). Considering the recorded spectra, the important differences were observed at the strong absorbing bands located at 1159 and 1099 cm^{-1}. These bands could be related to P=O stretching [2]. Indeed, this author reported that PO^{2-} and PO^{3-} compounds present bands in the 1323–1092 cm^{-1} and 1140 and 1055 cm^{-1} regions, respectively. On the other hand, SAV cheeses presented the higher intensity at 1408 cm^{-1}: this band has been assigned to the bending mode of C–C–H [41,43].

PCA was applied, separately, to the three spectral regions (3000–2800, 1700–1500 and 1500–900 cm^{-1}). From these spectral regions, the similarity maps defined by principal component 1 and principal component 2 did not show good discrimination between the four groups of cheeses (data not shown). In a second step, the ability of infrared data to differentiate between the four groups (SNF, SNM, SND and SAV) of cheeses was investigated by applying FDA to the first 20 PCs of the PCA performed on the three data tables.
Considering the 3000–2800 cm^{-1} spectral region, the map defined by discriminant factors 1 and 2 took into account 70.2 % of the total variance (fig.4a). SAV and SNM cheeses were clearly discriminated according to discriminant factor 1 which accounted for 36.4 % of the total variance while SNF and SND cheeses were separated along the discriminant factor 2

(33.8 % of total variance). In addition, the discriminant factor 3 (28.8% of total variance) essentially discriminated SND and SAV cheeses (data not shown).
The data allow a good classification of all the samples. 100% of good classification were observed for the calibration (data no shown) and validation (Tab. 2) spectral data sets, respectively.

Concerning the 1700–1500 cm^{-1} spectral region, the map defined by the discriminant factors 1 and 2 is shown in Figure 4b. This map shows that all the cheeses are well discriminated. The discriminant factor 1 discriminated SAV cheeses from the other cheeses, whereas the discriminant factors 2 and 3 principally separated the SNM and SND cheeses.
Complete classifications were obtained for the four cheese groups: the good classifications amounted to 100% for calibration (data no shown) and validation (Tab. 2) data sets.

Regarding the fingerprint region (1500-900 cm^{-1}), the map defined by the discriminant factors 1 and 2 (69% of total variance) showed that SAV and SND cheeses were well discriminated while others were not well grouped (data no shown). The discriminant factor 1 esentially separated SAV and SND cheeses. The SND cheeses had positive score values according to factor 1, while SAV cheeses presented positive value. In addition, the factor 3 (31% of the total variance) discriminated SNF and SNM cheeses (data not shown).

The good classifications were as for the two other mid infrared regions: 100% of the samples for the calibration and the validation (Tab. 2) data sets were well classified.

- Discrmination of cheeses from theirs fluorescence spectra
Recording of fluorescence spectra at different excitation and emission wavelengths was performed to investigate the differences between the four cheeses. Tryptophan, riboflavin and vitamin A fluorescence spectra were recorded on the investigated cheeses. Figures 2a and b showed riboflavin and vitamin A fluorescence spectra. From Figure 2, it can be made a visual discrimination between the four groups of cheeses as most of the spectra show different shapes.

Considering riboflavine fluorescence spectra (Fig. 2a), SNF and SND cheese spectra exhibited large differences from the other ones. The SNF cheeses presented two maxima at about 413 and 521 nm, whereas the maxima were at 413 and 518 nm for the SND cheeses.

However, two maxima located at 441 and at 518 nm and at 518 and 543 nm were observed for the SNM and SAV cheeses, respectively. These can be assigned to riboflavin degradion products [32]. Riboflavin, also known as vitamin B2, is a water soluble vitamin that occurs naturally in dairy products. It has a strong and broad fluorescence emission peak in the region 525–531 nm [32,47]. Riboflavin is stable to heat, oxidation, and acid, but unstable in the presence of alkali or light, especially ultraviolet light. The vitamin is photo-chemically degraded into different forms, i.e., lumichrome and lumiflavin. These two compounds are also fluorescent with emission maxima in the regions 444 to 479 nm and 516 to 522 nm, respectively [32,47].

When the excitation spectra were scanned from 280 to 350 nm with emission measured at 410 nm, two maxima located at 305 and 322 nm and a shoulder at 295 nm (Fig. 3) were observed. The SND cheeses had the higher intensity at 322 nm whereas the SNM cheeses presented the lowest ones. In addition, the shape of the spectra changes with the manufacturing process of the investigated cheeses. Recently, it has been reported that the shape of the vitamin A excitation spectrum is correlated with the physical state of the triglycerides in the fat globules [14,30].

Considering tryptophan fluorescence spectra (data no shown), the normalised emission spectra of cheeses exhibited a maximum at about 343 nm for SNF, SND and SAV cheeses, while SNM cheeses had a maximum at 345 nm. This shift indicates diversity for the environment of tryptophan residues between the SNM and the other cheeses. Herbert et al.(2000) [20] have investigated tryptophan fluorescence of cheeses in order to discriminate between eight marketed soft-cheeses varying by the manufacturing conditions. They demonstrated that the tryptophan fluorescence spectra of a cheese are fingerprints that allow its identification : using FDA, 95% of the spectra were well classified. It has also been shown that tryptophan fluorescence spectra can discriminate raw milk cheeses (Emmental) from pasteurized milk cheeses [29].

The discriminant ability of the 3 data sets was then investigated by applying FDA to the first 20 PCs. Figures 5a and 5b showed the maps defined by discriminant factors 1 and 2 for riboflavin (72 % of total variance) and vitamin A (74.2% of total variance) fluorescence spectra. In this two maps, the SNM and the SNF cheeses were well discriminated, while the SND and the SAV cheeses were also separated but formed closer groups. Considering

riboflavin and vitamin A data, the discriminant factors 3 discriminated SND from SAV cheeses (data not shown). The present results are in agreement with the results of [20] who found that vitamin A was a useful probe to discriminate soft cheeses as a function of ripening and/or of manufacturing process. In addition, tryptophan spectra showed similar discriminant ability (data no shown).

As shown in Table 2, 100% of correct classification were observed for the calibration (data no shown) and validation (Tab. 2) data sets for the 3 fluorophores.

CONCLUSION

As physico-chemical and rheology data only allowed 72.2 % and 91.7 % of good classification of the investigated cheeses, fluorescence and mid infrared spectra classified correctly all the investigated cheeses.

This study showed that simple and rapid spectroscopic methods such as the mid infrared and front-face fluorescence, combined with chemometric tools, offer a promising approach to the authentication of cheeses. While the use of infrared spectroscopy has widely spread across food analysis application during the last two decades, there is now attraction to develop new analytical techniques based on fluorescence spectroscopy. The recent development of very small spectrofluorimeter, as well as of fibre optic probes and LED emitting in the UV range, is leading to the development of integrated apparatus. The most attractive advantages of these laptop spectrofluorimeters are that no preliminary sample preparation is needed prior the measurement and results are obtained in a short period of time (a couple of seconds) compared with conventional techniques. Thus, front-face fluorescence technique in conjunction with chemometric tools has a great potential to become a useful quality control method in rapid online, as well as offline, analysis of dairy products.

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Table 1. Physico-chimical composition of the investigated Sanit Nectaire cheeses.

	Mean	SD*	CV* (%)
		SNF cheeses	
pH	5.8	0.03	0.52
Dry matter (% g/100g)	56.5	0.14	0.24
Fat (%g/100g)	30.6	0.20	0.67
TN* (%g/100g)	22.7	0.32	1.40
WSN* (% g/100g)	3.0	0.07	2.49
Ca (g/kg)	5.3	0.16	3.01
		SNM cheeses	
pH	5.9	0.05	0.86
Dry matter (% g/100g)	58.6	0.45	0.76
Fat (%g/100g)	30.8	0.20	0.66
TN* (%g/100g)	24.0	0.23	0.95
WSN* (% g/100g)	3.2	0.04	1.33
Ca (g/kg)	5.8	0.16	2.81
		SND cheeses	
pH	5.8	0.08	1.31
Dry matter (% g/100g)	55.2	0.11	0.20
Fat (%g/100g)	28.5	0.32	1.13
TN* (%g/100g)	23.9	0.24	0.99
WSN* (% g/100g)	3.4	0.13	3.70
Ca (g/kg)	5.6	0.13	2.23
		SAV cheeses	
pH	5.8	0.01	0.21
Dry matter (% g/100g)	54.2	0.18	0.34
Fat (%g/100g)	30.3	0.35	1.17
TN* (%g/100g)	22.0	0.25	1.15
WSN* (% g/100g)	2.3	0.08	3.37
Ca (g/kg)	5.5	0.32	5.95

* SD:standard deviation; CV:100%(SD/mean);TN:total nitrogen; WSN: water-soluble nitrogen

Table 2: Classification table of the cheeses using the full cross-validation from physico-chemical, rheology, and MIR and fluorescence spectral data.

Real[b] \ Predicted[a]	SNF	SNM	SND	SAV	% well classification
Physico-chimical data					
SNF	**6**	0	0	3	66.7
SNM	1	6	2	0	66.7
SND	1	1	7	0	77.8
SAV	2	0	0	**7**	77.8
Rheology data					
SNF	**9**	0	0	0	100.0
SNM	1	**7**	0	1	77.8
SND	0	0	**8**	1	88.9
SAV	0	0	0	**9**	100.0
3000–2800 cm^{-1} spectral region (MIR)					
SNF	**9**	0	0	0	100.0
SNM	0	**9**	0	0	100.0
SND	0	0	**9**	0	100.0
SAV	0	0	0	**9**	100.0
1700–1500 cm^{-1} spectral region (MIR)					
SNF	**9**	0	0	0	100.0
SNM	0	**9**	0	0	100.0
SND	0	0	**9**	0	100.0
SAV	0	0	0	**9**	100.0
1500–900 cm^{-1} spectral region (MIR)					
SNF	**9**	0	0	0	100.0
SNM	0	**9**	0	0	100.0
SND	0	0	**9**	0	100.0
SAV	0	0	0	**9**	100.0
Riboflavine emission fluorescence spectra					
SNF	**9**	0	0	0	100.0
SNM	0	**9**	0	0	100.0
SND	0	0	**9**	0	100.0
SAV	0	0	0	**9**	100.0
Vitamin A exitation fluorescence spectra					
SNF	**9**	0	0	0	100.0
SNM	0	**9**	0	0	100.0
SND	0	0	**9**	0	100.0
SAV	0	0	0	**9**	100.0
Tryptophan emission fluorescence spectra					
SNF	**9**	0	0	0	100.0
SNM	0	**9**	0	0	100.0
SND	0	0	**9**	0	100.0
SAV	0	0	0	**9**	100.0

[a] The number of cheeses predicted from the model. [b] The number of real cheeses.

CAPTIONS

Figure 1: Mid infrared spectra of cheeses: SNF (—), SNM (---), SND (····) and SAV (–·–). Spectral regions considered in the study: (***a***) 3000-2800 cm^{-1} and (***b***) 1700-1500 cm^{-1}.

Figure 2: Fluorescence spectra spectra of cheeses: (a) riboflavine emission spectra and (b) vitamin A excitation spectra. SNF (—), SNM (---), SND (····) and SAV (–·–).

Figure 3: Discriminant analysis similarity map determined by discriminant functions 1 and 2 for the rheology data (G', G'', tan δ, strain and η*) for the four groups of cheeses: (▽): SNF; (●): SNM; (■): SND; (❋): SAV.

Figure 4: Discriminant analysis similarity maps determined by discriminant functions 1 and 2 for mid infrared spectra: (***a***) 3000-2800 cm^{-1} and (***b***) 1700-1500 cm^{-1}. (▽): SNF; (●): SNM; (■): SND; (❋): SAV.

Figure 5: Discriminant analysis similarity maps determined by discriminant functions 1 and 2 for riboflavine (***a***) and vitamin A (***b***) spectral data: (▽): SNF; (●): SNM; (■): SND; (❋): SAV.

Figure 1: Boubellouta, Lebecque & Dufour.

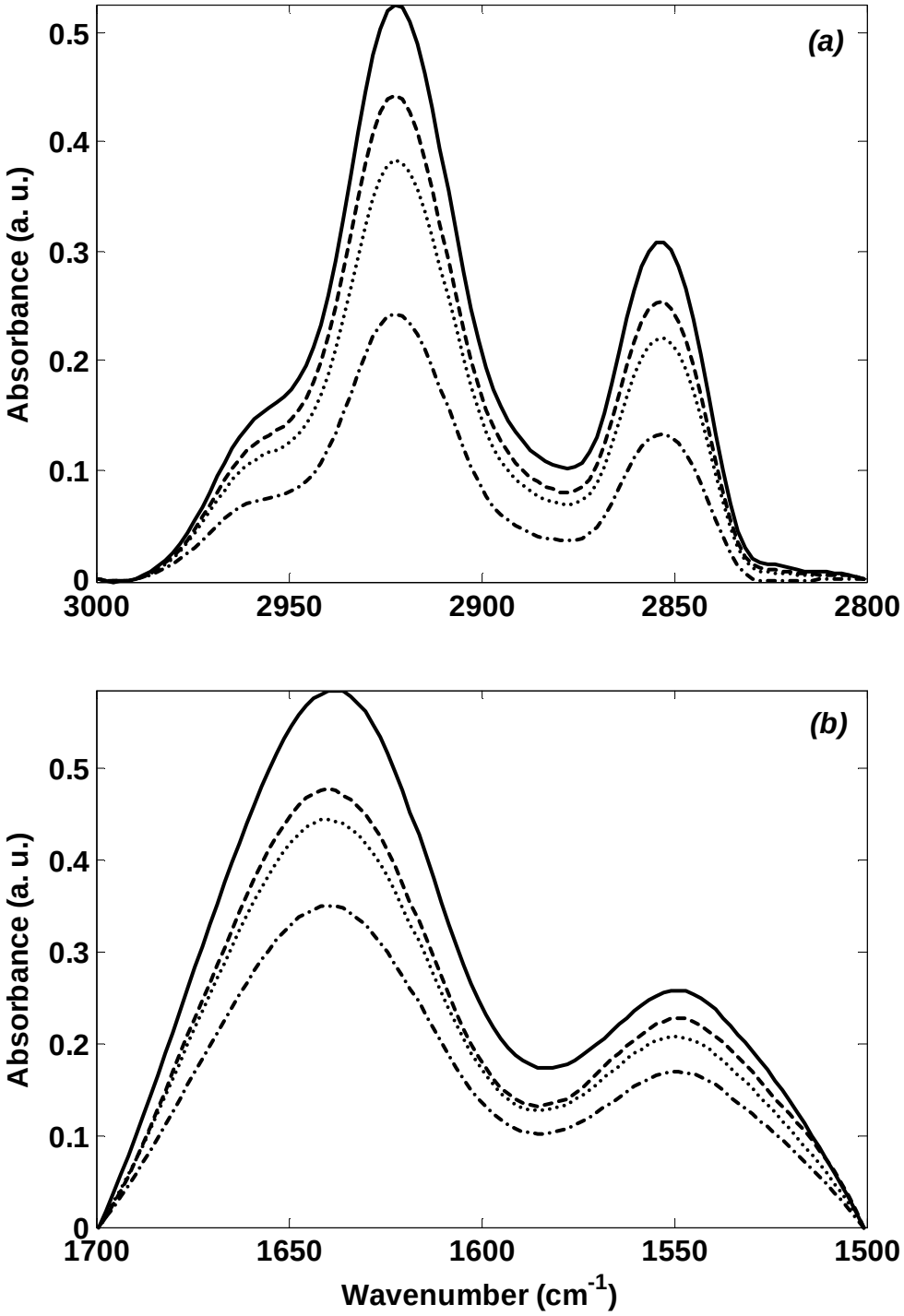


Figure 2: Boubellouta, Lebecque & Dufour.

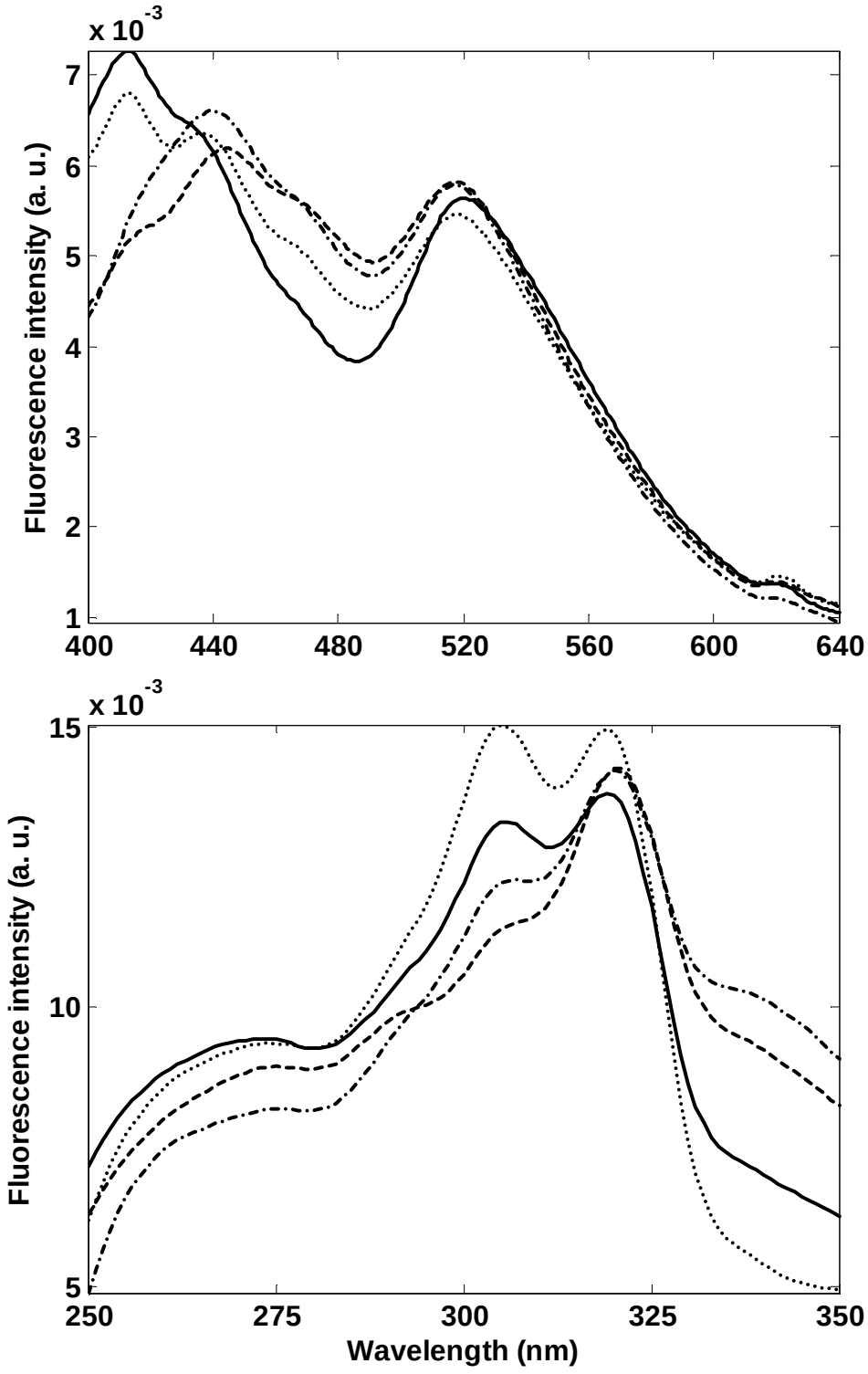


Figure 3: Boubellouta, Lebecque & Dufour.

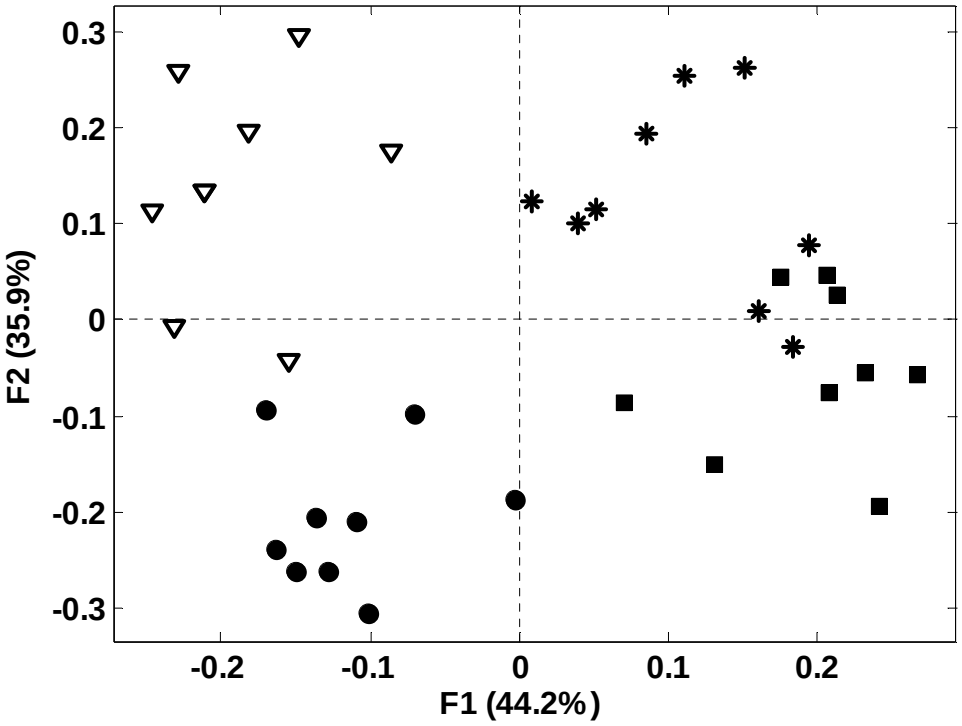


Figure 4: Boubellouta, Lebecque & Dufour

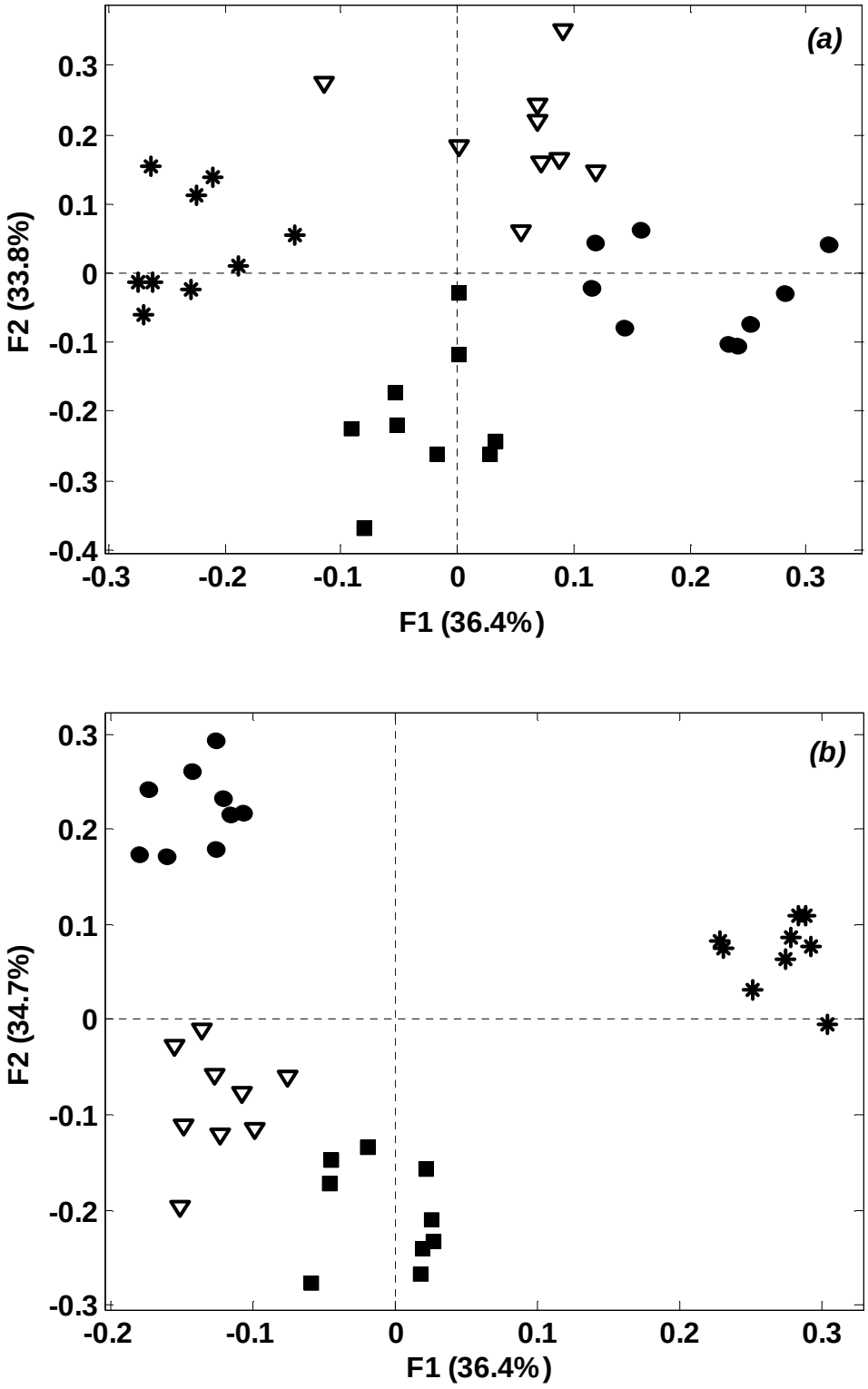
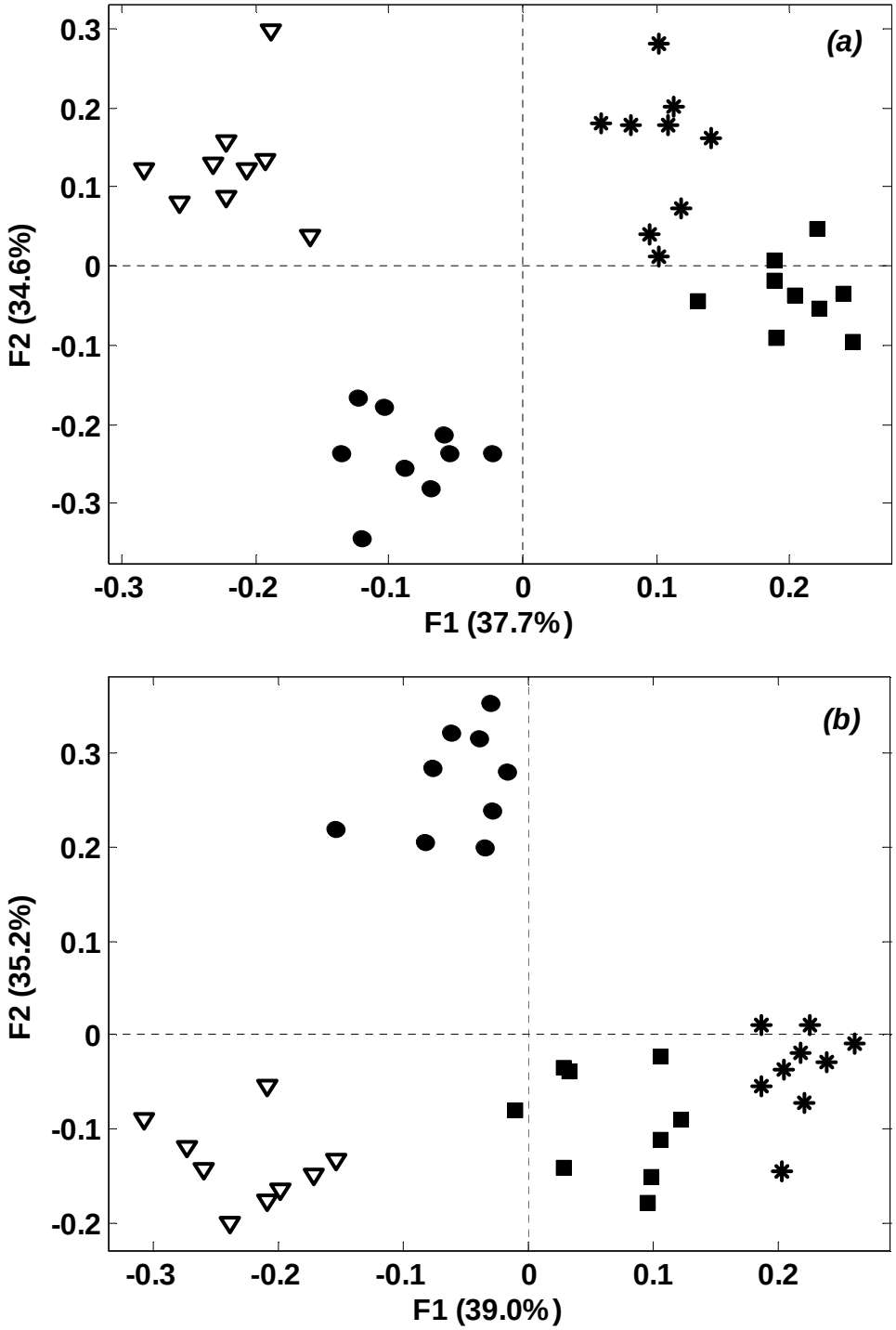


Figure 5: Boubellouta, Lebecque & Dufour.



Investigation of cheese-matrix rheology and structure during melting using dynamic testing rheology and mid infrared and synchronous front-face fluorescence spectroscopies coupled with chemometrics

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ABSTRACT

Trends in the texture and structure as a function of temperature were determined for Comté (hard cheese) and Raclette (semi-hard cheese) cheeses using dynamic testing rheology and, mid-infrared and synchronous front-face fluorescence spectroscopies. The storage modulus (G'), the loss modulus (G'') and the complex viscosity (η*) decreased while strain and tan δ increased as the temperature increased from 20 to 80 °C. SF (250-500 nm with Δλ=80) and MIR (3000-2800 (fat region), 1700-1500 (protein region and 1500-900 cm^{-1} (fingerprint region)) spectra were recorded on cheese samples at 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75 and 80 °C. The results showed that each spectroscopic technique provided relevant information related to the cheese protein and fat structures during melting allowing to investigate structural changes. In addition, the melting temperatures of cheese matrices and fats of the two cheeses were determined from the dynamic rheology data, SF spectra and MIR spectra. Similar temperatures were obtained whatever the technique, since values of about 60 °C and 31 °C were obtained for matrix and fat melting temperatures of Comté and Raclette cheeses, respectively.

Keywords: cheese, heating, melting, Mid-infrared, synchronous front-face fluorescence, rheology, chemometrics.

INTRODUCTION

Texture is an important criteria used to evaluate the quality of cheeses (Dufour et al., 2000) and textural properties play a key role in consumer acceptance of cheese (Ak & Gunasekaran, 1992; Bugaud et al., 2001; Jaros et al., 2000; Vassal et al., 1987). The rheological characterisation of cheeses is important as a mean of determining body and texture for quality and identity as a function of composition, processing techniques and storage conditions. Texture is a reflection of cheese structure at the microscopic and molecular levels. Structurally, cheese is a complex matrix of milk proteins, fats, minerals and other components including water. Cheese variety and composition influence component distribution which in turn largely determines the structural characteristics. It is generally assumed that at room temperature milk proteins contribute to firmness and milk fats provide smoothness to cheese: the higher the fat content, the softer the cheese (Lawrence et al., 1983). Moreover, cheese texture may change with the physical state of the fats, depending on temperature (Dufour et al., 2000). Concomitantly, there is a need to characterise cheese behaviour during heating, the change in the viscosity and the nature of protein-protein and protein-lipid interactions at elevated temperature.

Meltability is one of the most important physical properties of cheese at high temperatures (Karoui et al., 2003). The melting property of cheese is important not only for texture, but also for the mixing property of cheese in food preparation. It is affected by various technological factors of cheese manufacture such as fat and moisture contents, chymosin level and proteolysis (Kuo et al., 2000; Muthukumarappan et al., 1999). There are many methods available to study the phenomenon of cheese meltability (Arnott et al., 1957; Lee et al., 1978). Some of these methods are empirical and have a low repeatability like those based on measuring the change in the diameter or height of a cylindrical cheese sample after heating it in an oven (Arnott et al., 1957).

However, dynamic testing methods offer very rapid results with minimal chemical and physical changes (Konstance & Holsinger, 1992). Dynamic oscillatory method is the most common technique for the study of the viscoelastic behaviour of food (Bertola et al., 1992; Bowland & Foegeding, 2001; Campanella et al., 1987; Rosenberg et al., 1995).

Spectroscopic techniques are fast, relatively low-cost and provide a great deal of information with only one test (Birlouez-Aragon et al., 2002; Karoui et al., 2005). They are considered to be sensitive, non-destructive, rapid, environmentally friendly and non-invasive, which makes these methods suitable for on-line or at-line process control. In addition, these analytical tools require limited sample preparation. In the last decade, rapid spectroscopic measurements have

advanced in quality control in many areas of food production. Spectroscopic methods for measurements of food quality include ultraviolet and visual absorption, fluorescence emission, near-infrared and mid-infrared absorption, Raman scattering, nuclear magnetic resonance and microwave absorption. These spectroscopic techniques are based on different regions of the electromagnetic spectrum and different physical principles resulting in different sensing capabilities. The methods, however, share the ability to provide rapid multivariate information on the sample being monitored, which in turn makes it possible to simultaneously determine several quality parameters (Belton, 2000).

Mid-infrared spectroscopy is a method used for milk and dairy product analysis. Indeed, each chemical substance (apart from some salts and very simple chemical compounds) has its own distinctive spectrum. However substances occurring in a low concentration (below 0.1%) can be difficult to determine. An other disadvantage of mid-infrared spectroscopy is the strong absorptions of water present in many food products. The O-H bending band ~1650 cm^{-1} may effectively obscure potentially useful absorptions from proteins. However, it has been successfully used to investigate changes in protein interactions in cheese during ripening (Mazerolles et al., 2001).

Synchronous Fluorescence Spectroscopy (SFS) is a rapid and sensitive method for quantifying fluorescent components and characterising their molecular environments in all sorts of biological samples (Tang et al., 2008). This technique offers several inherent advantages for the characterisations of molecular interactions and reactions. Recently, SFS has been used to investigate the changes of milk component structures and interactions during milk heating and acidification (Boubellouta & Dufour, 2008). SFS presents an interesting advantage from our point of view. A synchronous fluorescence spectrum retains information related to several fluorophores, compared to a classical emission spectrum that is mainly specific of a sole fluorophore (Boubellouta & Dufour, 2008; Divya & Mishra, 2007).

The purpose of this study was to investigate the reliability and accuracy of dynamic testing rheology, mid-infrared and synchronous fluorescence spectroscopies coupled with chemometric methods to evaluate the meltability and the viscoelastic behaviour of a semi-hard cheese and a hard cheese. Another objective was to gain some insight into the structure of cheese matrix and to the relation between cheese matrix structure and cheese texture using chemometric methods.

MATERIAL and METHODS

- Cheese samples

Two different marketed cheeses, Comté (hard) cheese and (Raclette) semi-hard cheese, were purchased in a local supermarket. The durations of ripening were 5 months for Comté cheese and 8 weeks Raclette cheese. Slices were cut off in the middle of the cheese height 20 mm from the rind for mid-infrared, synchronous fluorescence and rheological analysis.

- Mid-infrared spectra

Infrared spectra were recorded between 3000 and 900 cm^{-1} at a resolution of 4 cm^{-1} on a Varian 3100 FT-IR Excalibur Series Fourier-transform spectrometer (Varian Inc., Palo Alto, USA) mounted with a thermostated ATR accessory equipped with a grip. The ATR cell is 6 reflections and was made of a horizontal ZnSe crystal which presented an incidence angle of 45°. The temperature was controlled by a Julabo temperature controller (Julabo, Germany).
Slices of cheeses were deposed on the crystal, a pressure on the grip ensuring a good contact between the two elements. In order to improve the signal to noise ratio, 32 scans were co-added for each spectrum. For each cheese sample, the spectra were recorded at 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75 and 80 °C in triplicate. For each cheese, three temperature kinetics were performed using different samples. Before each measurement, the spectrum of the ZnSe crystal was recorded in the conditions described above and used as background. Base line and ATR corrections were applied to the spectra using Varian Software (Resolution Pro 4.0).
The regions of the mid infrared spectra located between 3000 and 2800 cm^{-1} (fat region), 1700 and 1500 cm^{-1} (protein region) and 1500 and 900 cm^{-1} (fingerprint region) have been considered in this study. A second derivative was applied to the mid-infrared spectra (Dufour et al., 2000).

- Synchronous fluorescence spectra

Synchronous fluorescence spectra were recorded using a FluoroMax-2 spectrofluorimeter (Spex-Jobin Yvon, Longjumeau, France) mounted with a front-surface cuvette-holder and the incidence angle of the excitation radiation was set at 56° to ensure that reflected light, scattered radiation, and depolarisation phenomena were minimized. The spectrofluorimeter was equipped with a thermostatically controlled cuvette-holder and the temperature was controlled by a Haake temperature controller (Haake, Champlan, France).

Synchronous fluorescence spectra were collected in the 250–500 nm excitation wavelength range using offset of 80 nm between excitation and emission monochromators (Boubellouta & Dufour, 2008). For the experiments, the synchronous spectra of cheese samples (2 cm x 1 cm x 0.3 cm) placed in quartz cuvette were recorded at 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75 and 80 °C. For each cheese, three temperature kinetics were performed using different samples.

- Dynamic oscillatory experiments

Cheeses were sliced into thin disks (2 mm thick and 20 mm diameter) with a cheese slicer and the sliced samples were placed into plastic bags to prevent dehydration and stored at 5 °C until analysis. The dynamic oscillatory experiments were performed with a rheometer (CP 20, TA Instrument, Guyancourt, France) with plate geometry of 20 mm diameter. Oscillation experiments were performed in the linear viscoelastic region by applying a constant force of 0.5 N and a constant frequency of 1 Hz. All the experiments were carried out at temperatures ranging between 20 and 80 °C by applying a Peltier plate that provided very accurate and rapid temperature control. A temperature ramp of 35 min was selected, since it allowed an equilibration of the thin cheese samples, to obtain reproducible results and a discrimination of the different cheeses investigated. The data obtained included the two components of shear modulus G*, i.e., the elastic component G' (storage modulus) and the viscous component G" (loss modulus). The complex viscosity (η*) and tan δ were also measured. Data were recorded every 0.4 °C between 20 and 80 °C. For each cheese, three curves were recorded using different samples. Any slippage was detected for all the experiments. Plots of log (η*) versus temperature were obtained for the investigated cheeses. They showed two distinct linear regions and the intersection point was identified as the melting point of fats in the cheese (Karoui et al., 2003). The temperature for which delta is equal to 45° of angle (tan δ =1) indicates the moment when viscous properties prevail, and at which an increase in temperature involves primarily the melting of cheese (Famelart et al, 2002).

Chemometrics **:**

- *Principal Component Analysis (PCA)*

PCA is a multidimensional data treatment which provides a synthetic description of large data sets. When applied to spectral data, PCA allows similarity maps of the samples to be drawn and spectral patterns obtained (Bertrand et al., 1987). PCA was applied to the synchronous spectra in order to investigate differences in the spectra. This statistical multivariate treatment made it possible to draw similarity maps of the samples and to get spectral patterns (Jollife, 1986).

- Common Components and Specific Weight Analysis (CCSWA)

CCSWA was developed within a sensory framework as a tool to analyze several data sets (Qannari et al., 2000). The objective of CCSWA is to describe several data tables observed on the same *n* samples by recovering the maximum inertia (total variance) of each of them. It was subsequently applied in chemometrics for combining different kinds of measurements made on food products (Mazerolles et al., 2002; Pram Nielsen et al., 2001). The rationale behind this method is the existence of a common structure to the data tables. Therefore, the method determines a common space of representation for all the data sets. Each table is allowed having a specific weight (or salience) associated with each dimension of this common space (Mazerolles et al., 2006) .

Chemometric analyses were performed in MATLAB (The Mathworks Inc., Natic, MA, USA) using “Saisir” package available at the address: http://easy-chemometrics.fr.

RESULTS and DISCUSSION

- *Rheology data*

For the 2 cheeses, G', G" (Table 1) and η* (data not shown) decreased, but tan δ increased (data not shown) when the temperature increased. This result was in agreement with Karoui et al. (2003) and Rosenberg et al. (1995). Rosenberg et al. report that G' is affected by the temperature, the frequency and the cheese age. This can be attributed to the effect of temperature on fat and on protein-protein and protein-water interactions (Famelart et al., 2002; Rosenberg et al., 1995). It has been shown that the decrease in the temperature induces an increase in the yield of milk fat in the solid state and, as a consequence, results in an increase in G' and G" (Famelart et al., 2002; Foster et al., 2005).

From Table 1, it appears that during the increase in temperature, Comté cheese exhibited the highest values of G' and G" 20 °C 60 °C, whereas Raclette cheese showed the highest ones at 65, 70, 75 and 80 °C (Table 1). These differences may be explained by the manufacturing parameters and the gross composition of the cheeses. PCA was applied to cheese rheology data (G', G", tan δ, strain and η*) in order to investigate changes in the data in the 20–80 °C temperature range. The PCA results showed that the first two principal components took into account 99.3% of the total variance with a large predominance of principal component 1 (83%), discriminating the samples as a function of temperature (data not shown). The rheological values recorded at low temperature had positive scores according to the principal component 1, whereas negative scores were observed for the ones recorded at high temperatures.

- *Synchronous fluorescence spectra*

The synchronous scan only collects fluorescent emission from the wavelengths where the absorption and emission bands of a species overlap by the specified wavelength interval, Δλ (Moberg et al., 2001). As fluorescence spectra were recorded in triplicate for Δλ = 80 nm and different temperatures (20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75 and 80 °C), a total of 39 synchronous fluorescence spectra were recorded for each cheese.

Figure 1 presents Comté cheese synchronous spectra with an offset Δλ = 80 nm recorded at 20, 50 and 80 °C. Depending on the temperature values, bands appeared and disappeared on the spectra. The cheese synchronous spectrum recorded at 20 °C exhibited sharp and intense bands at 295 nm (emission at 370 nm) and 320 nm (emission at 400 nm) and a broader band centred at 460 nm (emission at 540 nm). The sharp intense band observed at 295 nm can be attributed to tryptophan residues of proteins. (Boubellouta & Dufour, 2008; Dufour & Riaublanc, 1997; Herbert et al., 2000; Herbert et al., 1999; Karoui et al., 2003). The band centred at 320 nm is similar to the one found in the vitamin A excitation spectrum recorded on cheese and milk (Boubellouta & Dufour, 2008; Dufour & Riaublanc, 1997). This spectrum also showed a broad band centred at 460 nm (emission at 540 nm) and a weak band at 360 nm (emission at 440 nm) which were attributed to riboflavin (Boubellouta & Dufour, 2008; Karoui et al., 2006).

An increase of temperature from 20 to 80 °C drastically changed the synchronous spectrum of investigated cheeses. The highest intensity observed for the band at 320 nm (emission at 400 nm) had totally disappeared at 80 °C. The other main changes was observed for tryptophan

residues fluorescence of proteins: the fluorescence intensity drastically decreased. In addition, a slight shift of the band was also observed when the temperature increased: the maxima at 295nm and 299 nm were observed for 20 °C and 80 °C, respectively.

PCA was applied to a subset containing the synchronous fluorescence spectra recorded on Comté cheese heated between 20 and 40 °C, a temperature range allowing to investigate the change in fat physical state (Karoui et al, 2003). The map defined by principal components 1 and 2 took into account 99% and 0.7% of the total variance (data not shown), respectively, and discriminated the samples according to temperature. The principal component 1 that explained most of the total inertia of the spectral data allowed discrimination of the spectra according to temperature. The examination of spectral pattern 1 (data not shown) showed negative peak at 322 nm corresponding to vitamin A. This pattern has been reported previously and agrees with the change in the physical state of triglycerides (Boubellouta & Dufour, 2008). Similar results were obtained following the analysis of the synchronous fluorescence spectra of Raclette cheese in the 20–40 °C temperature range. It suggests that vitamin A is a valuable probe for investigating the melting of cheeses in general, and of the fats in cheeses in particular.

Then PCA were applied to a subset containing the Comté synchronous spectra recorded in the temperatures range 45 - 80 °C to check the ability of synchronous spectra to retrieve information about the changes on the molecular structure during the melting of the cheese matrix. The first two principal components accounted for 98% of the total variance with a large predominance of the principal component 1 (91.7%). Considering the map defined by principal components 1 and 2, it appeared that spectra recorded at temperatures below 70 °C exhibited negative scores according to the first principal component, whereas those recorded at 70, 75 and 80 °C exhibited positive scores (Fig. 2A).

The examination of spectral pattern 1 showed an opposition between a negative peak at 299 nm corresponding to tryptophan residues and a positive broad band centered at 360 nm associated with riboflavin (Fig. 2B).

Considering the principal component 2, the associated spectral pattern showed an opposition between a negative peak at 322 nm corresponding to vitamin A and two positive bands located at 288 nm (tryptophan residues) and 460 nm (riboflavin) (Fig. 2B). This opposition between the bands at 288 nm and at 322 nm may be related to the changes (Herbert et al., 2000; Mazerolles et al., 2001) in protein-protein and protein-lipid interactions during heating

and to the different network structures resulting from cheese-matrix melting. In addition, two anti-parallel phases in the changes of cheese-matrix structure with temperature can be observed on the map according to PC2: the first one ranges between 45 and 65 °C and the second one is observed for temperatures between 70 and 80°C. The top of this bell-shaped curve was observed for 65 °C with is closed to the melting temperature (60.4 °C) determined from rheology data (see following sections).

Similar results were obtained following the analysis of Raclette cheese synchronous fluorescence spectra recorded in temperature range 45 – 80 °C.

- *Mid-infrared spectra*

The MIR spectrum of a cheese contains information for the compounds which are present at levels >0.1% (w/w) (Karoui et al., 2007). The absorption bands observed in the MIR (3000–900 cm^{-1}) region are associated with fundamental valence vibrations of functional groups of the molecule.

The 3000-2800 cm^{-1} spectral region (Fig. 3A) was dominated by two strong bands at 2920 and 2850 cm^{-1} assigned to the methylene anti-symmetric and symmetric stretching modes (Unemura et al., 1980), respectively. Weaker bands resulting from the asymmetric and symmetric stretching modes of the terminal methyl groups were also present at 2955 and 2872 cm^{-1}.

The spectra showed different shapes as a function of temperature. First, it was observed that absorbance of methylene bands decreased as temperature increased and $A_{\gamma CH2}/A_{\gamma CH3}$ values were different between the chesses spectra at 20 and 50 °C. Furthermore, a slight shift to higher wavelengths of the CH_2 stretching mode was observed when the temperature increases from 20 to 50 °C. The shifts of the maxima at about 2850 and 2920 cm^{-1} toward lower wavenumbers were observed. It is well known that the bands between 3000 and 2800 cm^{-1} associated with C-H stretching modes are sensitive to the physical state of lipids. The shift of the band at about 2850 cm^{-1} is commonly used to follow the phase transition of phospholipids in model systems. For example, the frequency of the band associated with the symmetric stretching mode of CH_2 groups ($\upsilon_S CH_2$) shifts from 2850 to 2853 cm^{-1} during the melting of pure dipalmityl-phosphatidyl-choline bilayers (Casal & Mantsch, 1984). A sharp transition of the $\upsilon_S CH_2$ frequency is observed for the melting temperature of this phospholipid. Considering cheeses, fat globules contain a huge number of different triacylglycerols. The large number of triacylglycerols of different fatty acid composition and

distribution induces a broadening of the MIR bands associated with C-H stretching in the spectra, limiting the observed shift of $\upsilon_S CH_2$ frequency.

PCA was performed on second-derivative of 3000–2800 cm^{-1} region spectra recorded on Comté cheese between 20 and 50 °C. The map (data not shown) defined by the first two PCs (97.5% and 2.4% of the total inertia, respectively) showed a discrimination of the spectra according to temperature. The spectral pattern associated with PC2 was characterized by the opposition of bands at 2847 and 2859 cm^{-1} and bands at 2914 and 2926 cm^{-1}. The pattern indicated a shift of the methylene bands of triglycerides as a function of temperature. This pattern is characteristic of the melting of triglycerides (Dufour et al., 2000). Similar results were obtained for Raclette cheese.

The 1700–1500 cm^{-1} region was characterised by the presence of bands containing some information on the proteins and on the interaction of these latter with other components, such as ions and water (Karoui et al., 2007). Figure 3B shows the shapes of the spectra of the Comté cheese at various temperatures. Maximum absorption of the amide I band, used to investigate the secondary structure of proteins, can be observed around 1633 $cm^{-1}$and the absorption band at 1547 cm^{-1} is generally assigned to the amide II vibrations.
In order to investigate the effect of temperature on the cheese spectra, PCA was performed on second-derivative 1700–1500 cm^{-1} region spectra recorded on Comté cheese between 45 and 80 °C. The map defined by PC1 and PC2 (98.1% and 1% of the total inertia, respectively) allowed to discriminate spectra according to temperature. It appeared that spectra recorded at temperature below 70 °C were mainly discriminated according to PC1, whereas those recorded at 70, 75 and 80 °C were essentially discriminated according to PC2 (Fig. 4A). This pattern derived from the analyse of spectra in the Amide I and II region suggested that at least two different phenomena occured when the temperature increased from 45°C to 80°C: the first one takes place before the melting of cheese and the second one is observed for temperatures above 70°C. The spectral pattern associated with PC1 was characterized by the opposition of a negative band at 1634 and two positive bands at 1576 and 1531 cm^{-1} (Fig. 4B). Spectral pattern 2 showed an opposition between negative bands around 1634, 1545 and 1520 cm^{-1} and a positive band at 1574 cm^{-1}. The investigation performed on the Raclette cheese spectra gave similar results (data no shown).

The region 1500–900 cm^{-1} is called the fingerprint region and numerous chemical bonds are absorbing in this region. Figure 2C presents the spectra recorded at 20, 50 and 80 °C for Comté cheese. As temperature increased, the intensity of all the bands, except one, decreased. Indeed, the absorbance of the band at about 1403 cm^{-1} increased from 20°C to 80°C. The origin of this weak band at 20°C is not known. The bands observed at 1458, 1414 and 1234 cm^{-1} (Fig. 2C) are due to bending modes of O–C–H, C–C–H and C–O–H as reported previously (Paradkar et al., 2003; Sivakesava & Irudayaraj, 2001). The strong bands located at 1159 and 1099 cm^{-1} could be related to P=O stretching (Bellamy, 1975). Indeed, the latter author reported that PO_2^- and PO_3^- compounds present bands in the 1323–1092 cm^{-1} and 1140 and 1055 cm^{-1} regions, respectively. The band located at 962 cm^{-1} is related to unsaturated fatty acids, due to CH out-of-plane deformation (Meurens et al., 2005).

The PCA was applied to the 1500–900 cm^{-1} region spectra recorded between 20 and 80 °C on Comté cheese. The map (data no shown) defined by PC1 and PC2 (96.6% and 3% of the total inertia, respectively) allowed to discriminate spectra according to temperature. The distribution of heated samples presented two directions: the first one (between 20 and 35 °C) was along PC1 and the second one (between 40 and 80 °C) along PC2.The spectral pattern of PC1 (data no shown) presented an opposition between 1477 and 1466 cm^{-1} and between 1146 and 1132 cm^{-1}. Mazerolles et al. (2006) and Paradkar et al. (2003) mention that these bands are from lactate (1130 cm^{-1}), to bending modes of O–C–H and C–C–H (1477 and 1466 cm^{-1}) in the cheeses. The opposition between the bands 1146 and 1132 cm^{-1} may be explained by the changes in the lactate ionisation induced by temperature increase. The spectral pattern of PC2 presented an opposition between 1369 and 1352 cm^{-1} and between 1427 and 1408 cm^{-1}. These bands could be associated to C–H bending. Raclette PCA had given same results (data no shown).

- *Determination of the melting temperatures of fats and of cheese-matrices from the rheology, synchronous fluorescence and mid-infrared data*

Milk fat globules contain hundreds of triacylglycerol species. Melting of these species occurs over a large temperature range, i.e., between -30 and + 40 °C. Based on the melting temperatures, three groups of triacylglycerol species are found in milk lipids. The first group has a low-fusion temperature (LFT; -30 – +10 °C), the second one shows a medium fusion

temperature (MFT; +10 – +20 °C) and, finally, the third group is characterized by a high-fusion temperature (HFT; +20 – +40 °C) (Timms, 1980).

In rheology, the melting temperature of fats in the cheese was calculated from the log (η*) plots. In each log (η*), two distinct linear regions and the intersection point was identified as the melting temperature of fats in the cheese.

Regarding fluorescence, it has been reported that the melting temperature of fats in cheeses can be derived by plotting F322 nm/F295 nm versus temperature (Karoui et al., 2003). Considering synchronous fluorescence, typical plots of the SF 322 nm versus temperature in the 20-50 °C range were drawn for each cheese (data no shown). The plots showed two distinct linear regions and the intersection point was identified as the melting temperature of fats in the cheese. For 3000-2800 cm^{-1} infrared region, two linear regions were observed by plotting absorption intensity at 2924 cm^{-1} versus temperature for the samples recorded between 20 and 50 °C.

Table 2 presents the fat melting-temperatures of the two cheeses derived from rheology, synchronous fluorescence and mid infrared (3000-2800 cm^{-1} region) data. Considering the rheology data, the difference observed between the results obtained for the two cheeses were not significant at a level of 5%. The Student test did not show differences between the results obtained with the three methods at a level effect of 5%.

In the rheology data, tan δ equal to 1 indicates that the cheese starts to melt (Famelart et al., 2002). For Comté cheese, this temperature was observed 60.4 ± 1.6 °C, while temperatures of 61.5 ± 1.2 °C were obtained for Raclette cheeses. This result is in agreement with (Famelart et al., 2002; Karoui et al., 2003) reporting that the melting temperatures of Comté and Raclette cheeses are at about 60 °C.

The determination of the cheese melting temperatures by the synchronous fluorescence and mid infrared (1700-1500 cm^{-1} region) data the temperatures in the 50-80 °C range were obtained by the applying the same approach as for the determination of the fat melting-temperatures. The synchronous fluorescence at 295 nm and the intensity at 1634 cm^{-1} were used. Derived cheese melting temperature were 60.5 ± 0.4 °Cand 61 ± 0.5 °C for Comté and Raclette cheeses, respectively. Table 3 shows the melting temperatures of the two cheese matrices derived from the three techniques. As for the fat melting temperatures, no significant difference was observed between the results obtained with the three methods (significance level of 5%).

The similar melting temperatures derived from rheology, synchronous fluorescence and mid infrared data also confirm the relationship between the structure of the cheese at the molecular level, as investigated by synchronous fluorescence and mid infrared spectroscopies, and the texture, assessed using dynamic testing rheology.

- *CCSWA of synchronous fluorescence, mid infrared and rheology data sets*

Applying CCSWA to these data tables makes it possible to determine common dimensions that can highlight how the characteristics of the cheeses are changing in the course of the heating process. CCSWA was applied to the five data tables corresponding to the three infrared regions (1500-900 cm^{-1} coded MIX, 1700- 500 cm^{-1} coded PRO and 3000-2800 cm^{-1} coded FAT), the synchronous fluorescence spectra (coded SFS) and rheology data (coded RHE). In each table, 78 items (three replicates for the two cheeses, at different temperatures) were considered.

The values of the saliences for the first five common components D1 to D5 are presented in Table 4. .The common component D1 explained more than 80% of the variability in the data tables (94.8%, 55.5%, 89%, 34.2% and 68.3% for MIX, PRO, FAT, SFS and RHE, respectively). Figure 5 depicts the relationships of the two cheese samples on the basis of the first two common components D1 and D2. It shows that the two cheese samples were discriminated along D1 according to heating temperatures. The samples heated at the lowest temperatures presented negative scores while the one heated at the highest temperatures had positive values. At least two continuous phenomenon, taking place during heating of the two cheeses, were observed. The first one was observed at about 30 °C and the second one at about 60 °C. These two phenomena could be related to the melting of the cheese fats and the melting of cheese matrices at about 30 and 60 °C, respectively.

The common component D2 allowed to discriminate the two cheeses. It mainly reflected the variability expressed in the data tables PRO (31.3%) and SFS (47.5%), whereas the three other tables exhibited lower saliences and were less important for this dimension. It is concluded that SFS and PRO spectra allowed to discriminate the two cheeses all along the melting process.

The loadings corresponding to the first common components for the tables MIX, PRO, FAT and SFS are presented in Figure 6. Considering the data table FAT (Fig. 6A), the loading was characterized by four important absorption bands at about 2853 cm^{-1} and around of 2922 cm^{-1}.

The shifts interpreted by the oppositions observed between the bands 2853 and 2855, and between 2922 and 2924. They indicated the changes in the fat physical state in cheese during heating. The highest intensities were located at around 2855 and 2924 cm^{-1} for the second loading.

Considering the data table PRO (Fig. 6B), the information related to the residual lactate was found. Indeed one of the major absorptions contributing to the loadings factor 1 was observed at about 1580 cm^{-1}.

The first loading of the data table MIX showed that the spectra of the cheeses were characterized by important absorption bands at about 1162 and 1130 cm^{-1} allowed to cheese fat and lactate (Mazerolles et al., 2002; Zhou et al., 2006). The band observed at 1460 cm^{-1} due to CH_2 vibrations.

In the SFS data table, the first and the second loadings presented two important bands at about 322 and 290 nm corresponding to vitamin A and tryptophan, respectively. These changes could be related to the melting of cheese fat (vitamin A) at temperature close to 30 °C and to the melting of the cheese matrix (tryptophan) at about 60 °C.

The relationships between all the five data tables mainly led to the assessment of two common components allowing an exhaustive characterization of the two cheese samples during heating. Each of these components was built using a set of information related to different characteristics of the two cheeses, but having a common meaning.

CONCLUSIONS

This study shows that mid infrared and synchronous fluorescence spectroscopies coupled with chemometrics are useful tools to delineate the structure changes of cheese matrices during melting. In addition, it has been possible to derive the melting temperatures of cheese fats and cheese matrices from both spectral and rheology data. The understanding of the changes of cheese rheology properties as a function of temperature is very important, since cheese is more and more often used as an ingredient for the manufacturing of elaborate food products. It is shown in this study that quality parameters of cheeses, such as rheology attributes, may be derived from mid infrared and synchronous fluorescence data. The coupling of different analytical techniques using appropriate chemometrics tools allows to cope with food complexity in a very efficient way.

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Table 1. Rheological parameters (G’ and G’’) measured for the two chesses between 20 and 80 °C.

	G' (Pa)						G" (Pa)					
	Comté			Raclette			Comté			Raclette		
T (°C)	Mean	SD**	CV*	Mean	SD**	CV*	Mean	SD**	CV*	Mean	SD**	CV*
20	232650	16650	7.2	91017	15595	17.1	91017	15595	17.1	12599	1565	12.4
25	191950	13250	6.9	76015	11966	15.7	76015	11966	15.7	10428	1281	12.3
30	131600	13900	10.6	53449	10659	19.9	53449	10659	19.9	9388	790	8.4
35	83690	8710	10.4	37138	7532	20.2	37138	7532	20.3	7051	363	5.2
40	48315	2605	5.4	24584	5242	21.3	24584	5242	21.3	5416	537	9.9
45	22480	1020	4.5	13215	1810	13.7	13215	1810	13.7	2692	265	9.8
50	8152	312	3.8	5036	346	6.9	5036	346	6.9	1959	83	4.3
55	3410	320	9.4	2309	322	13.9	2309	322	13.9	1452	115	7.9
60	1368	123	8.9	1363	117	8.5	1363	117	8.5	1360	154	11.3
65	645	48	7.4	784	31	3.9	784	31	3.9	1028	52	5.1
70	230	11	4.6	343	18	5.2	343	18	5.3	584	73	12.5
75	167	6	3.4	266	10	3.7	266	10	3.6	491	31	6.3
80	102	6	5.8	178	18	10.1	178	18	10.1	310	17	5.6

** SD: standard deviation; *CV: 100 x (SD/mean);

Table 2. Evaluation of the melting temperature of fats in cheeses using rhological data and synchronous fluorescence and mid infrared spectra.

	Fat melting temperatures (°C)								
	Rheology			SFS (322 nm)			Infrared (2924 cm^{-1})		
Cheese	Mean	SD**	CV*	Mean	SD**	CV*	Mean	SD**	CV*
Comté	31.6	0.27	0.87	31.6	0.02	0.05	30.5	0.03	0.11
Raclette	31.1	0.25	0.8	32.4	0.04	0.12	32.4	0.07	0.21

** SD: standard deviation; *CV: 100 x (SD/mean);

Table 3. Evaluation of the cheese melting temperatures using rhological data and synchronous fluorescence and mid infrared spectra.

	Cheeses melting temperatures (°C)								
	Rheology			SFS (295 nm)			MIR (1634 cm^{-1})		
Cheese	Mean	SD**	CV*	Mean	SD**	CV*	Mean	SD**	CV*
Comté	60.4	0.80	1.32	60.1	0.36	0.60	60.5	0.20	0.33
Raclette	61.5	0.61	0.99	61.3	0.31	0.51	61.2	0.26	0.43

** SD: standard deviation; *CV: 100 x (SD/mean);

Table 4. CCSWA saliences using the second derivative Mir infrared spectra regions and synchronous spectra and rheological data of the two cheeses.

	Components				
Tables*	D1	D2	D3	D4	D5
FAT	0.890	0.043	0.024	0.025	0.004
PRO	0.555	0.313	0.042	0.075	0.003
MIX	0.948	0.021	0.011	0.010	0.005
SFS	0.342	0.475	0.047	0.083	0.042
RHE	0.683	0.025	0.260	0.012	0.004

* FAT: fats region (3000-2800 cm-1), PRO: proteins region (1700-1500 cm-1), MIX: fingerprint region (1500-900 cm-1), SFS: synchronous spectra, RHE: rheology data.

CAPTIONS

Figure 1: Comté cheese synchronous fluorescence spectra ($\Delta\lambda$ = 80 nm) recorded at different temperatures: 20, 50 and 80 °C.

Figure 2: PCA performed on Comté synchronous fluorescence spectra recorded between 45 and 80 °C. (A): Similarity map defined by the PC1 and PC2; each label correspond to an infrared specrum recorded at the affiched temperature. (B): spectral patterns for the principal components 1 (—) and 2 (····).

Figure 3: Mid infrared spectra of Comté cheese recorded at different temperatures. Spectral regions considered in the study: (**A**) 3000-2800 cm^{-1}, (**B**) 1700-1500 cm^{-1} and (**C**) 1500-900 cm^{-1}.

Figure 4: PCA performed on Comté mid infrared spectra in the region 1700-1500 cm^{-1} recorded between 45 and 80 °C. (A): Similarity map defined by the PC1 and PC2; each label correspond to an infrared specrum recorded at the affiched temperature. (B): spectral patterns corresponding to the principal components 1(—) and 2 (····).

Figure 5: CCSWA similarity maps defined by the common components 1 and 2.

Figure 6: Loadings of the common components D1 and D2 calculated for the data tables FAT (A), PRO (B), MIX (C) and SFS (D).

CAPTIONS

Figure 1 : Boubellouta & Dufour.

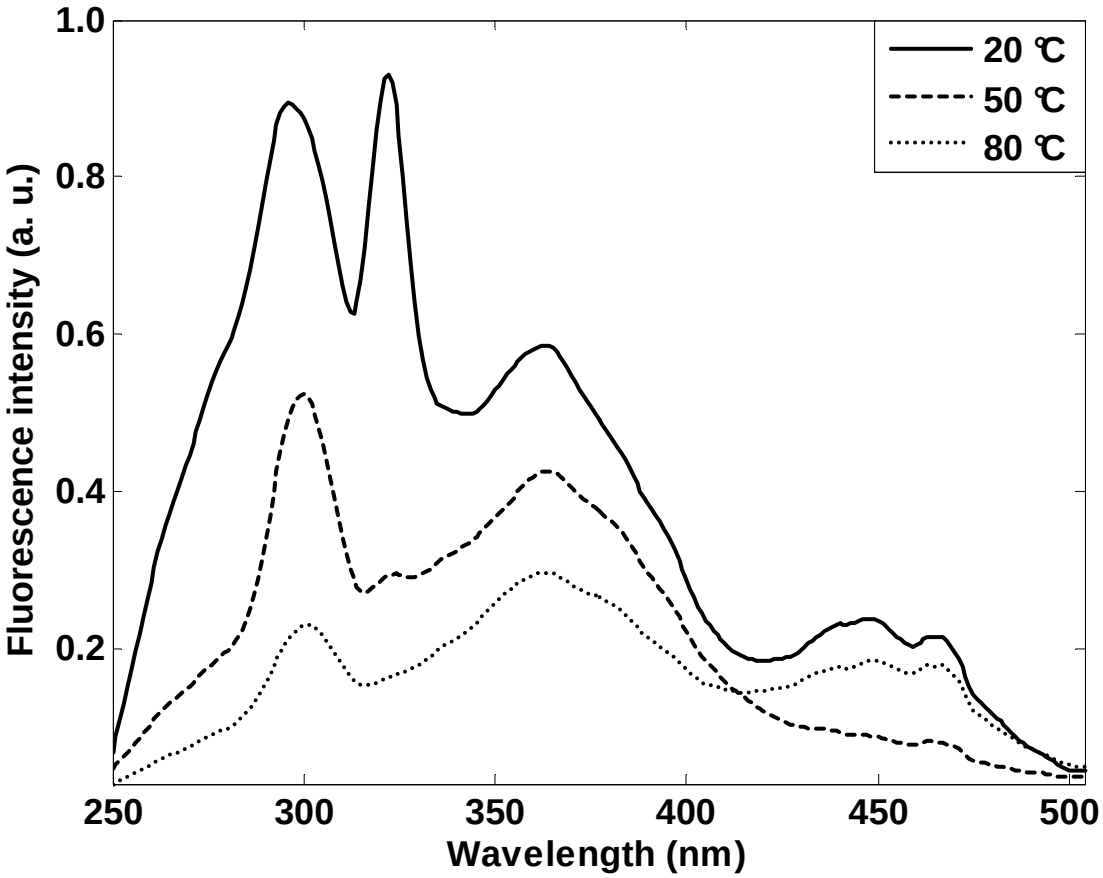


Figure 2: Boubellouta & Dufour..

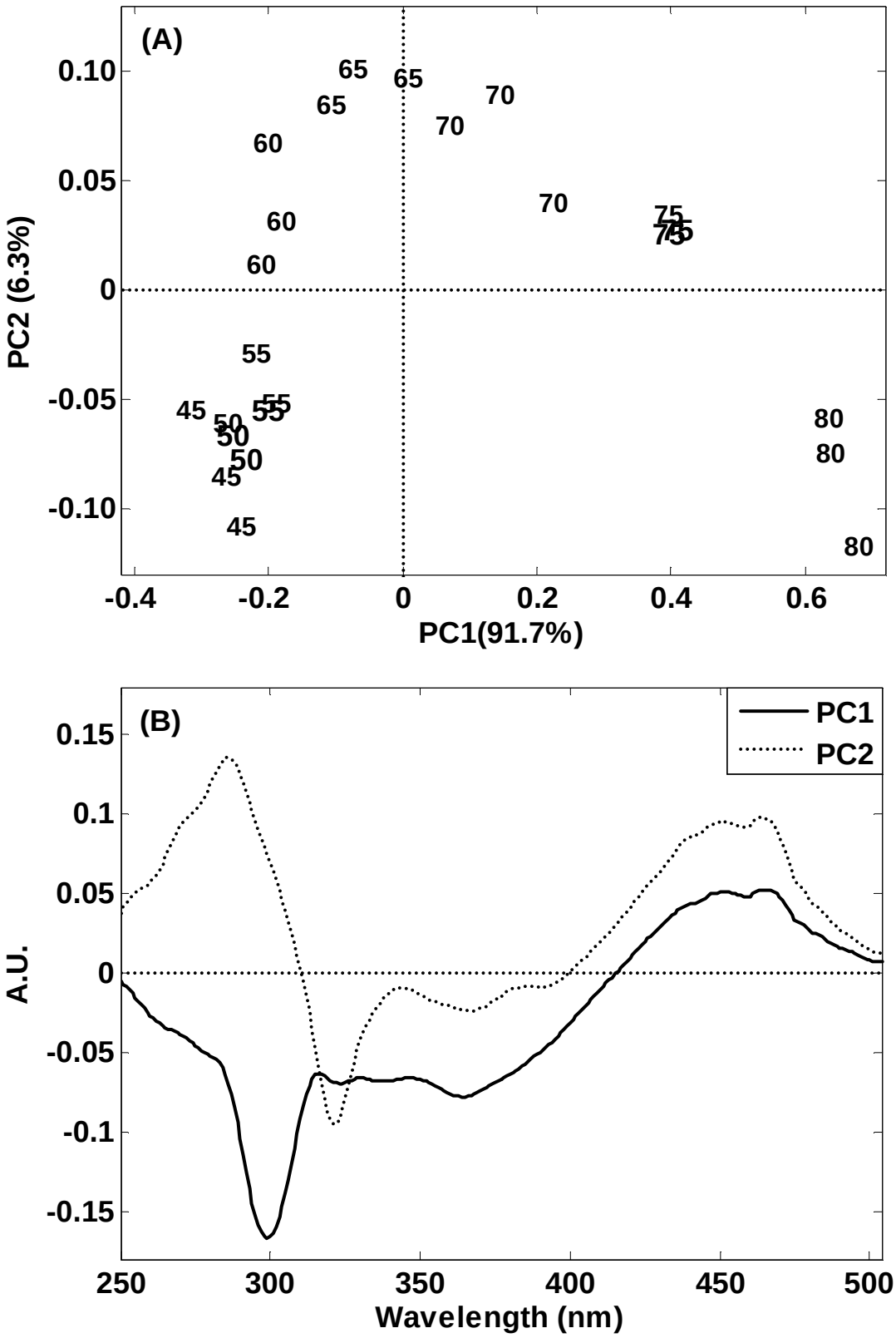


Figure 3 : Boubellouta & Dufour.

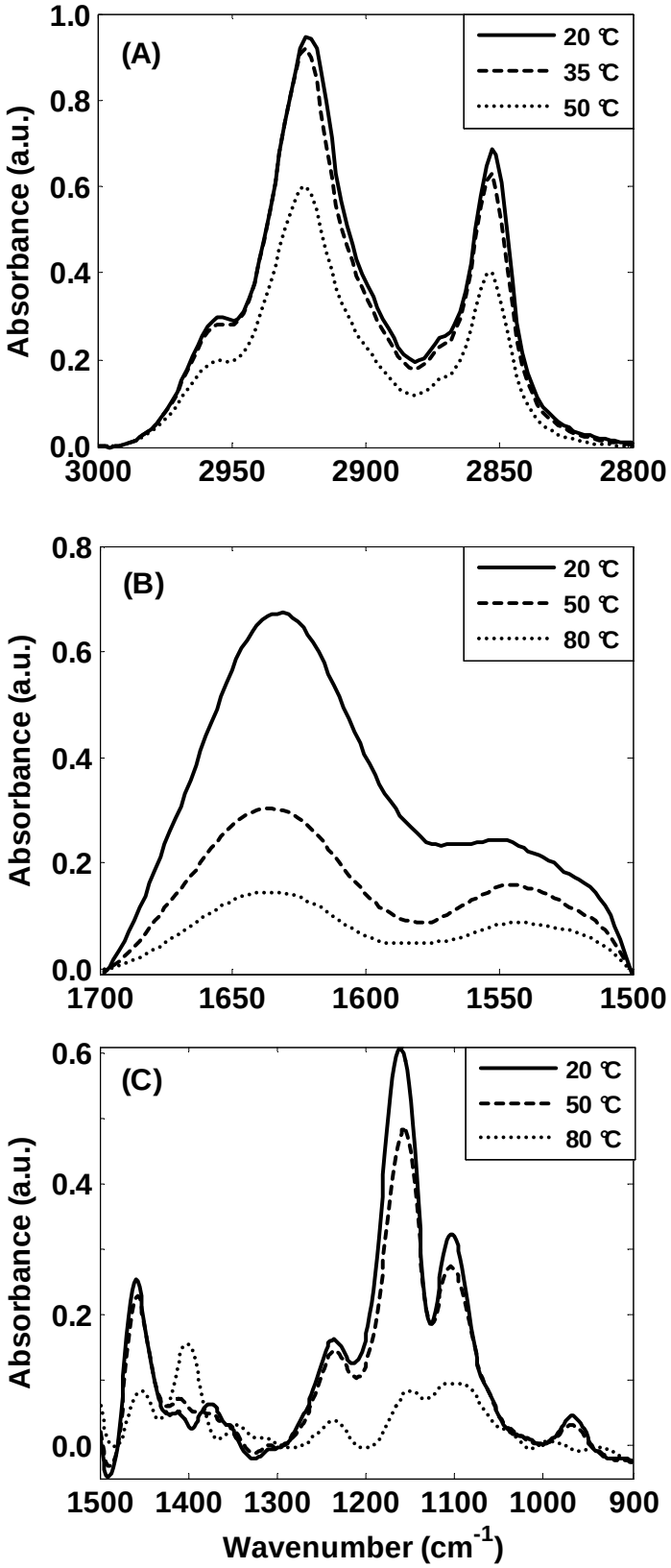


Figure 4: Boubellouta & Dufour.

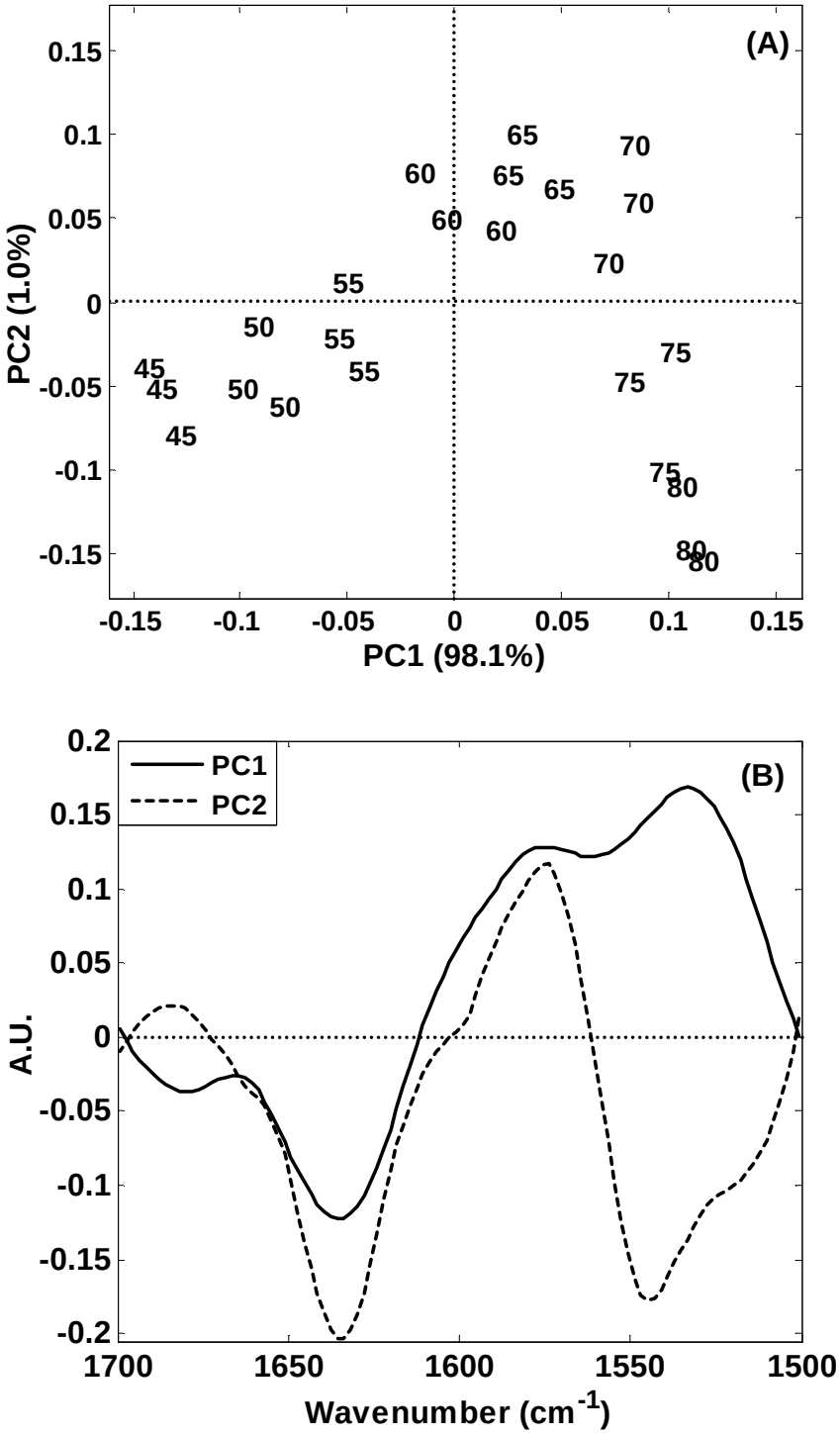


Figure 5: Boubellouta & Dufour.

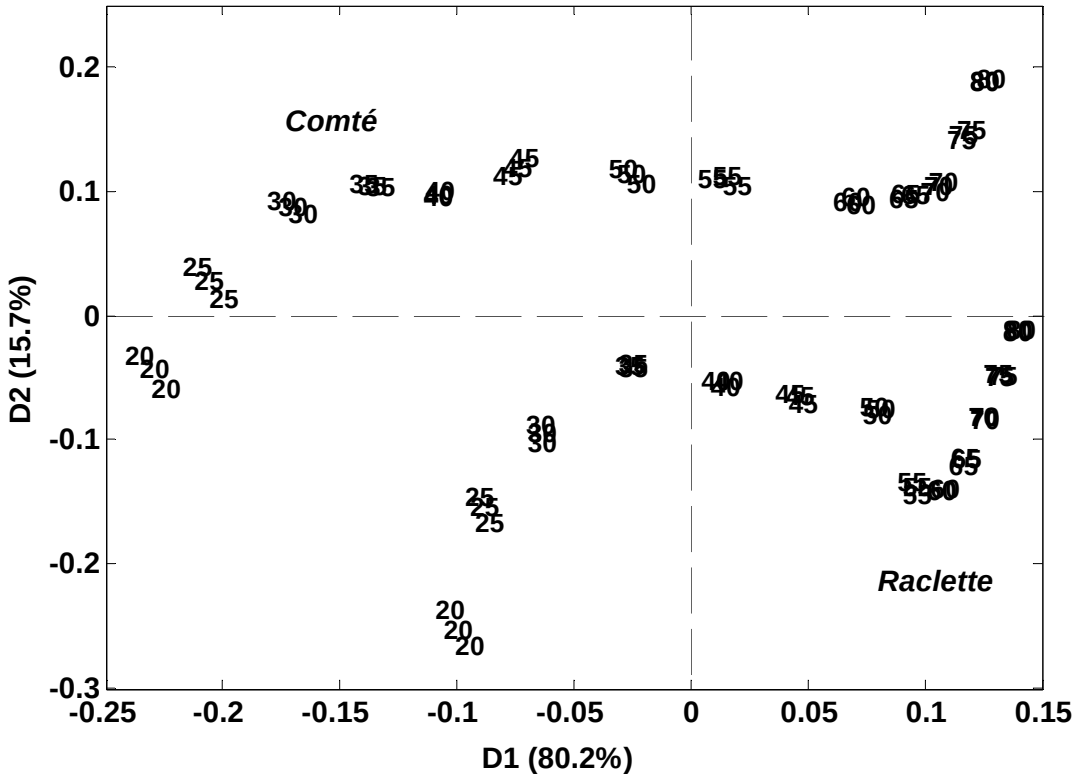
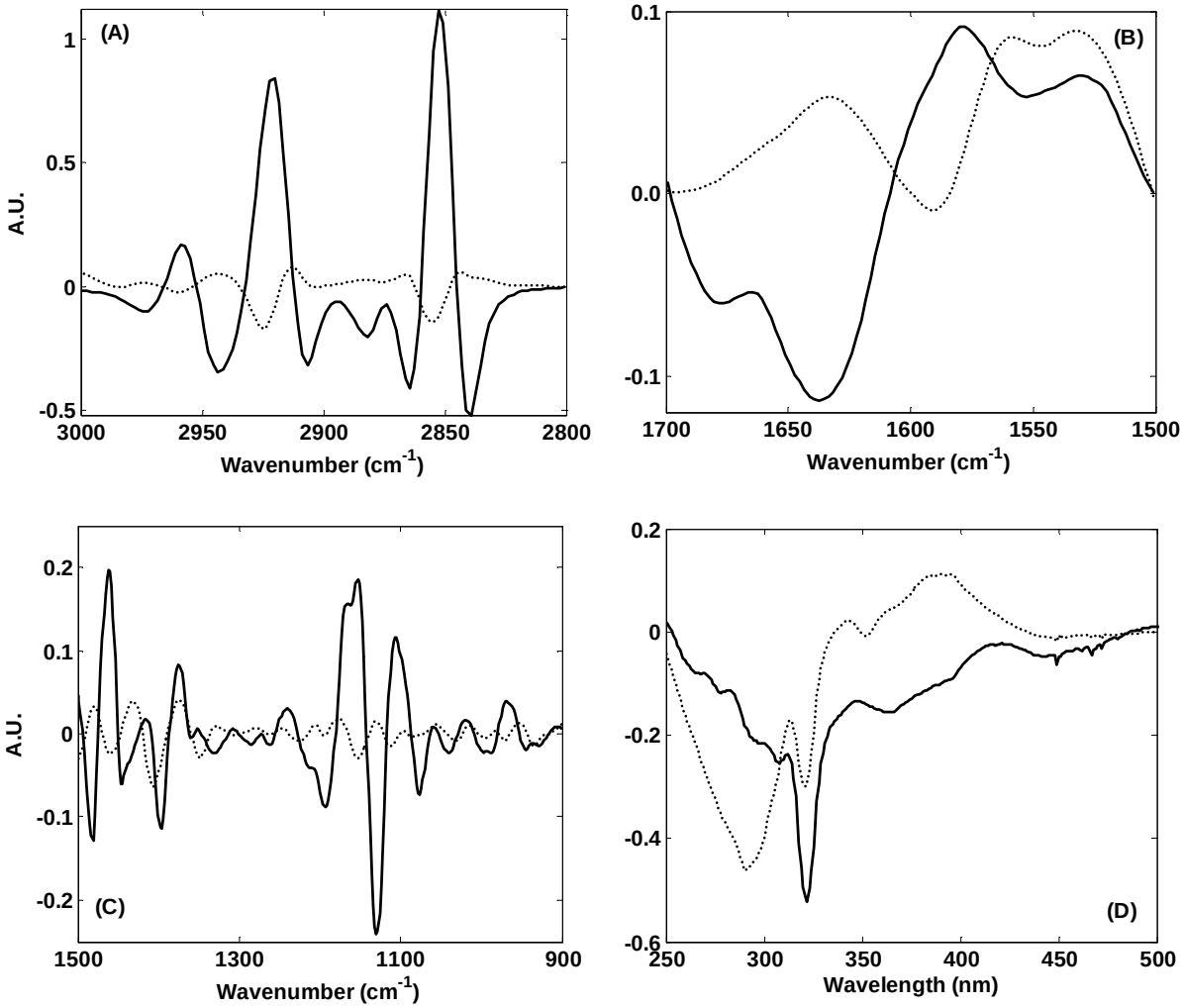


Figure 6: Boubellouta & Dufour.



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Original article

Investigation of the effects of season, milking region, sterilisation process and storage conditions on milk and UHT milk physico-chemical characteristics: a multidimensional statistical approach

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Abstract – Milk samples were collected in five dairy plants located in different regions of France (North, North-West, South-West and centre of France), during spring and autumn, at receipt (bulk-raw milk), and following pasteurisation and UHT sterilisation. Corresponding UHT milks were then stored at three temperatures (4, 20 and 40 °C) and analysed after different times (21, 42, 62, 90, 110 and 180 d). The physico-chemical characteristics of these different milks, including composition, micellar properties and stability as assessed by heat, ethanol and phosphate tests, were determined. The database was processed by principal component analysis and common components and specific weights analysis. The effects of season, milking zone, process and storage conditions were highlighted, and the involved physico-chemical characteristics were determined. For the region effect, numerous parameters related to the global composition and the casein micelles intervened. Some differences in milk stability as evaluated by the ethanol and phosphate tests were also observed. Considering the season, spring milks had higher values of pH, lactose, soluble phosphate and micellar hydration than milks collected in autumn. These spring milks also had lower values of fat and heat stability than autumn milks. The UHT process effect was observed through decreases in non-casein nitrogen content and in micellar hydration and by an increase in casein micelle size for UHT milks. The stability values derived from phosphate and ethanol tests were increased following the UHT process. Concerning storage conditions, the temperature of 40 °C led to a decrease in pH and increases in non-casein and non-protein nitrogen contents of milks. At 40 °C, low values of stability for the heat test and high values for the phosphate test were observed.

UHT milk / physico-chemical composition / stability / casein micelle / chemometry

摘要 – 应用多元统计方法研究季节、产地、杀菌方式和贮藏条件对巴氏杀菌奶和 UHT 奶物理化学性质的影响。分别在春季和秋季，对法国北部、西北、西南和中部四个地区的5个乳品厂的散装牛奶进行取样，杀菌方式为巴氏杀菌和超高温 (UHT) 杀菌。 UHT 奶贮藏温度分为 4、20 和 40 °C,

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贮藏时间为 21、42、62、90、110 和 180 d。测定了不同贮藏条件下 UHT 奶的组成, 研究了热处理对酪蛋白胶束特性和稳定性的影响, 以及牛奶的乙醇和磷酸盐试验。采用主成分分析、普通组成分析和权重分析法对试验数据进行统计分析。研究发现牛奶的生产季节、产地、杀菌方式和贮藏条件对牛奶理化性质有显著的影响。根据对所有成分的分析、与酪蛋白胶束相关的试验, 以及乙醇和磷酸盐试验等大量实验数据分析, 证明了不同地区牛奶的稳定性之间有一定的差异。在牛奶生产季节上的差异则是, 春季牛奶的 pH、乳糖含量、可溶性磷酸盐含量和酪蛋白胶束的水合能力均高于秋季牛奶, 春季牛奶的脂肪含量和热稳定性均低于秋季牛奶。对 UHT 奶, 通过降低非酪蛋白氮含量和酪蛋白胶束水合作用以及增大酪蛋白胶束尺寸的方法研究了 UHT 过程对其影响, 乙醇和磷酸盐试验证明经上述方法处理的 UHT 奶稳定性增加。在 40 °C下贮藏的牛奶 pH 降低, 而非酪蛋白氮和非蛋白氮含量增加; 并且在 40 °C下贮藏的牛奶热稳定性值较低, 但其磷酸盐试验的测定值较高。

UHT奶 / 物理化学性质 / 稳定性 / 酪蛋白胶束 / 化学计量

Résumé – Étude des effets saison, région laitière, traitement de stérilisation et conditions de stockage sur les caractéristiques physico-chimiques du lait et du lait UHT : une approche statistique multidimensionnelle. Des échantillons de lait ont été collectés dans cinq laiteries situées dans différentes régions de France (Nord, Nord-Ouest, Sud-Ouest et centre de la France), au printemps et en automne, à la réception (laits crus de mélange), après pasteurisation et après stérilisation UHT. Les laits UHT correspondants ont ensuite été placés à trois températures (4, 20 et 40 °C) et analysés après différents temps de stockage (21, 42, 62, 90, 110 et 180 j). Les caractéristiques physico-chimiques de ces différents laits, comprenant la composition, les propriétés micellaires et la stabilité évaluée à l'aide des tests à la chaleur, à l'alcool et au phosphate, ont été déterminées. Le jeu de données a été étudié par analyse en composantes principales et analyse en composantes communes et poids spécifiques. Les effets de la saison, de la région de collecte, du traitement technologique et des conditions de stockage ont été mis en évidence et les caractéristiques physico-chimiques impliquées ont été déterminées. Pour l'effet région, de nombreux paramètres liés à la composition globale et aux micelles de caséines intervenaient. Des différences de stabilité du lait, évaluée par les tests à l'alcool et au phosphate, ont aussi été observées. Considérant la saison, les laits de printemps avaient des valeurs de pH, de lactose, de phosphate soluble et d'hydratation micellaire plus grandes que les laits collectés en automne. Ces laits de printemps avaient aussi des valeurs plus faibles de matière grasse et de stabilité thermique que les laits d'automne. L'effet traitement UHT a été observé au travers d'une diminution de la teneur en azote non caséinique et de l'hydratation micellaire et par une augmentation de la taille des micelles. Les valeurs de stabilité des laits obtenues par les tests au phosphate et à l'alcool augmentaient après le traitement UHT. Concernant les conditions de stockage, la température de 40 °C induisait une diminution du pH et des augmentations des teneurs en azote non caséinique et non protéique des laits. À 40 °C, des valeurs faibles de stabilité dans le test à la chaleur et des valeurs élevées de stabilité dans le test au phosphate ont été observées.

lait UHT / composition physico-chimique / stabilité / micelle de caséine / chimiométrie

1. INTRODUCTION

Milk is a complex product, constituting three phases: a fat emulsion, a casein micelle dispersion and an aqueous phase containing mainly lactose, whey proteins and minerals [1]. Its composition can be influenced by numerous factors such as cow genetic (breed) [10], physiologic (animal medical state, lactation number and stage) [28, 29, 40] and zootechnic factors (feed, milking conditions, exercise) [5, 11]. Some of these factors may be correlated to other parameters such as region of production [22], season [3, 12, 18] or social practice in herd management [4], for example.

In addition to its natural and individual variability, milk is a sensitive product whose properties may be altered by various factors such as microbial contamination. To destroy micro-organisms and inactivate enzymes in order to increase milk shelf-life, technological treatments, and particularly heat treatments such as UHT

sterilisation, can be applied to milk. This last technique offers a "commercially sterile" product which can be stored at ambient temperature for several months. However, UHT processing and storage are not harmless and induce different changes in milk depending on the time-temperature parameters [8]. Sometimes these changes lead to UHT milk destabilisation during storage as a gel or a sediment [7, 25]. Parameters which affect milk stability have been reported such as composition parameters, process and storage conditions [27]. Nevertheless, all these studies about milk generally considered only one or few physicochemical characteristics and focused on one part of the life of the product (raw milk, manufacture or storage).

In the present study, the objective was to determine, on a large data set, by a multidimensional statistical approach, the possible effects of season, milking region, sterilisation process and storage conditions on milk and UHT milk physicochemical characteristics. For that, we analysed changes which occurred in five bulk-raw milks during UHT processing and storage for six months at three temperatures. These five bulk-raw milks were collected in dairy plants located in different regions of France. Sampling was performed in spring and autumn. The studied physico-chemical characteristics refered to global milk characteristics (pH, total solids, fat, lactose, proteins, nitrogen fractions such as NCN and NPN contents, concentrations in soluble calcium and phosphate), casein micelle properties (size, charge, hydration) and stability evaluated by three tests (heat, ethanol and phosphate tests).

2. MATERIALS AND METHODS

2.1. Milk collection

The study focused on five bulk-raw milks sampled in five dairy plants, called V, W, X, Y and Z. These dairy plants were located in different regions of France: North, North-West, South-West and centre, and the volumes of milk treated by each one comprised between 200 000 L and 900 000 L of milk per day. Milks mainly came from Prim'Holstein cows, but also from other breeds such as Pie Noire, Montbéliarde and Salers. According to the season, cows were fed with hay, corn grain or silage, oat, concentrates or were put on pasture. Milking was usually done twice a day. Raw milks were generally collected every 48 h, or at maximum, every 72 h. In the dairy plants, raw milks were stored in tanks of capacity between 100 000 and 250 000 L.

Concerning the manufacture of UHT milk in the dairy plants, it globally consisted of: (1) cooling of bulk-raw milk at 4 °C; (2) skimming between 30 and 50 °C; (3) pasteurisation (between 80 and 90 °C for 10 to 30 s, depending on the plant); (4) fat standardisation at about 15 $g \cdot kg^{-1}$; (5) cooling and storage of milk before homogenisation or direct homogenisation (between 25 and 200 bars at temperatures between 72 and 92 °C); (6) UHT sterilisation (140 °C for 3 to 6 s, depending on the plant), and (7) aseptic filling. In this study, milks were collected before any treatment (bulk-raw milks), after pasteurisation and then after UHT sterilisation and aseptic filling. Raw, pasteurised and freshly processed UHT milks were analysed and abbreviated as follows: "r", "p" and "u", respectively. The UHT milks corresponding to the bulk-raw milks initially considered and treated were semi-skimmed UHT milks. These UHT milks were stored at three temperatures (4, 20 and 40 °C) and analysed after different storage periods (21, 42, 62, 90, 110 and 180 d). This milk sampling was realised for two seasons, i.e. spring and autumn (cited as "1" and "2", respectively).

Bulk-raw and pasteurised milk samples were kept at 4 °C for a maximum

of 2 days before analyses and thimerosal 0.03% (w/w) (Sigma, Saint-Louis, USA) was added to prevent further microbial development. The temperature was raised to 20 °C just 1 h before analyses. Concerning UHT milks stored at 4 and 40 °C, samples were put at room temperature for equilibrium approximately 15 h before analysis. All UHT milks were checked for total mesophilic aerobic flora and psychrotrophic flora contaminations. Thimerosal 0.03% (w/w) was also added to all UHT milks after microbiological control in order to prevent further microbial contamination.

2.2. Physico-chemical analyses

2.2.1. Milk stability tests

Heat stability was valued as the Heat Clotting Time (HCT). It was determined on 5 mL of milk in sealed Pyrex tubes fitted into a thermostatically controlled oil bath at 140 °C ± 1 °C. The time (in min) necessary to obtain a visible destabilisation was considered as the result of the test [9].

The ethanol test (Alcohol) consisted of the addition of 1 mL of an ethanol solution to 1 mL of milk. Ethanol concentrations ranged from 50 to 95% (v/v) with increments of 5% (v/v). The mixture was stirred vigorously and the reading was done in 5 min. The lowest concentration (in %) giving a flocculation was defined as the result of the test [37]. Milks which were not destabilised by 95% of ethanol were considered as being 100%.

For the phosphate test (Ramsdell), milk samples (10 mL) were placed in sealed Pyrex tubes and volumes of KH_2PO_4 0.5 mol·L^{-1} (Panreac Quimica SA, Barcelona, Spain) were gradually added to each tube. After mixing, tubes were heated at 100 °C ± 1 °C for 10 min. After the heat treatment, the smallest volume of phosphate solution (in mL) resulting in milk destabilisation was considered as the result of the test [31].

2.2.2. Milk composition

Milk pH was measured using a HI 9024 pH-meter (Hanna Instruments, Vila do Conde, Portugal). Gross composition of milks such as concentrations of fat, lactose, proteins and total solids (g·kg^{-1}) was obtained by an infrared spectrometer Lactoscope (Delta Instruments, Drachten, Holland). Non-casein nitrogen (NCN) content was determined on the filtrate of milk acidified to pH 4.6 with a mixture of 10% (v/v) acetic acid and 1 mol·L^{-1} acetate buffer. Non-protein nitrogen (NPN) content was determined on the filtrate of 1:4 mixtures of milk and 15% (w/v) trichloroacetic acid solution (TCA). The filters used were Whatman® N°42 and 40 for NCN and NPN, respectively. Nitrogen content was determined by the Kjeldahl method (IDF standard 20B, 1993) and converted into equivalent protein contents (g·kg^{-1}) using 6.25 and 6.19 as converting factors for NCN and NPN contents, respectively. Concentration of soluble calcium was determined on the milk ultrafiltrate with an atomic absorption spectrometer (Varian 220FS spectrometer, Les Ulis, France) [6]. Concentration of soluble phosphate was determined on the milk ultrafiltrate using ion chromatography (Dionex DX 500, Dionex, Voisin-le-Bretonneux, France) [16]. Soluble concentrations were corrected by the factor 0.96 as determined by Pierre and Brulé [30]. Mineral concentrations were expressed in mmol·L^{-1}.

2.2.3. Particle characteristics

Hydrodynamic particle diameters (Size) were measured by dynamic light scattering on a Zetasizer 3000HS (Malvern Instruments, Worcestershire, UK) with a scattering angle of 90°, a wavelength of 633 nm and at 25 °C. Skimmed raw milks or semi-skimmed UHT milks, diluted in their corresponding ultrafiltrates, were filtered on a membrane Acrodisc® with a pore size

of 0.8 μm (Pall Corporation, Ann Arbor, USA) before measurements. The refractive index for the dispersive solution was set at 1.342 and its viscosity at $0.99{\cdot}10^{-3}$ Pa·s.

Zeta-potentials (Charge) of particles were determined with the same apparatus at 25 °C with a voltage of 125 V. Samples were prepared as for particle size determination and the viscosity of the dispersion solution was kept at $0.99.10^{-3}$ Pa·s.

Hydration of casein micelles corresponded to the water content of the pellet obtained after ultracentrifugation of milk for 1 h at $100\,000\times g$. Pellets were weighed and dried for 7 h at 103 °C. The difference between the weight before and after drying corresponded to the value of the water content of the ultracentrifugation pellet and was expressed in g of water per g of dry pellet.

2.3. Data analysis

Measurements were made in duplicate and inserted in the database. Then the data were centred and normalised. Two multivariate statistical methods were performed with the Matlab R2006a software (The Mathworks Inc., Natic, MA, USA), i.e., principal component analysis (PCA) and common components and specific weights analysis (CCSWA).

PCA is a common technique for finding patterns in data of high dimension and for representing the maximum of information in a synthetic graph called a similarity map [23]. PCA was applied to the data to investigate differences between the samples. This technique transforms the original variables into new axes called principal components (PCs), which are orthogonal, so that the data sets presented on these axes are uncorrelated with each other. Therefore, PCA expresses, as much as possible, the total variation in the data set in only a few PCs and each successively derived PC expresses decreasing amounts of the variance. This statistical multivariate treatment was earlier used to observe similarities among different soft cheeses [17, 21], reducing the dimension to two or three PCs, while keeping most of the original information found in the data.

The objective of CCSWA, presented in detail elsewhere [24], is to determine a space of representation common to all data tables, each table having a specific weight associated with each dimension of this common space. A custom-designed CCSWA algorithm programmed in MatLab was used in this study. This version of CCSWA allowed the calculation of common dimensions, latent variables related to each data table, and loadings of each variable in the data tables. Analysis of these CCSWA outputs made it possible to explore relations between the properties of the tables and to estimate the contribution of each property in the discrimination of milks observed in this study. Each table has a specific weight (or salience) indicating its contribution in each dimension of the common space. This technique is particularly interesting to determine influential factors implicated in a process, a physicochemical reaction or in the evolution of samples as a function of time or temperature [23, 39].

3. RESULTS AND DISCUSSION

PCA was firstly carried out on the whole data set containing 420 samples and the 15 variables (matrix of 420×15), corresponding to all the measured parameters on the ten milks (5 from spring and 5 from autumn) before and after processing, i.e., bulk-raw milks, pasteurised milks, freshly processed UHT milks and UHT milks stored for different periods at 4, 20 and 40 °C. The similarity map defined by principal components 1 and 2 showed a discrimination of samples according to the different stages of the process (results not shown). It appeared that

the UHT sterilisation process was the principal factor modifying milk characteristics. To improve the determination of this effect and the explanatory variables, the data set with the 420 samples and the 15 variables was split into 2 data sub-sets. One sub-set contained the data recorded on bulk-raw milks, pasteurised milks and freshly processed UHT milks of the five dairy plants and for the two seasons. It was assimilated to the data set of bulk-raw and processed milks. The second sub-set contained the data recorded on UHT sterilised milks during storage, i.e. UHT milks, from the five dairy plants and for the two seasons, stored at 4, 20 and 40 °C and analysed after 21, 42, 62, 90, 110 and 180 d. It was assimilated to the data set of UHT milks during storage.

3.1. Determination of the variables explaining the discrimination of investigated bulk-raw and processed milks

3.1.1. Results of the Principal Component Analysis

PCA was carried out on the sub-set including data recorded on raw, pasteurised and freshly processed UHT milks for the two seasons and for the different milking zones. Considering the PCA similarity map defined by principal components 1 and 2, raw, pasteurised and freshly processed UHT milks were well separated according to the principal component 1 (38.3% of the total variance; Fig. 1A). This component clearly showed the effect of the sterilisation process. Raw milks were located on the positive part of the similarity map, whereas UHT milks were on the negative part. The correlation circle (Fig. 1B) showed that raw milks were characterised by high contents of NCN, total solids and fat, and high values for the ultracentrifugation-pellet water content. Oppositely, UHT milks were characterised by large particle size, and exhibited higher values for phosphate and ethanol tests than raw milks. Raw milks also presented higher pH and contents of soluble calcium, phosphate and proteins than UHT milks, but the weights of these variables were less significant. Contents of lactose, NPN and micellar zeta-potential were not affected by this effect, since these variables were located close to the origin of the X-axis (Fig. 1B). Minimal and maximal values for these different variables and for raw, pasteurised and freshly processed UHT milks are given in Table I. The high concentrations of total solids, fat and proteins in raw milks agreed with natural concentrations of milk before standardisation performed during industrial UHT processing. High NCN contents for raw milks were also consistent with the high contents of native whey proteins which did not precipitate at pH 4.6 and remained in the analysed filtrate. From the correlation circle, it also appeared that raw milks had high values of ultracentrifugation-pellet water content and, oppositely, low values for heat-treated milks, confirming the findings of Ruegg et al. [34]. In the same way, particles were larger in UHT milks than in their corresponding raw milks. These particles would correspond better to casein micelles than to fat globules since the UHT process included a homogenisation stage which reduced fat globule size. The increase in particle size may originate from denaturation of whey proteins induced by UHT sterilisation and their association with casein micelles, leading to an increase in casein micelle size [2]. For both raw and heat-treated milks, a negative relationship was observed between hydration and size of casein micelles, in agreement with the work of O'Connell and Fox [26]. These authors showed an increase in hydration of sedimentable protein in heated milks (120 °C for 10 min) as the size of casein

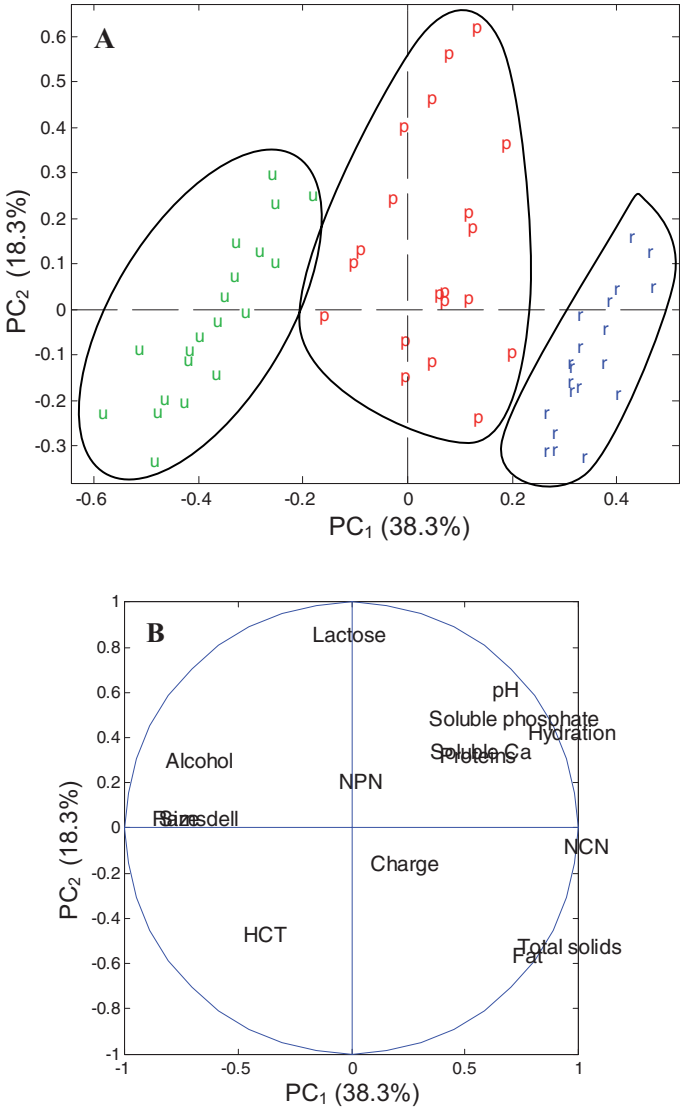

Figure 1. Principal component analysis (PCA) similarity map determined by principal components 1 (38.3%) and 2 (18.3%) of the PCA performed on data including raw (r), pasteurised (p) and freshly processed UHT milks (u). **A**: Effect of the process on the UHT milk shelf-life according to principal component 1. **B**: Correlation circle determined by the first two principal components of the PCA.

Table I. Minimal (min) and maximal (max) values observed for each analysed variable and each type of milk considered in the UHT processing effect. Both minimal and maximal values corresponded to the mean value of duplicates. Values were considered from the data sub-set of bulk-raw and processed milks.

	Raw milks		Pasteurised milks		Freshly processed UHT milks	
Values	min	max	min	max	min	max
pH	6.69	6.76	6.70	6.78	6.66	6.70
Total solids ($g \cdot kg^{-1}$)	119.8	129.9	92.3	114.4	102.0	105.1
Fat ($g \cdot kg^{-1}$)	31.4	42.4	2.0	26.4	15.2	16.4
Lactose ($g \cdot kg^{-1}$)	45.5	47.4	46.5	49.1	46.2	47.9
Proteins ($g \cdot kg^{-1}$)	33.5	34.8	33.2	34.9	33.0	34.9
NCN ($g \cdot kg^{-1}$)	7.15	7.80	4.12	7.13	2.53	3.22
NPN ($g \cdot kg^{-1}$)	1.40	1.87	1.41	1.88	1.22	2.43
Soluble calcium ($mmol \cdot L^{-1}$)	8.5	9.5	8.4	9.3	7.7	9.2
Soluble phosphate ($mmol \cdot L^{-1}$)	9.0	11.4	8.6	10.5	8.5	11.8
Size (nm)	173	212	181	251	232	294
Charge (mV)	–21.9	–13.3	–20.5	–18.3	–20.9	–18.0
Hydration (g of water$\cdot g^{-1}$ of dried pellet)	2.19	2.38	2.17	2.44	1.88	2.26
HCT (min at 140 °C)	0.5	8.5	0.5	12.0	3.0	18.0
Alcohol (% of ethanol)	50	65	50	100	75	100
Ramsdell (mL of KH_2PO_4 0.5 $mol \cdot L^{-1}$)	0.1	1.0	0.5	1.0	1.2	2.0

micelles decreased. However, it was expected that the increase in micellar size and the decrease in ultracentrifugation-pellet water content for UHT milks induced a decrease in heat stability. As observed on the correlation circle (Fig. 1B), the results of the heat stability test did not significantly explain the discrimination of the different groups as a function of their process stage. Nevertheless, this parameter was positioned in the negative part of the circle, like phosphate and ethanol stability tests. Considering the values obtained for the different stability tests, it could be concluded that heat-treated milks were more stable than raw milks, despite micellar changes induced by heat treatments being generally considered as negative for milk stability.

3.1.2. Results of the Common Components and Specific Weights Analysis

The present sub-set was organised into three standardised data tables, each one corresponding to raw, pasteurised and freshly processed UHT milks. These three tables were analysed by CCSWA. This analysis allows one to create a space of representation common to these three tables in order to determine the possible existence of common structures to these tables. To simplify, it corresponds to a re-organisation of the data in multi-tables with the objective of determining some effects which affect the data. The CCSWA similarity map defined by common components 1 and 2

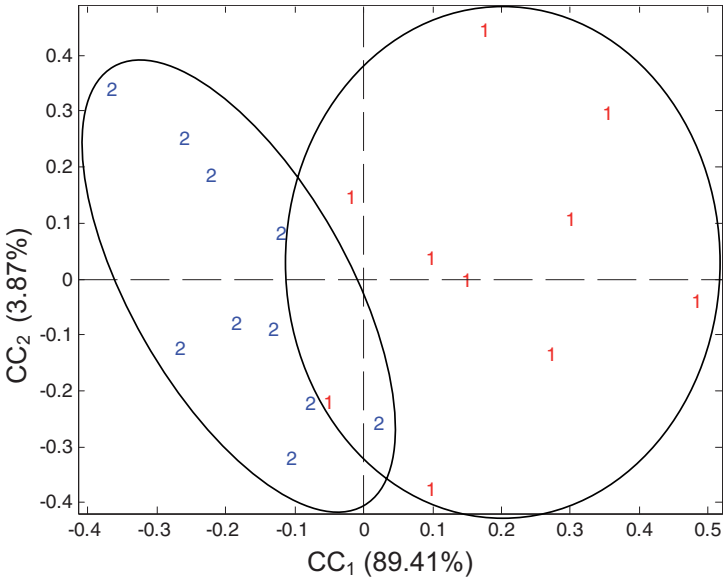

Figure 2. Common components and specific weights analysis (CCSWA) similarity map determined by common components 1 (89.41%) and 2 (3.87%), demonstrating the effect of season, of the CCSWA performed on the 3 data tables corresponding to raw, pasteurised and freshly processed UHT milk samples.

(Fig. 2) showed a discrimination of season 1, on the positive part of the map, from season 2, on the negative part of the map, according to the common component 1. The effect of season was clearly shown with this statistical analysis. The results of the weights of the 3 tables according to the first common component are presented in Table II. These weights (0.28, 0.36 and 0.25) were rather similar, indicating that raw, pasteurised and freshly processed UHT milk data contributed to the formation of the first common component. The factors responsible for this season effect can be determined according to their correlation coefficients (Tab. II). Milks collected in spring were characterised by high values of pH, lactose, soluble phosphate, micellar hydration and a low content of fat. Raw milks collected in spring also contained larger casein micelles than autumn raw milks. These parameters altered by the season affected milk stability, since lower values of HCT were observed for milks collected during spring. Oppositely, contents of total solids, proteins, NCN, NPN, soluble calcium and micellar charge were not involved in the season effect. Neither ethanol stability nor phosphate stability were significantly modified. Minimal and maximal values for these different variables and for milks collected during season 1 and season 2 are given in Table III. Some of these relationships have been reported in previous papers. Indeed, Rose [32] demonstrated that high natural pH did not necessarily lead to a milk of high heat stability. Moreover, only high increases in soluble phosphate concentrations may contribute to increase in the buffering capacity and thus to milk heat stability. In addition, O'Connell and

Table II. Correlation coefficients for the explanation of the first common component (CC_1) showing the effect of season and determined by CCSWA performed on process data arranged according to process stage: raw, pasteurised and freshly processed UHT milks. In bold type, coefficients exhibiting the heaviest weights.

Type of milk	pH	Total solids	Fat	Lactose	Proteins	NCN	NPN	Soluble calcium	Soluble phosphate	Size	Charge	Hydration	HCT	Alcohol	Ramsdell
Raw CC_1 (0.28)[1]	**+0.71**	–0.39	**–0.54**	**+0.84**	+0.18	–0.26	+0.26	+0.39	**+0.62**	**+0.75**	+0.44	**+0.73**	**–0.64**	–0.29	–0.10
Pasteurised CC_1 (0.36)[1]	**+0.84**	**–0.78**	**–0.81**	**+0.87**	+0.39	–0.02	+0.25	+0.41	**+0.74**	+0.09	–0.38	**+0.83**	**–0.59**	**+0.51**	**+0.61**
Freshly processed UHT CC_1 (0.25)[1]	**+0.69**	+0.33	**–0.56**	**+0.76**	+0.35	–0.03	+0.12	+0.36	**+0.86**	–0.10	–0.48	+0.35	**–0.80**	+0.33	–0.47

[1] Table weight for the first common component of the CCSWA.

Fox [26] showed that large casein micelles negatively affected milk in the heat stability test. Changes in casein micelle size [18], fat [29] and heat stability [19] as a function of season have also been reported earlier. All these seasonal variations may be linked to lactation stage, changes in feeding or environmental conditions such as hot temperature [1, 3, 20, 36]. Nevertheless, it was not possible to evaluate the weights of these parameters from this study because the investigated samples were bulk milks.

The obtained results allowed the demonstration of the effects of UHT processing and season and to determine the involved physico-chemical parameters.

3.2. Determination of the effects observed during UHT milk storage

3.2.1. Results of the Principal Component Analysis

The considered data set in this section corresponded to the parameters measured on UHT milks, collected in the two seasons and in the five dairy plants, after different storage periods (21, 42, 62, 90, 110 and 180 d) at different temperatures (4, 20 and 40 °C). A PCA was carried out on this data set and the PCA similarity map defined by the first two principal components is presented in Figure 3A. The first principal component (PC_1), which accounted for 23.6% of the total variance, discriminated UHT milks according to their storage temperature. The effect of storage temperature was clearly observed here. A net discrimination was observed between the group of milks stored at 40 °C, on the negative part of the PC_1, and the groups of milks stored at 4 and 20 °C, on the positive part of the PC_1. Milks stored at 40 °C were characterised by high contents of NCN and NPN and low values of pH (Fig. 3B). Minimal and maximal values for these different variables and for UHT milks stored at 4, 20 and 40 °C are given in Table IV. These changes, suggesting proteolysis and acidification, were in agreement with many physico-chemical and enzymatic reactions promoted by high storage temperatures [14]. Milks stored at 40 °C also presented low heat stability values but high stability values for the phosphate test (Fig. 3B, Tab. IV). This opposition in the results of stability tests may be related to the principle of each test. In the heat stability test, heat treatment at 140 °C induces acidification, whey protein denaturation, Maillard reaction and casein hydrolysis [13]. In the phosphate test, the addition of large amounts of phosphate before heat treatment at 100 °C leads to a decrease in pH, mineral imbalance, solubilisation of caseins from casein micelles, aggregation and changes in particle hydration [15, 33, 38]. It could be suggested that lactosylation of caseins in UHT milks stored at 40 °C may obstruct the mechanism of milk destabilisation in the phosphate test by changes in the particle hydration or by inhibition of particle aggregation, as proposed by Samel et al. [35]. This may explain why large volumes of phosphate were necessary to destabilise UHT milks stored at 40 °C and why the milk stability in the phosphate test was higher than the milk stability evaluated by the heat stability test.

Except for these changes in heat and phosphate stability test values and the modifications of pH and NCN and NPN contents, no other parameter seemed significantly involved in the storage-temperature effect.

3.2.2. Results of the Common Components and Specific Weights Analysis

In order to extract more information about the variables which could be responsible for alteration of UHT milks, the present data set was re-organised into three

Table III. Minimal (min) and maximal (max) values observed for each analysed variable and each type of milk considered in the season effect. Both minimal and maximal values corresponded to the mean value of duplicates. Values were considered from the data sub-set of bulk-raw and processed milks.

	Milks from season 1		Milks from season 2	
Values	min	max	min	max
pH	6.67	6.78	6.66	6.77
Total solids ($g \cdot kg^{-1}$)	92.3	129.6	102.6	129.9
Fat ($g \cdot kg^{-1}$)	2.0	41.4	15.3	42.4
Lactose ($g \cdot kg^{-1}$)	46.2	49.1	45.5	47.1
Proteins ($g \cdot kg^{-1}$)	33.4	34.9	33.0	34.8
NCN ($g \cdot kg^{-1}$)	2.53	7.80	2.65	7.73
NPN ($g \cdot kg^{-1}$)	1.55	2.09	1.22	2.43
Soluble calcium ($mmol \cdot L^{-1}$)	8.4	9.5	7.7	9.3
Soluble phosphate ($mmol \cdot L^{-1}$)	9.0	11.8	8.5	10.3
Size (nm)	186	272	173	294
Charge (mV)	–21.5	–13.3	–21.9	–17.2
Hydration (g of water·g^{-1} of dried pellet)	1.92	2.44	1.88	2.27
HCT (min at 140 °C)	0.5	7.0	4.5	18.0
Alcohol (% of ethanol)	50	100	50	100
Ramsdell (mL of KH_2PO_4 0.5 $mol \cdot L^{-1}$)	0.1	1.8	0.2	2.0

Table IV. Minimal (min) and maximal (max) values observed for each analysed variable and each type of milk considered in the storage temperature effect. Both minimal and maximal values corresponded to the mean value of duplicates. Values were considered from the data sub-set of UHT milks during storage.

	Milks stored at 4 °C		Milks stored at 20 °C		Milks stored at 40 °C	
Values	min	max	min	max	min	max
pH	6.60	6.75	6.57	6.72	6.19	6.64
Total solids ($g \cdot kg^{-1}$)	101.1	105.8	103.0	106.2	102.0	106.5
Fat ($g \cdot kg^{-1}$)	15.1	16.6	14.0	16.8	13.1	17.0
Lactose ($g \cdot kg^{-1}$)	46.1	48.5	46.5	48.5	45.8	48.1
Proteins ($g \cdot kg^{-1}$)	32.1	35.4	33.0	35.5	33.1	35.8
NCN ($g \cdot kg^{-1}$)	2.64	4.14	2.65	4.23	2.84	6.14
NPN ($g \cdot kg^{-1}$)	1.46	2.16	1.55	2.16	1.60	3.15
Soluble calcium ($mmol \cdot L^{-1}$)	6.4	11.1	6.2	11.1	6.1	11.2
Soluble phosphate ($mmol \cdot L^{-1}$)	6.6	16.7	7.3	12.9	7.5	15.0
Size (nm)	226	300	227	295	213	287
Charge (mV)	–20.8	–17.3	–22.5	–16.9	–21.0	–16.5
Hydration (g of water·g^{-1} of dried pellet)	1.75	2.40	1.71	2.30	1.77	2.34
HCT (min at 140 °C)	5.0	24.5	2.9	20.0	2.0	18.5
Alcohol (% of ethanol)	85	100	80	100	75	100
Ramsdell (mL of KH_2PO_4 0.5 $mol \cdot L^{-1}$)	1.4	2.6	1.5	2.9	1.2	3.9

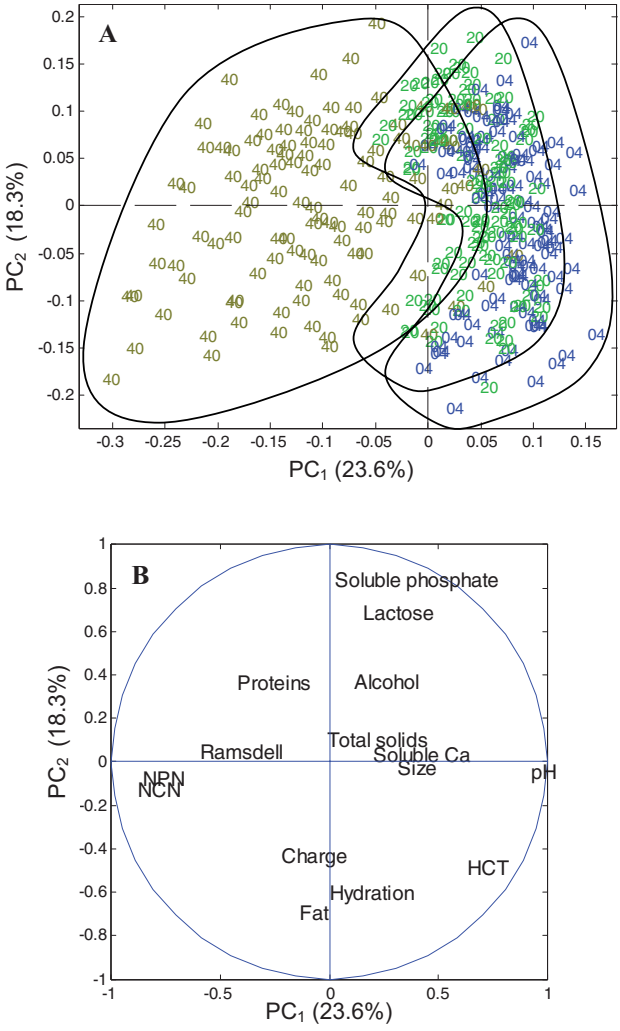

Figure 3. Principal component analysis (PCA) similarity map determined by principal components 1 (23.6%) and 2 (18.3%) of the PCA performed on storage data for UHT milk samples stored at 4, 20 and 40 °C for different storage periods. **A**: Effect of storage temperature according to principal component 1. **B**: Correlation circle determined by the first two principal components of the PCA.

normalised data tables, each one corresponding to UHT milks stored at a given temperature, i.e., 4, 20 and 40 °C. CCSWA was performed on these three data tables. The results (not shown) showed that the season effect was still observed during storage. This indicated that the initial differences in raw milks still remained in UHT milks, independently of storage conditions. In order to go further and more deeply into the evaluation of the data, this data set was divided into two sub-sets

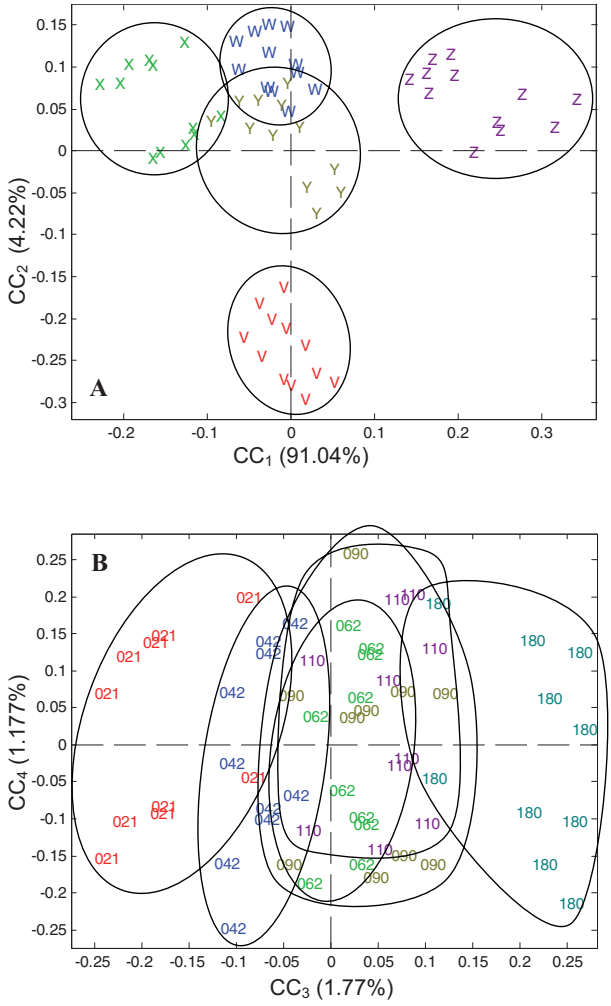

Figure 4. Common components and specific weights analysis (CCSWA) similarity maps determined by common components 1 (91.04%) and 2 (4.22%), demonstrating the effect of dairy plants (**A**), and by common components 3 (1.77%) and 4 (1.177%), demonstrating the effect of storage time (**B**), of the CCSWA performed on the 3 data tables corresponding to UHT milk samples stored at 4, 20 and 40 °C (for 21, 42, 62, 90, 110 and 180 d, season 2).

corresponding to season 1 and season 2. As similar results were obtained for seasons 1 and 2, we chose to present the results obtained from CCSWA performed on the data of season 2.

The CCSWA, performed on the data sub-set corresponding to UHT milks of season 2, from the five dairy plants and stored at 4, 20 and 40 °C for different times, showed a discrimination of the samples as a function of the dairy plants according to the first two common components (Fig. 4A). This effect of dairy plants, which could be associated

Table V. Correlation coefficients for the explanation of the first (CC_1), second (CC_2) and third (CC_3) common components showing the effects of dairy plants (CC_1 and CC_2) and of storage time (CC_3), and determined by CCSWA performed on storage data arranged according to storage temperature: 4, 20 and 40 °C. In bold type, coefficients exhibiting the heaviest weights.

Storage T °C	pH	Total solids	Fat	Lactose	Proteins	NCN	NPN	Soluble calcium	Soluble phosphate	Size	Charge	Hydration	HCT	Alcohol	Ramsdell
4 °C															
CC_1 (0.28)[1]	+0.18	**+0.78**	**+0.85**	+0.39	**+0.65**	**+0.71**	+0.36	**–0.65**	–0.45	–0.19	+0.18	**+0.86**	+0.16	+0.35	+0.02
CC_2 (0.20)[2]	–0.25	–0.13	+0.31	**–0.66**	–0.05	+0.10	**–0.61**	+0.28	–0.17	**–0.82**	+0.01	+0.29	–0.41	**–0.54**	**–0.84**
CC_3 (0.06)[3]	+0.16	–0.28	–0.25	+0.06	–0.40	**+0.54**	–0.14	+0.21	+0.17	+0.08	–0.15	+0.14	+0.25	–0.15	+0.06
20 °C															
CC_1 (0.31)[1]	+0.27	**+0.94**	**+0.86**	+0.35	**+0.76**	**+0.67**	+0.35	**–0.66**	–0.46	–0.20	+0.15	**+0.88**	+0.16	+0.35	–0.13
CC_2 (0.20)[2]	–0.23	–0.12	+0.34	**–0.76**	–0.01	+0.09	**–0.57**	+0.30	–0.22	**–0.80**	–0.04	+0.24	–0.11	**–0.53**	**–0.88**
CC_3 (0.06)[3]	+0.16	–0.08	–0.17	+0.38	–0.29	**+0.61**	+0.03	+0.23	+0.07	–0.07	–0.06	+0.21	–0.16	–0.26	+0.12
40 °C															
CC_1 (0.31)[1]	–0.03	**+0.92**	**+0.90**	+0.41	**+0.87**	+0.49	+0.30	**–0.67**	**–0.53**	–0.28	+0.33	**+0.79**	+0.09	+0.24	–0.44
CC_2 (0.15)[2]	+0.20	+0.01	+0.31	**–0.52**	+0.06	–0.13	–0.31	+0.29	–0.08	**–0.86**	–0.08	+0.23	–0.26	**–0.50**	**–0.72**
CC_3 (0.20)[3]	**–0.82**	–0.10	–0.03	–0.21	–0.05	**+0.83**	**+0.76**	+0.36	+0.14	–0.13	+0.25	+0.42	**–0.61**	–0.43	+0.07

[1,2,3] Table weights for the first, second and third common components of the CCSWA, respectively.

Table VI. Minimal (min) and maximal (max) values observed for each analysed variable and each type of milk considered in the dairy plants/milking region effect. Both minimal and maximal values corresponded to the mean value of duplicates. Values were considered from the data sub-set of UHT milks during storage, only for season 2.

	Milks collected in V		Milks collected in W		Milks collected in X		Milks collected in Y		Milks collected in Z	
Values	min	max	min	max	min	max	min	max	min	max
pH	6.23	6.74	6.26	6.74	6.32	6.68	6.27	6.70	6.26	6.75
Total solids ($g \cdot kg^{-1}$)	102.6	104.6	102.2	104.1	101.1	103.3	102.0	104.6	103.3	106.5
Fat ($g \cdot kg^{-1}$)	15.2	15.8	15.6	16.1	15.1	15.5	15.3	15.8	16.1	17.0
Lactose ($g \cdot kg^{-1}$)	46.5	48.1	46.1	47.1	46.1	47.4	45.8	47.5	46.4	47.8
Proteins ($g \cdot kg^{-1}$)	32.7	34.2	32.6	34.1	32.1	33.5	33.2	34.5	33.2	35.5
NCN ($g \cdot kg^{-1}$)	2.90	5.70	2.84	5.09	2.64	4.82	2.89	4.64	3.29	6.14
NPN ($g \cdot kg^{-1}$)	1.90	2.59	1.46	2.38	1.62	3.15	1.55	2.39	1.78	2.62
Soluble calcium ($mmol \cdot L^{-1}$)	6.4	8.7	7.4	10.4	8.2	11.2	6.6	9.2	6.3	7.8
Soluble phosphate ($mmol \cdot L^{-1}$)	7.3	9.2	6.6	8.6	9.1	11.4	8.1	10.0	7.8	8.6
Size (nm)	271	300	235	268	229	266	239	268	222	264
Charge (mV)	–19.6	–16.9	–20.1	–17.3	–19.8	–17.8	–20.8	–17.4	–20.3	–16.8
Hydration (g of water$\cdot g^{-1}$ of dried pellet)	1.80	2.11	1.92	2.19	1.78	2.04	1.86	2.14	2.09	2.40
HCT (min at 140 °C)	5.5	24.5	2.5	20.2	3.7	13.5	4.0	23.0	3.0	19.5
Alcohol (% of ethanol)	95	100	80	100	80	100	80	100	80	100
Ramsdell (mL of KH_2PO_4 0.5 $mol \cdot L^{-1}$)	2.1	3.9	1.6	2.8	1.7	3.1	1.7	2.9	1.2	2.6

with an effect of the milking region (cow breed, feed, herd management, climate) or of the processing conditions of each dairy plant (time/temperature pair for pasteurisation and sterilisation, pressure of homogenisation, industrial practice), was clearly observed here. Common component 1 (CC_1), accounting for 91.04% of the total variance, discriminated principally the dairy plant X, on the negative part of the map, from the dairy plant Z (Fig. 4A). The weights of the tables were similar (0.28, 0.31 and 0.31), as shown by the values of saliences reported in Table V, i.e., each table participated equally in the building of the common component 1. Samples of the dairy plant Z had higher contents of total solids, fat, proteins, NCN, higher values for micellar hydration and lower contents of soluble calcium than the ones from dairy plant X. No change in milk stability was observed according to CC_1 (Tab. V). Common component 2, which accounted for 4.22% of the total variance, discriminated samples of the dairy plant V, on the negative side of CC_2, from those of the dairy plant W, on the positive side of CC_2 (Fig. 4A). As the weights of the tables were similar (0.20, 0.20 and 0.15), it could be concluded that all the tables participated in the building of common component 2 (Tab. V). Samples from the dairy plant W had lower contents of lactose and NPN, and smaller casein micelles than those from the dairy plant V. These differences altered milk stability values in the tests, and samples of the dairy plant W had lower stability values in ethanol and phosphate tests than samples of dairy plant V (Tab. V). Minimal and maximal values for these different variables and for UHT milks from the dairy plants V, W, X, Y and Z are given in Table VI. All the physico-chemical characteristics studied were involved in this dairy plant effect with the exception of the parameters pH, content of soluble phosphate and micellar charge. Changes in physico-chemical characteristics resulted in changes in the ethanol and phosphate tests results but not in the heat stability test result.

As shown in Figure 4B, common component 3 discriminated UHT milks as a function of their storage time even if it accounted for only 1.77% of the total variance. The samples stored for 21 d were on the negative part of CC_3 and those stored for 180 d were on the positive part. Considering the values of saliences in the different data tables (Tab. V), it was noticed that the data table "40 °C" had a heavier weight than the two others (0.20 versus 0.06). Consequently, the table containing data on UHT milks stored at 40 °C mainly contributed to the building of common component 3 and explained the discrimination. UHT milks stored for long periods, and notably at 40 °C, were characterised by lower values of pH, higher contents of NCN and NPN and were easily destabilised when submitted to the heat stability test. Minimal and maximal values for these different variables and for UHT milks stored for 21, 42, 62, 90, 110 and 180 d are given in Table VII. These findings agreed with the results obtained for the temperature effect during storage reported in Section 3.2.1. The time/temperature pair has to be taken into account when the effect of storage on the quality of UHT milk is investigated.

4. CONCLUSION

To our knowledge, this study was the first one attempting to investigate the different effects which alter commercial UHT milk shelf-life, starting with the sampling of raw milks and ending with UHT processed milks stored for several months at 4, 20 and 40 °C. To investigate the characteristics of the samples as a function of the milking zone and the alterations in UHT milk samples during storage, the initial global and large data set was divided into smaller ones and analysed thanks to two multidimensional statistical tools, i.e.,

Table VII. Minimal (min) and maximal (max) values observed for each analysed variable and each type of milk considered in the storage time effect. Both minimal and maximal values corresponded to the mean value of duplicates. Values were considered from the data sub-set of UHT milks during storage, only for season 2.

	Milks stored for 21 d		Milks stored for 42 d		Milks stored for 62 d		Milks stored for 90 d		Milks stored for 110 d		Milks stored for 180 d	
Values	min	max	min	max	min	max	min	max	min	max	min	max
pH	6.55	6.70	6.50	6.69	6.48	6.75	6.46	6.74	6.39	6.72	6.23	6.71
Total solids ($g \cdot kg^{-1}$)	102.6	105.5	102.7	105.3	102.0	104.9	101.1	105.5	103.1	105.6	101.8	106.5
Fat ($g \cdot kg^{-1}$)	15.4	16.6	15.4	16.6	15.3	16.4	15.1	16.5	15.4	16.6	15.1	17.0
Lactose ($g \cdot kg^{-1}$)	46.4	47.6	46.1	47.7	45.8	47.6	46.1	48.0	46.5	48.1	46.2	48.1
Proteins ($g \cdot kg^{-1}$)	33.0	34.6	33.1	34.5	33.1	34.2	32.1	34.7	33.1	34.7	32.5	35.5
NCN ($g \cdot kg^{-1}$)	2.64	3.43	2.89	3.93	2.90	4.43	2.96	4.78	3.09	5.05	3.30	6.14
NPN ($g \cdot kg^{-1}$)	1.55	2.07	1.72	2.15	1.78	2.43	1.76	2.62	1.77	2.56	1.46	3.15
Soluble calcium ($mmol \cdot L^{-1}$)	6.8	9.1	7.0	9.9	6.4	10.4	6.3	9.9	6.6	10.6	6.5	11.2
Soluble phosphate ($mmol \cdot L^{-1}$)	6.6	11.4	7.0	10.5	8.0	9.8	7.3	10.3	7.5	9.6	8.3	10.0
Size (nm)	230	299	227	293	222	288	231	287	223	300	231	295
Charge (mV)	–20	–17.4	–19.5	–17.3	–19.5	–17.4	–20.3	–16.9	–20.1	–17.3	–20.8	–16.8
Hydration (g of water·g^{-1} of dried pellet)	1.78	2.27	1.83	2.33	1.86	2.40	1.89	2.37	1.93	2.40	1.88	2.34
HCT (min at 140 °C)	5.5	20.5	5.0	20.5	3.7	19.7	3.8	24.0	4.0	23.0	2.5	24.5
Alcohol (% of ethanol)	80	100	85	100	80	100	80	100	80	100	80	100
Ramsdell (mL of KH_2PO_4 0.5 $mol.L^{-1}$)	1.7	3.1	1.6	3.2	1.7	3.9	1.7	3.6	1.6	3.7	1.2	3.3

Table VIII. Summary of the main effects altering milk and UHT milk, and the involved physico-chemical characteristics. Relationships with milk stability evaluated by the three tests were also presented. Involvement of the factor is marked by *.

Factors	pH	Total solids	Fat	Lactose	Proteins	NCN	NPN	Soluble calcium	Soluble phosphate	Size	Charge	Hydration	HCT	Alcohol	Ramsdell
UHT process		*	*			*				*		*		*	*
Season	*		*	*					*	*		*	*		
Storage temperature and time	*					*	*								
Dairy plant		*	*	*	*	*	*	*		*		*		*	*

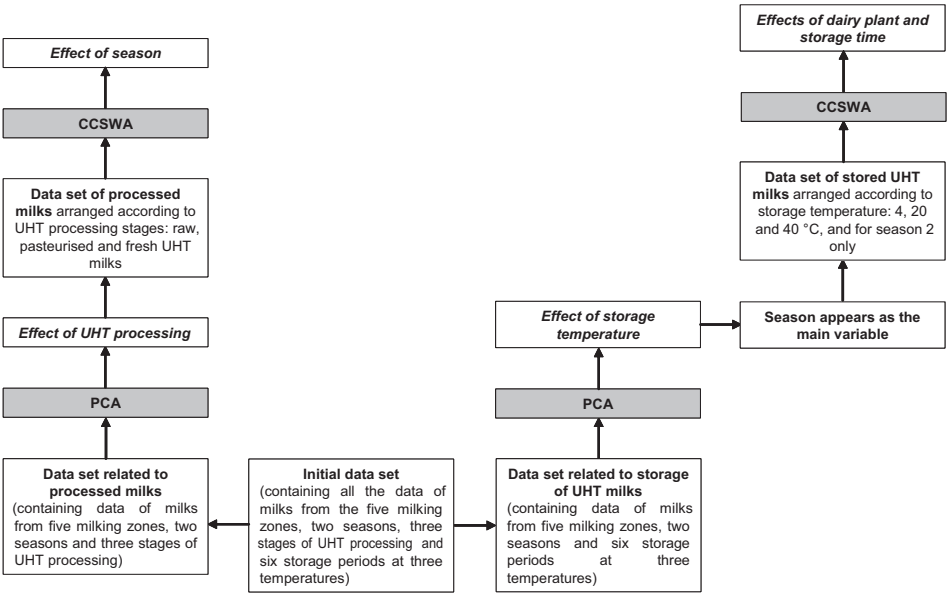

Figure 5. Recapitulative scheme of the different data sets analysed, the chemometric tools used, and the different alterations demonstrated in milk samples.

PCA and CCSWA. Figure 5 summarises the different data sets analysed, the chemometric tools used and the observed effects. These effects corresponded to UHT processing, season, storage conditions and milking zone/dairy plant. Table VIII summarises the physico-chemical characteristics involved in these different effects. This study, based on the use of chemometric tools allowing the investigation of the relations between several data tables, has brought out a global view of the parameters altering milk and UHT milk shelf-life. It also showed that the three milk stability tests did not evaluate milk stability in the same way, and that milk stability evaluated by these different tests was not always linked to the same changes in casein micelle properties. This study contributed to a better knowledge of effects which affect milk and UHT milk characteristics. It is important in order to improve quality of raw material and consequently those of final products. However, additional studies are needed. For a better knowledge of the parameters implicated in milk stability, it will be necessary to analyse other milk characteristics, and especially other casein micelle properties. In addition, some effects such as lactation stage, feed or environmental conditions should be taken into account for a better explanation of dairy plant/milking zone or season effect.

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Physico-chemical factors involved in the result of phosphate, ethanol and heat stability tests: a multidimensional statistical approach

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Summary

Milk stability to heat is a problem that can be observed in dairy industry. In the present study, we examined three tests generally applied to raw milks before heat treatments to evaluate their stability: heat, ethanol and phosphate tests. Thirty individual raw milks were thus tested for their stability using these three tests, and characterized for 34 zootechnical and physico-chemical factors. The data table was evaluated using partial least-squares discriminant analysis (PLS-DA). Among the 34 factors studied, this analysis emphasized 17 parameters, all stability tests considered. None of these parameters was common to the three tests and only eight factors were found common to two tests: lactation number, pH, size and hydration of casein micelle, contents in lactose, NPN, β- and α_{s1}-caseins. It was concluded that each test was multifactorial and that milk stability was influenced by casein micelle properties but also micelle environment. The few parameters common to these tests demonstrated that each test evaluate specific parameters of milk.

Keywords: individual milk, stability, phosphate test, alcohol test, heat test, chemometrics

In dairy industries, some tests are applied to raw milks in order to make a fast selection of milks susceptible to pass heating step. These main tests are based on the measurement of milk stability to heat (Davies & White, 1966), ethanol (Sommer & Binney, 1923) or phosphate (Ramsdell et al., 1931). They globally consist in a voluntary destabilization of the micelle and its environment by physico- and / or chemical treatments. Previous studies, when they exist, showed many contradictions on the parameters that influence milk stability in these tests (Davies & White, 1958; Thompson et al., 1969; Schmidt & Koops, 1977; Chavez et al. 2004) and different response can be given by these three tests for a same product. A real lack of knowledge exists about the different rapid methods used to detect unstable milks and about the factors which are involved in milk stability. Moreover, most of the previous studies were conducted on modified milks or model systems (Pierre, 1985; Horne, 1987; Pouliot & Boulet, 1991; Philippe et al., 2005). The number of factors studied was generally limited since statistical analyses mainly consisted in simple regression.

The originality of the present study corresponds in the examination of 30 individual raw whole milks on 34 physico-chemical, zootechnical and biological factors and the three stability tests. The data table thus obtained has been evaluated by a multivariate statistical analysis: Partial Least-Squares Discriminant-Analysis (PLS-DA) in order to highlight the factors which explain the results of phosphate, ethanol and heat stability tests.

Materials and Methods

Materials

Thirty fresh morning whole milks were collected from individual Prim'Holstein under various diets at the INRA Méjusseaume farm, at the end of June month. Number and stage of lactation for these cows were known and considered in the present study (abbreviated in the similarity maps *Lact nb* and *Lact stg*, respectively). Milks were kept at 4°C for one day in darkness before analyses. Then they were added with thimerosal (0.03% p/v) (Sigma, Saint-Louis, USA) to prevent microbial growth.

For each milk, an ultrafiltrate was recovered in two steps: milk was ultracentrifuged at 100,000 *g* for 1 h at 20°C (Sorvall Discovery 90 SE, Sorvall, Courtaboeuf, France) and the obtained supernatant was then centrifuged at 1,800 *g* for 2 h on a Vivaspin 20 concentrator (molecular mass cut-off: 10 kDa) (Vivascience, Palaiseau, France).

Milk stability tests

Phosphate (Ramsdell), ethanol and heat stability tests were performed as described in Gaucher et al (2008)

Analysis of milk composition

The pH, concentrations in fat, lactose and total solids (*TS*) were determined as described in Gaucher et al (2008).

Determination of nitrogen fractions and proteins

Total nitrogen content (coding in the text: *TN*) was determined by the Kjeldhal method (IDF standard 20B, 1993). Non casein nitrogen (*NCN*) and non protein nitrogen (*NPN*) contents were determined as described in Gaucher et al (2008). These nitrogen contents were used to calculate proteins (*PNs* = TN-NPN), caseins (*CNs* = TN-NCN) and whey proteins (*Whey P* = NCN-NPN) contents. Results were expressed as $g.kg^{-1}$ of milk.

The different proteins in milk, i.e., κ-+α_{s2}- (*TK- & A_{s2}-*), α_{s1}- (*TA_{s1}-*), β-caseins (*TB)*-and β-lactoglobulin *(TBLG*), were determined by reverse-phase high performance liquid chromatography. Milks were previously reduced by 0.01 M dithiotreitol in the presence of 8 M urea, 0.1 M Tris-HCl and 0.044 M trisodium citrate for 1 h at 37°C, and then acidified by trifluoroacetic acid (TFA) to pH ≈ 2 before injection of 100 µL of sample on the column. The reverse-phase column was a Vydac 214 TP 54 (C4, 5 µm, 4.6 mm internal diameter x 15 cm length) (Touzart & Matignon, Les Ulis, France) and the chromatographic conditions were as described by Jaubert and Martin (1992) but slightly modified using a linear gradient from 37 to 62 % of buffer B (80 % (v/v) acetonitrile and 0.106 % (v/v) TFA in water) for 58 min at a flow rate of 1 $mL.min^{-1}$. Detection was at 214 nm. Results were expressed as % of chromatogram area and converted in g / kg of milk by multiplying result by proteins content.

Mineral analysis

Concentrations in calcium (coding in the text: *Ca*), magnesium (*Mg*), sodium (*Na*) and potassium (*K*) were determined on an atomic absorption spectrometer (Varian 220FS spectrometer, Les Ulis, France) as described by Brulé et al. (1974). Concentrations in total and soluble cations were obtained from milks and ultrafiltrates, respectively. Concentrations in inorganic phosphate (*PO4*), citrate (*Cit*), chlorure (*Cl*) were determined by ion chromatography (Dionex DX 500, Dionex, Voisin-le-Bretonneux, France) (Gaucheron et al. 1996). Concentrations in total and diffusible anions were obtained from the supernatant of milk acidified at pH 4.6 and ultrafiltrates, respectively. Soluble concentrations were corrected

by the factor 0.96 as determined by Pierre and Brulé (1981). Colloidal calcium, phosphate and magnesium were calculated making the difference between total and soluble concentrations. Minerals concentrations were expressed in mM. In the similarity maps, concentrations in total, soluble and colloidal minerals were indicated as following for an element X: *TX*, *SX* and *CX*, respectively.

Determination of micellar characteristics

Hydrodynamic micelle diameter (*Size*), micelle zeta-potential (*Charge*) and micellar hydration (*Hydration*) were measured as described in Gaucher et al (2008).

Statistical analyses

The PLS-DA was performed on normalized and centred data using Matlab R2006a software (The Mathworks Inc., Natic, MA, USA) using "Saisir" package available at the address: http://easy-chemometrics.fr. The aim of this technique is to predict the membership of an individual to a qualitative group defined as a preliminary (Vigneau et al., 2006). For each stability test, samples were classified in three groups according to their stability e.g. low, medium and high stability. The PLS-DA assesses new synthetic variables called loading factors, which are linear combinations of the variables, and allows a better separation of the centre of gravity of the considered groups. In this study, the optimum number of loading factors used in the model was determined to be four. The method allows the individual samples to be reallocated within the various groups. Comparison of the predicted group to the real group is an indicator of the quality of the discrimination and it is valued as the percentage of correct classification. Individuals were represented on the similarity map defined by loading factors 1 and 2.

Linear regression was done in order to determine correlations between the results of the stability tests.

Results and discussion

Classification of milks according to their stability

Milks were arranged in three classes according to their stability in each test, e.g. low, medium and high. These classes were realized according to the values found in the literature and the distribution of individuals. For the phosphate test, only a limit of 1.6 mL of KH_2PO_4 was given to reject milks unable to pass heat treatment (Hélaine, 1981). Consequently, the classification for this test was realized according to the distribution of the population. Classes

of low, medium and high stability were thus realized for milks with a phosphate test value below 0.4 mL, between 0.5 and 0.8 mL, and above 0.9 mL of KH_2PO_4 0.5 M, respectively.

For the ethanol test, Mitamura (1937) found ethanol concentrations inducing milk destabilization ranging from 66 % to 94 % with an average of 80 %. For Pien (1972), Metro et al. (1979) and Molina et al. (2001), a concentration of 75 % of ethanol could be considered as a limit between acceptable and unacceptable milks submitted to heat treatment. Chavez et al. (2004) discriminated stable milks, which resist to ethanol 78 %, from unstable milks, which destabilized with ethanol 72 %. For the present study, classes of low (from 50 to 65 % of ethanol), medium (from 70 to 85 % of ethanol) and high stability to ethanol (from 90 to 100 % of ethanol) were created.

For the heat stability test, references in the literature to classify milks differed since authors worked in different conditions (temperature of boiling, volume of milk, pH-adjustment or not, tube agitation, etc). Classes were thus realized according to the distribution of the population. Classes of low, medium and high stability corresponded to milks which destabilized within 2 min at 140 °C, between 2 and 5 min and for milks which resisted for more than 5 min, respectively.

Table 1 presents the classification of milk samples according to their stability in the different tests. For the phosphate test, milks had values between 0 and 1.5 mL of KH_2PO_4 0.5 M, that were lower than those found by Ramsdell et al. (1931) and Demeaux et al. (1986) (between 0.8 and 2.7 mL of KH_2PO_4 0.5 M for individual milks, at their natural pH). However they determined these values with a heat treatment of 5 min and not 10 min as in the present study. The considered experimental conditions could partly explain the difference of range observed between our study and the literature. A large proportion of samples (50 %) were found to have low phosphate stability. The two other classes were rather homogeneous, with 18 and 12 samples for groups of medium and high stability, respectively.

For the alcohol test, milks began to destabilize with 65 % of ethanol and some were not affected by 95 % of ethanol. The range of destabilization for the ethanol test is large as observed by Davies and White (1958) for individual milks. Most of milks (38 samples) in this study destabilized with concentrations included between 70 and 85 % of alcohol. Six samples were found to have low ethanol stability and 16 with high ethanol stability.

For the heat stability test, half of milk samples destabilized within 2 min in the heat stability test. Milks with the highest heat stability presented maximum destabilization time of about 12 min. These data were in agreement with those determined by Rose (1961) and De

Koning et al. (1974) who found values ranging between 2 and 22 min. Groups of medium and high heat stability were rather homogeneous with 16 and 14 samples, respectively.

Prediction of the membership of an individual to a given group using PLS-DA and the physico-chemical data

The number of PLS-DA loading factors used in the models was four. The classification table with numbers of observed and predicted samples *per* class and test is presented in Table 2.

Correct classification was observed for 90.8 % of the samples according to the phosphate stability test. In detail, this percentage decreased to 83.3 % for samples of medium stability (group B) and rose 100 % for samples with low and high stability (groups A and C, respectively). Only three samples on a total of 60 were misclassified (Table 2).

Concerning the ethanol stability test, a correct classification was noticed for 81.6 % of the individuals. For medium- and high-values groups (classes E and F, respectively), classification was very good with 92.1 and 93.7 % of correct classification, respectively. The percentage of good classification decreased to 66.7 % for the low-values group (class D). The small number of individuals in this class and the misclassification of two samples on a total of six in the group E instead of D may be explained this low percentage.

For the heat stability test, correct classification was also observed for 81.6 % of the milks. A percentage of 90 and 100 % of correct classification were obtained for groups of low (group G) and medium stability (group H), respectively. This percentage decreased to 78.6 % for the class of high values of stability (group J).

As a conclusion, physico-chemical parameters globally allowed a good discrimination of milks in a PLS-DA performed using four loading factors. This discrimination is particularly well done for the phosphate test. Misclassification of some samples could be related to the difficulty to share samples in three homogenized classes and to the difficulty to determine with precision the result of the test. Destabilization could effectively appear under a gel, sediment or dual phases in some tests and as a function of the milk.

Determination of the parameters explaining milk stability values in the different tests

** Phosphate stability test*

The representation of individuals on the map defined by the loading factors 1 and 2 of the PLS-DA is presented in Figure 1.A. A pretty good separation of A, B and C groups was observed according to the loading factor 1 which accounted for 71.7 % of the total variance.

The Figure 1.B. shows the projection of the variables on the similarity map determined by loading factors 1 and 2 of the PLS-DA. The most influent parameters involved in the result of phosphate stability appeared to be casein micelle hydration, pH and lactation stage, located at the right extremity of the loading factor 1, and casein micelle size, content in soluble chloride and lactation number, situated in the left side of the similarity map. Milks with a relatively high pH (near 6.7), containing small casein micelles (near 128 nm) highly hydrated (near 2.46 g of water/g of dried pellet) (Table 3), had a good phosphate stability. Influence of casein-pellet hydration had already been demonstrated for heat stability (Thompson et al., 1969), but it is the first time regarding phosphate stability. Moreover, at the difference of Ramsdell et al. (1931), a positive influence of pH on phosphate stability was observed here. Positive relationship between casein micelle hydration and pH (Ahmad et al., 2008) and inverse correlation between size and hydration of casein micelle (O'Connell & Fox, 2000) were also confirmed by the present study. Concerning lactation parameters, phosphate stability decreased for milks collected from cows with a high lactation number (near 6 lactations), but increased for milks collected from cows in advanced lactation stage (near 295 days of lactation) (Table 3). This latest result differed from those found by Holm et al. (1932) who observed a tendency of phosphate stability result to decrease as the lactation stage progressed. Minerals were also involved in the result of phosphate stability since a high soluble chloride content negatively influenced milk stability evaluated by this test. Contribution of other minerals (phosphate, citrate, sodium and calcium) was minor since these variables were projected close to the origin point.

* *Ethanol stability test*

The map defined by the loading factors 1 and 2 of the PLS-DA performed on ethanol test data is presented in Figure 2.A. The six individuals forming the group of low stability (group D) were not well clustered on the map and consequently were not considered in the following discussion. Milks with medium-values of stability (group E) were rather well separated from milks with high-values of stability according to the loading factor 1 which accounted for 78.3 % of the total variance. The variables involved in the discrimination of these two groups were determined thanks Figure 2.B. For a long time, the main parameter cited as influencing the ethanol stability was ionic calcium content (Davies & White, 1958; Horne, 1987; Chavez et al., 2004; Tsioulpas et al., 2007). In the present study, this parameter was not measured, but little influence of total, soluble and colloidal calcium contents on ethanol stability was noticed as previously observed by Tsioulpas et al. (2007). Positive effect of high content of phosphate

on ethanol stability was confirmed here (Schmidt et al., 1979) but not for citrate content (Schmidt et al., 1979; Tsioulpas et al., 2007). As these minerals are chelating agents for ionic calcium, they should decrease ionic calcium concentration in milk, limit the impact of these ions on the charge and sensitivity of caseins to precipitation, and improve milk stability to ethanol (Horne, 1987). The large ranges of concentration for these minerals determined for the thirty milks investigated (Table 3) may explain these differences between previous studies and ours. The statistical analysis also revealed the influence of other parameters such as pH, casein micelle hydration, lactose, β- and α_{s1}-caseins contents, located at the right extremity of the map, as well as lactation number, somatic cell count, contents in NPN and NCN, placed at the left extremity of the map. Positive effects of pH and lactose concentration on ethanol stability were observed and agreed with previous studies (Metro et al., 1979; Horne, 1987; Chavez et al., 2004; Oliveira & Timm, 2006). Strong influence of nitrogen fractions on ethanol stability was also noticed here, with a positive effect of contents in β- and α_{s1}-caseins, and negative effect of NCN and NPN contents. These two last parameters globally correspond to whey proteins, peptides and small nitrogen molecules. They could consequently serve as indicators of casein degradation. Opposition between contents in some caseins and these two indicators in the map appeared rather logical and in agreement with a sensitivity of milk for destabilization in presence of ethanol. However, it was more surprising not to find κ-casein as the most important casein for ethanol stability. Indeed, the C-terminal part of this casein forms polyelectrolyte brush which mainly contributes to casein micelle stability by electrostatic repulsions and steric hindrance (Holt & Horne, 1996). The casein micelle hydration is moreover found to have a positive influence on ethanol stability. This absence of κ-casein among main factors influencing the ethanol stability could be explained by the co-elution of κ-casein with α_{s2}-casein in our RP-HPLC analyses. Regarding zootechnical and biological parameters, milks with medium stability had a high somatic cell count (near two millions of cells/mL) and were preferentially collected from cows with a high lactation number (near 6 lactations) in comparison to milks with high stability which had a low somatic cell count (about 18 000 cells/mL) and were collected from primiparous cows or with a small lactation number (Table 3). Even if these results differed from those of Chavez et al. (2004), they seemed rather logical. Indeed, a positive relationship between somatic cell count and lactation number exists (Vecht et al., 1989) and it is known that somatic cells contain several proteinases susceptible to degrade caseins (Fox & Kelly, 2006) and affect milk stability.

** Heat stability test*

Considering the map defined by loading factors 1 and 2 of the PLS-DA performed on the heat stability data (Figure 3.A.), samples of the groups G, H and J were superimposed. Only a tendency to the separation of group of low stability (group G) from groups of medium and high stability (groups H and J) according to the loading factor 1 (55.0 % of the total variance) and the separation of group J from group H according to the loading factor 2 (26.7 % of the total variance) could be distinguished. Projection of the variables on the similarity map (Figure 3.B.) showed factors involved in this discrimination. Samples of low heat stability (group G) were characterized by a high casein micelle charge, e.g., a charge which tends to positive values. In our study, the highest measured value of charge was -8.9 mV (Table 3). This result was in agreement with a decrease in electrostatic repulsions resulting in lower repulsions between casein micelles and in a loss of milk stability (Holt & Horne, 1996). At the other side of the similarity map (Figure 3.B.), the lactose content appeared to be in favour of a good heat stability that differed from findings of White and Davies (1958). Good heat stability was also related to a high level of non protein nitrogen content, which agreed with conclusion of Robertson and Dixon (1969) and which may be related to the level in urea. Concerning other nitrogen fractions, our results showed relationships between bad heat stability and high contents in total β- and α_{s1}-caseins, and between good heat stability and high contents in caseins and β-lactoglobulin. These findings also differed from those of White and Davies (1958). At a lesser degree and according to the loading factor 2, milks belonging to group H were characterized by a high casein micelle size in comparison to milks belonging to group J (Table 3). O'Connell and Fox (2000) explained this relation by the lower content in κ-casein in large micelles than in small micelles.

In conclusion, the present study reminds that several techniques exist to evaluate milk stability to heat, the main methods being heat, ethanol and phosphate stability tests. Thanks to PLS-DA, the results have demonstrated that each test is multifactorial. Particular interest was given to main factors involved in the result of the test, and these factors are summarized in Table 4. Among the 34 physico-chemical and zootechnical parameters initially studied, only the half appeared of prime interest. None of these factors was found common to the three tests and only eight were found common to two tests. These eight factors corresponded to lactation number, pH, casein micelle size and hydration, contents in lactose, NPN, β- and α_{s1}-caseins. These results showed great importance of casein micelle properties but also of its environment in milk stability. This study also confirmed that each test is correlated to certain physico-chemical characteristics of milk and evaluate milk stability to heat by different ways.

This may explain the small or inexistent correlations between tests. Correlation coefficients, investigated by linear regression on tests results, were equal to 0.01 between heat and phosphate tests, 5.10^{-5} between heat and ethanol tests, and 0.22 between phosphate and ethanol tests. However, the original approach presented in this study allowed a better knowledge of the factors involved in the different tests applied to raw milk to evaluate its stability to heat. The most influential parameters could help in the comprehension of casein micelle stability, and could be considered as the bases of a new stability test, more efficient to predict milk stability to heat.

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Table 1. Presentation of the different classes of stability and the corresponding number of samples for each test. The number of samples includes duplicates.

Stability tests	Stability level	low	medium	high
Phosphate stability test (mL of KH_2PO_4 0.5M)	Classes	A	B	C
	Range	0 – 0.4	0.5 – 0.8	≥ 0.9
	Number of samples	30	18	12
Ethanol stability test (% of ethanol)	Classes	D	E	F
	Range	50 - 65	70 - 85	90 - 100
	Number of samples	6	38	16
Heat stability test (min at 140°C)	Classes	G	H	J
	Range	0 - 2	2.1 - 5	≥ 5.1
	Number of samples	30	16	14

Table 2. Classification table of milk samples according to the test and the class of stability. The number of samples (predicted and observed) includes duplicates.

		observed†			
	predicted‡	A	B	C	% correct classification
Phosphate stability	A	30	0	0	100.0
	B	3	15	0	83.3
	C	0	0	12	100.0
	Total				90.8
		observed†			
	predicted‡	D	E	F	% correct classification
Ethanol stability	D	4	2	0	66.7
	E	0	35	3	92.1
	F	0	1	15	93.7
	Total				81.6
		observed†			
	predicted‡	G	H	J	% correct classification
Heat stability	G	27	1	2	90.0
	H	0	16	0	100.0
	J	1	2	11	78.6
	Total				81.6

(†)The number of observed samples. (‡) The number of predicted samples.

Table 3. Mean, standard deviation (SD) and range for the different physico-chemical and zootechnical parameters considered in the statistical analyses.

Parameter	Abbreviation	Mean ± SD	Range
Lactation number	Lact nb	3 ± 1	1 – 6
Lactation stage (days)	Lact stg	226 ± 49	111 – 295
Somatic cell count (cells/mL)	SCC	270300 ± 544851	18000 – 2172000
pH	pH	6.6 ± 0.0	6.5 – 6.7
Total solids concentration (g/kg)	TS	121.3 ± 8.2	110.6 – 152.6
Fat concentration (g/kg)	Fat	36.9 ± 7.3	28.2 – 62.6
Lactose concentration (g/kg)	Lactose	45.0 ± 2.5	37.9 – 48.8
Total nitrogen content (g/kg)	TN	30.8 ± 3.2	25.0 – 39.4
Non casein nitrogen content (g/kg)	NCN	7.2 ± 1.1	5.4 – 9.4
Non protein nitrogen content (g/kg)	NPN	1.7 ± 0.4	1.0 – 2.5
Proteins content (g/kg)	PNs	29.1 ± 3.0	23.5 – 37.1
Caseins content (g/kg)	CNs	23.6 ± 2.5	19.0 – 30.0
Whey proteins content (g/kg)	Whey P	5.5 ± 1.0	4.0 – 7.9
κ- and α_{s2}-caseins concentration (g/kg)	TK- & As2-	5.6 ± 2.1	2.1 – 12.9
α_{s1}-casein concentration (g/kg)	TAs1-	7.4 ± 1.9	2.3 – 11.7
β-casein concentration (g/kg)	TB-	10.1 ± 2.8	2.3 – 14.9
β-lactoglobulin concentration (g/kg)	TBLG	3.5 ± 1.4	1.3 – 7.2
Total calcium (mM)	TCa	31.1 ± 2.4	27.0 – 36.2
Total magnesium (mM)	TMg	4.8 ± 0.4	4.0 – 6.2
Total inorganic phosphate (mM)	TPO4	16.5 ± 1.8	13.8 – 22.4
Total citrate (mM)	TCit	10.1 ± 1.8	6.3 – 16.0
Soluble calcium (mM)	SCa	10.2 ± 1.5	7.6 – 14.2
Soluble magnesium (mM)	SMg	3.3 ± 0.4	2.6 – 4.4
Soluble sodium (mM)	SNa	19.5 ± 3.7	15.1 – 29.4
Soluble potassium (mM)	SK	41.5 ± 2.7	36.3 – 46.0
Soluble inorganic phosphate (mM)	SPO4	8.3 ± 1.2	6.0 – 11.2
Soluble citrate (mM)	SCit	8.6 ± 1.5	5.4 – 13.1
Soluble chloride (mM)	SCl	35.5 ± 5.0	24.7 – 46.0
Casein micelle hydration (g of water/g of dried pellet)	Hydration	2.14 ± 0.16	1.79 – 2.46
Casein micelle size (nm)	Size	188 ± 34	128 – 270
Casein micelle charge (mV)	Charge	-17.5 ± 2.2	-21.9 – -8.9
Colloidal calcium (mM)	CCa	20.8 ± 2.1	18.1 – 26.3
Colloidal inorganic phosphate (mM)	CPO4	8.2 ± 1.3	5.9 – 12.3
Colloidal magnesium (mM)	CMg	1.5 ± 0.5	0 – 3.2

Table 4. Comparison of the physico-chemical and zootechnical factors mainly involved in the phosphate, alcohol and heat stability. The effect of the factor on milk stability is indicated by + (positive effect) or – (negative effect).

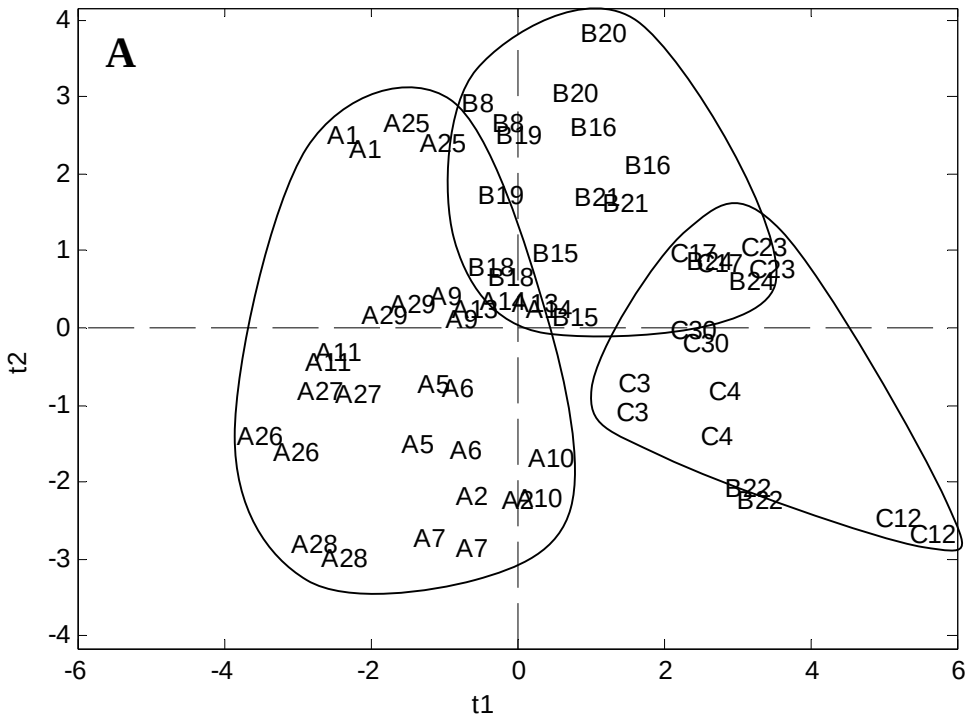
Parameter	Phosphate test	Alcohol test	Heat test
Lactation stage	+		
Lactation number	-	-	
Somatic cell count		-	
pH	+	+	
Micellar size	-		-
Micellar hydration	+	+	
Micellar charge			-
Soluble Chloride	-		
Phosphate (total and soluble)		+	
Citrate (total and soluble)		-	
Lactose		+	+
NCN		-	
NPN		-	+
Caseins			+
β-casein		+	-
α_{s1}-casein		+	-
β-lactoglobulin			+

Figures captions:

Fig. 1. Similarity map determined by loading factors 1 (71.7 %) and 2 (11.7 %) of the PLS-DA for phosphate stability (A: 0 – 0.4 mL of KH_2PO_4 0.5M; B: 0.5 – 0.8 mL of KH_2PO_4 0.5M; C: ≥ 0.9 mL of KH_2PO_4 0.5M). **A**. Projection of the individuals. **B.** Projection of the variables.

Fig. 2. Similarity map determined by loading factors 1 (78.3 %) and 2 (5.0 %) of the PLS-DA for ethanol stability (D: 50 – 65 % of ethanol; E: 70 – 85 % of ethanol; F: 90 -100 % of ethanol). **A.** Projection of the individuals. **B.** Projection of the variables.

Fig. 3. Similarity map determined by loading factors 1 (55.0 %) and 2 (26.7 %) of the PLS-DA for heat stability (G: 0 – 2 min at 140°C ; H: 2.1 – 5 min at 140°C ; J: ≥ 5.1 min at 140°C). **A.** Projection of the individuals. **B.** Projection of the variables.


A
t2
t1
B20
B8
A1
A25
B19
B16
B21
B15
C23
B24
A29
A9
A13
A14
C30
A11
A27
A5
A6
C3
C4
A26
A10
A2
B22
C12
A28
A7

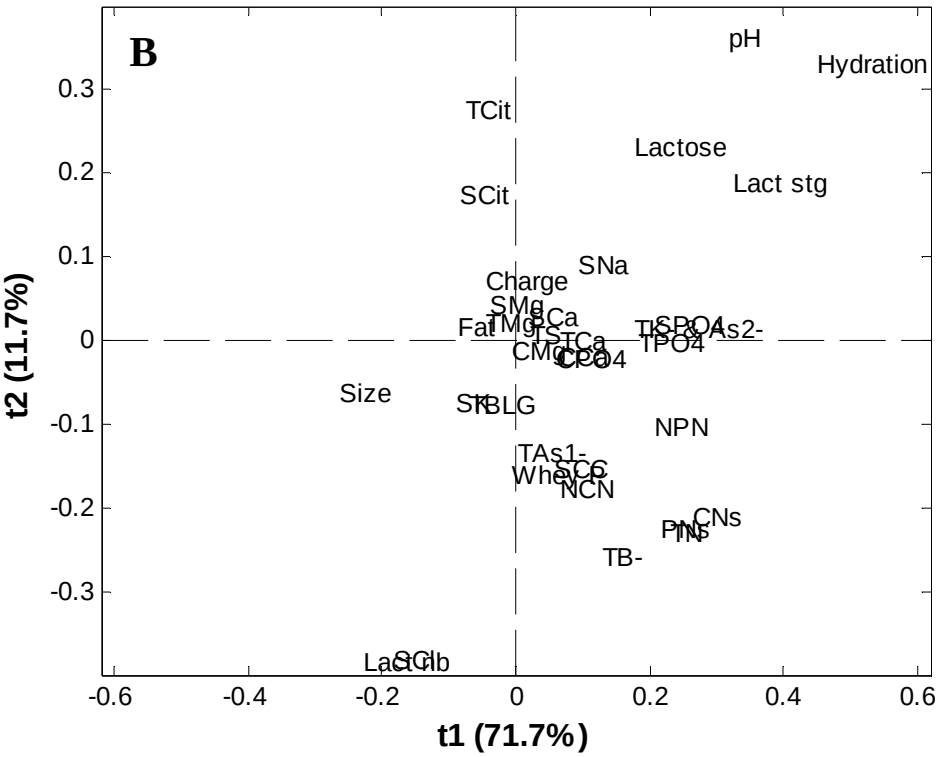
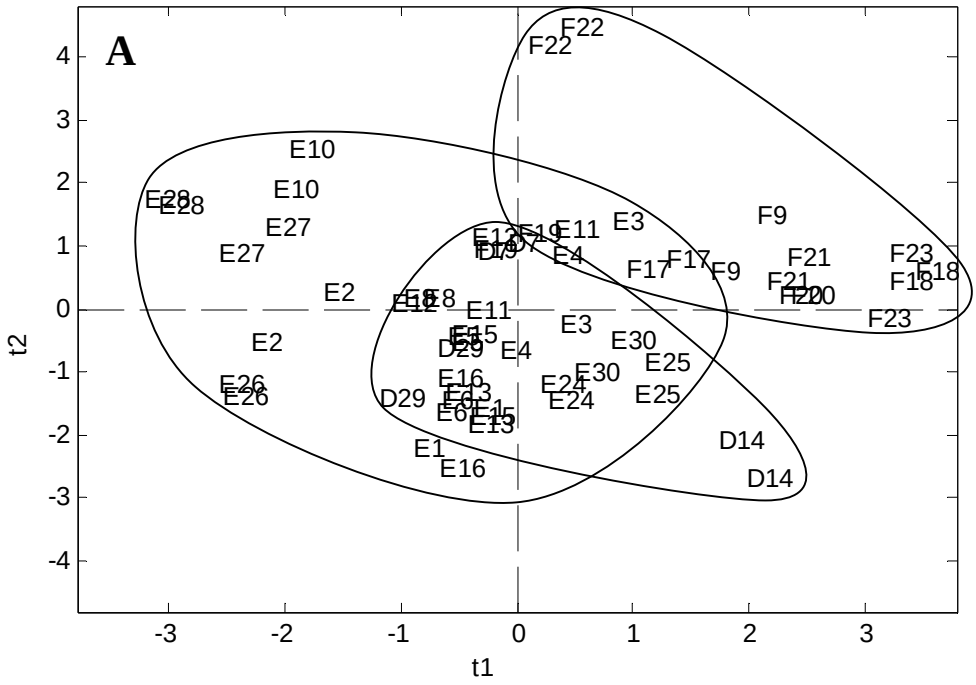

B
t2 (11.7%)
t1 (71.7%)
pH
Hydration
TCit
Lactose
Lact stg
SCit
SNa
Charge
SMg
Fat
Size
NPN
NCN
CNs
TB-
Lact-lb

Fig. 1.


A
t2
t1
F22
E10
E28
E27
E2
E11
E3
F9
F17
F21
F23
F18
E4
E30
E25
E24
E16
D29
D14
E1

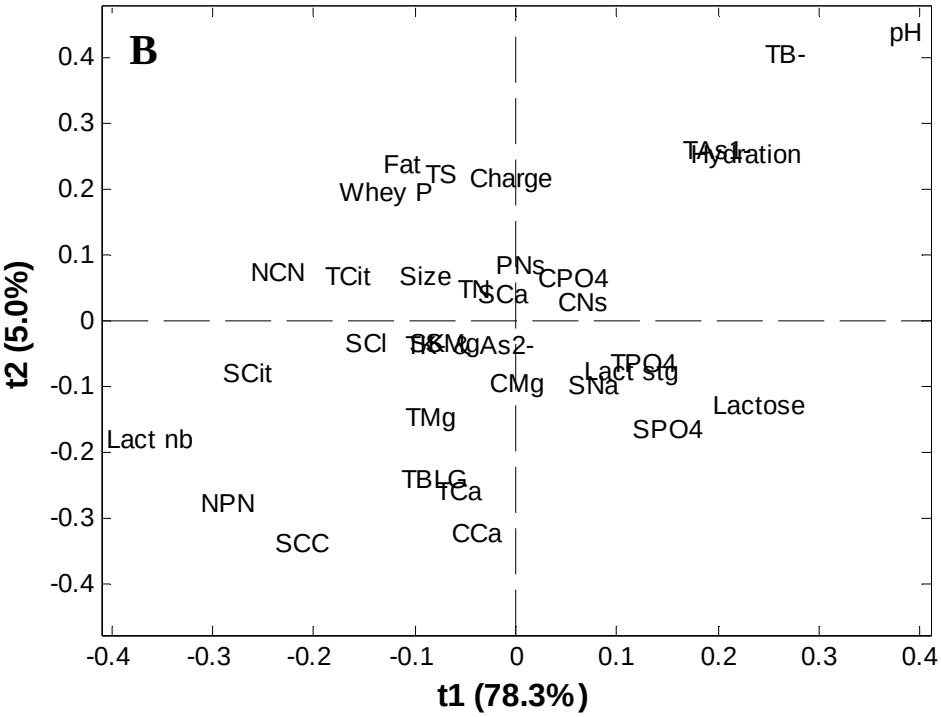
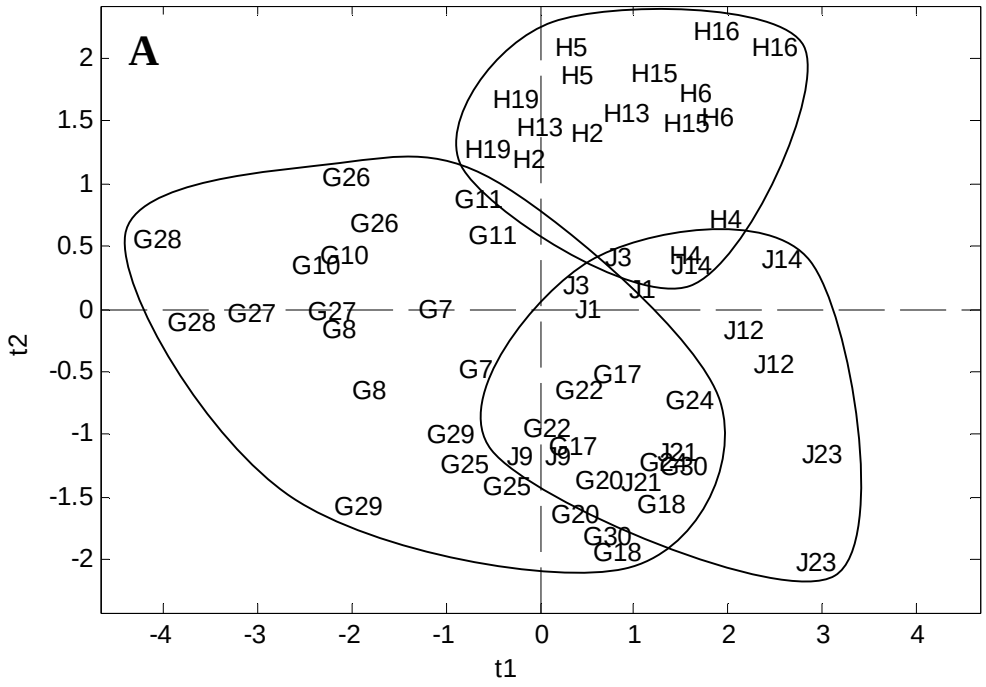

B
t2 (5.0%)
t1 (78.3%)
pH
TB-
Hydration
Fat
TS
Charge
Whey P
NCN
TCit
Size
PNs
CPO4
CNs
SCa
SCit
TPO4
Lact stg
CMg
SNa
Lactose
TMg
SPO4
Lact nb
TCa
NPN
SCC
CCa

Fig. 2.


A
t2
t1
H16
H16
H5
H5
H15
H6
H19
H13
H13
H15
H6
H13
H2
H19
H2
G26
G11
H4
G26
G28
G11
G10
J3
H4
J14
G10
J14
J3
J1
G28
G27
G27
G7
J1
J12
G8
G7
G17
J12
G8
G22
G24
G29
G22
G17
J21
J23
J9
J9
G25
G20
J21
G25
G18
G29
G20
G30
G18
J23

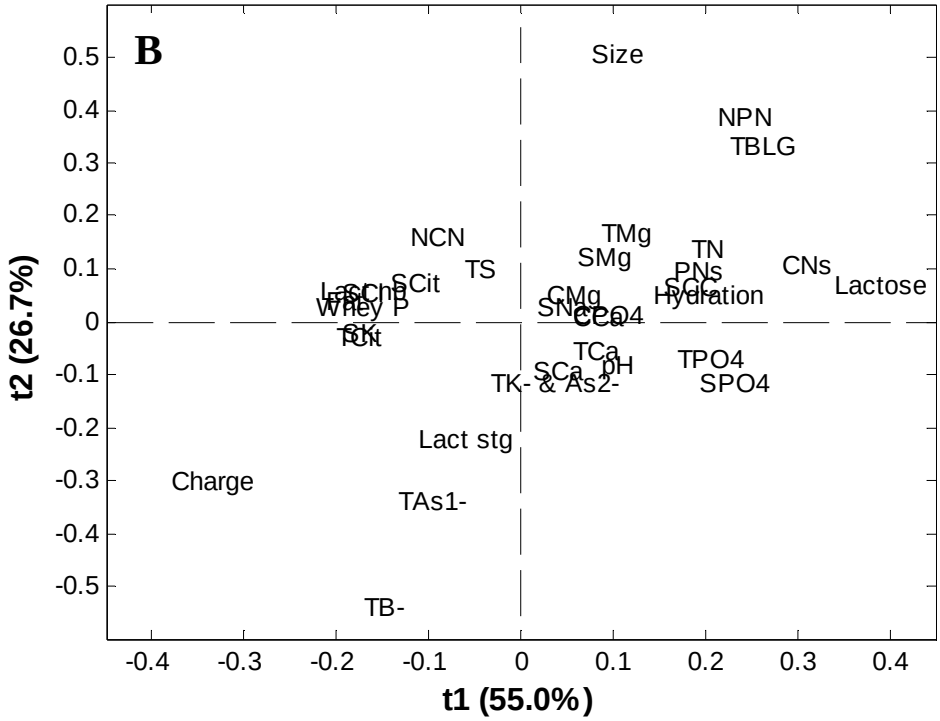

B
t2 (26.7%)
t1 (55.0%)
Size
NPN
TBLG
NCN
TMg
TN
SMg
TS
PNs
CNs
SCit
SCC
Lactose
Hydration
CMg
Whey P
TCa
TPO4
pH
SCa
TK- & As2-
SPO4
Lact stg
Charge
TAs1-
TB-

Fig. 3.

RESUME

La première partie de cette thèse porte sur la mise au point de la spectroscopie de fluorescence synchrone et frontale, avec pour modèles d'étude le lait chauffé ou amené à différents pH. L'analyse des matrices d'excitation-émission par PARAFAC a permis de décomposer les matrices de spectres synchrones et de modéliser les spectres d'excitation et d'émission des différents fluorophores présents dans le lait. Dans un deuxième temps, l'effet de l'addition des sels sur la structure des constituants du lait a été étudié. Ce même chapitre rapporte le suivi des cinétiques de coagulation du lait aux moyens du test de cisaillement dynamique et des spectroscopies de fluorescence et moyen infrarouge. Dans la troisième partie, les cinétiques de fusion de plusieurs fromages à pâte pressée cuite ou non (Comté, Raclette) ont été étudiées par techniques spectroscopiques. Enfin, nous avons montré que les techniques spectroscopiques peuvent être utilisées pour authentifier les fromages de type Saint-Nectraire.

ABSTRACT

The first part of this thesis focuses on the development of front-face synchronous fluorescence spectroscopy, using as models milk heated or acidified. The analysis of excitation-emission matrices using PARAFAC algorithm allowed to decompose the synchronous spectra matrices and to model the excitation and emission spectra of the different fluorophores found in milk. In a second part, the effect of salt addition on the structure of milk components has been studied. This chapter reports on the kinetics studies of milk coagulation by rennet or glucono-delta-lactone at different temperatures using dynamic testing rheology and fluorescence and mid-infrared spectroscopies. In the third part, the melting kinetics of several hard (Comté) or semi-hard (Raclette) cheeses have been studied using dynamic testing rheology and fluorescence and mid-infrared spectroscopies.. Finally, we have shown the ability of spectroscopic methods to authentify Saint-Nectaire cheeses.