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Evaluation of the Structural Changes of Cantal Cheese Throughout Ripening by Synchronous Fluorescence Spectroscopy and Rheology Methods

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ABSTRACT

The compositional, physical (colour and texture) and structural changes of 12 samples of Cantal cheese representative different ripening period (30, 120 and 200 days) were evaluated by chemical, rheology and synchronous fluorescence spectroscopy (SFS) methods. Synchronous fluorescence spectra were recorded on cheese samples from 250 to 500 nm with offset Δλ = 80 nm followed by a classification of samples using principal component analysis (PCA) and factorial discriminant analysis (FDA). All the compositional characteristics of Cantal cheese increased significantly (P<0.05) over ripening, except for the decrease in calcium and moisture contents. Proteolysis was the most important biochemical changes of Cantal cheese during ripening as revealed from the increase in the water soluble/total nitrogen ration (WSN/TN %). The watersoluble nitrogen to total nitrogen ratio increased significantly during the ripening period. The changes in the rheological characteristics and colour values reflected the biochemical changes in Cantal cheese. The G', G", tan δ and η* values of cheese increased significantly as the ripening processes, but exhibited an opposite trend over 120 days as compared to 200 days. Ripening led to a decrease of L* and b* values and a slight increase in a* value. The change in the fluorescence intensity at 29, 322 and 355 nm reflects the physicochemical changes of cheese matrix and, in particular, structural changes in the protein network throughout ripening period. The spectral pattern associated with the first two PCs shows the importance of the band with a maximum at 295, 322 and 355 nm which are the most suitable for separating the spectra. PCA and FDA show that SF spectra of Cantal cheese are clearly separated and the correct classification of 100% was observed. These results suggest that SFS in combination with multivariate data analysis could be considered as a fingerprint, allowing a good characterization and classification of cheese based on their structural changes throughout ripening period.

Key words: Cantal cheese, Texture, Rheology, Structure, Colour, synchronous fluorescence spectroscopy, chemometrics.

Introduction

Cantal cheese is a hard-uncooked, pressed cheese variety granted the status of a Protected Denomination of Origin (PDO) by European Commission and produced in the Auvergne region in France, with an annual production of 19 000 T (CNIEL, 2009). Its making process is very similar to Cheddar cheese. It is made from either raw or pasteurized cow’s milk and commercialized as “young” (ripened for at least one month), “between the two” (ripened for 2 to 6 months) or “old” (ripened for over 6 months). Cantal cheese is characterized as a cylinder-shaped (round wheels) cheese with a dry crust; its weight ranges between 35 to 40 kg, 40 cm height, 36 to 42 cm diameter. The dry matter content and the Fat/dry matter ratio must be, respectively, at least 57% and 45% resp.

Quality attributes of food products are closely related to structure. Cheese structure can be described as protein units (mostly caseins) held together by physical forces with fat, and moisture (contains minerals, vitamins and organic acids) dispersed throughout this structure (David and Auty, 2008). Much of the major changes in cheese structure, which ultimately affects final quality, occur during ripening process. Cheese ripening is complex process of physical, chemical and microbiological changes affecting the principal components (i.e., protein, fat, carbohydrate...etc) of cheese matrix that affect the structure and texture of cheese.

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Texture is the primary quality attribute of cheeses: it is a reflection of cheese structure at the microscopic and molecular levels (Dufour et al., 2001).

In cheese factories, evaluation of ripening stage is carried by the cheese maker on the basis of limited measurements (pH and weight) as well as on the basis of visual and tactile examination. In addition, several analytical techniques have been developed to follow cheese ripening at the laboratory level. All these methods are relatively expensive, time-consuming, require highly skilled operators and are not easily adapted to on-line monitoring (Karoui and De Baerdemaeker, 2007). For this reason, there is a need to develop new methods which are rapid, non-destructive, relatively low-cost and monitoring the cheese ripening process.

Rheological properties obtained in the linear viscoelastic region are useful tools for the food industry. Elastic and viscous contributions to the internal structure of the cheese can be obtained performing oscillatory measurements (Konstance and Holsinger, 1992). Such studies provide an insight into the fundamental nature of the physical basis of food texture (Gunasekaran and Ak, 2000).

Synchronous fluorescence is a type of spectroscopy which detects so-called fluorophores, molecules with a structure that allows emission of light when relaxing to the ground state from an excited singlet state. Synchronous fluorescence spectrum recorded on a cheese sample following excitation between 250-500 nm (offset 80 nm) gave information on several intrinsic fluorophores founded in cheese and may be considered as a characteristic fingerprint which allows the sample to be identified (Boubellouta and Dufour, 2010). The best known fluorescent molecules in dairy products include: tryptophan residues of proteins, vitamin A and riboflavin, which all have been reported to be affected during structural changes in cheese (Dufour et al., 2001). Tryptophan fluorescence spectra is often used as a reference group for protein structure changes, binding of ligands and protein-protein interactions (Herbert et al., 2000). Moreover, using vitamin A excitation, as an intrinsic fluorescent probe, can also provide information on the physical state of triglycerides and protein–lipid interactions (Dufour et al., 2000). Riboflavin can be used for the evaluation of oxidative changes in processed cheese during storage (Wold et al., 2002).

The objective of this research were to evaluate changes in compositional (pH value, moisture, protein, fat, WSN/TN%, salt, Ca and ash contents) and physical (color and texture) characteristics of Cantal cheese throughout ripening process. And to evaluate the potential of synchronous fluorescence spectroscopy to follow the ripening phenomena of Cantal cheese. In order to discriminate between these cheeses in term of ripening period, the principal component analysis (PCA) and factorial discriminant analysis (FDA) were applied to synchronous fluorescence data.

Materials and Methods

Cheese Samples:

Twelve samples of Cantal cheese varying in ripening period (30, 120 and 200 days) were supplied by two different cheese-manufacturing plants location in the Auvergne region in France. Samples (weighting 2-3 kg.) were cut off in the middle of the cheese height at 2 cm from the rind. About 900 g was grated and thoroughly homogenized for physico-chemical, rheological and synchronous fluorescence analysis.

Physicochemical Analysis:

pH values were measured by a pH meter (Schott, CG840, Paris, France) after grating 10 g of cheese and dispersing it in 50 ml of ionized water. The moisture content was determined by desiccation at 105°C for 24 h, and fat content was measured by Gerber method according to French standards (AFNOR, 2004). The total nitrogen was determined by Kjeldahl method (FIL-IDF standard 20B; IDF, 1993). Cheese extract for water-soluble nitrogen (WSN) was prepared according to (Bouton et al., 1994). Briefly, 3 g of cheese was homogenized with 50 ml of distilled water for 5 min with a laboratory blender (Stomacher MIX 1, AES Laboratoire, Combourg, France) and the resulting homogenate was maintained for 1 h in a water bath at 40°C. The insoluble material was centrifuged at 1200 for 30 min. at 4°C. The supernatant was filtered through glass wool, and nitrogen content was determined on a filtrate aliquot by (Kjeldahl method IDF, 1993). The salt content of cheese was determined according to French standard (AFNOR: NF ISO 5843) using an automatic titrator (TitroLine easy, Model III, Schott, France) which is based on Volhard titrimetric test according to (Marchall method IDF, 2003). The ash content was determined after incineration of a sample (5 g) in a muffle furnace at 550°C for 6 h. The total calcium of cheese was measured by using an atomic absorption spectroscopy as described by (IDF, 2003). All analyses were done in triplicate and the results reported as mean ± standard deviation.
Colour Measurements:

Cheese colour was measured using a colorimeter CR-400 (Konica Minolta, Tokyo, Japan). The $L^*$, $a^*$, and $b^*$ colour measurements were determined according to the CIELAB colour space (CIE, 1976) with reference to $D_65$ (natural daylight, the colour warmth of 6500K) and observation angle 10°. The following parameters were determined; $L^*$ (lightness or whiteness; $L^*$=0 for black and $L^*$=100 for white colour), $a^*$ (red-green components, $-a^*$=greenness and $+a^*$= redness) and $b^*$ (yellow-blue components, $-b^*$=blueness and $+b^*$= yellowness). The colorimeter was calibrated with a white standard plate 3.5 cm thick layer ($X=0.3155$, $Y=0.3319$, $Z=94.0$) before the measurements. Colour measurements were made 5 times, 1 on the middle and 4 on different parts of cheese surface after removing a 0.5 cm layer of upper surface.

Rheological Measurements:

For rheological characterization, cheeses were sliced into thin disks (2 mm thick and 20 mm diameter) with a cheese slicer. The dynamic oscillatory analyses were performed with a rheometer (CP 20, TA Instrument, Guyancourt, France) with a plate geometry of 20 mm diameter. Temperature sweep tests were used to determine the viscoelastic characteristics of the cheeses in the linear viscoelastic region by applying force (0.5 N) at a constant frequency of 1Hz as a function of temperature according to (Karoui et al., 2003) Parameters describes the viscoelastic characteristics of the cheeses included the elastic component $G'$ (storage modulus), the viscous component $G''$ (loss modulus), the phase angle ($\tan \delta$), and the complex viscosity ($\eta^*$). Three cylindrical specimens were tested for each cheese sample.

Synchronous Fluorescence Spectroscopy:

Synchronous fluorescence spectra were recorded using a FlyotoMax-2 spectrofluorimeter (Spex-Jobin Yvon, Longjumeau, France) mounted with a front-surface accessory. The incidence angle of the excitation radiation was set at 56° to ensure that reflected light, scattered radiation and depolarization phenomena were minimized. Spectra of cheese slices (2 cm long, 1 cm wide, 0.2 cm think) mounted between two quartz slides were recorded at 20°C with emission and excitation slits set at 4 nm SF spectra were recorded in the 250-500 nm excitation wavelength range using offsets of 80 nm (Boubellouta and Dufour, 2010) between excitation and emission monochromators. For each cheese sample, three spectra were recorded on 3 different slices.

Statistical Analysis:

One-Way ANOVA was carried out for the chemical and rheological data in order to assess significant differences among the samples throughout ripening and results reported as mean ± standard deviation. The Fisher least square difference (LSD) test was performed for each significant factor at a level significance of 5%. All calculations were carried out with XLSTAT software version 2007 (Addinsoft, France).

Principal components analysis (PCA) and Factorial discriminate analysis (FDA) were the two chemometric tools used in the multivariate evaluation of fluorescence data; both techniques based on a linear decomposition of data.

PCA (Wold et al., 1987) provides an approximation of a data matrix, $X$ into a few vectors, in terms of the product of two sets of vectors, $T$ (scores) and $P$ (loadings). These vectors capture the essential patterns of $X$, and are called latent variables or principal components (PC). PCA of the fluorescence data was applied in order to obtain the best possible overview of the spectral structure and distribution of samples. Score plots visualize the relationship between cheese samples for each PC, while loadings plots were used for interpretation of the corresponding spectral variation (Bertrand et al., 1987).

FDA technique (Safar et al., 1994) aim to predict the membership of an individual to a qualitative group defined as a preliminary. FDA assesses new synthetic variables called “discriminant factors”, which are linear combinations of the selected PCs, and allows a better separation of the centres of gravity of the considered groups. FDA was applied on the first 5 PCs performed on spectral data set to evaluate the potential of SFS to discriminate cheeses according to structural changes throughout ripening. A group was created for each ripening period (i.e. 30, 120 and 200 days). Synchronous fluorescence spectra were not subjected to any kind of preprocessing before analysis. PCA and FDA were performed by using MATLAB version 6.5 software (The Mathworks Inc., Natica, MA, USA).

Results and Discussion

Compositional Changes of Cantal Cheese Throughout Ripening Periods:

Table (1) indicated that as ripening progressed, fat, protein, salt, WSN/TN % and ash contents of Cantal cheese continuously increased, as a result of the significant decrease in the moisture content, whereas the calcium and fat in dry matter contents decreased. This can be related to cheese ripening, released amino acids...
raise pH value to a somewhat higher level (Waagner, 1993). The WSN/TN % of cheeses increased during the ripening period, indicating progressive proteolysis. It has also been reported that there is an appreciable reduction in the amount of calcium content in cheese during the ripening period because of the solubilization of colloidal Ca phosphate (CCP). The reduction in the amount of calcium associated with casein molecules (i.e., CCP) and hydrolysis of casein would be expected to alter cheese texture (Lucey et al., 2003; 2005).

Table 1: Mean (±SD) of chemical characteristics and texture of Cantal cheese throughout the ripening periods.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Cantal cheese</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Young (30 days)</td>
</tr>
<tr>
<td>pH (%)</td>
<td>5.23(±0.01)</td>
</tr>
<tr>
<td>Moisture (%)</td>
<td>42.92(±0.06)</td>
</tr>
<tr>
<td>Protein (%)</td>
<td>24.43(±0.08)</td>
</tr>
<tr>
<td>Fat (%)</td>
<td>29.92(±0.14)</td>
</tr>
<tr>
<td>Fat in dry matter (%)</td>
<td>52.41(±0.28)</td>
</tr>
<tr>
<td>WSN/TN (%)</td>
<td>1.34(±0.03)</td>
</tr>
<tr>
<td>Ash (%)</td>
<td>4.20(±0.03)</td>
</tr>
<tr>
<td>Total Ca (%)</td>
<td>0.765(±1.41)</td>
</tr>
<tr>
<td>Texture attributes</td>
<td></td>
</tr>
<tr>
<td>G' (KPa)</td>
<td>12.69(±2.09)</td>
</tr>
<tr>
<td>G'' (KPa)</td>
<td>4.36(±0.74)</td>
</tr>
<tr>
<td>η* (KPa.s)</td>
<td>0.34(±0.06)</td>
</tr>
</tbody>
</table>

One-Way ANOVA was applied to data and values in the same row with different superscript letter are significantly different (P < 0.05, LSD test)

Physical Characteristics:

The Changes of Colour Values in Cantal Cheese Throughout Ripening Periods:

No significant differences were observed in the colour values (L* and a* values) of Cantal cheese samples ripened for at 30, 120, and 200 days, although a slightly lower values for L* and a* values were found in aged samples (200 days) (Figure 1). The values of L* and b* indicate that the young cheeses had a light yellow colour which aquired more darker colour as the ripening progress. Regarding the a* parameter the cheeses had, in general, negative values. Negative numbers for the a* value indicate that cheeses are more green than red. The values of the b* confirms that the predominant colour of the cheeses was yellow.

The cheese whiteness is influenced by several factors including light scattering of fat and protein particles (Rudan et al., 1998) and whey pockets (Paulson et al., 1998). As ripening progressed, whey in serum pockets diffused, from cheese body out, as seen in moisture loss. The surface area occupied by light-scattering centers was therefore decreased. Thus, changes in Cantal colour throughout the ripening was probably and mainly attributed to the loss of moisture content which in turn increase the dry matter content and in parallel to changes in the decreased light scattering, and hence, lower L* and b* values.

Our results are in agreement with other who described a decrease in both lightness (L*) and yellowness (b*) and a slight increase in redness (a*) during cheese ripening (Rohm and Jaros, 1996; Pillonel et al., 2002).

Fig. 1: Changes in colour values (L*, a* and b*) of Cantal cheese ripened for 30, 120 and 200 days. Rheological changes in Cantal cheese throughout the ripening periods.

Changes in the rheological characteristics i.e. storage modulus (G'), loss modulus (G''), the phase angle (tan δ) and viscosity complex (η*) of Cantal cheese during the ripening are presented in Table (1) and fig. (2).
In general, at any ripening stage the $G'$ (index of the firmness), was always higher than $G''$ which indicates the predominating solid character of cheeses (Ustunol et al., 1995). Moreover, the value of $\tan \delta$ for Cantal cheese was less than 1.0 indicating that the elasticity nature ($G'$) of the samples was higher than their viscous nature ($G''$), an indication that the cheese exhibited solid-like behaviour (Tunick et al., 1993). One-way ANOVA showed that there were significant differences ($P < 0.05$) in all textural properties between the cheeses ripened for 30 and 120 days, but the further increase of the ripening period to 200 days slightly decreased in the $G'$ and $G''$ as compared to 120-days-old cheeses. The increase in the viscosity complex ($\eta^*$) observed throughout ripening can be attributed to degradation of protein (Visser, 1991; Olson et al., 1996).

These differences in rheological parameters could be explained by a magnitude of two opposite effects (weakening of cheese matrix due to proteolysis and firming effect due to moisture loss) throughout ripening period which would be predominant depending on the extent of proteolysis, pH and water content.

Differences between the cheese ripened for 30 and 120 days was probably attributed to, in the early stages of ripening time, the degree of curd fusion and contact area between casein particles was low which we believe to be responsible for the increase in the rheological parameters. The long-ripened cheese (120 and 200 days) the rheological parameters were decreased, but this decrease did not statistically important, due to extended proteolysis (Khosroshahi et al., 2006), a gradual breakage of the network calcium bonds (Ehsani et al., 1999) and the loss of water available of solvation of the protein chains and the consequent formation of a more compact cheese matrix (firmness cheese). Similar results were in agreement with those obtained for soft cheese (Karoui and Dufour, 2003) and Cheddar cheese (Wick et al., 2004).

**Synchronous fluorescence spectroscopy:**

The ripening of Cantal cheese was studied in terms of various structural changes at the molecular level-protein structure and interactions associated with protein and protein-lipids interactions by following the changes in the intrinsic fluorophores (tryptophan, vitamin A and riboflavin) exist in cheese by SFS.

The synchronous scans performed on Cantal cheese throughout the ripening period showed the presence of three major fluorophores, namely; 295, 322 and 355 nm, as shown in Figure (3). The synchronous fluorescence spectra showed slightly different shapes between the investigated cheeses and the fluorescence intensity decreased in accordance with the degree of ripening period.
From figure (3), for all cheeses, the highest synchronous fluorescence peak was obtained with excitation at 295 nm (emission at 375 nm), followed by that at 322 nm (emission at 402 nm), and that at 355 nm (emission at 435 nm). Apart from these three major peaks, smaller peaks were observed at around 449-490 nm. The band observed at 295 nm could be attributed to tryptophan residues of proteins (Karoui et al., 2004), while the band appeared at 322 nm (emission 402 nm) was probably related to vitamin A (Karoui, 2004) and the band appeared at 355 nm (emission at 435 nm) was probably related to riboflavin (Karoui et al., 2007c). Finally, the bands around 449-490 nm could be assigned to some coenzymes (e.g., NADH, FADH) (Kulmyrzaev et al., 2005), riboflavin found in milk (Boubellouta & Dufour, 2008) and Maillard-reaction products (Kristensen et al., 2001; Karoui et al., 2007).

The observed differences for tryptophan (band at 295 nm) and vitamin A fluorescence spectra (band at 322 nm) are consistent with changes of cheese matrix structure and lipid structure throughout the ripening period, respectively (Dufour and Riaublanc, 1997; Dufour et al., 2000). Concerning the changes in the band at 355 nm excitation, fluorescence spectra, this could be attributed to the lipid oxidation of cheeses throughout ripening which could contribute to the change observed on the riboflavin spectra.

The ability of synchronous spectra data to discriminate Cantal cheeses ripened for different periods was analyses by principal component analysis (PCA) and factor discriminant analysis (FDA), respectively. Firstly, PCA was applied to the set (24 objects and 251 variables) of synchronous fluorescence spectra recorded on Cantal cheese throughout the ripening period. The first two principal components accounted for 94 % of the total variance with a large predominance of the principal component 1 (explains 76.36%). Figure (4 a) shows the score plot of PC1 (explains 76.36% of total variance) versus PC2 (explains 17.92% of total variance) of PCA plot applied on the synchronous fluorescence spectra of young (30 days), between the two (120 days) and old (200 days) Cantal cheeses.

Three groups of cheese were observed; the first group (30 days) can be seen in the upper right quadrant which have high PC1 values; the second group (120 days) can be seen in the lower right quadrant of the low PC1 values and the third group (200 days) can be seen in the upper left quadrant according to PC2. It appeared that the first and second groups exhibited positive values according to PC1, the third group (200 days) showed negative values according to PC1 and positive values according to PC2 (Figure 4 a). These differences reflected changes in the structure of cheese matrix, the physical state of triglycerides and protein-lipid interactions throughout cheese ripening. It was concluded that one (or more) continuous phenomenon, taking place during the ripening, was detected when the fluorescence of the intrinsic was considered.

In order to point out which wavelengths were involved in the discrimination of the cheese samples, the factor loadings associated with the PC1 and PC2 were analyzed (Figure 4 b). The factor loadings for PC1 and PC2 shows the importance of the bands with maxima at 295 (assigned to tryptophan) at 322 (assigned to vitamin A) and 355 nm (assigned to riboflavin), and they describes changes in these bands throughout ripening.

Factor loadings 1 (Figure 4 b) characterized the samples on the right of the map (Figure 6 a) which it were characterized by a relatively higher fluorescence intensity than those on the left side. It indicated that during the ripening process, the main components of cheese (casein and fat) are subject to physical and chemical changes, which effect on the fluorescence intensity of tryptophan, vitamin A and riboflavin, resulting in changes in the structure of casein micelles. These structural changes can induce a more hydrophilic environment of the tryptophan of caseins in accordance with the red shift of the maximum for the older cheeses and change in the shape of vitamin A spectra which was found to correlate with lipid oxidation of cheese. Moreover, ripening involves mainly an increase in pH value, a change in protein-protein and the physical state of triglycerides and protein-lipid interactions. The pH of 30, 120 and 200 days-old cheeses were 5.23, 5.41 and 5.79, respectively (Table 1).

Factor loadings 2 (Figure 4 b) indicated that the shape of fluorescence spectra was larger for cheeses located on the positive side (30 and 120 days) than for those on the negative side (200 days). It appeared that changes in fluorescence spectra observed could be due to different protein-protein/fat interactions and different network structures resulting from the ripening process. Our results confirm previous findings (Herbert, 1999; Dufour et al., 2001; Mazerolles et al., 2001; Karoui et al., 2007; Karoui et al., 2007) reporting that three intrinsic fluorophores presented in the cheese could be considered as fingerprints allowing a good identification of changes in the cheeses as a function of their ripening time.

Secondly, in order to find out the differences between cheeses at the molecular level-protein structure and interactions throughout the ripening, the FDA was applied on the first 5 PCs of the PCA performed on the synchronous fluorescence spectra of Cantal cheese throughout ripening. The similarity map of the FDA allowed a good discrimination of the investigated cheeses. The map defined by the discriminant factors 1 and 2 represented 100 % of the total variance with discriminant factor 1 accounting for 82.10 % (Figure 5). Considering discriminant factor 1, Cantal cheeses ripened for 120 days and 200 days exhibited negative scores, whereas Cantal cheeses ripened for 30 days had positive score values. The discriminant factor 2 which took into
account 17.90% of the total variance differentiated between 120-days-old and 200-days-old Cantal cheeses. A correct classification of 100% was obtained.

Fig. 4: (a) Principal component analysis similarity map (score plot) determined by principal components 1 (PC1) and principal component 2 (PC2) and (b) factor loadings corresponding to PC1 and PC2 performed on the synchronous fluorescence spectra of the Cantal cheese ripened for 30, 120 and 200 days. The lines in (b) indicate: PC1 (solid) and PC2 (dotted).

Fig. 5: Discriminant analysis similarity map determined by discriminant factors 1 (F1) and 2 (F2) for the factorial discriminant analysis (FDA) performed on the first 5 principal components (PCs) of the principal component analysis (PCA) applied to the synchronous fluorescence spectra of Cantal cheeses ripened for 30, 120 and 200 days.

Conclusion:

The compositional characteristics (pH value, fat, protein, salt, WSN/TN% and ash contents) increased significantly during the ripening period but calcium and the moisture contents decreased to some extent. Ripening significantly influenced colour, resulting in a decrease of L* and b*, but it was observed a slight increase in a* value over ripening. Rheological characteristics increased with the ripening period, showing that ripening contributed to changes in the structure of cheese matrix, where the differences in G’ and G” were observed. The results of FDA performed on PCs, showed a good discrimination of the cheeses from their spectral data. Synchronous fluorescence spectroscopy presents a suitable alternative for monitoring changes in the chemical characteristics of Cantal cheese throughout the ripening period compared with the routine analysis. SFS could be considered as a fingerprint, allowing a good identification of cheese based on their structural changes throughout ripening period.
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