Original article

Infrared and fluorescence spectroscopy for monitoring protein structure and interaction changes during cheese ripening

Gérard MAZEROLLES[a*], Marie-Françoise DEVAUX[b], Gabriel DUBOZ[a],
Marie-Hélène DUPLOYER[a], Nadine MOUHOUS RIOU[c], Éric DUFOUR[d]

[a] Unité de Recherche en Technologie et Analyse Laitières, INRA, BP 89,
39801 Poligny Cedex, France
[b] Unité de Recherche sur les Polysaccharides, leurs Organisations et Interactions,
INRA, BP 71627, 44316 Nantes Cedex 03, France
c Unité d'Étude des Interactions des Molécules Alimentaires, INRA,
BP 71627, 44316 Nantes Cedex 03, France
[d] Département Qualité et Économie Alimentaires, ENITA de Clermont Ferrand, Marmilhat,
63370 Lempdes, France

Abstract — Sixteen experimental semi-hard cheeses, varying in moisture (42.1 to 49.8%), protein (20.2 to 25.9%) and fat (23.7 to 31.1%) content, were manufactured and ripened under controlled conditions. Fluorescence (tryptophan) and mid-infrared (Amide I and II regions) spectra were collected at 1, 21, 51 and 81 days of ripening in order to test the ability of spectroscopy to highlight the molecular changes that occur during this process. The mid-infrared and fluorescence spectral data from the experimental cheeses were analysed firstly by principal component analysis. Secondly, the correlations between the chemical domain and the spectral domains were studied by canonical correlation analysis methods. These analyses showed that each spectroscopic technique provided relevant information related to the cheese protein structure, which was used to discriminate each ripening stage. In addition, some spectral characteristics of ripened cheeses, linked to the initial chemical composition and the initial protein network structure, were detected at the early stage of ripening. Finally, a canonical correlation analysis between the two sets of spectroscopic data was performed and allowed to clearly discriminate each stage of ripening and each cheese at the 4 ripening stages. A molecular interpretation of these results involving the modifications of proteins, minerals and water interactions during ripening was attempted. This result demonstrated the interest of coupling two complementary spectroscopic techniques. Such coupling allowed the description of global characteristics of the investigated samples, which can be used for their characterisation.

cheese / ripening / protein / structure / spectroscopy

* Correspondence and reprints
Tel.: (33) 3 84 73 63 17; fax: (33) 3 84 37 81 81; e-mail: mazeroll@poligny.inra.fr

Résumé — Contribution de spectroscopie infrarouge et de la spectroscopie de fluorescence au suivi des modifications de la structure des protéines du fromage pendant son affinage. Seize fromages expérimentaux de type pâte pressée présentant des taux variables d'humidité (de 42,1 à 49,8 %), de matière grasse (de 23,7 à 31,1 %) et de protéines (de 20,2 à 25,9 %) ont été fabriqués et affinés en conditions contrôlées. Les spectres de fluorescence (tryptophane) et les spectres infrarouge (zone Amide I et II) de ces 16 fromages ont été enregistrés à 1, 21, 51 et 81 jours d'affinage, afin de tester l'aptitude de chaque technique à rendre compte de modifications moléculaires intervenant pendant cette période. Les deux bibliothèques spectrales ont été dans un premier temps analysées séparément par analyse en composantes principales. Dans un deuxième temps, les résultats d'analyses chimiques ont été confrontés aux données spectrales par analyse canonique. Ces traitements ont permis d'établir que chaque type de spectroscopie rend compte de modifications de la structure des protéines du fromage intervenant au cours de son affinage. Ces informations peuvent être utilisées pour discriminer chaque stade d'affinage étudié. D'autre part, certaines caractéristiques spectrales du fromage reliées à sa composition chimique peuvent être mises en évidence dès le début de l'affinage. En conclusion, l'analyse simultanée (analyse canonique) des deux bibliothèques spectrales a permis de caractériser chaque stade d'affinage étudié et chaque fromage aux 4 stades d'affinage. Différentes hypothèses moléculaires relatives aux modifications intervenant au sein du système protéines, eau et minéraux au cours de l'affinage du fromage ont pu être formulées pour interpréter ces résultats. Ce résultat démontre l'intérêt d'utiliser sur les mêmes échantillons deux types de spectroscopie complémentaires. Ce type de couplage permet de rendre compte de caractéristiques globales présentées par les échantillons étudiés, cette information pouvant être utilisée à des fins de caractérisation.

fromage / affinage / protéine / structure / spectroscopie

1. INTRODUCTION

Understanding the structure of cheese, particularly protein and fat structures, and the interactions between cheese components during and after manufacture, can provide useful information in determining what constitutes a quality product. Unfortunately, there is today a lack of non-destructive and non-invasive methods aiming at investigating cheese structure. In a previous paper [16], we have demonstrated that the coupling of two complementary spectroscopies (infrared and fluorescence) allows modifications in fat in semi-hard cheeses during ripening to be followed. The same methodology can be used to investigate modifications of protein structure and interactions which occur during ripening.

The aqueous phase of bovine milk contains six major proteins: β-lactoglobulin, α-lactalbumin, α_{s1}-, α_{s2}-, β- and κ-caseins. The amino acid composition of all these proteins includes at least one tryptophan residue [18]. Depending on its structure, each protein exhibits, following excitation in the region 280–295 nm, a characteristic fluorescence emission spectrum defined by its maximum emission wavelength and the tryptophan quantum yield [26]. Front face fluorescence spectroscopy allows investigation of the fluorescence of powdered, turbid, emulsified and concentrated samples [5, 13, 20, 24]. This fast method makes it possible to record spectra for cheese samples directly and it has been shown that information about the physical characteristics of proteins can be derived. Thus, the fluorescence of milk proteins has been used to monitor the structural changes in proteins and their physico-chemical environment during milk heating [12], milk coagulation [14, 22] and cheese manufacture [23]. Generally, the fluorescence spectrum must be considered as a fingerprint of the product, allowing its characterisation.

The development of Fourier transform infrared (FTIR) spectroscopy in recent years affords the possibility of obtaining

unique information about protein structure and protein-protein and protein-lipid interactions without introducing perturbing probe molecules [8]. The Amide I and II bands (1 700–1 500 cm^{-1}) are known to be sensitive to the conformation adopted by the protein backbone. The secondary structures of proteins can be deduced from their FT-IR spectra since there are good correlations between the Amide I band (1 700–1 600 cm^{-1}) [17] and the levels of α-helix, β-sheet and unordered structure in proteins [11, 27]. In H$_2$O solution, the maximum absorbances for α-helix and β-sheet in proteins are generally observed at about 1 655 cm^{-1} and 1 635 cm^{-1}, respectively. Random structure spectrum also shows a maximum at about 1 655 cm^{-1}, but the band is broader than for α-helix. β-Turns do not show a well-defined characteristic band in the Amide I region.

The development of the attenuated total reflectance (ATR) device allows the sampling problems encountered when collecting spectra from opaque and viscous samples to be overcome. This simple and reproducible method made it possible to investigate the aggregation and gelation kinetics of β-lactoglobulin at a molecular level [15].

Although the peptide bonds are essentially responsible for the absorbance of proteins in the 1 700–1 500 cm^{-1} region, the side chains of several amino acids (glutamic acid, aspartic acid, glutamine, asparagine, lysine, arginine and tyrosine) can contribute to the signal in the Amide II region [2, 21]. The carboxylate groups of the side chains of aspartic and glutamic acids absorb between 1 580 cm^{-1} and 1 520 cm^{-1}, the nature of anion interacting with the carboxylate group determining the exact location of the band within this interval. Thus, Byler and Farrell [7] showed, by infrared spectroscopy, that the O–C–O stretching vibrations of glutamate and aspartate residues shifted from 1 575 to 1 565 cm^{-1} when potassium ions bound to the proteins were replaced by calcium ions.

The purpose of this study was to investigate the changes of FTIR spectra in the Amide I and II regions and of tryptophan fluorescence spectra from 16 experimental semi-hard cheeses during ripening. The study serves as a general investigation of how front-face fluorescence and attenuated total reflexion mid-infrared spectroscopies can be used to characterise cheese products and processes at a molecular level. In addition, we show that the proposed approach could be used to predict several characteristics of the ripened cheeses at an early stage.

2. MATERIALS AND METHODS

2.1. Experimental design

Sixteen different cheeses were manufactured at the INRA pilot-scale dairy plant (Poligny). The gross composition of the cheeses was defined according to an experimental design [29] in order to obtain large variability and partial decorrelation for fat, protein and moisture contents for the whole set. Since only four cheeses could be manufactured in a day (four vats available at the dairy plant), four different milks (collected on four consecutive days from the same dairy farm) were used to make the whole set of experimental cheeses. Consequently, the experimental design took into account a block variable: "day of fabrication".

2.2. Cheese manufacturing, ripening and sampling

The experimental cheeses were made using a typical semi-hard cheese making scheme: after pasteurisation, milk, at 32 °C, was renneted. After gelation, the coagulum was stirred for a few minutes and lactose removed by curd washing. The coagulum was then heated at a temperature in

the range of 32.5 to 45 °C, depending on the moisture content required, and stirred again. In order to obtain the compositions in accordance with those of the experimental design, some process parameters were modified from one cheese to another. These modifications affected the size of the curd grains, the temperature and the duration of the second stirring. For each vat, corresponding to about 1.3 kg of the same curd, eight mini-cheeses were produced. Two of them were used for the first set of measurements. Slices, 8 cm long and 0.5 mm thick were cut from the middle of the cheese radius for infrared measurement. Close to this location, a piece of cheese was cut for fluorescence measurement. The remaining pieces were used for chemical analysis. The six remaining cheeses were salted in a brine bath and ripened at 13 °C. One and a half cheeses were used for each set of measurements, using the sampling protocol previously described.

Samples for spectroscopic and chemical analysis (pH, moisture, fat, nitrogen and mineral content) were taken after 1 (unsalted cheeses), 21, 51 and 81 d of ripening (labelled "d1", "d21", "d51" and "d81" in the text, respectively). The second sampling was performed after 21 d to allow the diffusion of salt into the cheeses [28].

2.3. Physico-chemical analysis

The methods used for the determination of pH, moisture (Moist.), fat, total nitrogen (TN), water-soluble nitrogen (WSN), phosphotungstic acid-soluble nitrogen (PTA-SN), sodium chloride (NaCl) and lactate (Lact.) content were described by Bouton et al. [6]. The total calcium content (Ca) was determined by the method described by Pearce [30]. All the results were expressed as percent of the mass of the cheese. The results of chemical analysis for groups "d1" and "d81" are presented in Table I.

2.4. Fluorescence spectroscopy

Fluorescence spectra were recorded using a SLM 4800C spectrofluorimeter (Bioritech, Chamarande, France) fitted with a thermostated front-surface accessory. The incidence angle of the excitation radiation was set at 56° to ensure that reflected light, scattered radiation and depolarisation phenomena were minimised. Spectra (resolution: 0.5 nm, averaging: 10) of cheese samples (4 cm × 1 cm × 0.3 cm) mounted between two quartz slides were recorded at 20 °C with emission and excitation slits set at 4 nm. The emission spectra of protein tryptophans (305–400 nm) were recorded with the excitation wavelength set at 290 nm. For each experimental cheese, three spectra were recorded using different samples. All spectra were corrected for instrumental distortions in excitation using a rhodamine cell in the reference channel. The data collection comprised of 192 fluorescence spectra was reduced to 189 items after elimination of three outliers.

2.5. Infrared spectroscopy

Infrared spectra were recorded between 3 000 and 900 cm^{-1} at a resolution of 4 cm^{-1} on a Fourier transform spectrometer 740 SX (Thermo Optek, Nicolet, Trappes, France) fitted with an ATR accessory equipped with a grip. The ATR cell was made of a horizontal ZnSe crystal which presented an incidence angle of 45°. The temperature of the cheese was set initially at 8 °C but reached 20 °C at the end of the record. 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, 250 scans were co-added for each spectrum. Before each measurement, the spectrum of the ZnSe crystal was recorded under the conditions described above and used as background. For each experimental cheese, three spectra were recorded using

Table I. Results from the chemical analysis of the cheeses of groups d1 (unsalted) and d81; n: cheese number; Moist.: moisture; TN: total nitrogen; WSN: water-soluble nitrogen; PTA-SN: phosphotungstic acid-soluble nitrogen; NaCl: sodium chloride; Ca: total calcium (all results except for WSN/TN ratio expressed in percent of mass of cheese).

Gr.	n	pH	Moist.	Fat	TN g·100 g^{-1}	WSN	TN-WSN	WSN/TN %	PTA-SN	NaCl g·100 g^{-1}	Ca
d1	1	5.23	43.78	31.15	3.46	0.15	3.31	0.04	--------	--------	0.66
d1	2	5.16	47.00	25.40	3.73	0.16	3.57	0.04	--------	--------	0.70
d1	3	5.55	42.11	28.01	4.06	0.13	3.93	0.03	--------	--------	0.85
d1	4	5.13	49.81	25.99	3.16	0.14	3.03	0.04	--------	--------	0.61
d1	5	5.38	46.77	24.95	3.86	0.12	3.74	0.03	--------	--------	0.79
d1	6	5.45	42.59	29.42	3.78	0.10	3.68	0.03	--------	--------	0.77
d1	7	5.17	49.67	25.58	3.25	0.15	3.10	0.05	--------	--------	0.65
d1	8	5.22	48.93	23.72	3.63	0.15	3.48	0.04	--------	--------	0.74
d1	9	5.18	48.86	25.90	3.38	0.14	3.25	0.04	--------	--------	0.67
d1	10	5.49	42.84	28.48	3.92	0.11	3.81	0.03	--------	--------	0.81
d1	11	5.41	45.88	26.12	3.80	0.11	3.69	0.03	--------	--------	0.79
d1	12	5.37	44.62	29.10	3.63	0.12	3.51	0.03	--------	--------	0.74
d1	13	5.35	44.29	27.94	3.76	0.15	3.61	0.04	--------	--------	0.75
d1	14	5.27	46.31	26.98	3.63	0.12	3.52	0.03	--------	--------	0.73
d1	15	5.31	46.59	26.62	3.61	0.12	3.49	0.03	--------	--------	0.73
d1	16	5.32	46.28	27.01	3.65	0.14	3.51	0.04	--------	--------	0.74
d81	1	5.31	43.50	30.61	3.38	0.67	2.71	0.20	0.93	0.90	0.62
d81	2	5.33	44.89	26.49	3.82	0.71	3.11	0.19	0.98	0.99	0.72
d81	3	5.76	40.45	28.85	4.16	0.82	3.34	0.20	1.10	0.73	0.86
d81	4	5.01	47.17	27.01	3.27	0.59	2.68	0.18	0.66	1.10	0.61
d81	5	5.59	44.68	25.57	3.95	0.75	3.21	0.19	1.13	0.92	0.79
d81	6	5.74	41.36	30.53	3.80	0.74	3.06	0.20	1.00	0.62	0.77
d81	7	5.24	47.34	26.82	3.38	0.64	2.74	0.19	1.05	0.95	0.64
d81	8	5.50	46.07	24.68	3.80	0.73	3.07	0.19	1.16	0.98	0.74
d81	9	5.22	47.05	26.46	3.45	0.64	2.81	0.19	1.08	1.08	0.66
d81	10	5.70	41.42	28.95	3.99	0.75	3.24	0.19	1.11	0.59	0.80
d81	11	5.64	43.73	26.68	3.92	0.72	3.20	0.18	1.12	0.81	0.79
d81	12	5.56	43.15	29.21	3.64	0.67	2.97	0.18	1.09	0.83	0.73
d81	13	5.56	43.06	28.44	3.78	0.76	3.02	0.20	1.13	0.89	0.75
d81	14	5.40	45.43	27.22	3.59	0.65	2.94	0.18	0.92	0.94	0.71
d81	15	5.53	44.10	27.35	3.72	0.70	3.03	0.19	1.13	0.91	0.74
d81	16	5.51	44.60	27.50	3.70	0.70	3.00	0.19	1.12	0.83	0.73

different samples. Baseline and ATR corrections, smoothing and water substraction were applied to the spectra using OMNIC Software (OMNIC 4.0, Nicolet, Trappes, France). The water substraction involved the combination band around 2 125 cm^{-1} and a mean spectrum of 27 individual spectra of distilled water recorded at the different periods of measurement. The data collection of 192 mid-infrared spectra was reduced to 189 to correspond exactly to the 189 fluorescence spectra recorded.

2.6. Mathematical treatments

Principal Component Analysis (PCA) and Canonical Correlation Analysis (CCA) were applied to normalised spectra in order to investigate only changes in the spectra. Normalisation was performed by reducing the area under each spectrum to a value of 1 according to Bertrand and Scotter [4].

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 [3, 25]. While the similarity maps allow the comparison of the spectra in such a way that two similar spectra are represented by two neighbouring points, the spectral patterns exhibit the absorption bands that explain the similarities observed on the maps.

CCA is a multivariate treatment which describes the correlations between two sets of variables [10, 32]. Linear combinations of the variables of each data set are assessed by CCA so that these linear combinations are as correlated as possible. The correlation coefficients between the homologous linear combination of each set of variables indicate the amount of correlation occurring between the 2 kinds of data. Similarity maps and spectral patterns can be derived and interpreted in the same way as for PCA.

PCA has been applied separately to the infrared and fluorescence spectra in order to visualise the information provided by each technique. Two CCA were performed, considering the infrared spectra jointly with the chemical variables described in Table I and the fluorescence spectra jointly with the chemical variables, respectively. The objectives were to determine to what extent the information described by the spectral data could be related to the chemical features of the cheese samples, being considered as reference data. A final CCA was applied between the infrared and fluorescence spectra in order to reveal the common information described by the 2 kinds of data.

3. RESULTS AND DISCUSSION

3.1. Effects of ripening on the infrared spectrum in the Amide I and II regions

A large number of proteins and peptides in cheese contributes to the Amide I and II bands. Thus, these bands contain global information on the proteins of cheese, but also on the interactions that they can develop with other components (water, ions, other proteins). In order to obtain a more detailed description of spectral variations during ripening, a second derivative was applied to the mean infrared spectra collected at each sampling period (spectra before normalisation). The results are presented in Figure 1. Contributions to the Amide I band can be observed around 1 675, 1 652, 1 640 and 1 627 cm^{-1}. As stated previously [11, 17, 27], this part of infrared spectra is used to investigate the secondary structures of several proteins. While the absorption band around 1 615 cm^{-1} has been assigned by Abott [1] to the side chains of protein, several other authors [9, 15] have observed that variations around 1 620 cm^{-1} occurred during protein aggre-
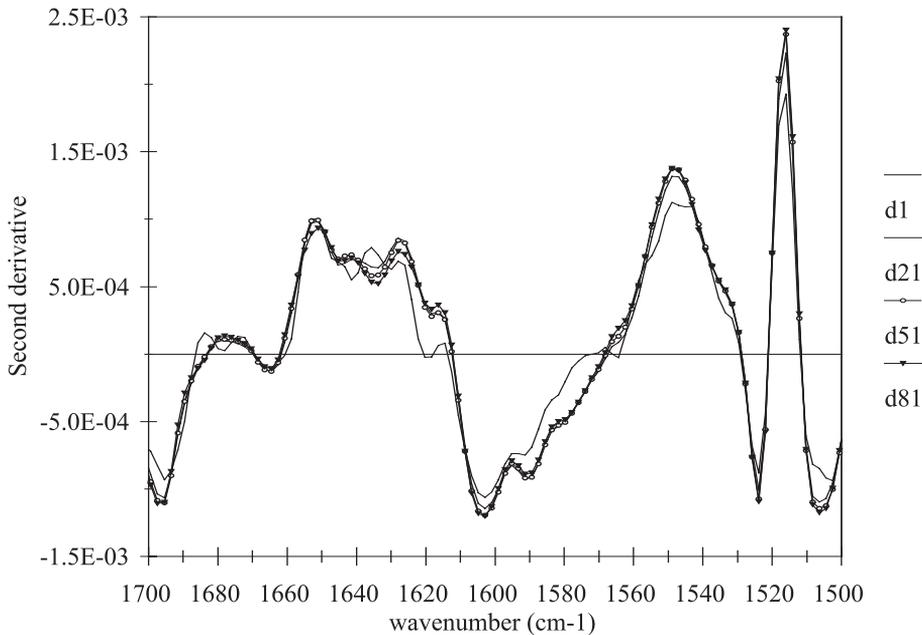
Figure 1. Averaged mid-infrared spectra (Amide I and II bands) for each stage of sampling. Second derivative data multiplied by –1.

gation. The absorption band at $1\ 535\ cm^{-1}$ is generally linked to Amide II vibration, as reported by Bellamy [2]. According to the same author, numerous small absorption bands between $1\ 600$ and $1\ 500\ cm^{-1}$ are attributed to the side chains of the amino acids (Glu, Asp) which can hold an electric charge at the pH of cheese. In addition, part of the information may be related to soluble carboxylic acids, such as lactates showing a characteristic absorption band around $1\ 575\ cm^{-1}$ [31].

Water also absorbs between $1\ 680$ and $1\ 620\ cm^{-1}$. For this reason, a spectrum of distilled water was subtracted from the spectra of all the cheeses (see Sect. 2). It must be kept in mind that pH and ionic strength of aqueous solutions modify slightly the infrared spectrum of water. Indeed, comparison of the spectra of distilled water and of NaCl and $CaCl_2$ solutions (pH 5.0, concentration comparable to

cheeses namely 10 and $8\ g\cdot L^{-1}$, respectively – data not shown) has shown differences in a sharp window centered on $1\ 635\ cm^{-1}$. In this part of the spectrum, interpretation must be made cautiously: spectral differences between subtracted spectra of cheeses may be due to differences between the pH and the ionic strength of their aqueous phase. Nevertheless, as the same mean spectrum of distilled water was subtracted from all the cheese spectra at each ripening stage, the differences between corrected spectra were analogous to the differences between raw spectra.

The mean spectrum of "d1" group was significantly different from the others (Fig. 1) which might be explained by the physico-chemical changes in the proteins occurring during the uptake of salt. Several frequencies showed variations of intensities which were related to ripening time: a continuous decrease in the band centered at
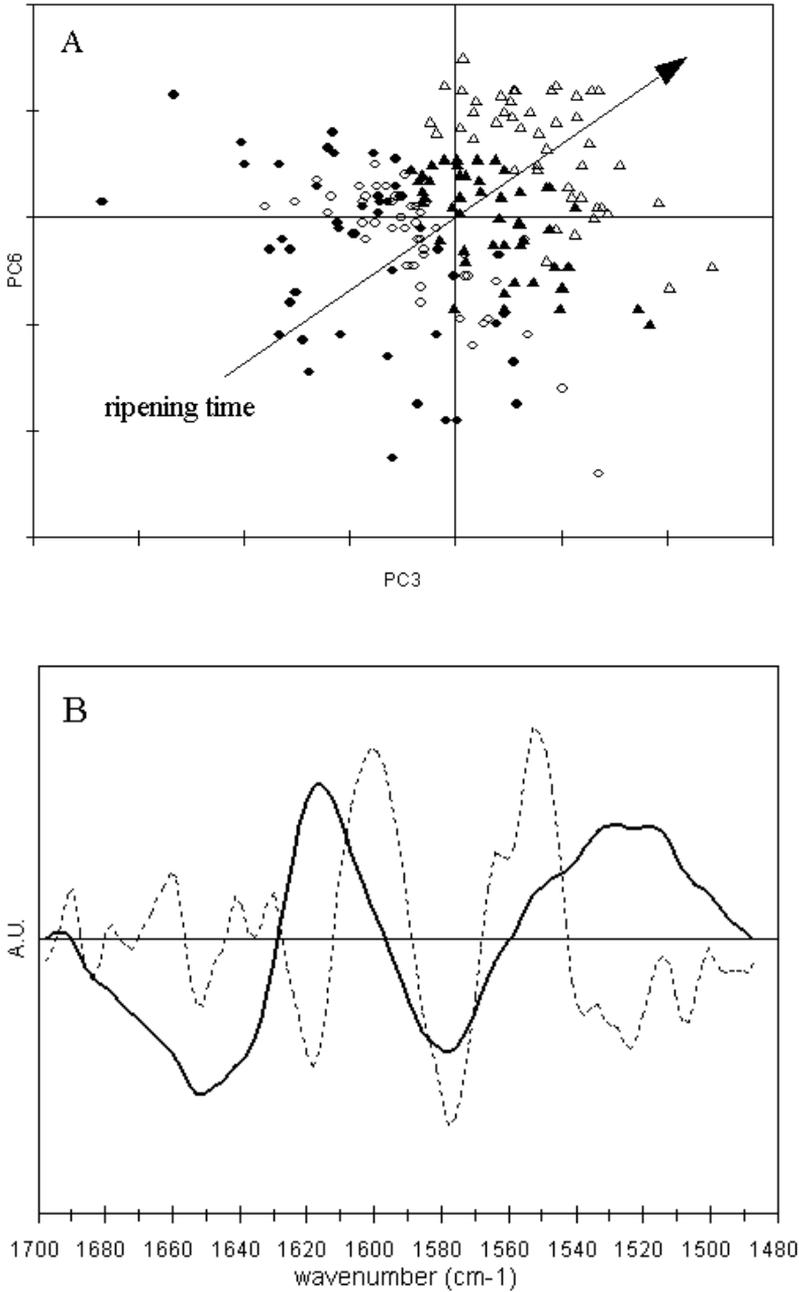
Figure 2. PCA of the infrared spectral data at the four stages of sampling. (A) Similarity map for the principal components 3 and 6; each label corresponds to an infrared spectrum: (●) d1, (○) d21, (▲) d51, (△) d81. (B) Spectral patterns for the principal components: 3 (—) and 6 (----).

$1\,652$ cm^{-1} was observed, whereas the bands at about $1\,620$ cm^{-1}, $1\,565$ cm^{-1}, $1\,550$ and $1\,515$ cm^{-1} increased continuously. However, none of them seemed to be able to clearly discriminate the 4 groups of samples.

PCA was applied to the whole library of normalised infrared spectra recorded in order to describe more efficiently their changes during ripening. This treatment has shown that the four ripening groups were ordered in accordance with time in the factorial map defined by the principal components 3 and 6 (7% and 0.17% of the total inertia, respectively). This map and the corresponding spectral patterns are presented in Figures 2A and 2B, respectively. Although groups "d1" and "d21" partly overlapped in Figure 2A, a good separation of groups "d21", "d51" and "d81" was observed. It was concluded that one (or several) continuous phenomenon, taking place during ripening of all the cheeses studied, was detected at the level of the Amide I and II absorptions bands. Spectral patterns corresponding to the principal components 3 and 6 (Fig. 2B) provided information about the characteristic absorption bands which explained the discrimination. Spectral pattern 3 showed an opposition between bands around $1\,650$ cm^{-1} and $1\,620$ cm^{-1}. They could explain the global modifications affecting the protein network during ripening. At the same time, oppositions can be seen between bands at $1\,580$ cm^{-1} and bands below $1\,560$ cm^{-1}. According to Byler [7], the decrease in the intensity of the band at $1\,580$ cm^{-1} could be attributed to a modification of the nature of the anion interacting with the carboxylic groups of aspartic and glutamic acids side chains in caseins. An opposition between bands at $1\,580$ cm^{-1} and $1\,555$ cm^{-1} was observed on spectral pattern 6, confirming that exchanges previously described happened during ripening. Nevertheless, as no other spectral information contained in spectral pattern 6 can be connected to proteins, it

was assumed that change of counter-ions may affect other kinds of (COO$^-$) groups. Lactate salts constitute the major part of the free carboxylic acids in cheese. Indeed, the spectrum of a reference solution of sodium lactate (same concentration as in cheese) recorded at pH 5 showed an absorption at $1\,580$ cm^{-1} (data not shown).

In the factorial map defined by the principal components 2 and 4 (respectively, 17.2% and 1.9% of the total inertia – data not shown), cheeses 4, 7 and 9 (highest moisture content, lowest pH, calcium and nitrogen content, respectively) were always opposed to cheeses 3, 6 and 10 (lowest moisture content, highest pH, calcium and nitrogen contents, repectively), whatever the stage of ripening. It was concluded that some characteristics of these cheeses observed in the infrared spectra just after manufacture were maintained until the end of ripening.

3.2. Effects of ripening on the fluorescence spectra of tryptophan

PCA was applied to the set of fluorescence spectra. The map defined by principal components 1 and 2 (respectively, 82.88 and 5.76% of the total inertia) and the corresponding spectral patterns are presented in Figures 3A and 3B, respectively. The results obtained were close to those obtained with infrared data. Although groups "d1" and "d21" were almost overlapping on the map shown in Figure 3A, a pretty good separation of the groups "d21", "d51" and "d81" was observed according to principal component 2. In fact, it appeared that (1) "d1" and "d21" groups exhibited positive values according to PC1 and negative values according to PC2, (2) "d51" group showed negative values according to PC1 and values close to zero according to PC2, and (3) "d81" group was characterised by values close to zero according to PC1 and positive values according to PC2 (Fig. 3A).
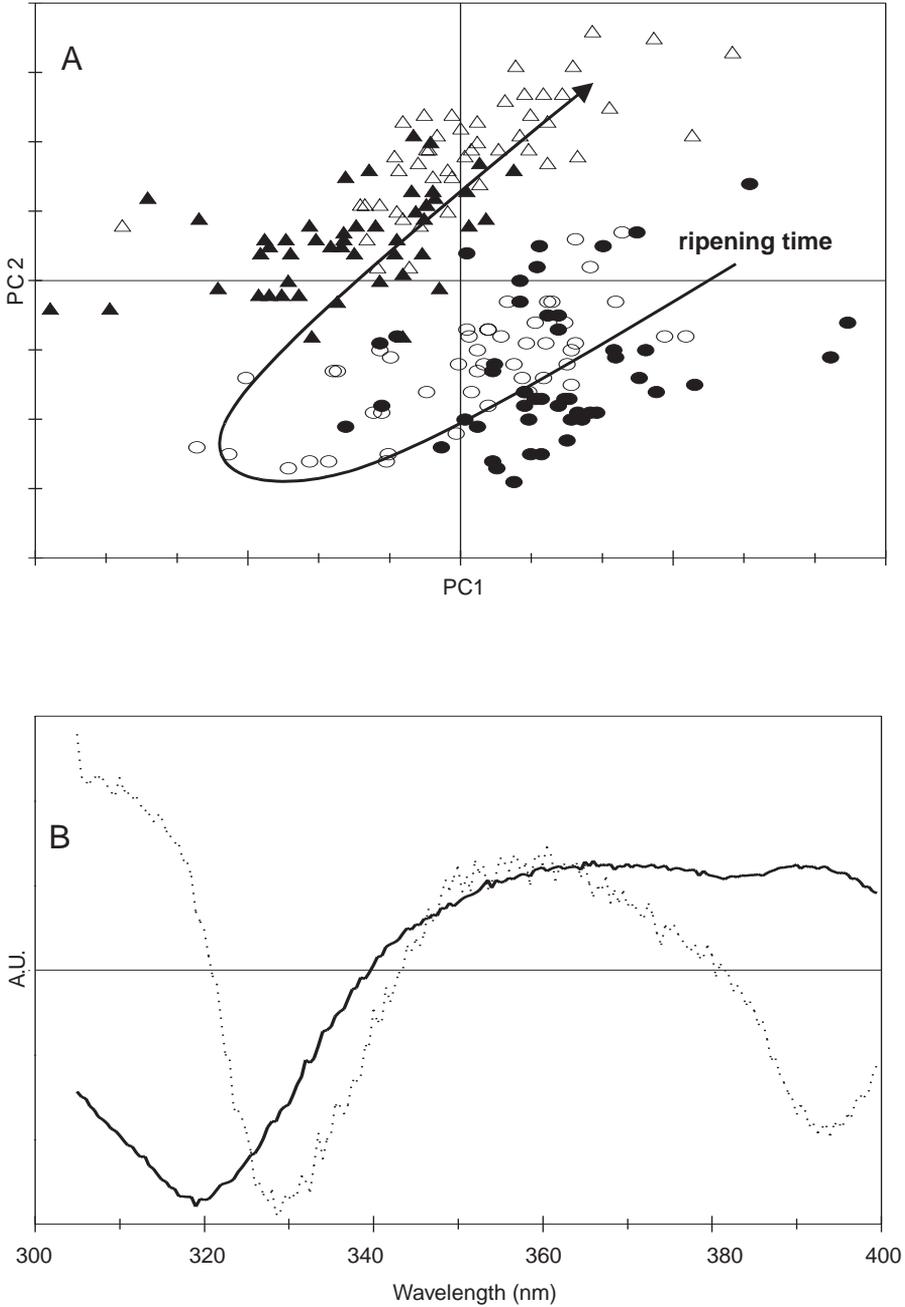
Figure 3. PCA of the fluorescence spectral data at the four stages of sampling. (A) Similarity map for the principal components 1 and 2; each label corresponds to a fluorescence spectrum: (●) d1, (○) d21, (▲) d51, (△) d81. (B) Spectral patterns for the principal components: 1 (—) and 2 (----).

It was concluded that one (or several) continuous phenomenon, taking place during the ripening, was detected when the fluorescence of the tryptophan residues was considered. Interpretation of the spectral pattern may permit explanation of the occurring phenomenon.

Spectral pattern 1 (Fig. 3B) characterised a shift of the emission maximum: the samples on the right of the map (Fig. 3A) were characterised by a maximum at a relatively higher wavelength than those on the left side. It indicated that the environment of tryptophan residues was relatively more hydrophilic for the cheeses located on the right of the map. As suggested in a previous paper [23], this phenomenon may be related to the partial proteolysis of caseins resulting in an increase in the exposure of tryptophan to the solvent. Another explanation would arise from salting, resulting in changes in the tertiary and quaternary structures of casein micelles. These structural changes can induce a more hydrophobic environment of the tryptophans of caseins in accordance with the blue shift of the maximum. Considering the similarity map (Fig. 3A), it was assumed that salting induced a shift of the maximum emission of protein tryptophans toward lower wavelengths, whereas proteolysis of the caseins between 51 and 81 d of ripening characterised the shift toward higher wavelengths.

Spectral pattern 2 (Fig. 3B) indicated that the shape of fluorescence spectra was larger for cheeses located on the positive side (mainly "d51" and "d81" groups) than for those on the negative side (mainly "d1" and "d21" groups). These spectroscopic differences could be due to different protein-protein interactions and different network structures resulting from the ripening process. It is well known that during cheese ripening, proteolysis generates partially hydrolysed caseins and peptides. It is suggested that native caseins and their degradation products increase the diversity of the environments of the tryptophan residues

[26] which explains the broadening of the emission spectrum of tryptophans between 1 and 81 d of cheese ripening.

3.3. Canonical correlation analysis of infrared and chemical data sets

The correlations between the chemical and the spectral domains have been taken into account in order to get a better insight into the relations between the molecular and spectroscopic characteristics of the experimental cheeses. Canonical correlation analysis can be applied when the same samples have been characterised by two different techniques. The method provides a measure of the link between the groups of variables and graphical representations of the correlation are revealed.

CCA was applied to the two data tables containing the normalised infrared spectra of the experimental cheeses and the chemical data. Ten pairs of canonical variates were used to study correlations between the two sets of data. These canonical variates were ranged in decreasing order of canonical correlation. The first 2 pairs were correlated with a squared canonical correlation coefficient equal to 0.93 and 0.92. These correlations indicated that the two first canonical variates provided a common description of the samples both from the infrared and chemical data. The similarity maps for chemical data and infrared spectral data are shown in Figures 4A and 4C, respectively. The correlation map for chemical data and the two first spectral patterns for infrared data are presented in Figures 4B and 4D, respectively. The similarity maps (Figs. 4A and 4C) for the infrared spectral and chemical data were similar, which strongly suggested that the chemical data and the infrared spectra set made it possible to observe the same phenomenon. As the infrared spectra were normalised, the spectral information involved in the canonical variates 1 and 2 did not address
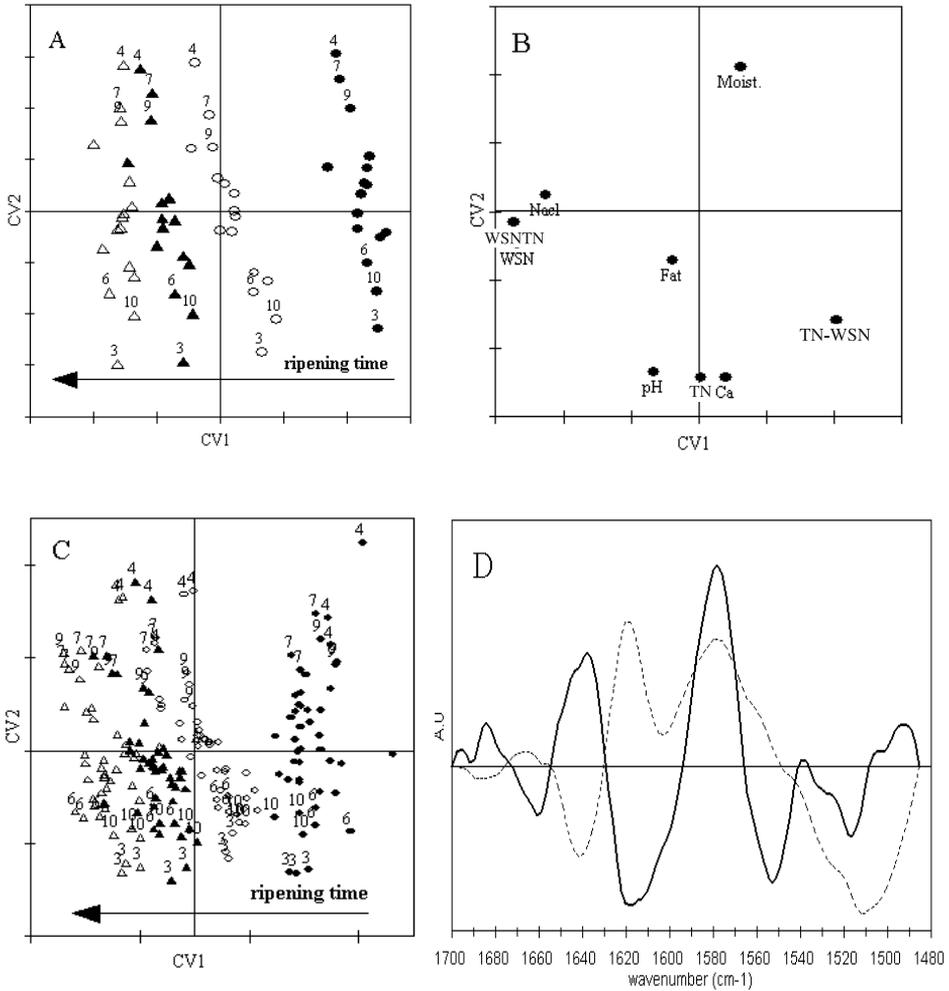
Figure 4. CCA for the infrared spectral data and the chemical data. (A) Similarity map defined by the canonical variates 1 and 2 for the chemical data; each label corresponds to a cheese: (●) d1, (○) d21, (▲) d51, (△) d81. (B) Correlation between chemical variables and canonical variates 1 and 2. (C) Similarity map defined by the canonical variates 1 and 2 for the infrared spectral data; each label corresponds to an infrared spectrum: (●) d1, (○) d21, (▲) d51, (△) d81. (D) Spectral patterns for the canonical variates 1 (—) and 2 (----) for infrared spectral data.

directly the variations of the chemical compositions of the experimental cheeses, but qualitative differences related to these variations such as protein structures and interactions in the cheese matrix.

Considering Figure 4B, it appeared that the chemical parameters relative to salting and proteolysis (NaCl, TN-WSN, WSN, WSN/TN) allowed the separation of the 4 stages of ripening according to the first

canonical variate: samples "d81" with the highest level of proteolysis showed negative scores. The chemical parameters expressing the differences between the initial composition of the cheeses (Moist., TN, pH, Ca) made it possible to discriminate the 16 experimental cheeses at all the ripening stages, according to the second canonical variate: cheese samples with the lowest moisture content, the highest pH, calcium and nitrogen contents showed negative scores. The nature of the chemical parameters linked to the two first canonical variates resulted from the manufacturing parameters and agreed with different structures of the curds among the experimental cheeses.

The most important result of the performed canonical analysis was the correlation between chemical data and infrared data, as shown in Figures 4C and 4D. Figure 4C shows that the first canonical variate totally discriminated the four groups of samples on the basis of their infrared spectra. It confirmed that spectral information present in the Amide I and II regions was connected to the continuous phenomena occurring during ripening time, allowing characterisation of the 4 stages.

Spectral pattern 1 (Fig. 4D) showed two majors features, previously observed in Figure 2B. The first feature was the appearance of an absorption at about 1 620 cm^{-1} during ripening which may be connected to modifications of the protein network. As previously reported [9, 15], the increase of the absorbance at about 1 620 cm^{-1} could be related to the changes in the interactions of the protein network during ripening time. On the other hand, this band has been correlated with differences of water activity in samples (Mazerolles, unpublished data). As water activity is rather constant during the ripening of cheese, this band might be connected to the modifications of the hydration of the proteins constituting the network during the ripening. It should be noticed that this absorption at about 1 620 cm^{-1} is particularly salient after salt-

ing (Fig. 1). But this band cannot be directly associated with the presence of sodium chloride as stated by comparison of the spectra of pure water and of NaCl solutions (pH 5.0, concentration comparable to cheeses namely 10 g·L^{-1} – data not shown). The good discrimination of "d1" group from "d21", "d51" and "d81" groups on the first canonical variate strongly supported the view that the salting of cheeses modified protein interaction and/or hydration. The high concentration of NaCl in cheese increased the screening effect towards the ionised groups and perturbed the hydration layers of proteins. However, further experiments would be necessary in order to get a better understanding of this important phenomenon occurring during cheese-making.

The second major feature of pattern 1 showed an opposition between bands at 1 580 and 1 555 cm^{-1}. The correlation between the absorption band at about 1 560 cm^{-1} and the decrease in absorption at about 1 580 cm^{-1} has already been described by Byler [7] studying the interactions of calcium ions with carboxylate groups of proteins. It was assumed that the calcium ions released following the proteolysis of proteins during cheese ripening would bind to the carboxylate groups of the side chains of aspartic and glutamic acids. Gagnaire and Leonil [19] and van Hooydonk et al. [33] have shown that part of the calcium released during hydrolysis remained in the casein micelles. In addition, van Hooydonk et al. [33] suggested that carboxylate groups significantly contributed to the binding of calcium since the binding was independent of pH in the 5.3–6.7 range. The pH independence of the binding of calcium was also found in the present study.

Spectral pattern 2 (Fig. 4D) discriminated cheese samples 4, 7 and 9 (the highest moisture content, and the lowest pH, calcium and nitrogen contents) from samples 3, 6 and 10 (the lowest moisture content, and the highest pH, calcium and nitrogen

contents), regardless of the ripening stage (see Fig. 4D). It was concluded that in the Amide I and II regions, each cheese showed several characteristic absorption bands, depending on the cheese manufacturing process, which were observed throughout the ripening time. This spectral information could be very useful for predicting at an early stage several characteristics related to the structure and properties of the ripened cheeses.

Spectral pattern 2 (Fig. 4D) was characterised by two bands located at about 1 580 and 1 620 cm⁻¹. As already presented, the first band could be assigned to carboxylate groups of aspartic and glutamic acid side chains bound to a counter ion other than calcium. Nevertheless, the absorption at 1 580 cm⁻¹ was stronger for cheeses 4, 7 and 9 which had the lowest nitrogen content and pH. This was quite surprising since the ionisation of the carboxylate groups of these cheeses should have been the lowest considering the pH. The strong band at 1 580 cm⁻¹ observed for cheeses 4, 7 and 9 may be explained by the presence of lactate which exhibits an absorption band close to that of proteins carboxylates. As a stronger acidification was observed during the manufacture of cheeses 4, 7 and 9, the concentration of lactate should be higher in these cheeses. This hypothesis was confirmed by measuring the lactate content for cheeses 4, 7, 9, 3, 6, 10 and 12, at "d1", "d21" and "d81" ripening stages. The results, presented in Table II, showed that (1) the level of lactate for all the cheeses was sufficient to generate an absorption on the infrared spectra, and (2) the differences of lactate content were significant among the cheeses investigated during ripening time. Considering the data, the highest levels of lactate were observed for cheeses 4, 7 and 9. It is concluded that this absorption band at 1 580 cm⁻¹ originates from lactate and can be considered as a marker for acidification.

Based on the observation of the absorption band near 1 620 cm⁻¹ in spectral pat-

Table II. Mean lactate content of cheeses 3, 6 and 10 (lact1) and cheeses 4, 7 and 9 (lact3) in d1, d21and d81 groups; lact2: lactate content for cheese 12; (all results expressed in percent of mass of cheese – see Sect. 3 for details).

Group	lact1	lact2	lact3
d1	2.25	2.59	2.94
d21	2.41	2.39	2.99
d81	2.19	2.33	3.08

tern 2, it was concluded that, after salting (stage of sampling where this band clearly appears – see Fig. 1), the protein structure in cheeses 4, 7 and 9 differed from that in cheeses 3, 6 and 10. These differences are related to the composition of the cheese and could be determined at the first step of measurement considering the absorption band at 1 580 cm⁻¹.

3.4. Canonical correlation analysis of fluorescence and chemical data sets

CCA was applied to the two data sets containing the normalised fluorescence spectra and the chemical data performed on the 16 experimental cheeses. Ten pairs of canonical variates were used to study correlations between the two data sets. Canonical variate 1, with a squared canonical correlation coefficient equal to 0.81, provided a common description of the samples both from the fluorescence and chemical data. The second canonical variate will not be interpreted, due to the weak correlation obtained. The similarity maps for chemical data and fluorescence spectral data are shown in Figures 5A and 5C, respectively. The correlation map for chemical data and the two first spectral patterns for fluorescence data are presented in Figures 5B and 5D, respectively.

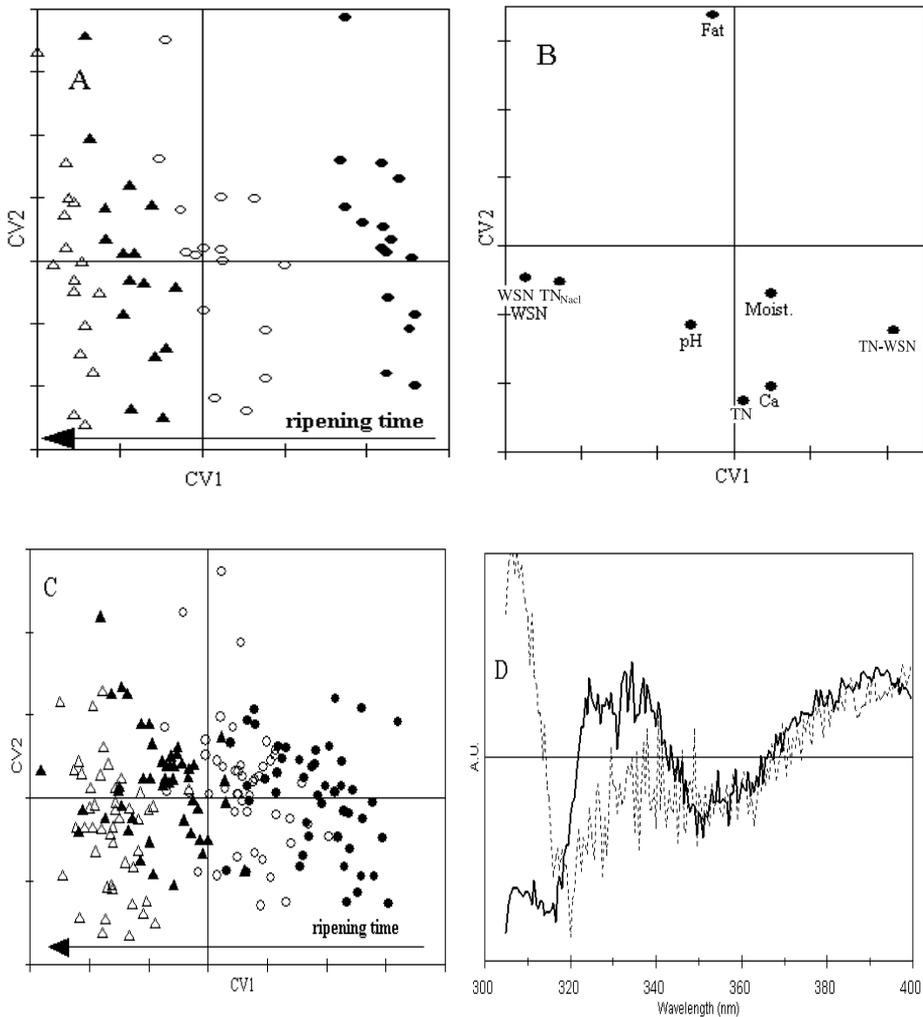Figures 5A and 5C show that the first canonical variates separated the four stages of

Figure 5. CCA for the fluorescence spectral data and the chemical data. (A) Similarity map defined by the canonical variates 1 and 2 for the chemical data; each label corresponds to a cheese: (●) d1, (○) d21, (▲) d51, (△) d81. (B) Correlation between chemical variables and canonical variates 1 and 2. (C) Similarity map defined by the canonical variates 1 and 2 for the fluorescence spectral data; each label corresponds to a fluorescence spectrum: (●) d1, (○) d21, (▲) d51, (△) d81. (D) Spectral patterns for the canonical variates 1 (—) and 2 (----) for fluorescence spectral data.

ripening. As in the preceding analysis, Figure 5B showed that the discriminant chemical parameters were salting and proteolysis (NaCl, TN-WSN, WSN, WSN/TN). Figure 5C confirmed that information contained in the fluorescence spectra of tryptophan was correlated to one (or more) continuous phenomenon taking place

during ripening and allowed characterisation of the 4 ripening stages. Spectral pattern 1 (Fig. 5D) indicated that the shape of fluorescence spectra was broader for ripened cheeses than for young cheeses. In fact spectral pattern 2 from PCA and spectral pattern 1 from CCA analyses on fluorescence spectra were similar. This result strongly suggested that the changes in the shape of fluorescence spectra were related to the changes of the protein network structure, resulting from the changes of the chemical characteristics of cheeses during ripening.

3.5. Canonical correlation analysis of infrared and fluorescence data set

As each spectral data set retained relevant information about the different stages of cheese ripening, the interest of analysing them jointly is obvious. This approach was attempted mainly in order to demonstrate the interest for combining the two spectroscopies. The question to be answered was: do the infrared and fluorescence spectral data contain complementary information permitting a full discrimination of the 4 stages of cheese ripening?

In this way, CCA was applied to the two data sets containing the normalised fluorescence and infrared spectra of the cheeses. Ten pairs of canonical variates were used to study correlations between the two data sets. The first 2 pairs were correlated with squared canonical correlation coefficients equal to 0.87 and 0.58, respectively. These correlations indicated that the first canonical variate provided a common description of the samples both from the infrared data and the fluorescence data. Even if the level of correlation was weaker for the second canonical variate, its interpretation remained possible. The similarity maps for fluorescence data and infrared spectral data are shown in Figures 6A and 6C, respectively. The first two spectral patterns for

fluorescence and infrared spectral data are presented in Figures 6B and 6D, respectively.

Figures 6A and 6C show that the first two canonical variates separated very clearly the four groups of samples on the basis of their infrared and fluorescence spectra. The effect of jointly analysing the two spectral data sets was to reveal that the characterisation of the ripening stages of cheeses can be obtained by two rapid and non-destructive techniques, without using any reference data such as the chemical features in the 2 preceding analyses. The similarity maps in Figures 6A and 6C must be compared to the principal component analyses (Figs. 2A and 3A). The 4 ripening stages were more clearly separated in the maps obtained by CCA and were found in the first variates instead of having to search the principal components of interest. On the other hand, the canonical variate 2 for the infrared data opposed samples 4, 7 and 9 to samples 3, 6 and 10 (Fig. 6C) regardless of their ripening stage. This opposition is less clear in the factorial map obtained for fluorescence (Fig. 6A), that can be evidenced considering the squared canonical correlation of 0.58 between infrared and fluorescence data for this second dimension. Nevertheless, even if infrared seems to be more appropriate for this task, the combination of the two techniques allows characterisation at an early stage of each kind of cheese among the 16 investigated, without any knowledge of its chemical composition.

Spectral pattern 1 for infrared data (Fig. 6D) was almost identical to the difference spectrum between Ca^{2+}-casein and K^{+}-casein presented by Byler [7]. It confirmed the hypothesis relative to the binding of calcium ions, released by proteolysis, to the (COO^{-}) groups of the side chains of aspartic and glutamic acids. This hypothesis is strengthened by the fact that these infrared results are highly correlated with fluorescence signals originating from proteins.
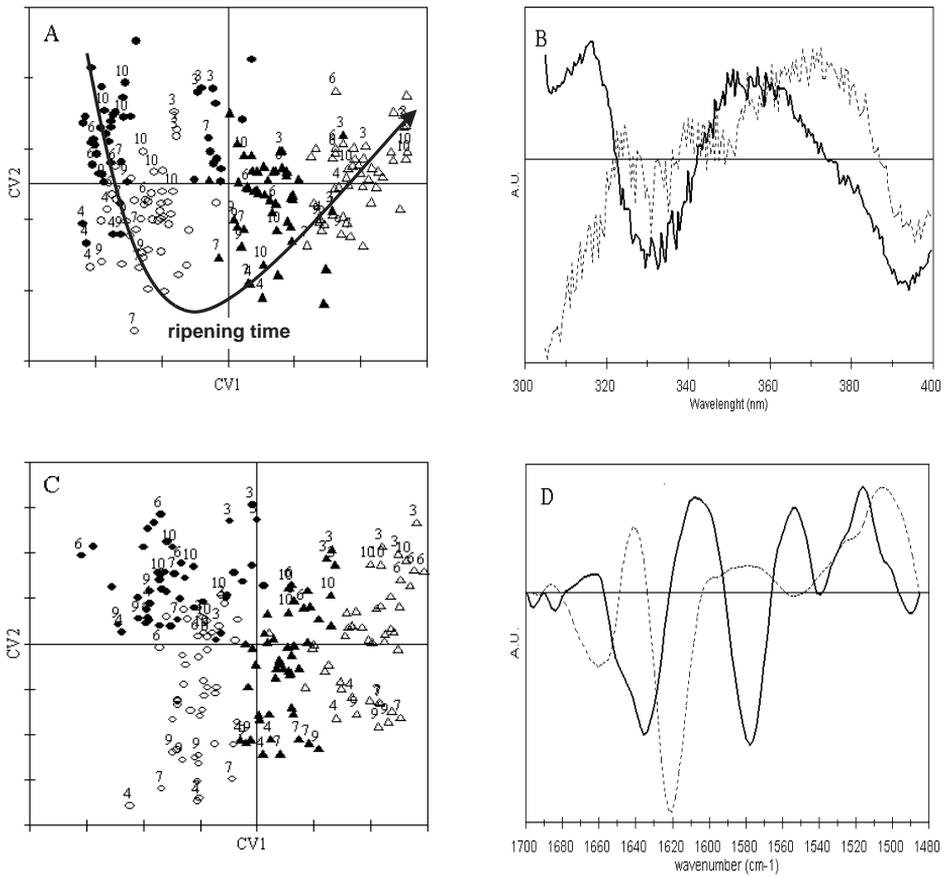
Figure 6. CCA for the fluorescence spectral data and the infrared spectral data. (A) Similarity map defined by the canonical variates 1 and 2 for the fluorescence data; each label corresponds to a fluorescence spectra: (●) d1, (○) d21, (▲) d51, (△) d81. (B) Spectral patterns for the canonical variates 1 (—) and 2 (----) for fluorescence spectral data. (C) Similarity map defined by the canonical variates 1 and 2 for the infrared spectral data; each label corresponds to an infrared spectrum: (●) d1, (○) d21, (▲) d51, (△) d81. (D) Spectral patterns for the canonical variates 1 (—) and 2 (----) for infrared spectral data.

4. CONCLUSION

This study shows that infrared and fluorescence spectroscopy are useful for characterising the changes in proteins during cheese ripening. As demonstrated using CCA, the shapes of the spectra are correlated to several characteristics of the protein network, as well as to the chemical composition of cheese. The information derived from the spectra is of two types: the first makes it possible to characterise the ripening stage of the cheese, and the second is relative to each cheese and may be used to predict at an early stage some characteristics of the ripened product. On the other hand, the interest of combining two kinds

of spectroscopies has been demonstrated. Applying CCA to the two sets of spectral data allows a complete discrimination of the processes and of the products, as well as an interpretation of the results at the molecular level. Fluorescence and infrared spectra provide different information related to the side chains of aromatic amino-acids and to the peptide bonds and the side chains of carboxylic amino acids, respectively, which complementarity makes it possible to characterise, in a global way, the 16 cheeses at the 4 stages of ripening.

REFERENCES

[1] Abott T.P., Wolf W.J., Wu Y.V., Butterfield R.O., Kleiman R., FTIR Analysis of Jojoba protein conformation in D_2O, Appl. Spectrosc. 45 (1991) 1665–1673.

[2] Bellamy L.J., The infrared spectra of complex molecules, John Wiley and Sons, Inc., New-York, 1975.

[3] Bertrand D., Lila M., Furstoss V., Robert P., Downey G., Application of Principal Component Analysis to the prediction of lucerne forage content and in vitro dry matter digestibility by NIR spectroscopy, J. Sci. Food Agric. 41 (1987) 299–307.

[4] Bertrand D., Scotter C.N.G., Application of multivariate analyses to NIR spectra of gelatinized starch, Appl. Spectrosc. 46 (1992) 1420–1425.

[5] Blumberg W.E., Doleiden F.H., Lamola A.A., Hemoglobin determined in 15 μL of whole blood by "front face" fluorometry, Clin. Chem. 26 (1980) 409–413.

[6] Bouton Y., Guyot P., Dasen A., Grappin R., Activité protéolytique de souches de lactobacilles thermophiles isolées de levain et de Comté. II. Applications en sites industriels, Lait 75 (1994) 33–46.

[7] Byler D.M., Farell H.M., Infrared spectroscopic evidence for calcium ion interaction with carboxylate groups of casein, J. Dairy Sci. 72 (1989) 1719–1723.

[8] Casal H.L., Mantsch H.H., Polymorphic phase behavior of phospholipid membranes studied by infrared spectroscopy, Biochim. Biophys. Acta 779 (1984) 382–401.

[9] de Collonque-Poyet B., Sebille B., Baron M.H., Chromatography of the interferon and the Analogue II: FTIR analysis, Biospectroscopy 2 (1996) 101–111.

[10] Devaux M.F., Robert P., Qannari M., Safar M., Vigneau E., Canonical correlation analysis of mid and near infrared oil spectra, Appl. Spectrosc. 47 (1993) 1024–1028.

[11] Dousseau F., Pézolet M., Determination of the secondary structure contents of proteins in aqueous solutions from their Amide I and Amide II infrared bands. Comparison between classical and partial least squares methods, Biochemistry 29 (1990) 8771–8779.

[12] Dufour E., Riaublanc A., Potentiality of spectroscopic methods for the characterisation of dairy products. I. Front-face fluorescence study of raw, heated and homogenised milks, Lait 77 (1997) 657–670.

[13] Dufour E., Dalgalarrondo M., Adam L., Conformation of β-lactoglobulin at an oil/water interface as determined from proteolysis and spectroscopic methods, J. Coll. Interface Sci. 207 (1998) 264–272.

[14] Dufour E., Lopez C., Riaublanc A., Mouhous Riou N., La spectroscopie de fluorescence frontale : une approche non invasive de la structure et des interactions entre les constituants des aliments, Agoral 10 (1998) 209–215.

[15] Dufour E., Robert P., Renard D., Llamas G., Investigation of β-lactoglobulin gelation in water/ethanol solutions, Int. Dairy J. 8 (1998) 87–93.

[16] Dufour E., Mazerolles G., Devaux M.F., Duboz G., Duployer M.-H., Mouhous-Riou N., Phase transition of triglycerides in fat globules during semi-hard cheese ripening, Int. Dairy J. 10 (2000) 87–99.

[17] Elliot A., Ambrose E.J., Structure of synthetic polypeptides, Nature 165 (1950) 921–922.

[18] Fox P.F., The milk protein system, in: Fox P.F. (Ed.), Developments in Dairy Chemistry– 4. Functional milk proteins, Elsevier Appl. Sci., New York, USA, 1989, pp. 1–54.

[19] Gagnaire V., Léonil J., Preferential sites of tryptic cleavage on the major bovine caseins within the micelle, Lait 78 (1998) 471–489.

[20] Genot C., Tonetti F., Montenay-Garestier T., Marion D., Drapon R., Front-face fluorescence applied to structural studies of proteins and lipid-protein interactions of visco-elastic food products. 2- Application to wheat gluten, Sci. Aliments 12 (1992) 687–704.

[21] Goormaghtigh E., Cabiaux V., Ruysschaert J.M., Determination of soluble and membrane protein structure by Fourier transform infrared spectroscopy. I. Assignments and model compounds, in: Hilderson H.J., Ralston G.B. (Eds.), Subcellular Biochemistry, Volume 23: Physicochemical Methods in the Study of Biomembranes, Plenum Press, New York, 1994, pp. 329–362.

[22] Herbert S., Bouchet B., Riaublanc A., Gallant D.J., Dufour E., Coagulation acide du lait étudiée par rhéologie, spectroscopie et microscopie, Cah. Rhéol. 15 (1997) 380–389.

[23] Herbert S., Mouhous Riou N., Devaux M.F., Riaublanc A., Bouchet B., Gallant D.J., Dufour E., Monitoring the identity and the structure of soft cheeses by fluorescence spectroscopy, Lait 80 (2000) 621–624.

[24] Hirsch R.E., Nagel R.L., Stopped flow front-face fluorometer: a prototype design to measure hemoglobin R->T transition kinetics, Anal. Biochem. 176 (1989) 19–21.

[25] Jollife I.T., Principal Component Analysis, Springer, New-York, USA, 1986.

[26] Lakowicz J.R., Protein fluorescence, in: Lakowicz J.R. (Ed.), Principles of fluorescence spectroscopy, Plenum Press, New York, NY, USA, 1983, pp. 341–389.

[27] Lee D.C., Haris P.I., Chapman D., Mitchell R.C., Determination of protein secondary structure using factor analysis of infrared spectra, Biochemistry 29 (1990) 9185–9193.

[28] Le Graet Y., Brulé G., Mineral migration in Camembert cheeses during ripening, Lait 68 (1988) 219–234.

[29] Mazerolles G., Duboz G., Phan-Tan-Luu R., Utilisation de la planification expérimentale pour le calibrage d'un appareil infrarouge destiné au dosage des principaux constituants d'un fromage de type pâte pressée, 5e journée Européenne "Agro-industrie et méthodes statistiques", 1997, p. 21.

[30] Pearce K.N., The complexometric determination of calcium in dairy products, N.Z.J. Dairy Sci. Technol. 12 (1977) 113–115.

[31] Picque D., Lefier D., Grappin R., Corrieu G., Monitoring of fermentation by infrared spectrometry – Alcoholic and lactic fermentations, Anal. Chim. Acta 279 (1993) 67–72.

[32] Saporta G., Probabilités – Analyse des données et statistique, Technip Ed., Paris, 1990.

[33] van Hooydonk A.C.M., Boerriger I.J., Hagedoorn H.G., pH-induced physico-chemical changes of casein micelles in milk and their effect on renneting. 2. Effect of acidification on physico-chemical properties, Neth. Milk Dairy J. 40 (1986) 281–296.