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Lipolysis in Picante cheese: Influence of milk type and ripening time on free fatty acid profile

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Abstract — Changes in the profile of the major free fatty acids (FFA) were assayed by HPLC up to 180 days of ripening in batches of cheeses manufactured with different proportions of ewe's and goat's milks. Evolution of lipolysis was also monitored by ethanolic titration, and good linear correlations were found between the two methods. For every batch of cheese, concentrations of all FFA exhibited continuous increases throughout the ripening period; levels of total FFA by 180 days of ripening ranged from 50.3 to 62.3 g/kg of fat depending on the cheesemilk composition; C_{10}, C_{16}, C_{18}, and $C_{18:1}$ were the most concentrated FFA throughout the whole ripening period. Values for the short chain FFA (C_4, C_6, and C_8) ranged from 2.8 to 4.5 g/kg of fat; such concentrations, which were not significantly different between batches with different proportions of ewe's and goat's milks, contributed considerably to the characteristic piquant flavor of Picante cheese. © Inra/Elsevier, Paris.

goat's cheese / ewe's cheese / lipase / free fatty acid

Résumé — Lipolyse dans le fromage Picante : influence du type de lait et du temps de maturation sur le profil d'acides gras libres. Les modifications du profil des acides gras libres majeurs (AGL) ont été analysées par CLHP au cours des 180 jours de l'affinage dans des fromages fabriqués avec différentes proportions de lait de brebis et de lait de chèvre. L'évolution de la lipolyse a aussi été déterminée par titrage éthanolique et de bonnes corrélations ont été obtenues entre les deux méthodes. Les concentrations de tous les AGL tendaient à croître au cours de l'affinage ; les niveaux d'AGL totaux à 180 jours étaient compris entre 50,3 et 62,3 g/kg de matière grasse, quelle que soit la proportion de lait de chèvre ; les AGL les plus abondants pendant l'affinage étaient C_{10}, C_{16}, C_{18}, et $C_{18:1}$. La concentration d'AGL à courte chaîne (C_4, C_6, et C_8) était comprise entre 2,8 et 4,5 $g.kg^{-1}$ de matière grasse ; ces concentrations, qui n'étaient pas significativement différentes entre les fromages fabriqués avec différentes proportions de lait de chèvre et de lait de brebis, doivent contribuer de façon considérable au goût piquant du fromage Picante. © Inra/Elsevier, Paris.

fromage de chèvre / fromage de brebis / lipase / acide gras libre

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1. INTRODUCTION

Cheese ripening is a complex process that encompasses breakdown of the curd via proteolysis, lipolysis, and other enzyme-catalyzed reactions which, as a whole, are responsible for flavor and textural changes that characterize the different cheese varieties. Lipolysis is a biochemical phenomenon of great importance; analysis of the profile of short and medium chain (i.e., C_4-C_{12}) free fatty acids (FFA) is often used in the chemical characterization of the extent of cheese ripening [4, 12, 19] because such FFA make significant contributions to the flavor of different types of cheese. Furthermore, FFA also act as precursors for chemical formation of such other important aroma components as esters, aldehydes, and ketones [10, 17].

Production of ewe's and goat's milk cheeses in Europe has been mainly concentrated in Mediterranean countries [3], and such countries have been consistently developing an interest towards characterization and improvement of such quota-free dairy products. Picante is one such cheese: it is a hard, salty, and spicy cheese originating in the Portuguese region of Castelo Branco (and possessing an 'Appellation d'Origine Contrôlée' status since 1988), and is traditionally manufactured on the farmhouse level only from mixtures of raw ewe's and goat's milks obtained from local herds. Coagulation is usually carried out at 29 °C for 50 min using animal rennet (without addition of a starter culture); cutting is often performed with 1.5 cm-spaced wires; moulding and pressing are generally done by hand using metallic cylindrical holders; and salting is made periodically throughout the ripening period using dry crude salt.

Some recent work has appeared in the literature concerning Picante cheese characterization, which has encompassed microbiological studies [5, 6] and physicochemical and proteolysis studies [7]. This paper attempts to complement such studies by tackling the lipolysis in Picante cheese during ripening and ascertaining the effect of using different proportions of ewe's and goat's milks for its manufacture.

2. MATERIALS AND METHODS

2.1. Manufacture, experimental design, and sampling

Five batches of mixtures of raw milks from Charnequeira goats and Frízia ewes at the volumetric percent ratios 0/100 (goat's/ewe's), 25/75, 50/50, 75/25, and 100/0 were prepared; these batches will be denoted hereafter as 0G, 25G, 50G, 75G, and 100G, respectively. Only one trial was considered within the lactation period, from flocks in certified excellent health condition, in the period when Picante cheese is known to possess the best organoleptic characteristics. At the time of milk collection (February), females were undergoing a feeding regime that consisted mainly of natural grass pasture and oats, and had given birth to calves 13 weeks before (on average). For each batch, 90 L of milk were used to manufacture 20 cheeses according to traditional procedures [7]. Cheeses from each batch were taken at random and sent under refrigerated conditions to our laboratory for analysis after 0, 9, 25, 40, 55, 83, 110, 140, and 180 days of ripening (counted from the time at which the first salting procedure was complete).

2.2. Physicochemical analyses

General milk physicochemical analyses were performed according to Kosikowski [11] and Richardson [16]. Fat content was determined by the Van Gulik method [1] using 40% H_2SO_4 and isoamyl alcohol (Pronalab, Lisbon, Portugal). The pH was measured with an electrode for solids (Ingold, Urdorf, Switzerland) connected to a potentiometer MicropH 2001 (Crison, Barcelona, Spain). The moisture content was determined gravimetrically after drying at 100 °C in an oven from Memmert (Schwabach, Germany). The NaCl content was determined by the modified Volhard method using $AgNO_3$ and KCNS (Merck, Darmstadt, Germany).

Fat extraction from cheese samples was carried out using diethyl ether according to Nuñez et al. [15]. The acidity index of the fat, expressed as mg of equivalent KOH per g of fat, was calculated from ethanolic titration following Nuñez et al. [15].

2.3. Assays for individual free fatty acids

Fatty acid standards, butylated hydroxyanisole (BHA), and *p*-bromophenacylbromide (PBPB) were obtained from Sigma (St. Louis MO, USA). Diethyl ether, anhydrous sodium sulfate, calcium carbonate, and 18-crown-6-ether were obtained from Merck. Chloroform, acetronitrile, methanol, and formic acid were purchased from Romil (Leicester, UK). A Milli-Q-Plus 185 water purification system was used to produce HPLC-grade water.

Resolution and quantification of free fatty acids (FFA) was performed according to Garcia et al. [8]. The HPLC equipment used (Beckman Instruments, San Ramon CA, USA) consisted of a solvent delivery system with two high pressure pumps, an automatic autosampler with temperature control for the column, a programmable multiwavelength spectrophotometer, and a software package for data acquisition and control. The column used was a Beckman C-18 reversed-phase (25 cm × 4.6 mm × 5 mm) coupled with a pre-column cartridge. The concentrations of each individual FFA in the different cheese samples were calculated using peak areas for the FFA normalized by those of the internal standards coupled with calibration curves previously prepared with chromatographic standards via the same protocol.

3. RESULTS AND DISCUSSION

In order to assess the reproducibility of the HPLC method, two separate extractions were performed in a sample of cheese manufactured with 50% ewe's and 50% goat's milks and ripened for 90 days. Two independent derivatizations were performed for each extraction, and two injections were carried out for each derivatization. A measure of the reproducibility of the HPLC method utilized for FFA assay, taken as the coefficient of variation of all analytical steps (extraction, derivatization, resolution, and detection), was ca. 5%; this value is comparable to those reported by Juárez et al. [9] for fresh cheese and by Woo and Lindsay [18] for Cheddar cheese.

The ethanolic titration and HPLC methods were compared in order to determine whether fat acidity and total FFA concentration, respectively, conveyed equivalent information about lipolysis in Picante cheese throughout ripening; linear regression of the results obtained from ethanolic titration (y) and from HPLC (x) for the various types of cheese (0G–100G) yielded means and standard deviations of 0.39 ± 0.06 for the slope and 2.18 ± 1.67 for the intercept, with associated Pearson's correlation coefficients not below 0.90. It could thus be concluded that ethanolic titration is an alternative, yet simpler method to assess extension of lipolysis in Picante cheese. Theoretical slopes in the range 0.25–0.26 (obtained from 56/Mw, where 56 is the molecular weight of KOH and Mw is the average molecular weight of free fatty acid residues, ranging from 217 to 222 throughout the ripening time) are in principle expected; although such values are virtually within the 95 % confidence interval of the aforementioned experimental slope (i.e., [0.26, 0.52]), it should be noted that acids other than free fatty acids might have interfered in the titration assay but not in the HPLC assay, thus leading to overestimation of fat acidity and, consequently, of the slope (it should be recalled here that the salt content did not apparently interfere with extraction degree of fatty acid because the amounts of acids other than fatty acids remained virtually constant as ripening time elapsed).

In general the total FFA concentration increased, as expected, throughout the

Table I. Evolution of mean percentage, and corresponding standard deviation, of individual FFA (% of total FFA), total FFA (g per kg of fat), fat content (%), pH, moisture content (%), and NaCl (%) over the ripening period for the different 0G, 25G, 50G, 75G and 100G cheeses.

Tableau I. Évolution des pourcentages moyens, et des écarts types correspondants, des AGL individuels (% des AGL totaux), AGL totaux (g/kg de graisse), taux de graisse (%), pH, humidité (%), et NaCl (%) pendant l'affinage pour les différents fromages 0G, 25G, 50G, 75G et 100G.

	Ripening time (days)								
Free fatty acid	0	9	25	40	55	83	110	140	180
Butyric acid ($C_{4:0}$) (%)	8 ± 1	7 ± 1	9 ± 1	12 ± 2	13 ± 2	10 ± 2	10 ± 2	7 ± 1	6 ± 1
Caproic acid ($C_{6:0}$) (%)	5 ± 0	4 ± 0	4 ± 0	4 ± 0	4 ± 0	4 ± 0	4 ± 0	5 ± 1	5 ± 1
Caprylic acid ($C_{8:0}$) (%)	9 ± 0	8 ± 1	7 ± 0	8 ± 1	7 ± 1	7 ± 0	7 ± 0	7 ± 1	7 ± 1
Capric acid ($C_{10:0}$) (%)	12 ± 0	12 ± 1	12 ± 1	12 ± 1	13 ± 1	14 ± 2	15 ± 1	15 ± 1	15 ± 1
Lauric acid ($C_{12:0}$) (%)	6 ± 1	7 ± 2	7 ± 2	6 ± 1	7 ± 2	7 ± 2	7 ± 1	7 ± 1	7 ± 1
Myristic acid ($C_{14:0}$) (%)	3 ± 1	4 ± 1	5 ± 2	3 ± 1	4 ± 1	5 ± 0	4 ± 0	5 ± 1	5 ± 1
Palmitic acid ($C_{16:0}$) (%)	21 ± 1	21 ± 1	19 ± 1	18 ± 1	17 ± 1	18 ± 1	18 ± 1	19 ± 1	19 ± 1
Stearic acid ($C_{18:0}$) (%)	22 ± 1	20 ± 2	15 ± 2	18 ± 2	16 ± 2	14 ± 2	16 ± 1	15 ± 1	15 ± 1
Oleic acid ($C_{18:1}$) (%)	14 ± 1	16 ± 2	21 ± 3	19 ± 2	20 ± 3	22 ± 1	19 ± 2	20 ± 2	21 ± 1
Total FFA (g/kg fat)	4.58 ± 0.99	4.71 ± 1.15	6.872 ± 1.79	14.82 ± 3.36	20.493 ± 4.19	23.03 ± 0.77	26.26 ± 2.87	36.95 ± 3.16	57.09 ± 4.55
pH	5.45 ± 0.21	4.98 ± 0.38	5.36 ± 0.20	5.38 ± 0.11	5.31 ± 0.08	5.72 ± 0.02	6.15 ± 0.11	6.06 ± 0.03	6.04 ± 0.05
Total fat (%)	20.3 ± 2.8	22.6 ± 2.7		24.2 ± 2.3		25.8 ± 1.7	27.6 ± 1.5		30.6 ± 2.0
Moisture content (%)	41.9 ± 1.7	48.5 ± 1.6	50.0 ± 0.5	50.4 ± 1.3	51.0 ± 0.8	50.7 ± 0.5	52.7 ± 0.5	56.4 ± 0.9	60.1 ± 0.7
NaCl (%)	3.5 ± 0.4	7.8 ± 0.5	7.6 ± 0.4	7.8 ± 0.2	7.9 ± 0.3	8.3 ± 0.2	8.6 ± 0.2	8.7 ± 0.3	11.7 ± 0.2

ripening period for all batches of cheese (*table I*); such increase was approximately linear and of the order of 0.3 g/kg of fat per day. Levels of total FFA concentration ranged from 5.3, 5.8, 4.6, 3.9, and 3.3 g/kg of fat at day 0 of ripening to 50.3, 55.8, 60.0, 62.3, and 57.1 g/kg of fat by 180 days of ripening for 0G, 25G, 50G, 75G and 100G cheeses, respectively. These values are of the order of magnitude of those found for Majorero cheese, a Spanish goat's milk cheese [12], and are an indication of high levels of lipolysis in Picante cheese despite the high levels of salt attained, viz. 10–12 % (*table I*). *Figure 1* shows that the effect of cheese milk composition on lipolysis in Picante cheese is not significant; however, higher proportions of caprine milk in the cheese milk were in general associated with higher total concentration of FFA, especially after 25 days of ripening, although cheeses manufactured with 75% of goat's milk exhibited the highest values by 180 days. These observations are probably a consequence of the fact that cheeses richer in goat's milk contain higher levels of fat [7], a somewhat unexpected observation since ewe's milk is often considered to possess higher fat content than goat's milk. In our case, such rule can also be applied: the fat contents of milk from Charnequeira goat (4.5–6.5 %) were essentially below those of milk from Frízia ewes (5.5–8.0 %), and so differences in the protein matrix of the cheese, or in the whey expression pattern during manufacture, might account for the aforementioned unexpected result.

The FFA profile varies slightly between the various types of cheese and throughout the ripening period; hence, only the mean percentage, and associated standard deviation, of each FFA for the various types of cheese are recorded in *table I*, as well as total FFA, fat content, pH, total solids content, and NaCl content. The main FFA present in the different samples and throughout the whole ripening period were capric ($C_{10:0}$), palmitic ($C_{16:0}$), stearic

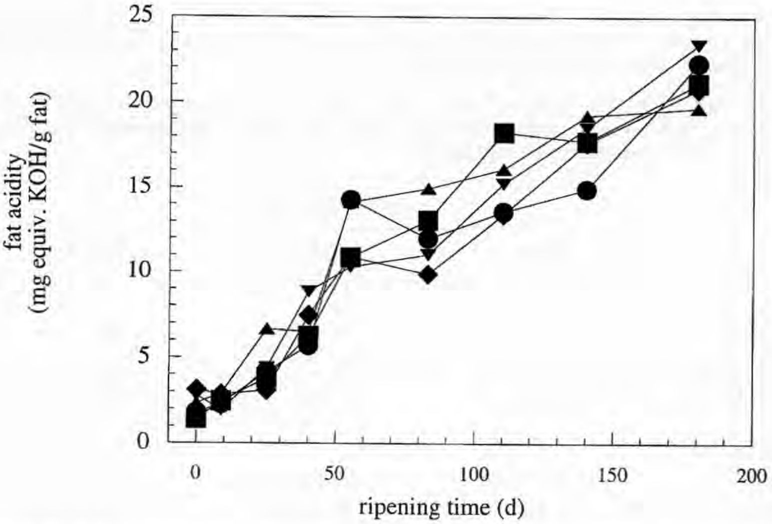

Figure 1. Evolution of fat acidity throughout ripening period for the various cheeses manufactured: (●) 0G, (■) 25G, (◆) 50G, (▲) 75G, and (▼) 100G.

Figure 1. Évolution de l'acidité grasse pendant l'affinage des fromages pour les différents fromages fabriqués : (●) 0G, (■) 25G, (◆) 50G, (▲) 75G, et (▼) 100G.

($C_{18:0}$), and oleic ($C_{18:1}$) acids; they accounted for ca. 68 ± 1 % of the total FFA pool, a figure that parallels that of the longer chain fatty acid residue counterparts esterified in the triglycerides of milk fat. In such cheeses as Idiazabal, Majorero, Roncal, and Roquefort [12], which are manufactured with ewe's and goat's milks, $C_{10:0}$, $C_{16:0}$ and $C_{18:1}$ also appear between the major FFA; a recent review [14] claims that $C_{16:0}$ and $C_{18:1}$ are consistently the dominating FFA in all types of cheeses assayed, with values between 19 and 31 % of total FFA. The percentage of goat's milk in the cheese milk mixture played a very limited role when compared with ripening time in terms of concentration of each one of those major free fatty acids (*table I*). Noteworthy differences in the FFA profile were noticed for $C_{16:0}$ and $C_{18:1}$ in the early ripening period for ewe's cheese where they appeared in higher relative percentage than in goat's cheese, and $C_{18:0}$ in early stages of ripening as well as $C_{18:1}$ in late stages for goat's cheese where their relative percentage were higher than in ewe's cheese (results not shown). In terms of medium chain FFA, myristic acid ($C_{14:0}$) was the medium chain FFA which had the highest percentual increment by 180 days of ripening; however, the low relative proportion of $C_{14:0}$ in Picante cheese throughout the whole ripening period contrasts with the usual claim that it is a representative FFA in ewe's and goat's milk cheeses. Similar conclusions were, nevertheless, obtained by Malcata [13] for Serra cheese (an ovine milk cheese) by 35 days of ripening.

Although $C_{16:0}$, $C_{18:0}$, and $C_{18:1}$ were between the most abundant FFA found throughout ripening for all experimental cheeses, they do not intrinsically contribute to cheese flavor quite as much as short chain fatty acids do [2]; even though inspection of *table II* indicates that short chain FFA are a minor family of FFA pre-

Table II. Percent values for short (SCFA, < C_{10}), medium (MCFA, ≥ C_{10}, ≤ C_{14}), and long chain (LCFA, > C_{14}) FFA in 180-day-old Picante cheese and in reference cheeses manufactured with plain ovine or caprine milk.

Tableau II. Proportion des AGL à chaîne courte (SCFA, < C_{10}), moyenne (MCFA, ≥ C_{10}, ≤ C_{14}) et longue (LCFA, > C_{14}) du fromage picante à 180 jours et des fromages de référence fabriqués avec du lait pur brebis ou pur chèvre.

Type	Free fatty acids		
of cheese	SCFA	MCFA	LCFA
Picante cheese			
Ovine (0G)	22	27	51
Caprine (100G)	17	26	56
0G–100G	18 ± 2	27 ± 1	54 ± 2
Reference cheeses*			
Ovine	8.2 ± 1.6	15.6 ± 3.8	63.8 ± 16.9
Caprine	8.4 ± 2.4	28.1 ± 2.9	63.2 ± 3.7

* Mean values and variation limits of free fatty acid percentages of ovine and caprine ripened cheeses as reported by Nájera et al. [14].

* Valeurs moyennes et limites de variation des pourcentages d'acides gras libres dans les fromages de brebis et de chèvre, d'après Nájera et al. [14].

sent by 180 days of ripening, their relative percentage is considerably higher than that reported by Nájera et al. [14] for similar cheeses, which could thus be one of the unique characteristics of Picante cheese. Values for butyric ($C_{4:0}$), caproic ($C_{6:0}$), and caprylic ($C_{8:0}$) acids in Picante cheeses by 180 days of ripening ranged from 2.8 to 4.5 g/kg of fat. These levels of short chain FFA, which are likely responsible for the strong and sharp characteristic flavor of this cheese and thus deserve special discussion, are not significantly different between cheeses manufactured with different cheese milk mixtures; nevertheless, *table II* suggests that a slight decrease in the percentage of short chain FFA occurs by 180 days when the proportion of goat's milk in the cheese milk mixture increases; such trend was enhanced for samples taken at shorter ripening times (results not shown). Data reported by Nájera et al. [14] indicate that $C_{10:0}$ and, to a lesser extent, $C_{4:0}$ are present at higher percentages in goat's than in ewe's milk cheeses; according to our results, no significant differences exist between 0G and 100G cheeses. In terms of short chain FFA, $C_{6:0}$ exhibited the highest and $C_{4:0}$ the lowest increment by 180 days of ripening. Considering that the rates of release of short chain fatty acids in Picante cheese are approximately two-fold those in ovine and caprine cheeses [14], this fact can be claimed as a characteristic feature of Picante cheese, together with the pH value close to 6 and the extremely high salt contents.

4. CONCLUSIONS

Good linear correlations exist between ethanolic titration and HPLC in the assay for the total concentration of FFA in Picante cheese. Ripening time is very important with respect to release of every individual FFA, whereas proportion of goat's and ewe's milks in the cheese milk is of a much lesser importance. The most concentrated FFA in ripened Picante cheese are $C_{16:0}$, $C_{18:0}$, and $C_{18:1}$, as expected from the high content of such fatty acid residues in milkfat; however, extensive and fast release of short chain FFA are mostly responsible for the piquant flavor of this cheese, which is enhanced by its high salt concentration.

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