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Lipolysis, proteolysis and sensory properties of ewe's raw milk cheese (Idiazabal) made with lipase addition

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ABSTRACT

In this paper, we describe the effect of the addition of pregastric lipase on the composition and sensory properties of Idiazabal cheese. Free fatty acids (FFA), partial glycerides, free amino acids (FAA), gross composition and sensory characteristics were determined at different ripening times in cheeses manufactured with three different amounts of commercial animal lipase or with lipase-containing artisanal lamb rennet paste. The addition of lipase increased the content of total FFA, particularly of short-chain FFA, and that of total partial glycerides in cheeses. Unexpectedly, lipase utilization significantly affected total FAA concentration, which decreased in cheeses elaborated with high lipase amount. In general, Val, Glu and Leu were the major FAA, and their concentrations depended, mainly, on ripening time. Lipase addition had significant influence on the sensory characteristics of the cheeses, increasing scores for most of the flavour and odour attributes of the cheese. Principal component analysis (PCA) was done including dry matter, FFA, FAA, partial glycerides and odour and flavour attributes of the cheeses. It indicated that aroma and flavour parameters of Idiazabal cheese and the content of short-chain FFA and diglycerides were highly correlated to first principal component (PC1), while texture parameters, compositional variables and FAA were correlated to the second principal component (PC2).

1. Introduction

The use of exogenous lipases (glycerol ester hydrolases EC 3.1.1.3) has been reported to accelerate ripening or to develop characteristic flavours for a variety of cheeses at the experimental level (Fox, 1993; Hernández et al., 2001, 2005; Kilcawley, Wilkinson, & Fox, 1998). In commercial cheeses the use of enzymes other than chymosin and pepsin is declining due to difficulties in selecting the most adequate enzyme or mixture of enzymes, or in adjusting the correct enzyme amount to be added in each case, or due to the presence of secondary enzyme activities in some of the commercial enzyme preparations (Law, 2001; Wilkinson, van den Berg, & Law, 2002). To the best of our knowledge, at present, lipases are sold almost exclusively for the commercial manufacture of certain Italian cheeses or for enzyme modified cheeses.

Lipolysis is usually understood as the accumulation of FFA during ripening, with most of the free fatty acids (FFA) being released from triglycerides. Total FFA concentration and short/long-chain FFA ratio have been related to the type and the amount of lipase used during cheese ripening, and to the sensory characteristics of the cheese (Fox, O'Connor, McSweeney, Guinee, & O'Brien, 1996. In some Mediterranean cheeses, like Provolone and Romano (Woo & Lindsay, 1984), Parmesan (Fox & Guinee, 1987), Idiazabal cheese (Hernández et al., 2001) or white pickled cheese (Akin, Aydemir, Koçak, & Yilduz, 2003), characteristic flavour is developed by the addition of pregastric lipase-containing lamb rennet paste. In Roquefort cheese, the fungal lipase produced by *Penicillium roqueforti* is necessary for the development of the characteristic flavour of this cheese variety. Lipolysis and β-oxidation, biochemical routes that are specially important in blue cheeses due to the activity of the thiol ester hydrolase for β-keto-acyl-CoA (Law, 1981), are sources of compounds like FFA and ketones, (2-heptanone and other methyl ketones). FFA and alcohols can be converted to short-chain fatty acid esters, like ethyl acetate, or ethyl butyrate by *Lactococcus lactis* EstA esterase (Nardi, Fiez-Vandal, Tailliez, & Monnet, 2002).

Proteolysis is another major process with high impact on cheese flavour during cheese ripening. Very briefly, hydrolysis of milk pro-

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teins during cheese ripening occurs in different steps (for an extend review see Fox et al., 1996. Proteolysis starts when milk proteins (mainly caseins) are hydrolyzed by the chymosin and the pepsin added with the rennet, releasing high molecular weight peptides, that are degraded to smaller peptides by bacterial extracellular peptidases (Visser, 1993). These peptides are taken up by bacteria and further hydrolyzed to amino acids by microbial exopeptidases, amino and carboxy-peptidases and di/tri peptidases, during cheese ripening (Kunji, Mierau, Hagting, Poolman, & Konings, 1996). Finally, free amino acids (FAA) are metabolised by bacteria producing volatile compounds like 3-methylbutanal from isoleucine or methanethiol from methionine, compounds with a very low odour threshold and a very high sensory impact (for a recent review see Smit, Smit, & Engels, 2005, and references therein).

Thus, proteolysis and lipolysis are major sources of cheese flavour and odour compounds. These enzymatic processes must occur in a coordinated way to give each cheese type its unique and appreciated sensory characteristics. In most cheese types, milk fat lipolysis does not occur to a large extent, and many researchers have considered proteolysis and FAA catabolism as the main processes in the production of impact-aromatic compounds during cheese ripening (Fox et al., 1996; Smit et al., 2005). In consequence, most reports about flavour development in cheese study the relation between proteolysis and sensory properties, disregarding lipolysis.

Idiazabal cheese is a traditional semi-hard cheese from the Basque Country region of Northern Spain, manufactured with sheep's raw milk, and protected by the Denomination of Origin Idiazabal cheese (BOE, 1993). Idiazabal cheese is manufactured under a strictly controlled procedure, with a minimum ripening time of 60 days. In a previous article, Hernández et al. (2005) described an extensive study of the influence of three different commercial lipases on the FFA content of cheeses and their sensory characteristics. In the present paper we focus on the lipase that gave best sensory results for Idiazabal cheese and describe the effect of adding this exogenous lipase on the content of free amino acids and partial glycerides, in addition to the FFA content, and explore the relationship between lipolytic, proteolytic and sensory parameters in a principal component analysis.

2. Materials and methods

2.1. Measure of coagulating and lipase activities

Coagulating activity of the rennet was measured as described (IDF, 1987) using standardised milk as substrate (INRA, Dijon, France). One rennet unit (RU) is the amount of enzyme necessary to coagulate 10 ml of standardised milk in 100 s. Lipase activity was measured with emulsified tributyrin as substrate, at pH 6.2, as described (Barton, O'Connor & Turner, 1996) with the modifications described by Svensson, Hernández, Virto, and de Renobales (2006). One lipase unit (LU) is defined as the amount of enzyme necessary to produce 1 μmol of n-butanoic acid per minute, considering that 1 μmol of NaOH is equivalent to 1 μmol of n-butanoic acid under these reaction conditions.

2.2. Experimental design

The experimental design is summarized in Table 1. Four batches of cheeses were made on the same day in 50 L vats in the pilot plant of Queserías Araia (Araia, Alava, Spain) from bulk raw ewe's milk according to the method approved by the Denomination of Origin (BOE, 1993). Bulk raw ewe's milk was a mixture of milks collected from several flocks, in early lactation, and kept refrigerated at 4 °C for up to 24 h until cheese manufacture. One batch of cheeses was made using commercial bovine rennet (Marschall powder, Rhône-Poulenc Texel, Dangé St. Romain, France, 1028 RU/g and no lipase activity), and one batch was made using artisanal rennet paste (206 RU/g and 2.8 LU/g), prepared from lamb abomasa as described before (Virto et al., 2003). The other two batches were made with a mixture of commercial bovine rennet and commercial pregastric lipase (animal lipase, Christian Hansen Lacta, Madrid, 25 LU/g): 4.8 g/50 L (120 LU/50 L) added to one vat and 9.7 g (242 LU/50 L) added to the other vat. A homofermentative starter culture (EZAL, Rhône Poulenc Texel) was added to all vats. Lipase was added with the rennet after heating the milk at 30 °C. After moulding and pressing, cheeses were brined for 16 h at 10–12 °C in a saturated solution of table salt. Duplicate sets of fabrications were made in consecutive weeks. Cheeses were ripened at 8–10 °C and 85% relative humidity for up to 180 days. Cheeses were cylindrically shaped and weighed 1.1 ± 0.1 kg. After 45, 90 and 180 ripening days, two cheeses from each vat were sampled at random. Cheeses were cut into triangular sections weighing around 100 g each, sections were wrapped in plastic film and aluminum foil and frozen at −80 °C until analysed. One cheese section was not frozen and used for the analysis of pH and dry matter. Before analysis, cheese sections were defrosted for 24 h at 7–8 °C and the rind (approx. 0.5 cm) was removed. All physicochemical analyses were made in duplicate. One whole cheese from each vat was kept at 4–6 °C for up to 24 h and used for sensory analysis after 90 and 180 ripening days.

Table 1
Experimental design used for cheese manufacture with different amount of lipase added.

Rennet	Coagulating activity (RU/50 L)	Added lipase		
		Type	Amount (g/50 L)	Activity (LU/50 L)
Commercial[a]	2000	0	0	0
Commercial[a]	2000	Commercial[b]	4.8	120
Commercial[a]	2000	Commercial[b]	9.7	242
Artisanal[c]	2581	Endogenous[c]	12.5	35

[a] Bovine rennet (Marshall powder).
[b] Animal lipase (Christian Hansen).
[c] Artisanal rennet was prepared from lamb abomasa as described in Hernández et al. (2005).

2.3. Physico-chemical analysis

Total nitrogen content of the cheese and nitrogen fractions were determined by the Kjeldahl method (IDF, 1993). The pH of cheeses was measured by inserting a combination electrode into the sample. Fat content was determined by the VanGulik method (ISO, 1975), and dry matter was determined as described in IDF standard 4A (IDF, 1982).

Free fatty acids (FFA) were analyzed as described previously by Chávarri et al. (1997). Gas-liquid chromatography was carried out in a 5890 series II gas chromatograph (Hewlett-Packard, Madrid, Spain) equipped with a flame ionization detector, and FFA were separated on a fused-silica capillary column (HP-FFAP, 25 m × 0.32 mm) coated with FFA phase (cross-linked polyethylene glycol, 0.52 μm layer thickness). Quantification was done with n-pentanoic, n-nonanoic and n-heptadecanoic acids (Sigma–Aldrich) as internal standards added to the cheese sample at the time of extraction. The results were expressed as μmol of FFA per kg of cheese.

Partial glycerides were extracted and analyzed by HPLC with a light scattering detector as described by Barrón et al. (2004). Quantification was done with hexadecanediol (Sigma–Aldrich) as

internal standard added to cheese samples at the time of extraction. The results were expressed as mg of partial glycerides per 100 g of cheese.

Free amino acids (FAA) were extracted as described (Bütikofer, Fuchs, Bosset, & Gmür, 1991). FAA were derivatized with AccQ.Fluor Reagent® (Waters, Barcelona, Spain), according to the manufacturer's procedure, and HPLC analysis was carried out as described (Bustamante et al., 2003). Norleucine was added at the beginning of the extraction procedure as internal standard. The results were expressed as μmol of FAA per g of cheese.

2.4. Sensory analysis

Sensory analysis was performed on cheeses after 90 and 180 ripening days. After reception, cheeses were kept at 4–6 °C and sensory analysis was performed within 48 h by a trained analytical panel of 8 members, using a seven-point scale (Barcenas, Perez-Elortondo, Salmerón, & Albisu, 1999; Barcenas, Perez-Elortondo, & Albisu, 2000). Odour attributes were overall intensity, sharp, brine, rennet, milk, butter and toasty. Flavour attributes were overall intensity, butyric, piquant, rennet, buttery, nutty, sweet, acid, salty and bitter. Texture attributes were firmness, graininess, elasticity, surface roughness, surface moisture, friability, adhesiveness, solubility and moisture in mouth.

2.5. Statistical analysis

The SPSS software, version 14.0 (SPSS, Chicago, IL, USA) was used for the statistical analysis. Two-way analysis of variance (ANOVA) was done to establish the presence or absence of significant differences ($P \leqslant 0.05$) in compositional and sensory parameters of the cheeses according to the factors 'amount of lipase' and 'ripening time'. When the interaction term was significant, one-way analysis of variance (ANOVA) was used to determine the presence or absence of significant differences ($P \leqslant 0.05$) in compositional and sensory parameters among the PDO cheeses made with different amounts of lipase at the same ripening time. Student's t test was used to evaluate the significance of the differences ($P \leqslant 0.05$) in compositional and sensory parameters between cheeses made with the same amount of lipase at different ripening times (90 and 180 days). Linear regression analysis was used to study the evolution of FFA content with ripening time (from 45 to 180 days). Linear regression models were also applied to study the relationship between lipolysis and proteolysis indexes, and between sensory and compositional variables of the cheeses. Principal component analysis (PCA) was applied to compositional and sensory variables of the cheeses made with different amounts of lipase. Only variables with communality higher than 0.5 were included. The Kaiser criterion was established to select principal components. Factors were rotated using the Varimax method for ease of interpretation (Hair, Tatham, Anderson, & Black, 1998).

3. Results and discussion

3.1. Gross composition

After 180 days of ripening, total protein content was between 22.4% and 23.9% in all cheeses regardless of the amount of lipase added. Fat proportion was between 35.0 and 35.5%, and dry matter percentage between 67.1% and 68.6%. After 90 ripening days, the percentage of dry matter was lower in the cheese made without lipase (63.2 ± 0.6) than in the cheeses made with commercial lipase added (average 66.1 ± 0.4). However, this difference disappeared after 180 ripening days.

After 180 days of ripening, the pH decreased to 4.8 in most cheeses. This pH value was low when compared with strongly lipolysed cheeses like Fiore Sardo (pH = 5.1–5.3) or Pecorino Romano (pH = 5.3–5.4) (Gobbetti, 2004), but pH values below 5.0 have been reported in Idiazabal cheese (Virto et al., 2003).

Total nitrogen values in dry matter were between 3.9% and 4.0% in all cheeses after 180 days of ripening and between 3.6% and 4.0% after 90 days. These values were lower than those obtained for Idiazabal cheese by Bustamante et al. (2003). These differences could be attributed to the lactation period of the ewes, because the milk protein content is considerably lower at the beginning of the lactation period (cheeses reported herein) than at the end of this period (cheeses reported by Bustamante et al., 2003).

3.2. Lipolysis

After 180 ripening days, total FFA varied from 8.72 ± 0.72 μmol/g cheese in cheeses without lipase up to 54.71 ± 3.00 μmol/g cheese in cheeses with high amount of commercial lipase (Table 2). Total FFA concentration was significantly ($P \leqslant 0.05$) different in cheeses made with different amount of lipase after 45, 90 or 180 ripening days, with the highest content of total FFA

Table 2
Mean values and standard deviations of the concentrations of FFA (μmol/g cheese) in cheeses made with different amount of added lipase after 45 and 180 days of ripening.

FFA	No lipase added		Lamb rennet lipase		Comercial lipase			
	0 LU/50 L		35 LU/50 L		120 LU/50 L		242 LU/50 L	
	45 days	180 days	45 days	180 days	45 days	180 days	45 days	180 days
C4	0.61 ± 0.02^{a}	1.77 ± 0.32^{a}	2.31 ± 0.15^{b}	5.01 ± 1.01^{b}	8.24 ± 0.74^{c}	17.31 ± 0.99^{c}	13.17 ± 0.62^{d}	24.78 ± 1.44^{d}
C6	0.20 ± 0.01^{a}	0.52 ± 0.01^{a}	0.78 ± 0.03^{b}	1.56 ± 0.33^{b}	3.11 ± 0.32^{c}	6.27 ± 0.42^{c}	4.89 ± 0.23^{d}	8.53 ± 0.50^{d}
C8	0.24 ± 0.01^{a}	0.53 ± 0.01^{a}	0.44 ± 0.01^{b}	0.87 ± 0.20^{b}	1.34 ± 0.13^{c}	2.85 ± 0.26^{c}	1.97 ± 0.12^{d}	3.62 ± 0.23^{d}
C10	0.54 ± 0.02^{a}	1.20 ± 0.21^{a}	0.92 ± 0.02^{b}	1.86 ± 0.44^{b}	2.64 ± 0.22^{c}	5.95 ± 0.38^{c}	4.19 ± 0.33^{d}	7.65 ± 0.33^{d}
C12	0.23 ± 0.02^{a}	0.44 ± 0.01^{a}	0.34 ± 0.01^{b}	0.70 ± 0.14^{b}	0.92 ± 0.11^{c}	1.97 ± 0.22^{c}	1.41 ± 0.11^{d}	2.66 ± 0.12^{d}
C14	0.33 ± 0.04^{a}	0.67 ± 0.15^{a}	0.37 ± 0.01^{a}	0.79 ± 0.17^{a}	0.68 ± 0.07^{b}	1.65 ± 0.20^{b}	0.96 ± 0.10^{c}	2.08 ± 0.11^{b}
C16	0.73 ± 0.06^{a}	1.35 ± 0.21^{a}	0.75 ± 0.01^{a}	1.33 ± 0.27^{a}	0.91 ± 0.04^{b}	2.25 ± 0.14^{b}	1.10 ± 0.01^{c}	2.43 ± 0.16^{b}
C18:0	0.23 ± 0.01^{a}	0.39 ± 0.05^{a}	0.22 ± 0.01^{a}	0.38 ± 0.05^{a}	0.25 ± 0.04^{a,b}	0.51 ± 0.04^{b}	0.25 ± 0.01^{b}	0.49 ± 0.06^{b}
C18:1	0.75 ± 0.05^{a}	1.61 ± 0.28^{a}	0.79 ± 0.03^{a}	1.56 ± 0.37^{a}	0.88 ± 0.10^{a,b}	2.17 ± 0.16^{b}	0.96 ± 0.04^{b}	2.14 ± 0.32^{b}
C18:2	0.12 ± 0.01^{a}	0.25 ± 0.05^{a}	0.15 ± 0.01^{b}	0.23 ± 0.06^{a}	0.15 ± 0.01^{b}	0.34 ± 0.02^{b}	0.16 ± 0.01^{b}	0.33 ± 0.08^{b}
Short-chain	1.59 ± 0.05^{a}	4.02 ± 0.71^{a}	4.46 ± 0.19^{b}	9.30 ± 1.97^{b}	15.33 ± 1.39^{c}	32.38 ± 1.48^{c}	24.24 ± 0.85^{d}	44.58 ± 2.14^{d}
Medium-chain	0.56 ± 0.06^{a}	1.10 ± 0.25^{a}	0.70 ± 0.02^{b}	1.49 ± 0.31^{a}	1.60 ± 0.18^{c}	3.65 ± 0.42^{b}	2.37 ± 0.21^{d}	4.74 ± 0.23^{c}
Long-chain	1.83 ± 0.06^{a}	3.60 ± 0.58^{a}	1.91 ± 0.05^{b}	3.50 ± 0.76^{a}	2.19 ± 0.17^{c}	5.27 ± 0.32^{b}	2.47 ± 0.11^{d}	5.39 ± 0.60^{b}
Total	3.98 ± 1.53^{a}	8.72 ± 1.52^{a}	7.07 ± 0.25^{b}	14.30 ± 3.01^{b}	19.12 ± 1.45^{c}	41.28 ± 1.86^{c}	29.07 ± 1.11^{d}	54.71 ± 2.84^{d}

$^{a–d}$Means within rows with different superscripts are significantly different ($p < 0.05$) for the same ripening time. C4: butyric acid, C6: n-hexanoic acid, C8: noctanoic acid, C10: n-decanoic acid, C12: n-dodecanoic acid, C14: n-tetradecanoic acid, C16: n-hexadecanoic acid, C18: n-octadecanoic acid, C18:1: noctadecenoic acid, C18:2: n-octadecadienoic acid; Short-chain: C4–C10, Medium-chain: C12–C14, Long-chain: C16–C18:2.

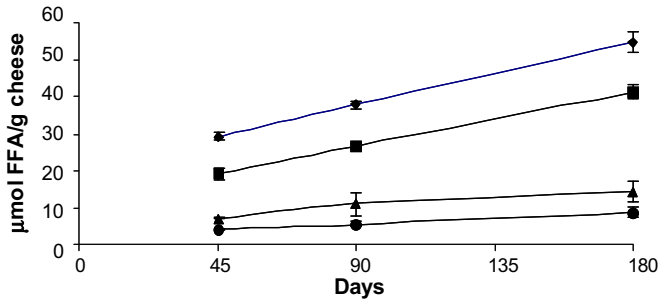

Fig. 1. Total FFA content (µmol FFA/kg cheese) at different ripening times in cheeses made with different amounts of lipase: ● 0 LU/50 L, ▲ 35 LU/50 L, ■ 120 LU/50 L and ♦ 242 LU/50 L.

found in the cheeses made with the highest amount of lipase (Fig. 1). Total FFA concentration linearly increased in all cheeses throughout ripening, but the rate of FFA accumulation was considerably higher in cheeses made with commercial lipase. The linear increase ($R^2 > 0.96$ in all cases) indicated that FFA accumulation was constant throughout ripening time, and was consistent with added pregastric lipase being active after 180 ripening days, as recently described (Svensson et al., 2006).

Lipoprotein lipase (LPL) and microbial lipases were most likely responsible for the low lipolysis level found in cheeses without added lipase (Chávarri et al., 1999; Chávarri, Santisteban, Virto, & de Renobales, 1998; Collins, McSweeney, & Wilkinson, 2003). In addition, a linear correlation ($R^2 > 0.95$) between the lipase amount used for cheese manufacture and total FFA concentration was observed after 45, 90 or 180 ripening days. This relationship can be useful to predict the FFA concentration in such types of cheese depending on the amount of pregastric lipase used.

Table 2 shows the content of individual FFA in the cheeses made with different amount of added lipase after 45 and 180 ripening days. The addition of pregastric lipase had a considerable effect on the accumulation of short-, medium- and long-chain FFA during cheese ripening after, 45, 90 (data not shown) or 180 ripening days. Cheeses with commercial lipase added had significantly higher amounts of short-, medium-, and long-chain FFA than cheeses made without lipase at all sampling times. As observed in Table 2, the increase in the lipolytic activity during ripening was primarily related to an increase in the content of short-chain FFA (butyric, n-hexanoic, n-octanoic and n-decanoic acids). However, the differences observed in the content of medium- and long-chain FFA among the cheeses made with different amount of lipase were smaller for long-chain FFA than for medium-chain FFA (Table 2).

Partial glycerides are derived from the hydrolysis of triacylglycerols by lipases. The concentration of various diglycerides (DG) in cheeses made with different amount of added lipase is shown in Table 3. With the exception of cheeses made without lipase added, (1,2+2,3)-DG were, by far, the major partial glycerides in all cheeses. Monoglycerides were not detected in any of the cheeses, regardless of the amount of lipase added. As it was expected, the highest total DG content occurred in the cheeses made with the highest amount of commercial lipase, particularly after 180 days of ripening. Although cheeses made with lamb rennet paste (35 LU/50 L) showed higher content of total DG than cheeses made without lipase addition, these differences were not statistically significant ($P > 0.05$). Commercial lipase addition caused a significant ($P \leqslant 0.05$) increase in total DG content after 90 or 180 ripening days. Statistically significant ($P \leqslant 0.05$) differences in the content of (1,2+2,3)-DG and 1,3-DG were found only after 180 days between cheeses made with high and low amount of commercial lipase. The amount of 1,3-DG increased significantly ($P \leqslant 0.05$) during ripening in cheeses made with high amount of commercial lipase, whereas no statistically significant differences ($P > 0.05$) were observed for (1,2+2,3)-DG.

Results reported herein in cheeses are consistent with the substrate specificity exhibited by pregastric lipases using model TG "in vitro". Previously, it has been reported that pregastric lipases release preferentially short-chain FA and have *sn*-3 stereospecificity (Lai & O'Connor, 2000; O'Connor, Bang, Taylor & Brimble, 2001; O'Connor, Manuel & Turner, 1993). In ewe's milk fat, short-chain FA are esterified mainly on the *sn*-3 position of the triglyceride molecule (Anifantakis, 1986; Ha & Lindsay, 1993; O'Connor et al., 2001). Thus, milk fat hydrolysis by pregastric lipases yields short-chain FFA and (1,2+2,3)-DG (Hernández et al., 2001; Lai, Mackenzie, O'Connor & Turner, 1997). These results are also in accordance with those obtained by Barrón et al. (2004) in Idiazabal cheeses made with addition of different types of lipases.

3.3. Proteolysis

Total FAA concentration in the different cheeses varied from 29.9 ± 3.6 µmol/g cheese to 39.7 ± 4.6 µmol/g cheese (43.3–62.8 µmol/g DM) after 90 ripening days, and between 54.1 ± 4.4 and 69.9 ± 7.1 µmol/g cheese after 180 days (79.9–104.2 µmol/g DM) (Table 4). These concentrations were lower than those reported before in Idiazabal cheese (around 170 µmol/g DM after 90 ripening days (Bustamante et al. 2003), in Ossau-Iraty after 120 days (155 µmol/g DM; Izco, Torrre, & Barcina, 2000b) or in Emmental, Gruyere and Appenzeller cheeses (Steffen, Flueckiger, Bosset, & Ruegg, 1987). Differences in FAA content between different cheeses are difficult to interpret, and total FAA concentration can vary widely even within the same cheese type (Frau, Massanet, Rosello, Simal, & Cañellas, 1997; Innocente, 1997). Total FAA concentration increased with ripening in all types of cheeses (Table 4). This result agrees with those of Vicente, Ibanez, Barcina, & Barron (2001a) and of Bustamante et al. (2003), who reported a lin-

Table 3
Mean values and standard deviations for the concentrations of partial glycerides (DG) (mg/100 g cheese) in cheeses made with different amount of added lipase after 90 and 180 days of ripening.

Glyceride	Days	No lipase	Lamb rennet paste	Commercial lipase	
		0 LU/50 L	35 LU/50 L	120 LU/50 L	242 LU/50 L
(1,2+2,3)-DG	90	96.7 ± 8.2^{a}	260.8 ± 46.81^{c}	592.9 ± 74.8^{b}	723.2 ± 99.9^{b}
	180	104.7 ± 42.7^{a}	238.6 ± 63.7^{a}	556.0 ± 44.3^{b}	897.2 ± 121.1^{c}
1,3-DG	90	99.1 ± 16.6^{a,*}	82.7 ± 6.3^{a,b,*}	62.5 ± 15.7^{b}	61.8 ± 5.3^{b,*}
	180	155.4 ± 35.2^{a}	99.4 ± 11.2^{a,b}	74.7 ± 8.1^{b}	236.0 ± 46.3^{c}
Total DG	90	195.9 ± 18.3^{a}	343.5 ± 44.5^{a}	655.4 ± 88.4^{b}	784.9 ± 100.6^{b,*}
	180	260.2 ± 73.0	338.0 ± 58.9^{a}	630.7 ± 51.5^{b}	1133.1 ± 135.7^{c}

$^{a-c}$Means within rows with a different superscript are significantly different ($P \leqslant 0.05$).
* Means significantly ($P \leqslant 0.05$) different between ripening days for each cheese batch.

Table 4
Mean values and standard deviations of the concentrations of FAA (μmol/g cheese) in ewe's raw milk cheeses made with different amounts of lipase after 90 and 180 days of ripening.

FAA	No lipase added		Lamb rennet paste		Commercial lipase			
	0 LU/50 L		35 LU/50 L		120 LU/50 L		242 LU/50 L	
	90 days	180 days	90 days	180 days	90 days	180 days	90 days	180 days
HPro	ND	ND	0.32 ± 0.06^{b}	ND	0.17 ± 0.07^{a}	ND	ND	ND
Asp	1.66 ± 0.39^{a}	3.47 ± 0.71^{*,a}	2.00 ± 0.43^{a}	4.04 ± 1.21^{*,a}	1.67 ± 0.24^{a}	3.63 ± 0.78^{*,a}	1.39 ± 0.25^{a}	2.84 ± 0.24^{*,a}
Ser-Asn	0.93 ± 0.60^{a}	1.66 ± 1.12^{a}	0.13 ± 0.11^{b}	0.34 ± 0.27^{b}	0.13 ± 0.08^{b}	0.51 ± 0.30^{*,a,b}	ND^{b}	0.58 ± 0.35^{*,a,b}
Glu	6.12±0.58^{a,b}	11.60 ± 0.96^{*,a}	6.32 ± 0.49^{b}	12.40 ± 2.36^{*,a}	5.24 ± 0.09^{a}	10.80 ± 1.40^{*,a}	5.31 ± 0.46^{a}	9.92 ± 0.42^{*,a}
Gly	ND	0.51 ± 0.28^{*,a}	ND	0.34 ± 0.09^{*,a}	ND	0.39 ± 0.26^{*,a}	ND	0.11 ± 0.08^{*,a}
His-Gln	1.15 ± 1.35^{a}	1.06 ± 0.90^{a}	0.75 ± 0.71^{a}	0.80 ± 0.83^{a}	0.53 ± 0.37^{a}	0.84 ± 0.62^{a}	0.37 ± 0.23^{a}	0.70 ± 0.74^{a}
Tau	ND	ND	0.06 ± 0.04^{a}	ND	0.05 ± 0.03^{a}	0.09 ± 0.13^{a}	ND	0.01 ± 0.01^{a}
Arg	0.68 ± 0.32^{a}	1.46 ± 0.44^{*,a}	0.81 ± 0.44^{a}	1.53 ± 0.61^{a}	0.70 ± 0.26^{a}	1.32 ± 0.44^{a}	0.62 ± 0.16^{a}	1.06 ± 0.11^{*,a}
Cit-Thr	1.11 ± 0.44^{a,a}	2.21 ± 0.70*	1.01 ± 0.10^{a}	1.89 ± 0.43^{*,a}	0.93 ± 0.26^{a}	2.30 ± 0.95^{*,a}	0.71 ± 0.34^{a}	1.48 ± 0.15^{*,a}
Ala	1.42 ± 0.30^{a}	2.50 ± 0.51^{*,a}	1.41 ± 0.24^{a}	2.18 ± 0.18^{*,a}	1.37 ± 0.27^{a}	2.50 ± 0.23^{*,a}	1.24 ± 0.41^{a}	2.01 ± 0.5^{a}
Pro	1.85 ± 0.71^{a}	3.33 ± 1.32^{a}	1.91 ± 0.29^{a}	2.36 ± 0.61^{a}	2.00 ± 0.5^{a}	3.97 ± 1.47^{*,a}	1.44 ± 0.22^{a}	2.81 ± 0.73^{*,a}
GABA	0.65 ± 0.49^{a}	0.44 ± 0.29^{a}	0.44 ± 0.50^{a}	0.65 ± 0.53^{a}	0.39 ± 0.32^{a}	0.60 ± 0.51^{a}	0.35 ± 0.14^{a}	0.31 ± 0.15^{a}
AABA	0.31 ± 0.12^{a,b}	0.50 ± 0.20^{a}	ND	0.62 ± 0.20^{*,a}	0.46 ± 0.11^{b}	0.66 ± 0.19^{*,a}	0.23 ± 0.11^{a,b,b}	0.41 ± 0.04^{*,a}
Cys2	3.98 ± 1.42^{a}	6.83 ± 1.90^{a}	1.27 ± 0.40^{b}	4.35 ± 0.52^{*,a}	3.11 ± 0.46^{a}	6.60 ± 1.13^{*,a}	1.08 ± 0.19^{b}	5.30 ± 1.16^{*,a}
Tyr	0.40 ± 0.30^{a}	0.76 ± 0.41^{a}	0.23 ± 0.16^{a}	2.25 ± 3.28^{a}	0.21 ± 0.16^{a}	0.63 ± 0.72^{a}	0.31 ± 0.20^{a}	0.29 ± 0.07^{a}
Val	4.85 ± 0.42^{a}	7.46 ± 0.64^{*,a}	5.19 ± 0.49^{a}	7.85 ± 0.85^{*,a}	4.93 ± 0.40^{a,b}	7.41 ± 0.80^{*,a}	4.20 ± 0.41^{b}	6.55 ± 0.20^{*,a}
Met	0.35 ± 0.37^{a}	1.17 ± 0.83*	0.25 ± 0.07^{a}	1.00 ± 0.36^{*,a}	0.71 ± 0.57^{a}	1.25 ± 0.60^{a}	0.65 ± 0.29^{a}	0.64 ± 0.04^{a}
Orn	1.75 ± 0.40^{a}	2.73 ± 0.48^{*,a}	2.83 ± 0.19^{b}	3.50 ± 0.26^{*,a}	1.72 ± 0.44^{a}	2.71 ± 0.45^{*,a}	1.31 ± 0.46^{a}	1.77 ± 0.27^{b}
Lys	2.11 ± 0.51^{a,b}	4.68 ± 0.28^{*,a,b}	2.65 ± 0.45^{a}	5.37 ± 0.90^{*,a}	1.82 ± 0.30^{b}	4.61 ± 0.70^{*,a,b}	1.81 ± 0.33^{a,b}	3.52 ± 0.09^{*,b}
Ile	1.15 ± 0.19^{a}	2.26 ± 0.30^{*,a}	1.12 ± 0.09^{a}	2.26 ± 0.26^{*,a}	0.93 ± 0.11^{a}	1.91 ± 0.07^{*,a}	0.94 ± 0.18^{a}	1.85 ± 0.20^{*,a}
Leu	5.80 ± 0.63^{a}	9.42 ± 0.77^{*,a,b}	7.03 ± 1.02^{a}	11.56 ± 1.82^{*,a}	5.64 ± 0.30^{a}	9.46 ± 1.55^{*,a,b}	5.08 ± 0.45^{a}	7.85 ± 0.59^{*,b}
Phe	2.72 ± 0.28^{a}	4.13 ± 1.01^{*,a}	2.40 ± 0.35^{a}	3.99 ± 0.43^{*,a}	2.57 ± 0.38^{a}	4.30 ± 0.58^{*,a}	2.37 ± 0.61^{a}	3.34 ± 0.28^{*,a}
Trp	0.66 ± 0.16^{a}	0.66 ± 0.17^{a}	0.50 ± 0.09^{a}	0.64 ± 0.14^{a}	0.52 ± 0.07^{a}	0.52 ± 0.11^{a}	0.53 ± 0.15^{a}	0.79 ±0.10^{*,a}
Total	39.70 ± 4.6^{a}	68.90 ± 9.7^{*,a}	38.60 ± 3.4^{a}	69.90 ± 7.1^{*,a}	35.80 ± 2.2^{a}	67.00 ± 6.1^{*,a,b}	29.90 ± 3.6 ^{b}	54.10 ± 4.4^{*,b}

ND: Not detected.
HPro: Hidroxyproline; Ser-Asn: Serine-Asparagine; Glu: Glutamic Acid; Gly: Glycine; His-Gln: Histidine-Glutamine; Tau: Taurine; Arg: Arginine; Cit-Thr: Citrulline-Threonine; Ala: Alanine; Pro: Proline; GABA: γ-Amino-butyric acid; AABA: α-amino-butyric acid; Cys2: Cystine; Tyr: Tyrosine; Val: Valine; Met: Methionine; Orn: Ornithine; Lys: Lysine; Ile: Isoleucine; Leu: Leucine; Phe: Phenylalanine; Trp: Tryptophan.
[a–d]Means were significantly ($p < 0.05$) different among cheese batches (amount of lipase added) for the same ripening time.
[*] Means were significantly ($p < 0.05$) different between ripening days for each cheese batch.

ear response between the content of FAA and ripening time for Idiazabal cheese.

Unexpectedly, there was a clear inverse relationship between the amount of added lipase and the FAA content of the cheeses. As shown in Table 4, cheeses with commercial lipase added showed statistically significant lower total FAA concentration, after 90 or 180 ripening days, than cheeses made with no lipase. This difference was more pronounced for cheeses made with high amount of lipase. However, there were no significant ($P > 0.05$) differences in total FAA content between cheeses made with the lowest amount of added lipase (as part of the artisanal lamb rennet) and cheeses made with no added lipase (Table 4). Thus, small differences in coagulating and lipolytic activities (as those between commercial and artisanal rennets) do not have a significant effect on FAA release. In a previous work (Bustamante et al., 2003), we demonstrated that total FAA content was not affected by the type of rennet when comparable coagulating and lipolytic activities were used.

If proteolysis and lipolysis produced during cheese ripening are studied jointly, a linear, inverse, relationship between total FAA and total FFA content could be established in cheeses made with commercial lipase. It was found that the higher the FFA content, the lower the FAA content, with a linear relation having similar slopes (−0.3 μmol FAA/μmol FFA) after 90 or 180 days (Fig. 2). Most likely, the release of FAA is mainly due to proteolytic activities of lactic acid bacteria and other bacterial species (Smit et al., 2005), and thus, the relationship between proteolysis and lipolysis indicated herein could be related to bacterial enzymatic activities. Sun, O'Connor and Roberton (2002), using pregastric lipase-hydrolyzed milk fat, inhibited the *in vitro* growth of Gram positive and Gram negative bacteria when short-chain FFA concentration was

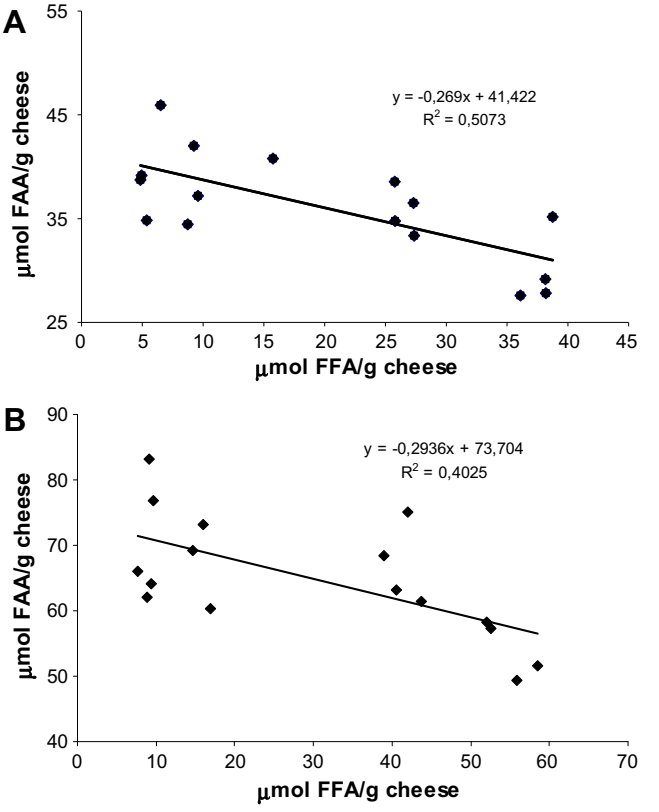

Fig. 2. Linear relationship between the content of FFA and FAA after 90 (A) or 180 (B) ripening days.

over 18.5 mM, indicating that milk-fat hydrolyzed with pregastric lipase can inhibit bacterial growth. The concentration of short-chain FFA in Idiazabal cheese could have similar effect if we consider that, after 90 ripening days, short-chain FFA concentration was 21.5 μmol/g in cheeses made with 120 LU/50 L. Nair et al. (2005) reported the use of FFA in milk as a bacterial growth inhibitor agent, with short-chain FFA being more effective than long chain FFA for inhibition of bacterial growth. Kinderlerer, Matthias, and Finner (1996) proposed that high concentration of medium-chain FFA could inhibit the growth of *Listeria monocytogenes* in mould ripened cheeses, and the growth of *Salmonella typhi* and *Escherichia coli* was inhibited by lipase-hydrolyzed cow's milk *in vitro* (Orozco, Ogura, Hirosawa, Garduno, & Kubo, 2007). In addition, these researchers showed that *Helicobacter pylori* became nonculturable in a medium containing short-chain fatty acids (C4 to C10) from lipase-hydrolyzed milk. Finally, Vicente, Ibañez, Barcina, and Barron (2001b) described that in the absence of starter bacteria FAA accumulation in cheese was reduced.

In the literature there are few references reporting FAA decreasing concentration in dairy products made with different concentrations of lipase. Vicente, Ibanez, Barcina, and Barron (2001a) found significant differences ($P < 0.05$) in FAA concentrations between cheeses elaborated with artisanal lamb rennet (presumably with lipase) and those made with commercial rennet, but they did not report the lipolytic activity for the artisanal lamb rennet. However, work done with different fungal lipases gave contradictory results. Izco, Irigoyen, Torre, and Barcina (2000a) found that cheeses made with commercial fungal lipase Lipomod™ 187 presented lower FAA concentration than cheeses made without added lipase, whereas Fernandez-Garcia, Ramos, Polo, Juarez, and Olano (1988) found that Manchego cheeses made with commercial fungal lipase (Palatase 750L®) presented higher FAA content than Manchego cheeses made without added lipase. Taking into account the above mentioned possible antimicrobial effects of short-chain FFA, the content of FFA should be considered when comparing FAA levels between very different types of cheeses or when FAA concentration is used as ripening index (Farkye & Fox, 1990; Sousa, Ardo, & McSweeney, 2001).

Glutamic acid, valine and leucine were the major FAA after 90 or 180 ripening days, representing together around 44% of total FAA content after 180 days (Table 4). Histidine-glutamine, tyrosine, methionine and tryptophan were minor compounds (all of them below 5% of the total FAA content). Other authors have found similar major FAA in other types of cheeses (Hayaloglu, Guven, Fox, & McSweeney, 2005; Steffen et al., 1987) and in Idiazabal cheese (Barcina, Ibañez, & Ordoñez, 1995; Bustamante et al., 2003; Mendia, Ibanez, Torre, & Barcina, 2000). As it was expected, the content of most FAA increased with ripening time (Table 4). Between 90 and 180 ripening days, the contents of the following amino acids increased rapidly: glutamic from 0.051 to 0.067 μmol/g/day; cystine from 0.032 to 0.047 μmol/g /day and leucine from 0.031 to 0.050 μmol/g/day. However, the content of histidine-glutamine, taurine, GABA, tyrosine and tryptophan did not depend on the ripening time (Table 4). These results were in accordance with those found by other authors (Frau et al., 1997; Mayer, Rockenbauer, & Mlcak, 1998). The presence of GABA was described before in raw milk Idiazabal cheese (in some cases over 1.6 μmol/g DM) but not in pasteurized milk cheese (Mendia et al., 2000). GABA concentrations reported herein are lower than this value. The content of most of the major FAA was statistically influenced by the amount of lipase after 90 (Glu, Val, Lys) or 180 (Leu) ripening days (Table 4). In these cases, the lowest FAA content is present in cheeses with the highest lipase amount. The concentration of the minor FAA did not vary in cheese elaborated with different lipase amounts.

3.4. Relation between physicochemical parameters and sensory properties

In this paper we demonstrated that lipase utilization has a clear effect on the FFA composition and on the FAA accumulation during cheese ripening. However, in order to describe the general effect of lipase utilization, other parameters have to be considered. Sensory properties of the cheeses were investigated by means of determining 8 odour, 10 flavour and 10 texture attributes after 90 and 180 ripening days.

Increasing amount of lipase resulted in significantly ($P < 0.05$) higher scores for odour and flavour intensities, sharp odour, pungent flavour, lamb rennet paste odour, nutty flavour and bitterness. In contrast, low amount of lipase resulted in statistically significant ($P < 0.05$) increases for attributes such as milky, butter and toasty odours, usually associated with mild odour notes, in accordance with Bustamante et al. (2003). All odour and flavour attributes were statistically ($P < 0.05$) affected by the amount of lipase, ripening time or/and the interaction of both factors. As ripening time increased, firmness and graininess increased in all the cheeses, while elasticity decreased. Other texture attributes did not show a significant ($P > 0.05$) correlation with the studied factors.

Principal component analysis (PCA) was applied to sensory attributes and compositional variables that were affected by the amount of lipase or the ripening time, such as short-, medium-, and long-chain FFA, total FFA, FAA and partial glycerides. Other variables such as dry matter were also included. The only texture attributes included were elasticity, firmness and graininess. Two PC (Fig. 3) explained 83.5% of the total variance (55.9% and 27.6% for the first and the second component, respectively). The first factor (PC1) was highly correlated (positive loadings > 0.90) with short- and medium-FFA and total DG. PC1 also had high positive loadings (>0.75) with the following sensory attributes: rennet odour, overall flavour and odour intensities, sharp, rennet and brine odours, and butyric, piquant and bitter flavours. Short- and medium-chain FFA, rennet odour and piquant flavour have been associated with the use of animal pregastric lipase during cheese making (Bustamante et al., 2003; Barron et al., 2005; Fox & Guinee, 1987; Hernández et al., 2001). In consequence, this factor was defined as "pregastric lipase factor", which correlated positively with

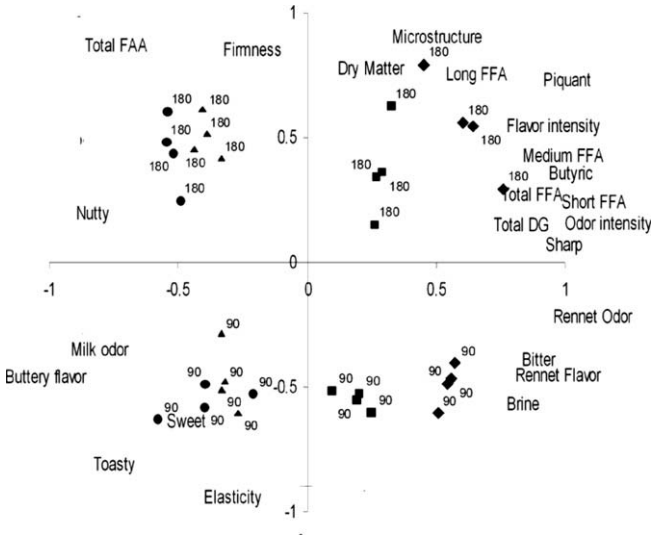

Fig. 3. Plot depicting sample distribution (factor score mean values) from PCA including physico-chemical variables and sensory attributes of Idiazabal cheeses made with different amount of lipase. PC1: horizontal axis; PC2: vertical axis. ●: 0 LU/50 L; ▲: 35 LU/50 L; ■: 120 LU/50 L; ♦: LU/50 L.

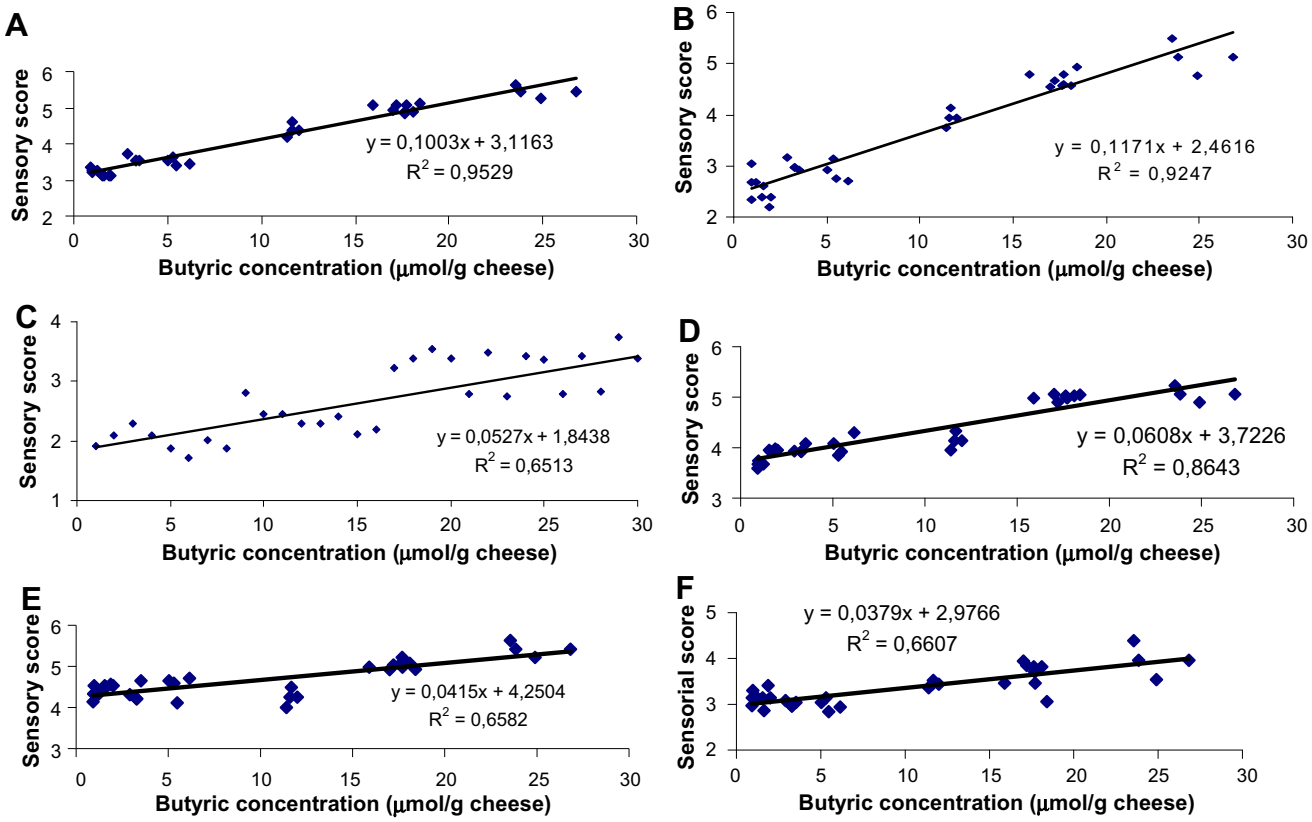

Fig. 4. Relationship between scores of sensory flavour or odour attributes and n-butanoic acid concentration in Idiazabal cheeses made with different amounts of lipase. A: Overall odour intensity; B: sharp odour; C: Rennet odour; D: Butyric flavour; E: Overall flavour intensity; F: Piquant flavour.

most of the "strong notes" in Idiazabal cheese flavour. Mild sensory attributes such as milky, buttery and toasty odours and nutty flavour showed high negative loadings (>−0.75) with "pregastric lipase factor". In order to investigate the incidence of each individual short-chain FFA on the sensory parameters conferring "strong notes" to the cheese, a linear regression analysis was done. "Strong notes" sensory parameters described in PC1 (loadings > |0.75|) correlated with the concentration of butyric acid, but not with any other short-chain FFA ($R^2 < 0.4$) (Fig. 4), probably due to the fact that butyric acid represented between 52% and 55% of the short-chain FFA (Table 2) in cheeses made with added lipase. All slopes were significantly different from zero ($P < 0.001$). These results indicate that changes in the concentration of butyric acid between 5 and 25 µmol/g cheese were necessary to cause changes between 3 and 5 in the scores of these sensory parameters. A similar relationship between flavour intensity and short-chain FFA content was also found in other cheeses (Akin et al., 2003; Woo and Lindsay, 1984).

Compositional variables such as total FAA, long-chain FFA, dry matter and texture parameters such as firmness and graininess, showed positive loadings (>0.69) with PC2, whereas elasticity showed a high negative loading with this factor (Fig. 4). PC2 was defined as "ripening factor" because it contained compositional variables which are strongly influenced by ripening time. Long-chain FFA are not released from milk fat by pregastric lipase (Lai, Mackenzie, O'Connor, & Turner, 1997) and their content increased with ripening time in batches without lipase added (Chávarri et al., 1999). Other parameters, such as dry matter, are clearly ripening time-dependent. Only nutty flavour and milk odour, which usually present low scores in Idiazabal cheese (Bustamante et al., 2003), were correlated with PC2.

All cheese samples in this study were accordingly distributed in the bi-plot coordinate system corresponding to PC1 and PC2 (Fig. 3). The "pregastric lipase factor" (PC1, horizontal axis) differentiated cheese samples with different amount of lipase. Cheese samples made with addition of commercial lipase were separately distributed from cheese samples made without lipase or with artisanal lamb rennet. Also, cheese samples made with low or high amount of lipase were separated by PC1. The "ripening factor" (PC2, vertical axis) distinguished clearly between cheeses after 90 and 180 days of ripening, regardless of the amount of lipase added (Fig. 3).

4. Conclusions

We conclude that the use of pregastric lipase (animal lipase) had an overall effect both on cheese proteolysis and lipolysis during ripening. Secondary proteolysis was affected by high lipase amounts, reducing total FAA concentration, but maintaining the individual FAA profile. Lipase addition increased mainly short-chain FFA concentration, modifying the FFA profile and conferring a characteristic fatty acid balance. At the same time, pregastric lipase activity resulted in a increase of (1,2+2,3)-DG partial glycerides. From the sensory point of view, desirable attributes for Idiazabal cheese, like odour intensity, butyric flavour or rennet flavour, were positively influenced by lipase utilization. Finally, among the short-chain FFA, the content of butyric acid was instrumental to impart "strong notes" on the odour and flavour of Idiazabal cheese.

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References

Akin, N., Aydemir, S., Koçak, C., & Yilduz, M. A. (2003). Changes of free fatty acids contents and sensory properties of white pickled cheese during ripening. *Food Chemistry*(80), 77–83.

Anifantakis, E. M. (1986). Comparison of the physicochemical properties of ewes' and cows' milk. *Bulletin of the International Dairy Federation, 202*, 42–53 (Prod. Util. Ewe's Goat's Milk).

Barcenas, P., Perez-Elortondo, F. J., & Albisu, M. (2000). Selection and screening of a descriptive panel for ewés milk sensory profiling. *Journal of Sensory Studies*(15), 79–99.

Barcenas, P., Perez-Elortondo, F. J., Salmerón, J., & Albisu, M. (1999). Development of a preliminary sensory lexicon and standard references of ewés milk cheese aided by multivariant statistical procedures. *Journal of Sensory Studies*(14), 161–179.

Barcina, Y., Ibañez, F. C., & Ordoñez, A. I. (1995). Evolution of free amino acids during Idiazabal cheese ripening. *Food Control*(3), 161–164.

Barrón, L. J., Hernández, I., Bilbao, A., Flanagan, C. E., Najera, A. I., Virto, M., et al. (2004). Changes in lipid fractions and sensory properties of Idiazabal cheese induced by lipase addition. *Journal of Dairy Research, 71*(3), 372–379.

Barron, L. J. R., Redondo, Y., Flanagan, C. E., Perez-Elortondo, F. J., Marta, A., et al. (2005). Comparison of the volatile composition and sensory characteristics of Spanish PDO cheeses manufactured from ewes' raw milk and animal rennet. *International Dairy Journal, 15*(4), 371–382.

Barton, R. H., O'Connor, C. J., & Turner, K. W. (1996). Characteristics of tributyroylglycerol hydrolysis mediated by a partially purified lamb pregastric lipase. *Journal of Dairy Science, 79*(1), 27–32.

BOE (1993). Reglamento de la denominación de origen Idiazabal y de su consejo regulador. *Boletin Oficial del Estado* (30 de Noviembre de 1993).

Bustamante, M. A., Virto, M., Aramburu, M., Barron, L. J. R., Perez-Elortondo, F. J., Albisu, M., et al. (2003). Lamb rennet paste in ovine cheese (Idiazabal) manufacture. Proteolysis and relationship between analytical and sensory parameters. *International Dairy Journal, 13*(7), 547–557.

Bütikofer, U., Fuchs, D., Bosset, J., & Gmür, W. (1991). Automated HPLC-amino acid determination of protein hydrolysates by precolumn derivatization with OPA and FMOC and comparison with classical ion exchange chromatography. *Chromatographia, 31*(9–10), 441–447.

Chávarri, F., Bustamante, M. A., Santisteban, A., Virto, M., Barrón, L. J. R., & de Renobales, M. (1999). Changes in free fatty acids during ripening of Idiazabal cheese manufactured at different times of the year. *Journal of Dairy Science, 82*(5), 885–890.

Chávarri, F., Santisteban, A., Virto, M., & de Renobales, M. (1998). Alkaline phosphatase, acid phosphatase, lactoperoxidase, and lipoprotein lipase activities in industrial ewe's milk and cheese. *Journal of Agricultural and Food Chemistry, 46*(8), 2926–2932.

Chávarri, F., Virto, M., Martin, C., Najera, A. I., Santisteban, A., Barrón, L. J. R., et al. (1997). Determination of free fatty acids in cheese: Comparison of two analytical methods. *Journal of Dairy Research, 64*(3), 445–452.

Collins, Y. F., McSweeney, P. L., & Wilkinson, M. G. (2003). Evidence of a relationship between autolysis of starter bacteria and lipolysis in Cheddar cheese during ripening. *Journal of Dairy Research, 70*, 105–113.

Farkye, N. Y., & Fox, P. F. (1990). Objective indices of cheese ripening. *Trends in Food Science and Technology, 1*, 37–40.

Fernandez-Garcia, E., Ramos, M., Polo, C., Juarez, M., & Olano, A. (1988). Enzyme accelerated ripening of Spanish hard cheese. *Food Chemistry, 28*, 63–80.

Fox, P. F. (1993). Exogenous enzymes in dairy technology – A review. *Journal of Food Biochemistry, 17*(3), 173–199.

Fox, P. F., & Guinee, T. P. (1987). Italian cheeses. In P. Fox (Ed.). *Cheese, chemistry, physics and microbiology* (Vol. 2, pp. 221–255). Elsevier.

Fox, P. F., O'Connor, T. P., McSweeney, P. L., Guinee, T. P., & O'Brien, N. M. (1996). *Cheese: Physical biochemical and nutritional aspects. Advances in food and nutritional research* (Vol. 39). Academic Press.

Frau, M., Massanet, J., Rosello, C., Simal, S., & Cañellas, J. (1997). Evolution of free amino acids content during ripening of Mahon Cheese. *Food Chemistry*(60), 651–657.

Gobbetti, M. (2004). Extra-hard varieties. In P. L. McSweeney, T. M. Cogan, T. P. Guinee, & P. F. Fox (Eds.). *Cheese chemistry, physics and microbiology* (Vol. 2, pp. 51–70). Elsevier.

Ha, J. K., & Lindsay, R. C. (1993). Release of volatile branched-chain and other fatty acids from ruminant milk fats by various lipases. *Journal of Dairy Science, 76*(3), 677–690.

Hair, J. F., Tatham, R. L., Anderson, R. E., & Black, W. (1998). *Multivariate data analysis*. London: Prentice-Hall.

Hayaloglu, A. A., Guven, M., Fox, P. F., & McSweeney, P. L. (2005). Influence of starters on chemical, biochemical, and sensory changes in Turkish White-brined cheese during ripening. *Journal of Dairy Science, 88*(10), 3460–3474.

Hernández, I., de Renobales, M., Virto, M., Pérez-Elortondo, F. J., Barron, L. J. R., Flanagan, C., et al. (2005). Assessment of industrial lipases for flavour development in commercial Idiazabal (ewe's raw milk) cheese. *Enzyme and Microbial Technology*(36), 870–879.

Hernández, I., Virto, M., Chavarri, F., Ceballos, G., Santisteban, A., Elortondo, F. J. P., et al. (2001). Development of characteristic flavor in Idiazabal cheese adding commercial lipases. In: Spanier, A. M., Shahidi, F., Parliment, T. H., Mussinan, C., Ho, C. T. & Contis, E. T. (Eds.), *Food flavors and chemistry: Advances of the new millenium* (pp. 151–159). Cambridge, United Kingdom: The Royal Society of Chemistry.

IDF (1982). *Standard 4A: Cheese & processed cheese. Determination of total solids content*. Brussels: International Dairy Federation [IDF-FIL Standard 4A].

IDF (1987). *Standard 110A: Calf rennet and adult rennet determination of chymosin and bovine pepsin contents (chromatographic method)*. Brussels, Belgium: International Dairy Federation [IDF-FIL Standard 110A].

IDF (1993). *Standard 20B: Determination of the nitrogen (Kjeldahl method) and calculation of the crude protein content*. Brussels, Belgium: International Dairy Federation [IDF-FIL standard 20B].

Innocente, N. (1997). Free amino acids and water-soluble nitrogen as ripening indices in Montasio cheese. *Lait*(77), 359–369.

ISO (1975). Determination of fat content. *Van Gulik Method*. Geneva (ISO 33433).

Izco, J. M., Irigoyen, A., Torre, P., & Barcina, Y. (2000a). Effect of added enzymes on the free amino acids and sensory characteristics in Ossau-Iraty cheese. *Food Control, 11*, 201–207.

Izco, J. M., Torrre, P., & Barcina, T. (2000b). Ripening of Ossau-Iraty cheese: Determination of free amino acids by RP-HPLC and of total free amino acids by the TNBS method. *Food Control*(11), 7–11.

Kilcawley, K. N., Wilkinson, M. G., & Fox, P. F. (1998). Enzyme-modified cheese. *International Dairy Journal, 8*(1), 1–10.

Kinderlerer, J., Matthias, H., & Finner, P. (1996). Effect of medium-chain fatty acids in mould ripened cheese on the growth of Listeria monocytogenes. *Journal Dairy Research, 4*(63), 593–606.

Kunji, E. R., Mierau, I., Hagting, A., Poolman, B., & Konings, W. N. (1996). The proteolytic systems of lactic acid bacteria. *Antonie Van Leeuwenhoek, 70*(2–4), 187–221.

Lai, D. T., & O'Connor, C. J. (2000). Synergistic effects of surfactants on kid pregastric lipase catalyzed hydrolysis reactions. *Langmuir* 16(1), 115–121.

Lai, D. T., Mackenzie, A. D., O'Connor, C. J., & Turner, K. W. (1997). Hydrolysis characteristics of bovine milk fat and monoacid triglycerides mediated by pregastric lipase from goats and kids. *Journal of Dairy Science, 80*(10), 2249–2257.

Law, B. A. (1981). The formation of aroma and flavour compounds in fermented dairy products. *Dairy Science Abstract, 43*(3), 143–154.

Law, B. A. (2001). Controlled and accelerated cheese ripening: The research base for new technologies. *International Dairy Journal*(11), 383–398.

Mayer, H. K., Rockenbauer, C., & Mlcak, H. (1998). Evaluation of proteolysis in Parmesan cheese using electrophoresis and HPLC. *Lait, 78*(4), 425–438.

Mendia, C., Ibanez, F. C., Torre, P., & Barcina, Y. (2000). Effect of pasteurization and use of a native starter culture on protolysis in a ewe's milk cheese. *Food Control*(11), 195–200.

Nair, M. K., Joy, J., Vasudevan, P., Hinckley, L., Hoagland, T. A., & Venkitanarayanan, K. S. (2005). Antibacterial effect of caprylic acid and monocaprylin on major bacterial mastitis pathogens. *Journal Dairy Science, 88*(10), 3488–3495.

Nardi, M., Fiez-Vandal, C., Tailliez, P., & Monnet, V. (2002). The EstA esterase is responsible for the main capacity of *Lactococcus lactis* to synthesize short chain fatty acid esters in vitro. *Journal of Applied Microbiology, 93*(6), 994–1002.

O'Connor, C. J., Bang, K.-A., Taylor, C. M., & Brimble, M. A. (2001). Determining the regio- and typo-selectivity of calf pregastric lipase. *Journal of Molecular Catalysis B: Enzymatic, 16*(3–4), 147–157.

O'Connor, C. J., Manuel, R. D., & Turner, K. W. (1993). Calf and lamb lingual lipases as catalysts for the hydrolysis of tributyrin, triolein, and 4-nitrophenylacetate. *Journal of Dairy Science, 76*(12), 3674–3682.

Orozco, A., Ogura, T., Hirosawa, T., Garduno, R., & Kubo, I. (2007). In hydrolyzed cow's milk Helicobacter pylori becomes nonculturable and the growth of Salmonella typhi and Escherichia coli is inhibited. *Journal of Food Science, 72*(8), M306–M309.

Smit, G., Smit, B. A., & Engels, W. J. (2005). Flavour formation by lactic acid bacteria and biochemical flavour profiling of cheese products. *FEMS Microbiology Review, 29*(3), 591–610.

Sousa, M. J., Ardo, Y., & McSweeney, P. L. H. (2001). Advances in the study of proteolysis during cheese ripening. *International Dairy Journal, 11*(4-7), 327–345.

Steffen, C., Flueckiger, E., Bosset, J. O., & Ruegg, M. (1987). Swiss-type varieties. In P. F. Fox (Ed.). *Cheese: Chemistry, physics and microbiology* (Vol. 2, pp. 93–120). Elsevier Applied Science Publishers LTD..

Sun, C. Q., O'Connor, C. J., & Roberton, A. M. (2002). The antimicrobial properties of milkfat after partial hydrolysis by calf pregastric lipase. *Chemico-Biological Interaction, 140*(2), 185–198.

Svensson, I., Hernández, I., Virto, M., & de Renobales, M. (2006). Determination of lipase activity in cheese using trivalerin as substrate. *International Dairy Journal, 16*(5), 423–430.

Vicente, M. S., Ibanez, F. C., Barcina, Y., & Barron, L. J. R. (2001a). Changes in the free amino acid content during ripening of Idiazabal cheese: Influence of starter and rennet type. *Food Chemistry, 72*(3), 309–317.

Vicente, M. S., Ibañez, F. C., Barcina, Y., & Barron, L. J. R. (2001b). Changes in the free amino acid content during ripening of Idiazabal cheese: Influence of starter and rennet type. *Food Chemistry, 72*(3), 309–317.

Virto, M., Chavarri, F., Bustamante, M. A., Barron, L. J. R., Aramburu, M., Vicente, M. S., Perez-Elortondo, F. J., Albisu, M., & de Renobales, M. (2003). Lamb rennet paste in ovine cheese manufacture. Lipolysis and flavour. *International Dairy Journal, 13*(5), 391–399.

Visser, S. (1993). Proteolytic enzymes and their relation to cheese ripening and flavor: An overview. *Journal Dairy Science, 76*(1), 329–350.

Wilkinson, M. G., van den Berg, G., & Law, B. A. (2002). Technological properties of commercially-available enzyme preparations other than coagulants. *Bulletin of the IDF, 371*, 16–19.

Woo, A. H., & Lindsay, R. C. (1984). Concentrations of major free fatty acids and flavor development in Italian cheese varieties. *Journal of Dairy Science, 67*(5), 960–968.