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Original article

Monitoring the identity and the structure of soft cheeses by fluorescence spectroscopy

Sophie HERBERT[a, b], Nadine MOUHOUS RIOU[a], Marie Françoise DEVAUX[b], Alain RIAUBLANC[a], Brigitte BOUCHET[b], Daniel J. GALLANT[b], Éric DUFOUR[c]*

[a] UEIMA, INRA, BP 71627, 44316 Nantes Cedex 13, France
[b] URPOI, INRA, BP 71627, 44316 Nantes Cedex 3, France
[c] Département Qualité et Économie Alimentaires, ENITA de Clermont-Ferrand, 63370 Lempdes, France

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Abstract — Soft cheeses exhibit a wide range of textures and, as a consequence, of structures. The objective of the present study was to investigate intrinsic fluorophores of cheese in order to discriminate between eight different soft cheeses. Protein tryptophan emission spectra and vitamin A excitation spectra were recorded directly on cheese samples using front face fluorescence spectroscopy. The eight soft cheeses were discriminated using their spectra by applying multivariate statistical methods such as principal component analysis and factorial discriminant analysis. From the tryptophan fluorescence data set, a good classification was observed for 95% and 92% of the principal and test samples, respectively. A better classification (96% and 93% for principal and test samples) was obtained from the vitamin A spectra. The spectral pattern associated with the principal components provides characteristic wavelengths which are the most suitable for separating the spectra. They allow information on the protein structure at the molecular level to be derived, in relation with cheese texture.

cheese / identification / structure / fluorescence / protein

Résumé — La spectroscopie de fluorescence frontale pour identifier et caractériser la structure de fromages à pâte molle. Les fromages à pâte molle présentent une large gamme de texture et, en conséquence, de structure. L'objectif de cette étude était d'évaluer le potentiel des fluorophores intrinsèques du fromage dans la discrimination de 8 types de fromages à pâte molle. Les spectres d'émission des tryptophanes de protéines et les spectres d'excitation de la vitamine A ont été enregistrés directement sur des fromages au moyen de la fluorescence frontale. L'analyse des collections de spectres par des méthodes statistiques multidimensionnelles telles l'analyse en composantes

* Correspondence and reprints
Tel.: (33) 4 73 98 13 78; fax: (33) 4 73 98 13 90; e-mail: dufour@gentiane.enitac.fr

principales et l'analyse factorielle discriminante a permis de discriminer les 8 fromages. Respectivement 95 % et 92 % des échantillons principaux et de vérification ont été bien classés à partir des spectres de fluorescence des tryptophanes. Un meilleur résultat (respectivement 96 % et 93 %) a été obtenu à partir des spectres de la vitamine A. Par ailleurs, les profils spectraux associés aux composantes principales présentent les longueurs d'onde les plus discriminantes de la collection spectrale. Ils permettent d'obtenir des informations sur la structure des protéines à l'échelle moléculaire, en relation avec la texture des fromages.

fromage / identification / structure / fluorescence / protéine

1. INTRODUCTION

Texture is an important criteria used to evaluate the quality of cheeses. It is a reflection of their 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 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 of cheese, the softer the cheese [31]. Understanding the structure of cheeses, particularly protein and fat structures and the interactions between cheese components during and after manufacture, can provide information useful in determining what constitutes a quality product.

It is generally assumed that a good curd is a prerequisite to obtain a quality cheese. This fact implies that milk coagulation is a major step in cheese manufacturing, largely determining the texture of the product. The main parameters that have an effect on the physical properties of the curd are the milk composition, the acidification kinetic and the amount of rennet. For example, a change in fat content affects protein aggregation and can modify the physical properties of cheese.

The pH lowering and rennetting induce solubilisation of colloidal calcium phosphate and structural changes of proteins [6, 7, 38], and as a consequence, decrease the stability of the casein micelles [4] resulting in milk coagulation. The kinetics of coagulation and the structural aspects of protein-protein and protein-fat globule interactions generally determine the rheological properties of curds and thus their syneresis behaviour [26]. As the decrease of the pH still continues after coagulation and curd cutting, structural changes of micelles and shrinkage of curd grains are observed. Brining modifies the structure of the protein in cheese increasing the density of the protein matrix. The degree of proteolysis, the storage time and the storage temperature also influence the structure of proteins and the texture of cheese [15].

Few techniques enable the monitoring, at a molecular level, of the structural evolution of food samples. Fluorescence spectroscopy, which is a sensitive, rapid and non-invasive analytical technique that provides information on the presence of fluorescent molecules and their environment in biological samples, may be a good candidate for this purpose. For instance, fluorescence properties of aromatic amino-acids of proteins [9, 30, 32] can be used to study protein structure and protein interactions with hydrophobic molecules or ions. The aqueous phase of bovine milk contains six major proteins: β-lactoglobulin, α-lactalbumin, α_{s1} and α_{s2} caseins, β-casein and κ-casein. The amino-acid compositions of all these proteins include at least one tryptophan residue [16, 17]. Depending on their structures, 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 [30]. Milk retains vitamin A located in the core and in the membrane of the fat globule [21]. Due to its conjugated double bonds, vitamin A is a good fluorescent probe with excitation and emission wavelengths at about 330 and 450 nm, respectively [8]. In addition, it has been shown that the shape of the vitamin A excitation spectrum is correlated with the physical state of the triglycerides in the fat globule [11]. The lipids of milk fat globules contain hundreds of triacylglycerol species [28], for which melting occurs over a large temperature range, i.e., between –30 and +40 °C. Solid fat content is an important quality control parameter in the edible fats and oils industry. The organoleptic characteristics of creams, butters, margarines, cheeses and other fat blends depend partly on the value of this physical parameter.

Most fluorescence experiments are done on dilute solutions with absorbance of the sample below 0.1; it is classical right-angle fluorescence spectroscopy. When the absorbance of the sample is higher than 0.1, the screening effect (or inner filter effect) induces a decrease of fluorescence intensity and a distortion of excitation spectra [19]. To avoid these problems, an alternative method – frontal illumination fluorescence spectroscopy, has been developed [36]. Front face fluorescence allows investigation of the fluorescence of powdered, turbid, emulsified and concentrated samples [3, 10, 20, 27]. For example, the fluorescence of milk proteins allows the monitoring of the structural modifications of proteins and their physico-chemical environment during milk heating [8] or milk coagulation [11, 24, 25]. Front-face fluorescence spectroscopy was also used to investigate the protein's structure of wheat gluten [20].

Cheese is a complex product exhibiting simultaneously emulsion, gel and solution phases. Today there is a lack of non-destructive and non-invasive methods aiming at the investigation of cheese structure. In this paper, the focus is mainly on the study of the intrinsic fluorophores of cheese in order to record fluorescence spectra of "real" cheese samples and to discriminate between different soft cheeses by applying principal component analysis and factorial discriminant analysis to the fluorescence spectra.

2. MATERIALS AND METHODS

2.1. Cheeses

This study was performed on the six following marketed soft cheeses manufactured by different processes: four different mesophilic-processed cheeses, one thermophilic-processed cheese and an ultrafiltered-processed cheese. Among the mesophilic-processed cheeses, M1 and M2 cheeses are white mould with an acidic curd. M3 and M4 cheeses correspond to red smear cheeses characterised by a renneted curd. The cheeses were provided by the Bongrain group and were selected in order to investigate a wide range of structure and texture. They were studied at an early stage of ripening (25 d), except for the M1 and M2 cheeses which were also studied at a late stage of ripening (45 d). The sample coding is shown Table I. For each type of soft cheese, three different production batches were analysed. Each batch included three individual cheeses.

2.2. Fluorescence spectroscopy

Fluorescence spectra were recorded using a SLM 4800C spectrofluorimeter (Bioritech, Chamarande, France) mounted with a thermostatted 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. The spectra of cheese

Table I. Coding of the soft cheeses.
Tableau I. Codage des fromages.

Cheeses	Ripening stage	
	Young	Old
Mesophilic I	M1y	M1o
Mesophilic II	M2y	M2o
Mesophilic III	M3	–
Mesophilic IV	M4	–
Thermophilic	TH	–
Ultrafiltrated	UF	–

samples (3 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 (resolution: 0.5 or 1 nm, averaging: 10). The emission spectra of tryptophan residues (305–400 nm) were recorded with the excitation wavelength set at 290 nm, and the excitation spectra of vitamin A (260–350 nm) were recorded with the emission wavelength set at 410 nm. All spectra were corrected for instrumental distortions in excitation using a rhodamine cell in reference channel.

Slices of 3 cm length and 0.3 cm thickness were cut up in the middle of the cheese's radius. For each individual cheese, three spectra were recorded using different samples. The data collection set included 216 tryptophan emission spectra and 216 vitamin-A excitation spectra.

2.3. Mathematical treatment of data

In order to reduce scattering effects, the data were normalised by reducing the area under each spectrum to a value of 1 according to Bertrand and Scotter [1]. Principal Component Analysis (PCA) was applied to the normalised spectra in order to investigate changes in them. This statistical multivariate treatment makes it possible to draw similarity maps of the samples and to get spectral patterns [2, 29]. While the similarity maps allow the comparison of the spectra in such a way that two neighbouring points represent two similar spectra, the spectral patterns exhibit the absorption bands that explain the similarities observed on the maps [12]. The PCA software was written in the C language in the laboratory. PCA was applied on the spectral collection divided into two data sets for calibration and validation. The two data sets were obtained by splitting the spectral collection, i.e. for each batch (composed of three cheeses), two cheeses were put in the calibration group and the other cheese was used to create the validation group.

The ability of the data to describe the different kinds of cheese was investigated by applying discriminant analysis on the principal components. A group was created for each manufacturing process and ripening stage. In a first step, a step-wise discriminant analysis was performed to select the variables (principal components) the most relevant for the discrimination of the spectra following the eight qualitative groups initially defined. The variable selection was realised following the classification percentage of the samples. The selection of variables ended when the addition of a supplementary variable did not improve significantly the classification percentage.

From the selected variables, factorial discriminant analysis (FDA) assessed new synthetic variables called discriminant factors, which were not correlated and allowed the best separation of the qualitative groups. Similarity maps and patterns can be drawn, in analogy to those for PCA.

3. RESULTS AND DISCUSSION

3.1. Fluorescence spectra of the studied cheeses

Since the eight commercial soft cheeses investigated in this study were manufactured

by different processes leading to different textures, it was assumed that their structures, and as a consequence the environment of the intrinsic cheese fluorophores, were different. The tryptophan emission-spectra and vitamin-A excitation-spectra of

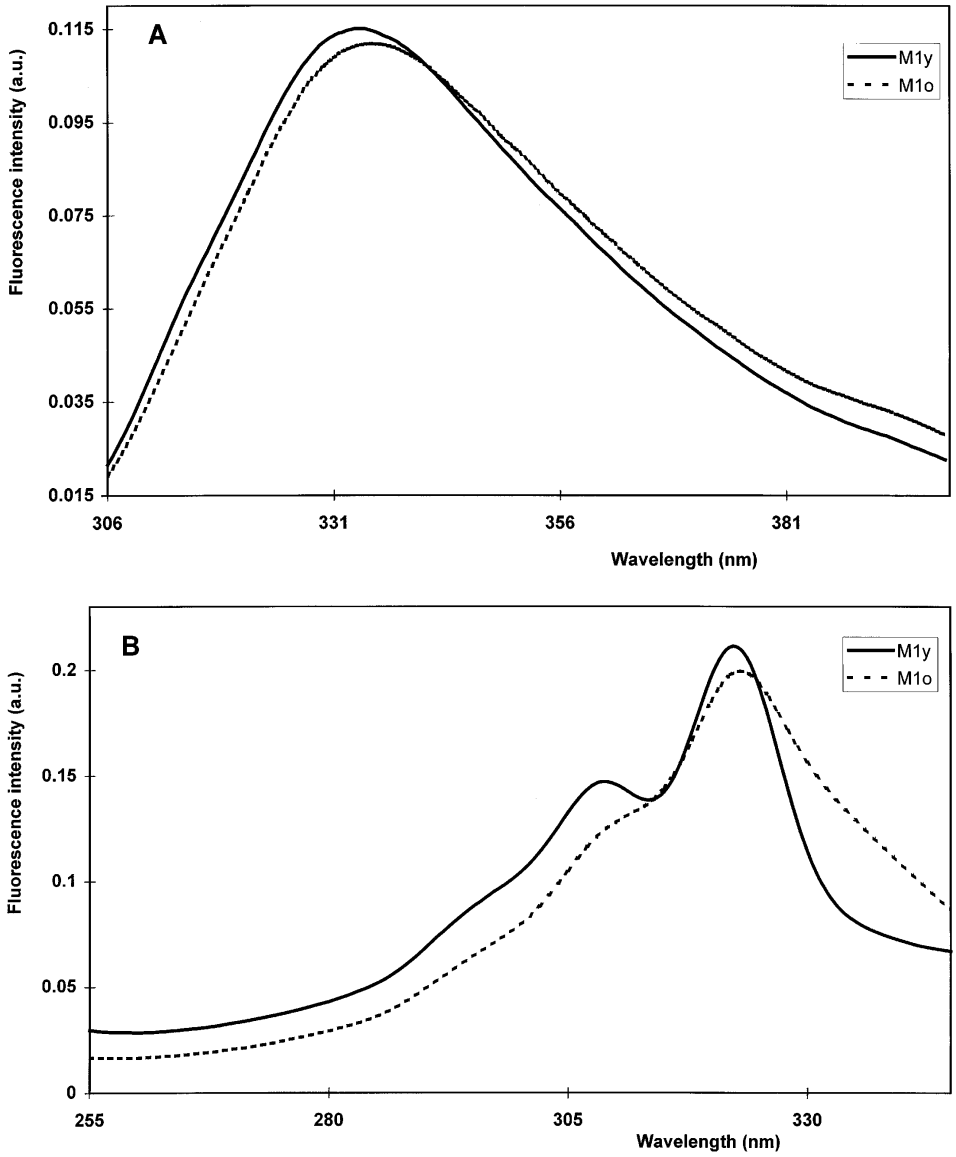

Figure 1. Fluorescence spectra of cheeses: (**A**) Tryptophan emission and (**B**) Vitamin A excitation. (——) Mesophilic-I young, (- - - - -) mesophilic-I old cheeses.

Figure 1. Spectres de fluorescence des fromages : (**A**) émission des tryptophanes et (**B**) excitation de la vitamine A. Fromages mésophile-I jeune (——), mésophile-I vieux (- - - - -).

two different cheeses are presented in Figures 1A and 1B, respectively. The tryptophan presented a maximum located at about 332 nm, the location of which slightly varied from one cheese to another. Generally, excitation spectra of vitamin A showed a maximum at 322 nm and two shoulders at 305 nm and 295 nm. More interestingly, the shape of the spectra changed with the type of cheese.

Although the different cheeses exhibit only slightly different fluorescence spectra, univariate analysis is not really appropriate for the study of complex spectra. Multivariate analysis techniques such as PCA and FDA appear necessary in this case. For this purpose 216 tryptophan spectra and 216 vitamin A spectra were collected from the eight types of soft cheeses.

3.2. Cheeses discrimination from their tryptophan spectra

In order to compare the set of fluorescence spectra and to emphasise the similarities and the differences underlined previously, principal component analysis was performed on the spectra to describe the main variations between the different cheeses. The first two principal components took into account 90.6% and 4.1% of the total variance. The map defined by the principal components 1 and 2 showed some differences between the eight cheeses (data not shown).

In a second step the ability of the data to identify the eight groups was investigated by applying discriminant analysis on the principal components. A correct classification was observed for 95% and 92% of the calibration and validation samples, respectively. It was concluded that tryptophan fluorescence spectra are fingerprints allowing the identification of the cheeses. Table II gives the classification of the validation spectra into the eight groups. This table illustrated that M1 and M2 cheeses at the young and old stages, the TH and the UF cheeses were well discriminated, while some misclassification occurred between M3 and M4 cheeses. The map defined by the discriminant factors 1 and 2 (Fig. 2) shows that the tryptophan emission spectra were well suited for the soft cheese discrimination. Considering discriminant factor 1, M2y and M2o cheeses were observed on the far right, whereas M1o, M1y, UF and TH are located on the far left. The red smear cheeses M3 and M4 exhibited coordinates close to the origin. The cluster formed by M1y, M1o, UF and TH included the cheeses with the highest fat yield (between 32.5 and 36 g·100 g^{-1} of

Table II. Classification table of the spectra for the validation group (trypophan spectra).
Table II. Classification des spectres de la collection de vérification (spectres des tryptophanes).

Observed \ Predicted	M1y	M1o	M2y	M2o	M3	M4	TH	UF
M1y	**9**	**–**	–	–	–	–	–	–
M1o	–	**7**	**–**	–	–	–	1	1
M2y	–	–	**8**	1	–	–	–	–
M2o	–	–	–	**9**	**–**	–	–	–
M3	–	–	–	–	**7**	2	–	–
M4	–	–	–	–	1	**8**	**–**	–
TH	–	–	–	–	–	–	**9**	**–**
UF	–	–	–	–	–	–	–	**9**

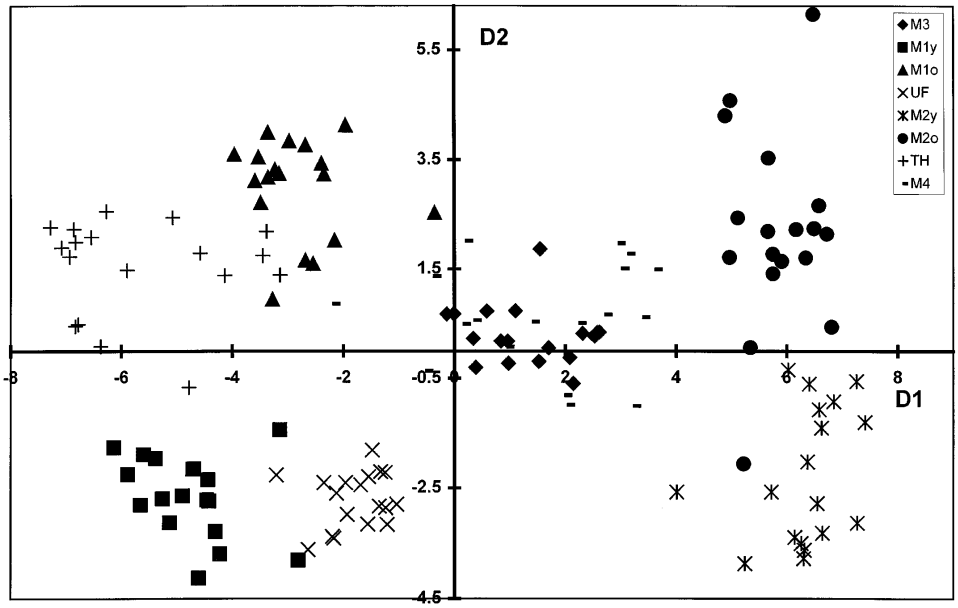

Figure 2. Discriminant analysis similarity map determined by discriminant functions 1 and 2 for the tryptophan spectra of the eight cheeses.

Figure 2. Carte factorielle 1–2 de l'analyse discriminante réalisée sur les spectres de fluorescence des tryptophanes des 8 fromages.

cheese), whereas M2y and M2o exhibited about 23 g of fat per 100 g of cheese [23]. In addition, M1y and M2y cheeses had negative scores according to discriminant factor 2, whereas M1o and M2o had positive scores. Fluorescence allowed the changes of cheese structure during ripening to be discriminated.

From the similarity map (Fig. 2), it appears also that the fluorescent properties of a ripened cheese are close to those of this cheese at a young stage. It suggests that the structure of a ripened cheese is highly correlated to the structure of the young cheese. This assumption has been confirmed by the spectroscopic experiments performed on 16 different experimental semi-hard cheeses [14, 35]. It was shown that the 16 cheeses were discriminated from their fluorescence and infrared spectra at 1 day old and that the spectrum of a given cheese at 81 days old can be predicted from its spectrum recorded at 1 day old.

Generally, it is assumed that cheese texture is correlated to its structure at molecular and microscopic levels. For example, the close location on the map (Fig. 2) of M1o and TH was in agreement with their texture properties as shown by texture sensory analysis [23]. In addition, the investigation of the protein network of TH and M1o cheeses by confocal laser scanning microscopy indicated similar microstructures exhibiting smooth protein networks [23]. Whereas the fluorescence images of M1y and M1o showed different protein networks [23], i.e. granular for M1y and smooth of M1o. These results indicate clearly that there is a relation between the structure at a molecular level, as measured by fluorescence, the microstructure and the texture of the soft cheeses.

Table III. Classification table of the spectra for the validation group (vitamin A spectra).
Table III. Classification des spectres de la collection de vérification (spectres de la vitamine A).

Observed \ Predicted	M1y	M1o	M2y	M2o	M3	M4	TH	UF
M1y	**9**	**–**	–	–	–	–	–	–
M1o	–	**6**	**–**	2	1	–	–	–
M2y	–	–	**8**	1	–	–	–	–
M2o	–	–	–	**9**	**–**	–	–	–
M3	–	–	–	–	**9**	**–**	–	–
M4	–	–	–	–	1	**8**	**–**	–
TH	–	–	–	–	–	–	**9**	**–**
UF	–	–	–	–	–	–	–	**9**

3.3. Fluorescence properties of vitamin A in the fat globules of the eight cheeses

PCA was applied to the set of vitamin A excitation spectra (216 spectra). The similarity map defined by principal components 1 and 2 allowed a discrimination of the eight cheeses (data not shown). In a second step, discriminant analysis was applied on the principal components.

A good classification amounted to 96% and 93% observed for the calibration and validation samples, respectively. Table III gives the classification of the spectra belonging to the validation samples according to the factorial discriminant analysis. This table illustrates that all cheeses, but M1o, were well discriminated.

The percentage of good classifications were better with the vitamin A spectra than with tryptophan spectra. These results confirm that vitamin A is a useful probe to discriminate milk products since its fluorescence spectra are sensitive to the process used and the aging of the product.

The map defined by discriminant factors 1 and 2 is shown in Figure 3. It showed that TH, UF, M1y, M2o and M2y cheeses were well separated, while M3 and M4 cheeses were somewhat confused on the map. A good discrimination of M3 and M4 cheeses was observed according to discriminant factors 3 and 4. M1o cheese spectra were widely distributed on the map. At the moment we can't explain the spectra distribution for this cheese.

The differences observed for vitamin A fluorescence spectra are consistent with changes of lipid structure, but the interpretation at the molecular level is more difficult. Indeed, much less is known on the relations between the shape of vitamin A spectra and the organisation of lipids than between the shape of tryptophan fluorescence spectra and protein structure. Nevertheless, it is well known that the fluorescent properties of fluorophores are very sensitive to the changes of the solvent viscosity [34]. Recently, correlations between the shape of vitamin A spectra and the viscosity of lipids in fat globules have been reported [14, 23]. For example, the shape of the vitamin A excitation spectrum was correlated with the physical state of the triglycerides in the fat globules of an emulsion measured by differential scanning calorimetry [11]. From the investigation of vitamin A fluorescence during semi-hard cheese ripening, it was also concluded that the viscosity of lipids changed with time and that a partial crystallisation of lipids took place [14].

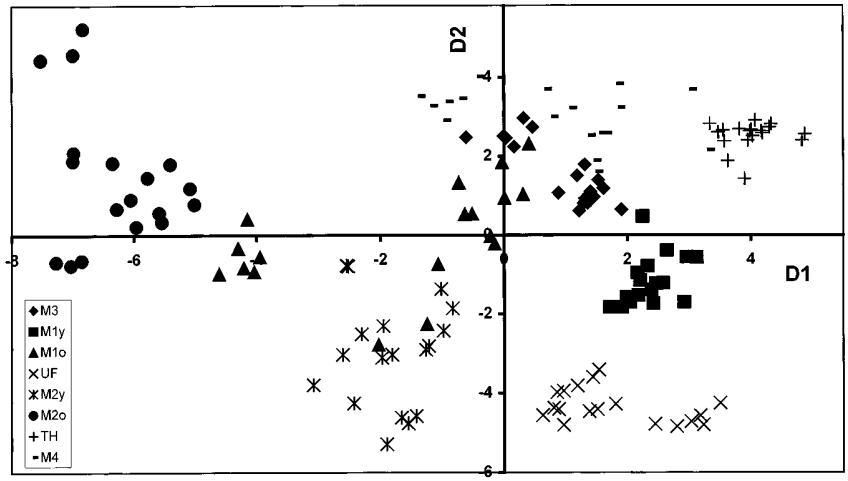

Figure 3. Discriminant analysis similarity map determined by discriminant functions 1 and 2 for the vitamin-A spectra of the eight cheeses.

Figure 3. Carte factorielle 1–2 de l'analyse discriminante réalisée sur les spectres de fluorescence de la vitamine A des 8 fromages.

On the other hand, the changes in the shape of vitamin A excitation spectra recorded during milk coagulation have been correlated to the changes of fat globule/protein interactions [23]. The different phases of milk coagulations induced by rennet, acidification plus rennet or acidification can be discriminated from the vitamin A excitation spectra. The results of the multidimensional statistical analysis of the spectral data show that protein/fat globule interactions develop in the coagulums. Moreover, the development of interactions between the protein network and the fat globules in coagulums have been demonstrated by fluorescence transfer between tryptophan and vitamin A in the case of the coagulation of reconstituted milks [33].

Considering the soft cheeses, the interactions between the protein network and the fat globule vary depending on the manufacturing process (heating, acidification rate, etc.), and modify in different ways the vitamin A spectra [11, 23, 25]. Crystallisation of the triglycerides during ripening time may also alter the shape of the vitamin A fluorescence spectra [14, 33]. However, further experiments are required in order to conclude on this point.

3.4. Differences between cheeses at the molecular level – protein structure and interactions

In order to investigate more precisely the reason for the discrimination of the manufacturing process and the ripening stage, some focus was given to the subset containing the tryptophan emission spectra of M1y, M1o, M2y and M2o cheeses. The aim of this analysis was to investigate the effects of two different manufacturing processes at two different ripening stages on the structure of cheeses at the molecular level.

The similarity map defined by the principal components 1 and 2 allowed a good discrimination of the four cheeses (Fig. 4). The principal component 1 separated the cheeses as a function of the ripening time,

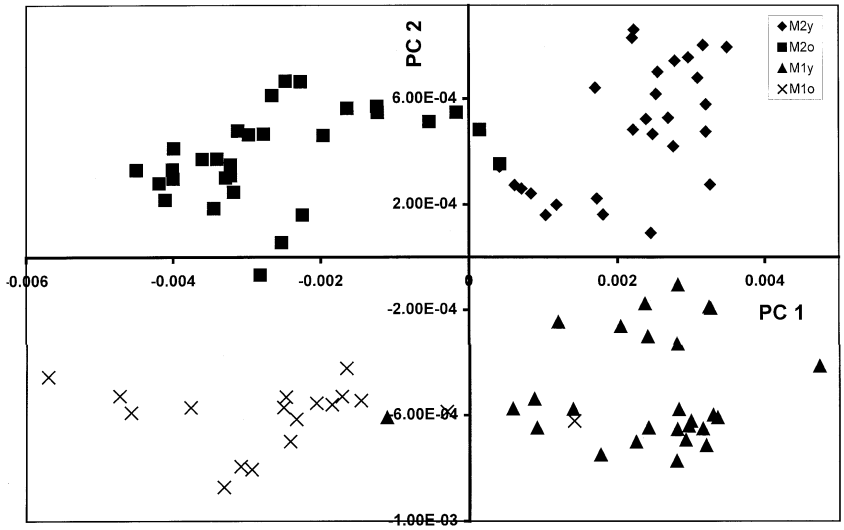

Figure 4. Principal component analysis similarity map determined by principal components 1 and 2 for the tryptophan spectra of mesophilic-I young, mesophilic-I old, mesophilic-II young and mesophilic-II old cheeses.

Figure 4. Carte factorielle 1–2 de l'analyse en composantes principales réalisée sur les spectres de fluorescence des tryptophanes des fromages mésophile-I jeune, mésophile-I vieux, mésophile-II jeune et mésophile-II vieux.

while a clustering depending on the process was observed according to the principal component 2. The spectral patterns associated with the principal components could be drawn. They reveal the characteristic wavelengths for which large differences are observed in the spectra. They are similar to spectra and can be used to derive structural information at the molecular level [13, 25].

Figure 5 shows the spectral patterns 1 and 2 associated with the principal components 1 and 2. The spectral pattern 1 characterised a shift toward higher wavelengths of the emission maximum during ripening of M1 and M2 cheeses. It indicated that the environment of tryptophan residues was relatively more hydrophilic for the old cheeses. This phenomenon may be related to the partial proteolysis of caseins resulting in an increase of tryptophan exposure to the solvent. Another explanation arises from the increase of pH during ripening, modifying the tertiary and quaternary structures of casein micelles. The pH of M1y, M2y, M1o and M2o were 4.97 ± 0.06, 5.87 ± 0.10, 7.07 ± 0.17 and 7.64 ± 0.11, respectively [23].

From the spectral patterns for principal component 1 presented in Figures 5 and 6, it appeared that the modifications in the fluorescence properties of milk proteins during coagulation [25] can be paralleled with the changes in fluorescence spectra observed during cheese ripening. In the two cases, the spectral pattern was characterised by a shift towards lower wavelengths of the emission maximum for pH decreasing from 7 to 4.5. It confirmed that the structure of casein was modified by the pH increase during ripening. As it was observed during milk coagulation, these structural changes are characterised by a more hydrophilic environment of tryptophan residues in proteins when the pH is close to the neutral pH. Herbert et al. [25] concluded that the changes of micelle conformation and hydration as a function of pH derived by voluminosity

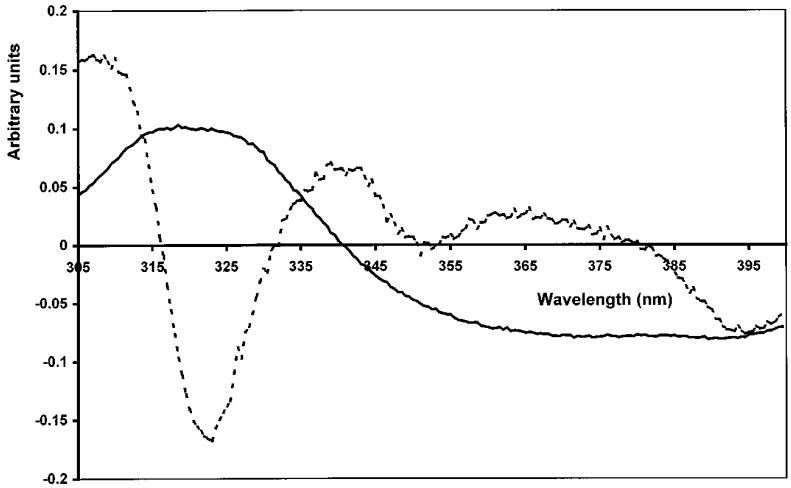

Figure 5. Spectral patterns corresponding to the principal components 1 (——) and 2 (----) of tryptophan data recorded on cheeses (Fig. 4).

Figure 5. Vecteurs propres correspondant aux composantes principales 1 (——) et 2 (----) pour les données de fluorescence des tryptophanes enregistrées sur les fromages (Fig. 4).

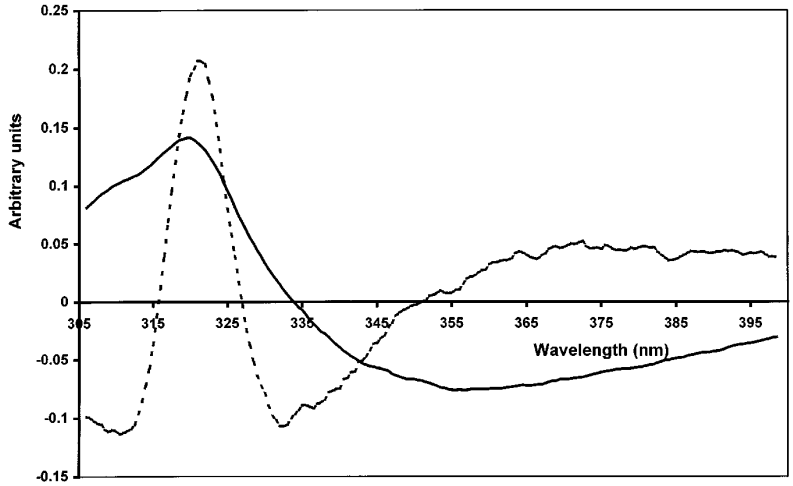

Figure 6. Spectral patterns 1 and 2 corresponding to the principal component 1 (——) and 2 (----) of the principal component analysis performed on the spectral data recorded during rennet, acid-rennet and acid coagulations of milk*.
* The spectral data collected during acid- and rennet-coagulation kinetics of milk [25] were re-analysed by PCA allowing drawing of the eigenvectors 1 and 2.

Figure 6. Vecteurs propres correspondant aux composantes principales 1 (——) et 2 (----) de l'analyse en composantes principales réalisées sur les données spectrales enregistrées au cours de la coagulation du lait par la présure, acidification + présure et acidification*.
* Les données spectrales enregistrées au cours de la coagulation du lait par la présure, acidification + présure et acidification [25] ont été re-analysées par ACP dont les résultats permettent de tracer les vecteurs propres 1 et 2.

measurements [5, 18, 22, 37, 38] agreed well with their fluorescence data. It appears that the fluorescence of protein tryptophan recorded by front face fluorescence spectroscopy is a valuable tool to investigate the changes in the protein network structure and protein/water interactions induced by pH alterations.

The spectral pattern for component 2 (Fig. 5) indicated that the shape of fluorescence spectra is larger for M1 cheeses than for M2 ones. These spectroscopic differences result from different protein-protein interactions and different network structures. They are the consequences of the processes used to manufacture the cheeses. This assumption is in agreement with the result obtained on coagulums [25]: the shapes of the spectral patterns for component 2 for coagulums (Fig. 6) and cheeses (Fig. 5) are similar. Considering the rennet, acid-rennet and acid coagulation kinetics, the principal component 2 discriminated the spectra as a function of gelation [25]. The shape of the spectra is sharper after 300 min coagulation than after 100 min. For cheeses, the shape of M1 cheese spectra is larger than M2 ones indicating that the structures of the protein networks are different for the two types of cheese and depend on the manufacturing processes. This hypothesis has been confirmed by the results of the texture analysis performed on the protein network images of M1 and M2 cheeses [23].

Although fluorescence spectroscopy is a technique whose theory and methodology have been extensively exploited for studies of both chemistry and biochemistry, the utility of fluorescence spectroscopy for molecular studies has not been yet fully recognized in food science. Fluorescence spectroscopy has the same potential to address molecular problems in food science as in biochemical field science, because the scientific questions that need to be answered are closely related.

4. CONCLUSION

Fluorescent properties of fluorophores are very sensitive to changes in their environment [34]. Using front face fluorescence in combination with chemometric methods, we have developed methods allowing the identification of cheeses and the characterisation at the molecular level of dairy products. The tryptophan fluorescence spectrum of a cheese, as well as its vitamin A fluorescence spectrum, are fingerprints that allow identification of the cheese. We have also shown that the different textures among investigated cheeses are related to the unique molecular structure of each cheese resulting from the process and the ripening time. This rapid technique based on surface fluorescence of the product may also have broad application in the field of authentication.

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