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Potentiality of spectroscopic methods for the characterisation of dairy products. I. Front-face fluorescence study of raw, heated and homogenised milks

E Dufour, A Riaublanc

Laboratoire d'étude des interactions des molécules alimentaires, Inra, BP 71627, 44316 Nantes cedex 03, France

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Summary — The fluorescence spectra of raw (NHO), heated (NHP), homogenised (HOM) and homogenised + heated (HOP) milks were recorded using a variable angle front-surface accessory. The emission fluorescence spectra of tryptophans in proteins, vitamin A and anilino-naphthalene sulfonic acid and the excitation fluorescence spectra of vitamin A and anilino-naphthalene sulfonic acid were collected. The spectra showed that the treatments applied to milk induced changes in the fluorescence characteristics of the probes. Principal component analysis was applied to the normalised fluorescence spectral data in order to distinguish between milk samples. It was shown that the map defined by principal components 1 and 2 discriminated NHO, NHP, HOM and HOP samples as a function of homogenisation and heating, respectively. The potential of fluorescence spectroscopy in combination with a chemometric method to discriminate between heated and homogenised milk samples was demonstrated.

milk / protein / vitamin A / front face fluorescence / multivariate analysis

Résumé — Intérêts des méthodes spectroscopiques pour la caractérisation des produits laitiers. I. Utilisation de la fluorescence frontale pour la caractérisation de laits natif, chauffé et homogénéisé. Les spectres de fluorescence de lait natif (NHO), chauffé (NHP), homogénéisé (HOM) et homogénéisé + chauffé (HOP) ont été enregistrés au moyen d'un accessoire de fluorescence frontale. Les spectres d'émission de fluorescence des tryptophanes, de la vitamine A et de l'acide anilinonaphtalène sulfonique, ainsi que les spectres d'excitation de la vitamine A et de l'acide anilinonaphtalène sulfonique, ont été enregistrés. Les traitements appliqués au lait induisent des modifications dans les spectres de fluorescence. L'analyse en composante principale a été appliquée sur les spectres de fluorescence normés. La carte factorielle 1-2 permet de séparer les échantillons en fonction du traitement appliqué au lait.

lait / protéine / vitamine A / fluorescence frontale / analyse multivariée

INTRODUCTION

Fluorescence spectroscopy, a very sensitive technique, has been used for a long time as a powerful analytical tool in many chemical, biochemical and environmental studies. The purpose of these studies is to find pairs of emission-excitation wavelengths with maximum intensity or to record fluorescence emission and excitation spectra. Fluorescence spectroscopy provides information on the presence of fluorescent molecules and their environment in biological samples. For instance, fluorescence properties of aromatic amino acids of proteins (Longworth, 1971; Lakowicz, 1983; Dalgalarrondo et al, 1992; Dufour et al, 1994), retinol (Dufour and Haertlé, 1990) or extrinsic fluorescent probes added to the sample can be used to study protein structure and protein-hydrophobic molecule interactions (Dufour et al, 1994).

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 (Fox, 1989). 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 (Lakowicz, 1983). Milk retains also fat-soluble vitamins such as vitamins A, D, E and K. Vitamin A occurs in more than one form but is generally found as retinol. Because of its alcohol group, retinol readily forms esters. In milk, almost all the vitamin occurs in the palmitate or acetate ester forms. Vitamin A (about 1 μmol/L in bovine milk) is located in the core and in the membrane of the fat globule (Hartman and Dryden, 1978). Due to its conjugated double bonds, retinol is a good fluorescent probe with excitation and emission wavelengths at about 330 and 450 nm, respectively. The fluorescence properties of retinol change as a function of the environment. A very weak fluorescence is observed for aqueous solution of retinol, but its quantum yield is drastically enhanced in an apolar environment (Dufour et al, 1994).

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 (Genot et al, 1992a). To avoid these problems, an alternative method, frontal illumination fluorescence spectroscopy, has been developed (Parker, 1968). Front face fluorescence allows investigation of the fluorescence of powdered, turbid and concentrated samples. The method has been used to quantitatively determine hemoglobin in undiluted blood (Blumberg et al, 1980), to study hemoglobin R->T transition kinetics (Hirsch and Nagel, 1989) or proteins in wheat gluten (Genot et al, 1992b). However, literature searches encountered very few papers dealing with the application of front face fluorescence in the characterisation of food products. This could be explained by the fact that food products are complex products containing numerous fluorescent compounds. In such a case the signals of the different chromophores may overlap and, for example, it becomes difficult to predict the concentration of one particular compound. However, fluorescence spectroscopy in combination with multivariate statistical methods has been used for predicting the concentrations of two component synthetic mixtures (Lindberg et al, 1983).

Milk is a complex product exhibiting simultaneously emulsion, colloidal and solution phases. Various chemical and spectro-

scopic methods have been used to characterise milk samples. Tedious and time-consuming chemical methods are more and more often replaced by more rapid and non-invasive spectroscopic methods. In this paper, the focus is mainly on the study of milk intrinsic fluorophores in order to record fluorescence spectra of 'real' milk samples and to discriminate between four milk samples (raw, homogenised, heated and homogenised + heated milks) by applying principal component analysis to the whole fluorescence spectra. The study serves as a general investigation of how to enhance the potential of fluorescence spectroscopy by statistical methods, as well as an investigation of how front face fluorescence spectroscopy in combination with other methods can be used to characterise food products and processes.

MATERIALS AND METHODS

1-anilinonaphthalene-8-sulfonic acid (ANS) was from Sigma. ANS stock solution (10 mmol/L) was prepared in methanol.

Milk samples

Raw pooled milk (10 L), provided by a dairy plant, was divided in four parts. Two of them were homogenised in a two-stage laboratory homogeniser (25 and 4.5 MPa). Then one raw and one homogenised milk sample were heated in a water bath during 20 min at 70 °C. Samples were coded NHO, NHP, HOM and HOP for raw, heated, homogenised and homogenised + heated milks, respectively.

Washed creams were extracted from an aliquot of each sample by centrifugation in high density medium as described by Patton and Huston (1986).

Fat globule size

A Malvern Mastersizer (Malvern Instruments Ltd, Malvern, UK) with optical parameters defined by the manufacturer's presentation code 0505 was used to determine the fat globule-size distribution, the weight-average diameters (d_{43}), the volume-surface average diameter (d_{32}) and the total interfacial area per unit volume of milk. The measures were made in triplicate. Water was used to disperse the milk.

Electrophoresis

Proteins adsorbed at the oil/water interface were characterised and quantified by electrophoresis for the four samples. 150 μL of washed cream was mixed with 50 μL of dissolution buffer (Tris-HCl 150 mmol/L pH 7, SDS 12%, mercaptoethanol 6%, glycerol 30%, Serva blue G 0.05%) and heated for 5 min at 90 °C. This stage broke emulsion and desorbed proteins in the water phase. Proteins were then separated on a 20% homogeneous acrylamide gel according to Laemmli (1970). Protein bands were stained by Coomassie blue and quantified using a Bio Profil densitometer (Vilber Lourmat, Marne la Vallée, France). Purified α-lactalbumin was used on the gel as internal standard for quantification. Lipid yields in creams were measured gravimetrically after solvent extraction and drying. Results were expressed in μg of adsorbed protein by mg of lipids.

Fluorescence spectroscopy

Fluorescence spectra were recorded using a SLM 4800C spectrofluorimeter (Bioritech, Chamarande, France) mounted with a variable angle front-surface accessory. The incidence angle of the excitation radiation was set at 56 ° to ensure that reflected light, scattered radiation and depolarisation phenomena are minimised. Emission and excitation spectra (resolution: 1 nm, averaging: 10) were recorded at 22 °C with emission and excitation slits set at 4 nm. All spectra were corrected for instrumental distortions in excitation using a rhodamine cell in reference channel. The spectrum of each sample was recorded six times using different aliquots. When ANS fluorescence was considered, 10 μL of ANS stock solution were added to the milk samples (1 mL) and the final concentration of ANS in the cuvette was 0.1 mmol/L.

The emission spectra of tryptophan (305–400 nm), vitamin A (350–500 nm) and ANS (400–600

nm) were recorded with excitation wavelengths set at 290, 321 and 370 nm, respectively and the excitation spectra of vitamin A (260–350 nm) and ANS (250–450 nm) were recorded with emission wavelengths set at 410 and 466 nm, respectively.

For washed creams, the emission spectra of protein tryptophans were recorded for the four samples (six times for each sample).

Mathematical processing and principal component analysis

In order to reduce scattering effects, the data have been normalised by reducing the area under each spectrum to a value of 1 according to the formula (Bertrand and Scotter, 1992):

$$c_i = F_i / norm \quad (1)$$

and

$$norm = \sqrt{\sum_{j=1}^{n} Fj^2} \quad (2)$$

where c_i is the corrected value at wavelength i, F_i is the fluorescence intensity at emission wavelength i. Fj is the fluorescence at wavelength j and n is the number of data points for each spectrum.

Principal component analysis (PCA) was applied to the normalised data. PCA is a multidimensional statistical method which optimises the description of the data with a minimum loss of information (Jolliffe, 1986). From a data set, PCA assesses principal components and their corresponding eigenvectors. The principal components are used to draw maps that describe the physical and chemical variations observed between the samples and make it possible to study them without any calibration step (Bertrand et al, 1987). Moreover, the eigenvectors are homologous to spectra and are called spectral patterns. Both positive and negative peaks of the spectral pattern can be interpreted as characteristic emission or excitation wavelengths of chemical constituents.

The PCA software was written by D Bertrand (LTAN, Inra, Nantes, France) and is described elsewhere (Bertrand et al, 1987).

RESULTS AND DISCUSSION

Fluorescence spectra of milks

A milk sample was poured into a 1 × 1 cm cuvette and the cuvette was placed in a variable angle front-surface accessory set at 56°. Actually, the pathlength of the cuvette does not matter for front face fluorescence since it is the fluorescence of the surface of the sample which is investigated (Genot et al, 1992a): all the excitation photons are absorbed within the first few micrometers. Figure 1 shows the unprocessed excitation and emission fluorescence spectra of raw (NHO), pasteurised (NHP), homogenised (HOM) and homogenised + pasteurised (HOP) milks. Considering the intrinsic fluorescence of proteins, tryptophans were excited at 295 nm and the emission spectra were recorded between 305 and 400 nm. The greatest difference was observed between homogenised and non-homogenised samples: following homogenisation, the emission fluorescence intensity increased by 6% (fig 1A). It appeared also that the tryptophan fluorescence intensities of heated samples (NHP and HOP) were slightly lower than those of non-heated milks (NHO and HOM). All the maxima of tryptophan emission peaks, except that of NHO sample (333 nm), were located at 332 nm. The excitation and emission fluorescence spectra of vitamin A for NHO, NHP, HOM and HOP samples are shown in figure 1B. The excitation spectra were characterised by a maximum located at 322 nm and two shoulders. The locations of the shoulders depended on the applied treatment. The first shoulder was observed at 292 nm for the homogenised milks and at 293 nm for non-homogenised samples. The locations of the second shoulder were 308, 309, 308 and 307 nm for NHO, NHP, HOM and HOP milks, respectively. The shapes of the spectra were overall similar, varying mainly in the maximum/shoulders intensity ratios. In the vitamin A emission spectra, the fluo-

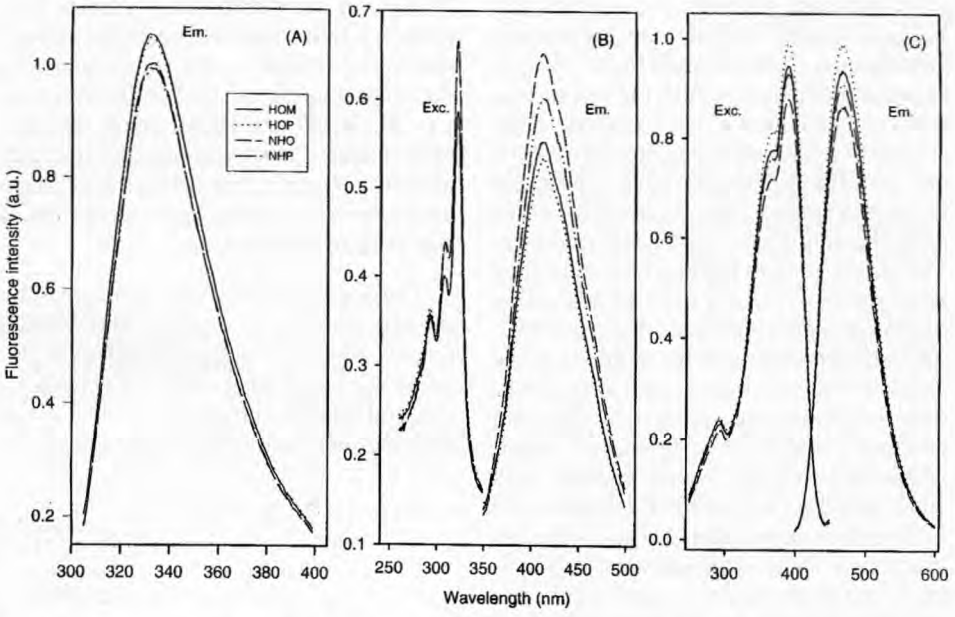

Fig 1. Fluorescence spectra of NHO, NHP, HOM and HOP milk samples. **A.** Tryptophan emission spectra. **B.** Excitation and emission spectra of vitamin A. **C.** Excitation and emission spectra of ANS. Exc, excitation spectrum; Em, emission spectrum. See *Materials and methods* for details.

*Spectres de fluorescence des échantillons NHO, NHP, HOM et HOP. **A.** Spectres d'émission des tryptophanes. **B.** Spectres d'excitation et d'émission de la vitamine A. **C.** Spectres d'excitation et d'émission de l'ANS. Exc: spectre d'excitation; Em: spectre d'émission. Se reporter à* « Materials and methods » *pour les détails.*

rescence intensities showed, however, larger differences. The highest fluorescence intensity was observed for NHO milk and treated milks were characterised by lower retinol fluorescence: the order was NHO > NHP > HOM > HOP. In addition, the emission maxima were at 412 nm and 413 nm for non-homogenised and homogenised milks, respectively. In addition to these intrinsic probes, an extrinsic fluorophore (ANS), was used in this study. ANS is well known to bind specifically to the hydrophobic pockets of proteins (Matarella and Richardson, 1983). Excitation and emission spectra of ANS added to the various milks samples are shown in figure 1C. In both excitation and emission spectra, the fluorescence yield increased in the order: NHO < NHP < HOM < HOP. The maxima for the emission spectra were observed at 468, 467, 466 and 467 nm for NHO, NHP, HOM and HOP samples, respectively. In fluorescence excitation, the spectra were characterised by a maximum located at about 388 nm and two shoulders. The shoulder closest to the maximum was located at 367 nm for all the samples. The second one was observed at 292, 294, 290 and 291 nm for NHO, NHP, HOM and HOP milks, respectively.

Characterisation of milk fat globule surface and size

The treatments applied to milk are well known to modify protein structure, fat glo-

bule shape and protein/lipid interactions. Indeed, heating may denature proteins. Denaturation is characterised by the changes of protein structure modifying tryptophan quantum yield, and by the exposure to the surface of hydrophobic regions where ANS can bind. Homogenisation breaks up fat globules into smaller ones, increasing drastically the area of the lipid/water interface. The stabilisation of the interface created by homogenisation results from the adsorption of proteins at the interface (Walstra, 1995). The adsorption of proteins at the interface changes their structure, as well as their binding and fluorescence properties (Castelain and Genot, 1994). In addition, homogenisation should modify retinol quantum yield since the structures of the fat globule and of its membrane are altered. The differences observed in the reported spectra clearly indicate that the physical treatments applied to the milk modified the characteristics of the fluorescent probes investigated. The opposite trends of tryptophan and ANS quantum yields upon heating suggest that the thermal treatment of the samples partly denatured the milk proteins. Based on the fluorescence data only, it appears more difficult, however, to explain, at a molecular level, the effects of homogenisation on milk components. In this case, the modification of fat globule size may also perturb light scattering and induce changes in the fluorescence spectra which would not be related to the quantum yield of the probes. In order to get more explanations on the observed fluorescence modifications of milk samples following physical treatments, tryptophan fluorescence of washed creams, fat globule size and the kind of proteins adsorbed at the surface of the fat globule were studied.

Figure 2 shows tryptophan emission spectra of the washed creams of the four milk samples. It appeared that NHO displayed the weakest fluorescence intensity. The proteins associated with the native membrane of the fat globule are found in low amounts (Walstra, 1995). After heating of raw milk, the fluorescence intensity doubled, suggesting that heat treatment induces binding of proteins to the fat globules. As shown in figure 2, the highest fluorescence was observed for the HOM sample. The dramatic increase of the fluorescence intensity at about 330 nm indicates that the interface created by the homogenisation of milk is stabilised by proteins.

These assumptions were confirmed by the electrophoresis study of the washed creams. Electrophoresis of purified fat globules is a convenient method to characterise and quantify proteins adsorbed at the oil/water interface (Sharma and Dalgleish,

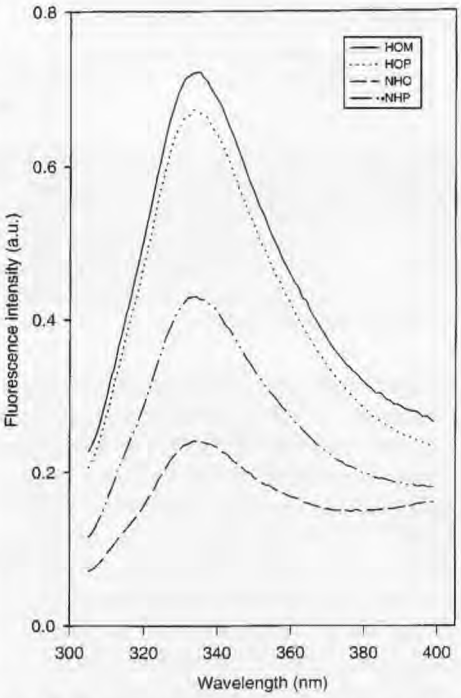

Fig 2. Tryptophan fluorescence spectra of the creams prepared from NHO, NHP, HOM and HOP milk samples. See *Materials and methods* for details.

Spectres de fluorescence des tryptophanes des crèmes provenant des échantillons de lait NHO, NHP, HOM et HOP. Se reporter à « Materials and methods » *pour les détails.*

Table I. Protein yield bound to the fat globule.
Quantité de protéine fixée sur le globule gras.

	Protein (μg/mg of lipids)				
	β + κ-caseins	*α-casein*	*α-lactalbumin*	*β-lactoglobulin*	*total*
NHO	0	0	0	0	0
NHP	8.4	5.6	4.7	0	18.7
HOM	25.0	8.8	6.5	0	40.3
HOP	48.6	19.3	7.1	6.4	81.4

NHO, raw milk; NHP, heat treated milk; HOM, homogenised milk; HOP, homogenised + heated milk.
NHO, lait cru; NHP, lait chauffé; HOM, lait homogénéisé; HOP, lait homogénéisé et chauffé.

1993). Data reported in table I indicate that no casein, nor whey proteins, were adsorbed at the surface of raw-milk fat globule. This is in agreement with the literature. Upon homogenisation, caseins adsorbed preferentially at the lipid/water interface. In this case, bound α-lactalbumin accounted for 16% of the total interfacial proteins. Heat treatment also induced the interaction of proteins with the fat globules. The amount of bound proteins (per mg of lipids) was twice as low for heated raw milk than for homogenised milk. Considering HOP samples, a synergistic effect of technological treatments (homogenisation and then heating) was observed (table I). The data reported in table I are somewhat different from the data found in the literature (McPherson et al, 1984; Houlilan et al, 1992a, b; Kim and Jimenez-Flores, 1995). The main differences affect the amounts of β + κ-caseins and α-caseins which should be about the same. The other concern is the lack of β-lactoglobulin in NHP and HOM cream samples. But one should remark that there is no general agreement in the literature about the amount of protein bound at the fat globule interface. In fact, the nature of proteins adsorbed at the interface depends greatly on the heating temperature of the milk (see below). The electrophoresis results however show that more proteins bind to the fat globule during homogenisation than heating (table I). The differences in the amount of bound proteins as a function of the applied treatment suggest that the fluorescence spectra of the different milk samples should exhibit different shapes. For example, it is well known that the fluorescence properties (maximum wavelength, quantum yield) of proteins depend on their environment (Lakowicz, 1983).

There are several reasons that can explain the adsorption of proteins to the fat globule surface following the heat treatment. They may result from the denaturation of proteins, the changes in casein micelle structure or from the higher fluidity of the fat globule phospholipids at 70 °C. Indeed, the sol-to-gel transition of fat globule phospholipids occurs at about 30 °C and the binding of proteins to phospholipids bilayers is enhanced for lipids in gel phase (Subirade et al, 1995). It is generally claimed that all the milk proteins, except β-lactoglobulin, are not denatured at 70 °C. It has been reported that, at pH 7, β-lactoglobulin dilute solution denatures at 73 °C (Kella and Kinsella, 1988), a temperature slightly higher than the temperature applied to the samples in this study. In addition, it is well known that the temperature of β-lactoglobulin denaturation depends on the protein environment. Anema and McKenna (1996) showed that

β-lactoglobulin in reconstituted whole milk was not or slightly denatured at 70 and 75 °C. On the contrary, it was reported that α-lactalbumin is partly denatured at 70 °C (Anema and McKenna, 1996). Whatever the conditions studied, electrophoresis data showed, however, that β-lactoglobulin bound weakly to the fat globule. This protein was detected at the lipid/water interface only for HOP sample. It suggests that β-lactoglobulin is not denatured by most of the treatments investigated in this study. It is generally assumed that native β-lactoglobulin does not adsorb to the interface and that only denatured β-lactoglobulin interacts with the fat globule membrane (McKenzie et al, 1972; Sharma and Dalgleish, 1993). It has also been reported that β-lactoglobulin interacted with milk fat globule membrane after heating of the milk at 87 °C, but only slightly after heating at 72 °C (Kim and Jimenez-Flores, 1995).The major proteins found at the fat globule surface were caseins. These flexible and hydrophobic proteins move easily to the lipid/water interface upon heating and homogenisation of the milk.

The results of the study of fat globule size are shown in table II. The weight-average diameters (d_{43}) of raw and pasteurised milks were similar. Homogenised samples showed lower d_{43} ranging between 1.3 and 0.85 μm. On the one hand, the decrease of the fat globule size induced an increase of the interfacial area. The specific surface area was three times larger for homogenised samples than for raw milk. This interface created by the homogenisation of milk was stabilised by the adsorption of amphipathic proteins, such as caseins. On the other hand, the change of the fat globule size might modify the light scattering and could partly be responsible for the differences observed in the fluorescence spectra. Nevertheless, the scattering effect alone cannot explain all the modifications reported in the fluorescence spectra. Indeed, non-heated and heated samples showed different fluorescence spectra but similar sizes for the fat globules.

Table II. Fat globule properties determined with the granulometer.

Caractéristiques des globules gras déterminée au moyen d'un granulomètre.

	$d_{4,3}$ (μm)	$d_{3,2}$ (μm)	*Sp S A (m^2/cc)
NHO	6.02	2.14	2.8
NHP	5.84	1.88	3.2
HOM	1.3	0.64	9.3
HOP	0.85	0.61	9.85

NHO, raw milk; NHP, heat treated milk; HOM, homogenised milk; HOP, homogenised + heated milk. * Sp, SA, the total interfacial area per unit volume of the fat contained in the milk.

*NHO, lait cru; NHP, lait chauffé; HOM, lait homogénéisé; HOP, lait homogénéisé et chauffé. * Sp, SA, surface interfaciale totale par unité de volume de la matière grasse contenue dans le lait.*

The characterisation of the creams by fluorescence, electrophoresis and granulometry indicated that the size and the surface of fat globule, as well as the structure of proteins, was modified by homogenisation and heating of milk samples.

Multivariate analysis of milk fluorescence spectra

The data show that the fluorescence spectra of proteins, fat-globule vitamin A and ANS are different for NHO, NHP, HOM and HOP samples. The applied treatments modify the environments of the fluorophores and, consequently, their fluorescence properties (quantum yield, anisotropy and lifetime) (Lakowicz, 1983). Despite most of the fluorescence studies only focus on emission spectra, it can be interesting to consider excitation fluorescence spectra too. Principal component analysis, a multivariate technique, was applied to the full emission or excitation fluorescence spectra data in order to discriminate between the milk samples. This method is well suited to optimise the

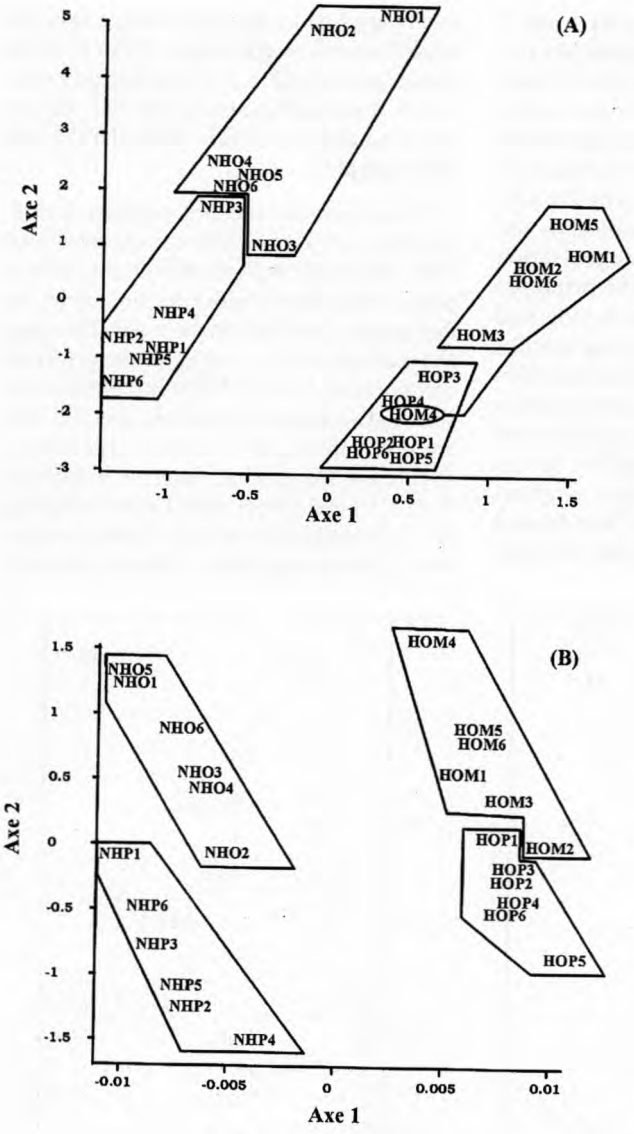

Fig 3. PCA similarity map defined by the principal components 1 and 2 for (**A**) tryptophan emission and (**B**) vitamin A excitation fluorescence spectral data. Each label corresponds to a spectrum. See *Materials and methods* for details.

*Carte factorielle 1-2 de l'analyse en composantes principales réalisée sur les spectres d'émission des tryptophanes (**A**) et d'excitation de la vitamine A (**B**). Chaque étiquette correspond à un spectre. Se reporter à* « Materials and methods » *pour les détails.*

description of the data collection with a minimum loss of information.

As suggested earlier, the distinct fat-globule sizes of the samples may induce differences in the fluorescence intensities. In order to reduce the scattering effect, the spectra were normalised using equation 1 (Bertrand and Scotter, 1992): the area under each spectrum was reduced to a value of 1. In this way, only the shapes of the spectra were considered in the analyses. PCA was applied separately on the three collections of 24 normalised spectra corresponding to tryptophan emission fluorescence, vitamin A excitation fluorescence and ANS excitation fluorescence. For the PCA results, the maps

defined by principal components 1 and 2 for tryptophan emission and vitamin A excitation fluorescence data are shown in figure 3A and 3B, respectively. The first two principal components took into account 96.9% (tryptophan data) and 99.3% (vitamin A data) of the total variability. For the two data collections, a discrimination of the samples as a function of homogenisation was observed according to the principal component 1: non-homogenised milks had negative scores, whereas homogenised milks showed positive scores. For principal component 2, a discrimination of the samples as a function of heat treatment was observed for both data collections: negative scores are observed for heated samples, whereas positive scores characterised non-heated samples. These results indicate that the treatments applied to the milk induce specific modifications in the shape of the fluorescence spectra and that tryptophan and vitamin A fluorescence spectra allow to discriminate between NHO, NHP, HOM and HOP samples.

Moreover, the spectral patterns corresponding to the principal components provide information about the characteristic peaks which are the most discriminating for the samples observed on the maps. The spectral patterns corresponding to the principal components 1 and 2 for tryptophan emission and vitamin A excitation spectral data are given in figures 4 and 5, respectively. In figure 4, the spectral pattern 1, associated with principal component 1 discriminating the tryptophan fluorescence spectra according to homogenisation, showed an oppo-

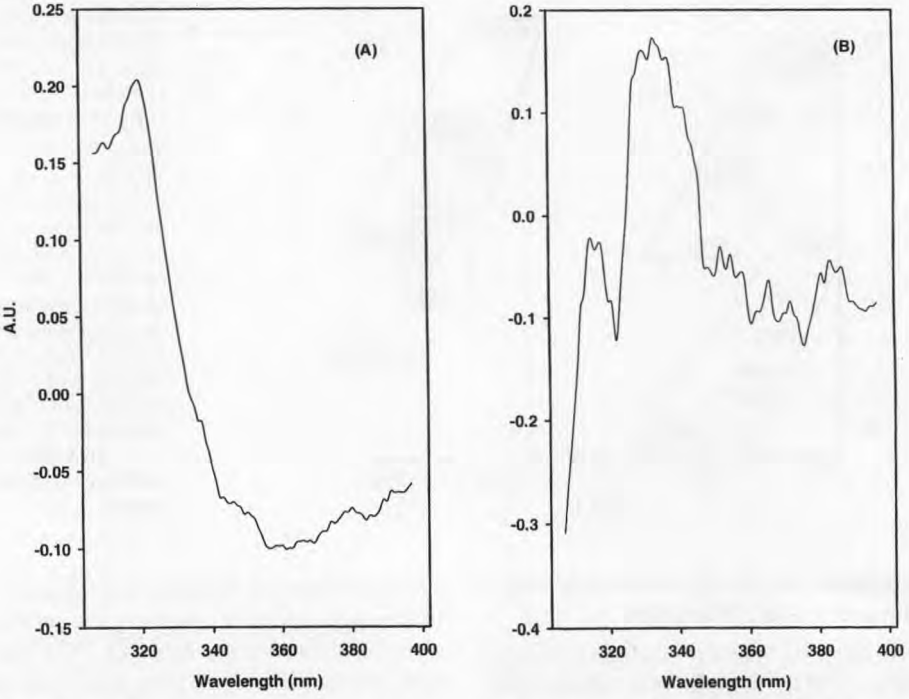

Fig 4. Spectral patterns corresponding to the principal component 1 (**A**) and 2 (**B**). Fluorescence emission spectra of tryptophans.

*Vecteurs propres 1 (**A**) et 2 (**B**) des analyses en composantes principales réalisées sur les données spectrales correspondant aux spectres d'émission de fluorescence des tryptophanes.*

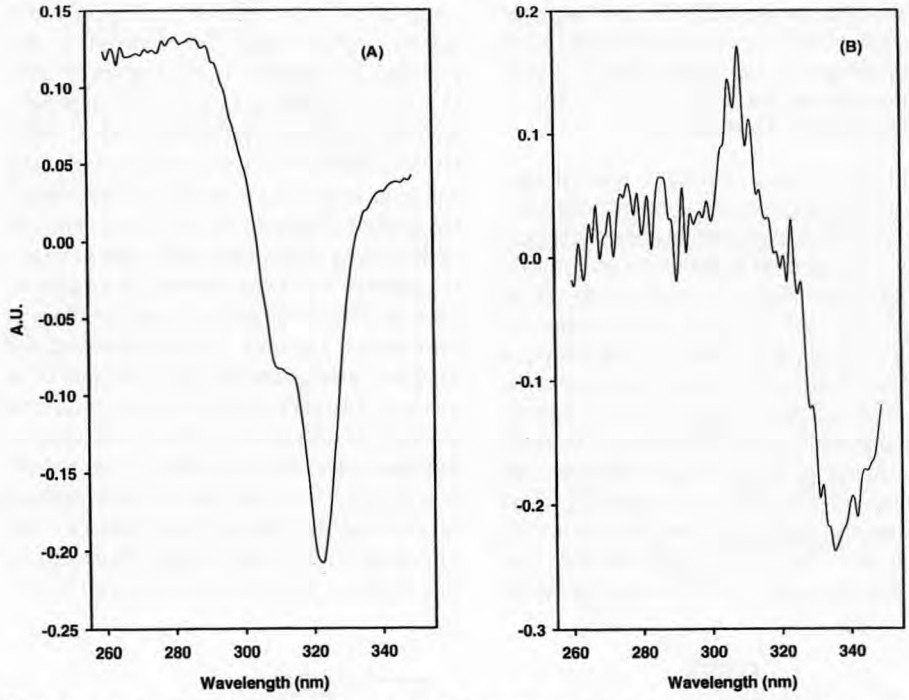

Fig 5. Spectral patterns corresponding to the principal component 1 (**A**) and 2 (**B**). Fluorescence excitation spectra of vitamin A.

*Vecteurs propres 1 (**A**) et 2 (**B**) des analyses en composantes principales réalisées sur les données spectrales correspondant aux spectres d'excitation de fluorescence de la vitamine A.*

sition between a negative peak at 360 nm and a positive one at 320 nm: a blue shift of the maximum emission of proteins was induced by homogenisation. It confirms that the environment of the tryptophans of proteins adsorbed at the surface of the fat globule becomes more apolar and that the quantum yield of tryptophans is increased (fig 1A). The spectral pattern 2 is noisy and more difficult to analyse. It however exhibits a positive maximum at 333 nm suggesting that the spectra of the heated samples are somewhat larger than the non-heated one. For the vitamin A data shown in figure 5A, the spectral pattern of the first principal component presented an opposition between a large positive band at about 290 nm and a negative peak (and a shoulder) at 322 nm. The positions of these three peaks correspond to the maximum and the two shoulders described above for the excitation spectra of vitamin A (fig 2B). Spectral pattern 1 also indicated that 322 nm/290 nm intensity ratios were modified by homogenisation. Spectral pattern 2 was noisy, but it showed two well defined peaks: a maximum at 310 nm and a minimum at 335 nm (fig 5B). This opposition between the two bands suggests that a red shift of the maximum emission located at about 322 nm occurs in the spectra upon heating of the milk samples.

The examination of the map defined by principal component 1 and 2 corresponding to the principal component analysis of ANS normalised spectra showed no discernible pattern according to the applied treatments (data not shown). Despite the differences observed in ANS fluorescence intensity for

the four milk samples (fig 1C), the shapes of the spectra are overall similar since, after normalisation, it was impossible to discriminate between the samples according to homogenisation and heating.

In a second step, the three spectral collections (tryptophan emission, retinol excitation and ANS excitation fluorescence spectra) were gathered together in one matrix and this new table was analysed by PCA. The aim of this approach was to improve the discrimination of the samples using a number of different fluorescence spectra. The PCA similarity map defined by principal components 1 and 2 is shown in figure 6. The first two principal components took into account 98.45% of the total variability. This map and the maps described above for tryptophan and vitamin A fluorescence data showed similar trends: a discrimination of the samples as a function of homogenisation and heating was observed according to the principal component 1 and 2, respectively. In addition, the comparison of the maps showed that the four populations, NHO, NHP, HOM and HOP, are better discriminated using the large data matrix than considering tryptophan or vitamin A data alone. The use of ANS data in this study also appeared useful (data not shown). Indeed, the addition of ANS data to the matrix made up of tryptophan and vitamin A data improved the discrimination of the samples, although PCA analysis of ANS excitation spectra alone showed no discernible pattern according to heating and homogenisation. The results demonstrate that the fluorescence spectra recorded reflect the physico-chemical characteristics of the milk sample. The analysis of combined fluorescence spectra (trypto-

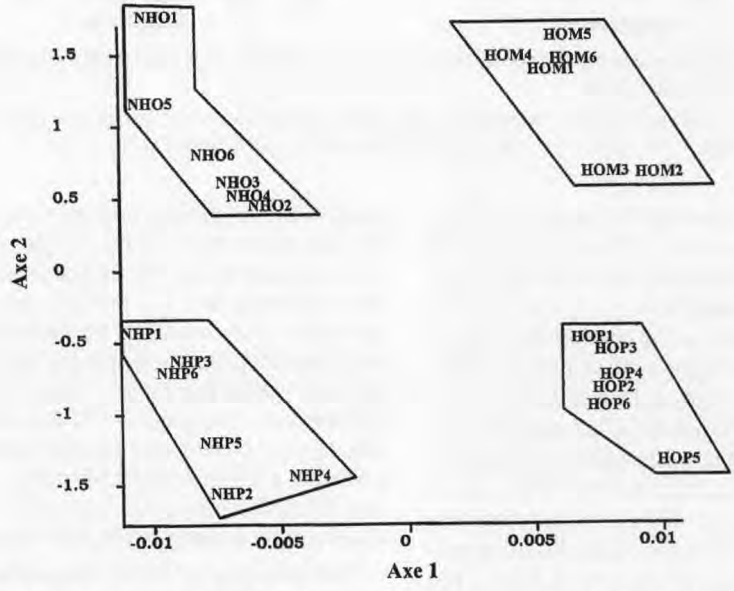

Fig 6. PCA similarity map defined by the principal components 1 and 2 for the data table including tryptophan emission, retinol excitation and ANS excitation spectra. Each label corresponds to a spectrum. See *Materials and methods* for details.

Carte factorielle 1–2 de l'analyse en composantes principales réalisée sur le tableau de données contenant les spectres d'émission des tryptophanes, d'excitation de la vitamine A et d'excitation de l'ANS. Chaque étiquette correspond à un spectre. Se reporter à « Materials and methods » *pour les détails.*

phan, vitamin A and ANS), recorded on the same sample, with chemometric methods should allow the determination of the physical treatments (homogenisation and/or heating) applied to milk.

Front face fluorescence is a very useful technique to record the excitation and emission spectra of powdered, turbid and concentrated samples. The potential of fluorescence spectroscopy in combination with chemometric methods to discriminate between milk samples has been demonstrated. A search of the literature showed that there are very few papers reporting the use of fluorescence to characterise food composition or process (Norgaard, 1996; Novales et al, 1996). Fluorescence, a sensitive and rapid technique, could, however, be used for the development of fast at-line or on-line analyse methods in the food industry (Marangoni, 1992). The development of fluorescence analytical tools implies, firstly, the characterisation of the fluorescence properties of the probes, ie, excitation and emission maximum wavelengths and, secondly, the recording of excitation and emission spectra. Multivariate statistical methods applied to spectra are useful to describe variations between samples and to derive from the spectral patterns the characteristic wavelengths of the samples. In general, spectrometric methods and, in particular, front face fluorescence, in combination with multivariate statistical analyses, have a huge potential in the development of fast analyses and in the quality control applications to food systems.

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