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Changes in lipolysis and volatile fraction of a goat cheese manufactured employing a hygienized rennet paste and a defined strain starter

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Abstract

The effect of a hygienized rennet paste (HRP) and a defined strain starter IFPL on the volatile fraction of goat cheese (Majorero) was examined. Three batches were made and those cheeses produced either industrially (IL) or in artisanal manner (AL) were compared with the experimental lot (EL), which included both HRP and IFPL starter in its manufacture. Analysis of the volatile fraction was by static headspace connected to GC–MS and this disclosed a total of 28 components belonging to the following chemical families: fatty acids, esters, ketones, aldehydes and alcohols. Most of the volatile components identified appeared in all lots but at different concentrations. IL cheeses were distinguished from the other lots, including HRP in their manufacture, essentially by a lower presence of esters and FFA (both branched-chain and aliphatic acids). The use of HRP and a defined strain starter culture (including *Lb. casei* IFPL 731 as adjunct) directly affected lipolysis degree. This was reflected in an increase in the mono-and diglyceride concentrations and in a greater content of short-chain FFA, particularly butanoic acid, an important flavour component, which imparts a desirable sharp, ''piquant'' taste to Majorero cheese. The IFPL starter culture also had a significant effect on concentrations of compounds such as 2-methyl-1-propanol, 3 methyl-1-butanol, isoamyl butyrate and acetoin.

Keywords: Rennet paste; Goat cheese flavor; Lipolysis; Volatile fraction

1. Introduction

Cheese ripening is a slow process, involving a concerted series of microbiological, biochemical and chemical reactions. Primary degradation of milk constituents by glycolysis, lipolysis, and proteolysis leads to the formation of a whole range of precursors of flavour compounds. These changes are followed and/or overlapped by a series of secondary catabolic reactions, which are responsible for the unique aroma profile of a particular variety of cheese (Marilley & Casey, 2004). Due to the growing interest in characterization of traditional products protected by a designation of origin, the volatile fractions of some Spanish cheeses, such as Manchego (Martínez-Castro, Sanz, Amigo, Ramos, & Martín-Alvarez, 1991; Villaseñor, Valero, Sanz, & Martínez-Castro, 2000), Mahón (Mulet, Escriche, Rossello, & Tarrazó, 1999), Roncal (Izco & Torre, 2000), Idiazabal (Larráyoz, Addis, Gauch, & Bosset, 2001), La Serena (Carbonell, Núñez, & Fernández-García, 2002), Palmero (Guillén, Ibargoitia, Sopelana, Palencia, & Fresno, 2004), and Zamorano (Fernández-García, Carbonell, Gaya, & Nuñez, 2004) have been determined. Recently,

Abbreviations: HRP, hygienized rennet paste; IL, industrial lot; EL, experimental lot; AL, artisanal lot; FFA, free fatty acids; BCFA, branched-chain fatty acids; MG, monoglycerides; DG, diglycerides; TG, triglycerides; LMW-TG, low molecular weight triglycerides; MMW-TG, medium molecular weight triglycerides; CN, carbon number.

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Barron et al. (2005), have also compared the volatile composition and sensory characteristics of some of these cheeses. Despite Majorero cheese being greatly appreciated for its organoleptic properties its volatile compounds have never been well characterized. Majorero cheese has traditionally been made using raw milk and artisanal rennet pastes, but aspects related essentially to the microbial safety have popularized the use of pasteurized milk as well as commercial rennet. Pasteurization causes denaturation of the native enzymes in the milk and also lowers the concentration of the indigenous microflora, including non-starter lactic acid bacteria (McSweeney, Fox, Lucey, Jordan, & Cogan, 1993). Thus, to produce cheeses with the same characteristics as cheeses made from raw milk, replenishment of the beneficial microorganism destroyed by heating is necessary (Mendía, Ibañez, Torre, & Barcina, 1999).

As a result of characterization of the native flora of Majorero cheese, a defined strain starter (IFPL starter), including *Lactobaccillus casei* subsp. *casei* IFPL731 as an adjunct was successfully developed (Requena, de la Fuente, Fernández de Palencia, Juárez, & Peláez, 1992). The multiple enzymatic system of this microorganism has recently been reviewed (Martínez-Cuesta, Fernández de Palencia, Requena, & Peláez, 2001), confirming its marked ability to influence the development of cheese flavour. Employment of this IFPL starter, along with a hygienized rennet paste (HRP) obtained in our laboratory (Calvo & Fontecha, 2004), has been a recently considered technique for improvement of Majorero cheese (Calvo, Castillo, Díaz-Barcos, Requena, & Fontecha, submitted for publication). Unlike most cheese varieties, where relatively little lipolysis occurs during ripening, an extensive lipolytic activity is desirable as a part of overall flavour development in certain cheeses, such as Majorero and Idiazabal and some hard Italian cheeses (Parmesan, Romano and Provolone). The flavour and aroma characteristic of these cheeses are due to FA released by the action of lipolytic enzymes present in the rennet pastes added during the elaboration process (Fox & Stepaniak, 1993). In addition to their direct impact on cheese flavour, FFAs also act as precursor molecules, which lead to the production of other flavour compounds, such as methylketones, esters, fatty acid lactones and alcohols (McSweeney & Sousa, 2000).

Short-chain fatty acids, even at low concentrations, play a key role in cheese flavour (Urbach, 1993). We considered that the intracellular esterase purified from *Lb. casei* IFPL731 (Castillo, Requena, Fernández de Palencia, Fontecha, & Gobbetti, 1999) and the pregastric esterase isolated from HRP (Calvo & Fontecha, 2004), both showing a marked specificity for triglycerides containing short-chain fatty acids, may contribute positively to cheese flavour development.

The objective of this study was to identify the volatile compounds characteristic of Majorero cheese throughout the entire ripening period. Simultaneously, the influence of the addition both a HRP and a defined strain starter IFPL (including *Lb.casei* IFPL731 as adjunct) on lipolysis degree and hence on the volatile fraction was also analyzed. Possible implications for the dairy industry were also considered.

2. Materials and methods

2.1. Hygienization process of rennet paste

Rennet paste was hygienized, following the general purification scheme described by Calvo and Fontecha (2004). Briefly, suckling kids stomachs (full of milk) and salted in brine for at least 2 months were washed and sectioned in order to extract their contents, which were minced and homogenized in Milli-Q water until a semi-liquid homogeneous paste was obtained. The crude homogenate was first centrifuged (15,000*g*, 4 °C for 30 min) to remove tissue remains as well as non-soluble particles. The supernatant was submitted to vacuum filtration in a Millipore unit, coupled to an Eyela pump, model A-3S (Tokyo Rikakikai Co.) Whatman-1 filters being employed to eliminate particles in suspension. Subsequently, the solution was filtered through 0.22 μm pore-size filters (Millipore) in order to avoid bacterial contamination of the samples. The final product, labelled as HRP (Hygienized Rennet Paste), displayed values of 2.66 and 0.79 ± 0.01 U/mg for clotting and lipase activities, respectively.

2.2. Starter culture preparation

The defined strain starter culture (IFPL) employed in this study had been developed in our laboratory (Requena et al., 1992) and included microorganisms previously isolated from Majorera raw goat milk. This IFPL starter contained the isolate *Lactococcus lactis* subsp. *lactis* IFPL359, and the adjunct *L. casei* subsp. *casei* IFPL731, *Lactobacillus plantarum* IFPL935, *Leuconostoc mesenteroides* subsp. *dextranicum* IFPL709 and *Leuconostoc paramesenteroides* IFPL705. Strains were grown separately in milk and then were combined and inoculated together in sterile skimmed milk at a final concentration of 1%.

2.3. Cheese manufacture

Three different cheese lots were manufactured using Majorera goat's milk from flocks of Fuerteventura island. Twelve cheeses were obtained from each lot. A total number of 36 cheeses were analyzed throughout the ripening. The industrial lot (IL) was manufactured using pasteurized milk (72–76 °C, 20 s) and a commercial starter from Larbus S.A (Madrid, Spain). Commercial rennet (strength 1:150,000) was employed for milk curdling. The artisanal lot (AL) was elaborated according to the traditional manner and no starter culture was added for cheesemaking because raw milk was employed. A hygienized rennet paste (1 ml/1 l milk), obtained according to the procedure described by Calvo and Fontecha (2004), was used as coagulant. For manufacturing of the experimental lot

(EL), pasteurized milk (72–76 °C, 20 s), a specific starter culture (IFPL) for Majorero cheese, as mentioned above, and the hygienized rennet paste (1 ml/1 l milk) were employed. Briefly, general cheesemaking procedure was applied as follows: milk was heated to 32 °C and $CaCl_2$ (0.2 g/l) and starter cultures (AL and EL) were added and left until milk pH decreased to 6.5. After the rennet was added, coagulation took place over 35 min for IL and 1 h and 15 min for AL and EL. Once the curd was cut, vat temperature was increased to 36 °C. The curds were placed into moulds of 3.5 kg and pressed until the pH was 5.2. Cheeses IL and EL were salted in brine (20° Be) and AL was salted on surface. Cheeses were ripened at 15–17 °C and ~85% HR for 60 days. Totally 36 cheeses were analyzed.

2.4. Analysis of triglycerides

The fat from goat's milk and from the different lots of cheese (at 7, 15, 30 and 60 ripening days), was extracted according to the procedure normalized by IDF (1965). Then samples were stored at −20 °C until their analysis by gas chromatography (GC).

The triglyceride analyses were performed on an Autosystem gas chromatograph (Perkin–Elmer Beaconsfield, UK) equipped to an automatic injector (split/splitless) and programmed temperature. For the analysis of total triglycerides (TGs) in cheese, 20 mg of the extracted fat were dissolved in 0.5 ml of hexane and 0.2 μl were injected in the split mode (split ratio was 1:4) into a capillary column, (30 m × 0.22μm i.d., Rtx-65 TG, containing 35% dimethyl, 65% diphenyl polysiloxane as stationary phase), supplied by Restek (Bellefonte, PA). The oven temperature was programmed as follows: the initial temperature (150 °C) was raised to 250 °C at a rate of 15 °C/min^{-1}, then increasing at 10 °C/min to 355 °C and then held at this temperature for 22 min. The injector and detector temperatures were 355 and 370 °C, respectively. The pressure at the top of the column was 0.17 MPa and helium was used as carrier gas.

Trinanoin (CN27, Sigma, Chemical Co., St. Louis, MO) was employed as internal standard (0.5 μl of a solution 10.1 mg/ml in chloroform) in quantitative studies. A mixture of synthetic triglycerides (tristearin, tripalmitin, trimyristin, trilaurin, tricaprin and tricaprylin), diglycerides (diestearin, dipalmitin and diolein) and monoglycerides (monoesterarin, monopalmitin and monoolein) (CN27, Sigma, Chemical Co., St. Louis, MO) was first analyzed to determine both the best chromatographic conditions and the retention time of these components.

A reference anhydrous butter fat, with known TG composition, BCR-519 (reference material, EC; Fedelco S.L., Madrid, BCR authorized distributor), was used to determine the response factors for quantitative studies of TG. TGs were quantified in weight percentage of the fat molecules of different size (CN20–CN54) using the even CN numbers only ($2n$). TGs with odd acyl-c number ($2n + 1$) were combined with the preceding even-numbered triglyceride.

Lipolysis degree in the cheeses was estimated by the increase in low molecular weight triglycerides (LMW-TG) and medium molecular weight triglyceride (MMW-TG) content during ripening, taking as reference the initial content of TGs in cheese milk. The method is based on the non- existence of DG containing fatty acids shorter than lauric acid (C12) (Precht & Abd-el Salam, 1985). Therefore, the increase in relative percentage of TGs smaller than 24 carbon atoms (CN20–CN22), i.e. LMW-TG, was calculated as the increase in MG. Otherwise, the increase in DG was estimated by the increase in MMW-TG fraction (CN24–CN36) in the ripened cheese.

2.5. Analysis of volatile compounds

Analysis of the volatile fraction was performed by the headspace gas chromatographic–mass spectrometric (GC–MS) method described by Alonso, Fontecha, and Juárez (1999).

To 10 g of cheese, previously homogenized, 80 μl of an aqueous solution of propionic acid ethyl ester (1.14 mg/ml) as internal standard and anhydrous sodium sulphate (10 g), to retain water, were added. Individual standard dilutions in aqueous solution were prepared and were stored in hermetically sealed vials at −20 °C prior to their use.

Prior to analysis in a static headspace apparatus (Model HSS 19395; Hewlett–Packard), the samples were maintained at 80 °C for 60 min until the sample and gaseous phase reached thermodynamic equilibrium. The apparatus was programmed as follows: 5 s pressurization, equilibrium and filling and 2 min for injection. Helium was employed as carrier gas at a flow rate of 17.5 ml/min. A Hewlett–Packard GC Model 5890, coupled to a selective MS Model 5972, was employed for volatile compounds analysis. Samples were injected in the split mode (split rate of 7:1) on a capillary silica column with polyethylene glycol (HP Innovas, 60 m, 0.25 mm ID, 0.25 μm film thickness, Hewlett–Packard). Helium was used as carrier gas, at a flow rate of 36.5 cm/s. The column temperature programme was: 33 °C for 5 min, increase at 1 °C/min up to 38 °C and then at 7 °C/min up to 210 °C, and held for 10 min. Injection was carried out at 200 °C and the interface line of MS at 280 °C. Electronic ionisation energy and photo-multiplier voltage were 70 eV and 1647 V, respectively.

2.6. Statistical analysis

The SPSS package (SPSS 11.5 for Windows, SPSS Inc., Chicago, IL, USA) was used for statistical analysis of the results. Analysis of variance (ANOVA) was undertaken and the meaningful significance level was established at $P \leqslant 0.05$. Mean comparisons were performed with Tukey's honestly significant differences (HDS) test. Thus, the a, b, c superscripts were used to state significant differences between lots for the same ripening time.

3. Results and discussion

3.1. Analysis of cheese triglycerides

Cheese fat lipolysis depends on type of rennet used, on the microbial flora and on the maturation period and its contribution to the final taste/flavour has been extensively discussed in the literature (Collins, McSweeney, & Wilkinson, 2003). Study of TG composition, due to the great preponderance of this lipid fraction in milk fat, provides limited information on cheese lipolysis level. Nevertheless, the slight differences detected in TG contents during the cheese ripening period allow us to predict important changes in relative amounts of DG and MG.

The TG composition values of the goat's milk used for manufacture of IL, EL and AL of Majorero cheese were in the same ranges that reported by Fontecha, Díaz, Fraga, and Juárez (1998) for goat's milk obtained from five herds (see Table 1). The three lots of cheese showed similar moisture content (40.84%, 40.77% and 43.34% for IL, EL, and AL, respectively). The total content of cheese fat (55%, 56% and 53% for IL, EL, and AL, respectively), expressed as percentage of TS, were in accordance with those found in a previous characterization of Majorero cheese performed in our laboratory (Fontecha et al., 1990; Martín-Hernández, Juárez, & Ramos, 1992).

Using the method previously described by Precht and Abd-el Salam (1985) we could determine lipolysis degree by calculation of the increase in LMW-TG (related to MG) and MMW-TG (related to DG) contents, at different stages of ripening (Fig. 1).

Fig. 1A shows the percentage of increase in LMW-TG at 7, 15, 30 and 60 ripening days. Industrial cheeses showed the lowest MGs contents throughout the maturation period, attaining 0.8% after 60 ripening days while, at this point, the contents in AL and EL were 1.7% and 2.3%, respectively. Due to the great rate of lipolysis characteristic of Majorero cheese, DGs are rapidly formed but are also hydrolyzed to FFA. This fact could explain the great oscillations detected in the DG contents (Fig. 1B) over the ripening. Despite these great variations, the results also indicate slightly higher levels of lipolysis in EL than IL cheeses (4.4% vs 3.7%) at 60 ripening days. These results were in accordance with the increment in FFAs found during the analysis of volatile compounds. Differences in lipolysis level found between lots are probably related to the use of HRP in EL and AL manufacture, which provide a potent lipolytic system, essential in development of the characteristic aroma of the Majorero cheese.

Amounts of MG detected in AL and EL cheeses were comparable to those found in Cheddar cheese with a medium (1.5%) and long (3%) maturation period (De Man, 1966). This author, however, found, in Cheddar cheese, amounts of DGs higher (8% and 12%) than those observed in our study. Vujicic and De Man (1967) calculated the MG, DG and TG content in 16 different cows' milk cheeses at the end of ripening and the amounts obtained ranged from 3.9% to 8.5% in DGs and from 0.5% to 1.4% in MGs.

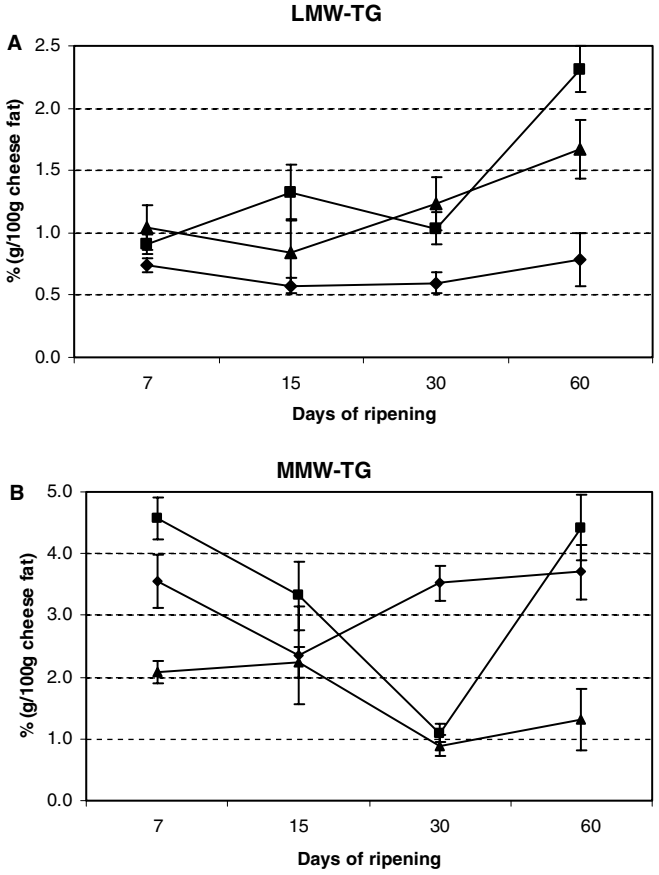

Fig. 1. Content of LMW-TGs (TG with CN < 24) (A) and MMWTGs (TG with CN24–CN36) (B) in cheeses from industrial lot (◆), artisanal lot (▲) and experimental lot (■) after 7, 15, 30 and 60 ripening days. CN = Carbon number; LMW-TG = Low molecular weight triglycerides; MMW-TG = Medium molecular weight triglycerides.

Table 1
The TG composition values of the goat's milk and the manufactured cheeses (IL, EL and AL) after 60 ripening days

TG(CN)	Milk	Cheese at 60 days of ripening		
		IL	AL	EL
<C24	0.50 ± 0.15	0.78 ± 0.09	1.67 ± 0.20	2.31 ± 0.21
C24	0.20 ± 0.03	0.27 ± 0.01	0.36 ± 0.03	0.41 ± 0.02
C26	0.33 ± 0.03	0.39 ± 0.02	0.38 ± 0.01	0.51 ± 0.04
C28	1.16 ± 0.10	1.35 ± 0.04	1.21 ± 0.04	1.51 ± 0.11
C30	1.99 ± 0.18	2.34 ± 0.07	2.14 ± 0.05	2.61 ± 0.16
C32	3.20 ± 0.25	4.05 ± 0.07	3.50 ± 0.13	4.11 ± 0.27
C34	5.50 ± 0.36	6.49 ± 0.22	5.73 ± 0.15	6.41 ± 0.47
C36	9.90 ± 0.33	11.1 ± 0.28	10.3 ± 0.39	11.2 ± 0.70
C38	14.9 ± 0.06	14.2 ± 0.33	14.1 ± 0.62	14.6 ± 0.66
C40	14.6 ± 0.04	15.4 ± 0.38	14.9 ± 0.28	15.9 ± 0.53
C42	14.0 ± 0.75	14.7 ± 0.98	14.5 ± 0.17	15.1 ± 0.99
C44	10.92 ± 1.43	9.58 ± 0.59	11.6 ± 0.37	9.74 ± 0.84
C46	7.86 ± 0.48	7.72 ± 0.37	8.14 ± 0.58	6.38 ± 0.69
C48	6.02 ± 0.27	4.45 ± 0.94	4.73 ± 0.94	3.19 ± 1.29
C50	5.54 ± 0.18	3.55 ± 0.56	3.78 ± 0.35	3.15 ± 0.80
C52	2.88 ± 0.39	2.99 ± 0.13	2.33 ± 0.57	2.19 ± 0.45
C54	0.57 ± 0.17	0.63 ± 0.29	0.74 ± 0.29	0.69 ± 0.15

CN = carbon number.

Table 2
Mean content of volatile compounds (μg/100g) in the three-studied systems of Majorero cheese

PN	Compounds	7 days			15 days			30 days			60 days		
		IL	AL	EL	IL	AL	EL	IL	AL	EL	IL	AL	EL
Ketones													
1	2-Propanone	342^{a}	368^{a}	190^{b}	446^{a}	383^{a}	320^{b}	883^{b}	597^{c}	1022^{a}	1248^{a}	788^{c}	928^{b}
3	2-Butanone	13^{a}	19^{a}	14^{a}	19^{a}	22^{a}	15^{a}	24^{a}	23^{a}	27^{a}	32^{b}	49^{b}	33^{a}
7	2-Pentanone	134^{a}	130^{a}	92^{b}	186^{a}	148^{b}	131^{b}	689^{b}	636^{b}	843^{a}	1471^{a}	687^{c}	929^{b}
8	2-3 Butanedione	268^{b}	304^{a}	311^{a}	205^{b}	224^{b}	670^{a}	175^{b}	192^{b}	1198^{a}	151^{b}	186^{b}	856^{a}
13	2-Heptanone	77^{a}	84^{a}	87^{a}	106^{a}	97^{a}	91^{a}	284^{b}	284^{b}	355^{a}	737^{a}	267^{a}	387^{b}
17	3-Hydroxy-2-butanone	1292^{b}	1773^{a}	1762^{a}	825^{b}	976^{b}	2437^{a}	399^{c}	758^{b}	2745^{a}	375^{c}	849^{b}	1986^{a}
19	2-Nonanone	17^{b}	22^{b}	33^{a}	20^{a}	23^{a}	26^{a}	44^{b}	56^{b}	82^{a}	92^{a}	44^{c}	74^{b}
Aldehydes													
4	3-Methyl butanal	140^{b}	106^{c}	182^{a}	363^{b}	287^{c}	408^{a}	530^{b}	323^{c}	725^{a}	1219^{a}	1011^{b}	799^{c}
12	Hexanal	4^{b}	3^{b}	12^{a}	4^{c}	25^{a}	13^{b}	6^{c}	43^{a}	17^{b}	10^{b}	51^{a}	19^{b}
21	Heptanal	n.d.	n.d.	4	n.d.	n.d.	10	n.d.	2	12	1^{b}	1^{b}	19^{a}
Alcohols													
5	2-Propanol	36^{b}	47^{a}	20^{c}	49^{b}	103^{a}	29^{c}	270^{a}	168^{b}	78^{c}	398^{a}	396^{a}	128^{b}
6	Ethanol	1711^{c}	2713^{b}	3766^{a}	4877^{b}	6095^{a}	4044^{c}	5193^{b}	7400^{a}	4579^{c}	5731^{ab}	6952^{a}	4226^{b}
10	2-Methyl-1-propanol	5^{c}	43^{b}	270^{a}	8^{c}	51^{b}	293^{a}	9^{c}	47^{b}	415^{a}	13^{b}	45^{b}	388^{a}
11	2-Pentanol	3^{a}	5^{a}	8^{a}	10^{c}	48^{a}	21^{b}	56^{b}	179^{a}	51^{b}	439^{a}	181^{b}	86^{c}
14	3-Methyl-1-butanol	178^{c}	268^{b}	1762^{a}	23^{c}	281^{b}	1987^{a}	41^{c}	289^{b}	2195^{a}	56^{c}	193^{b}	1894^{a}
18	2-Heptanol	3^{b}	4^{b}	12^{a}	6^{b}	7^{b}	11^{a}	17^{c}	21^{b}	30^{a}	50^{a}	25^{b}	41^{a}
Fatty acids													
22	Acetic acid	96^{b}	345^{a}	347^{a}	116^{c}	557^{a}	493^{b}	261^{b}	1240^{a}	1326^{a}	1018^{c}	3233^{a}	2680^{b}
23	Propanoic acid	2^{a}	2^{a}	4^{a}	3	2^{b}	13^{a}	5^{b}	2^{b}	15^{a}	9^{b}	2^{c}	17^{a}
24	2-Methyl propanoic acid	61^{c}	173^{a}	101^{b}	9^{c}	114^{b}	166^{a}	13^{c}	224^{a}	179^{b}	21^{c}	128^{a}	110^{b}
25	Butanoic acid	793^{c}	2280^{a}	1520^{b}	1100^{b}	4260^{a}	3895^{a}	1786^{b}	7960^{a}	7998^{a}	3255^{b}	16,079^{a}	17,255^{a}
26	3-Methyl butanoic	98^{b}	286^{a}	116^{b}	24^{c}	221^{a}	191^{b}	23^{c}	404^{a}	265^{b}	36^{b}	203^{a}	199^{a}
27	Pentanoic acid	n.d	3	2	n.d.	4	13	n.d.	10	22	2	25^{a}	36^{a}
28	Hexanoic acid	200^{b}	1641^{a}	237^{b}	468^{b}	1536^{a}	1502^{a}	760^{b}	2859^{a}	2012^{b}	1580^{c}	3950^{a}	2996^{b}
Esters													
2	Ethyl acetate	4^{b}	9^{b}	63^{a}	9^{c}	24^{b}	139^{a}	46^{b}	62^{b}	225^{a}	21^{c}	66^{b}	263^{a}
9	Ethyl butanoate	6^{c}	35^{b}	63^{a}	13^{b}	109^{a}	122^{a}	19^{c}	240^{a}	187^{b}	40^{c}	378^{a}	235^{b}
15	Ethyl hexanoate	20^{a}	19^{a}	24^{a}	6^{b}	54^{a}	58^{a}	10^{c}	128^{a}	90^{b}	20^{c}	282^{a}	118^{b}
20	Ethyl octanoate	n.d.	n.d.	4	n.d.	n.d.	15	17	n.d.	12	1^{c}	36^{a}	16^{b}
16	3-Methyl-butyl butanoate	9^{a}	7^{a}	12^{a}	6^{b}	8^{b}	22^{a}	50^{a}	13^{b}	13^{b}	12^{b}	10^{b}	42^{a}
	Total	5512	8901	11,555	10,688	15,659	24,172	36,117	11,018	17,125	26,760	36,715	36,715

P.N., peak numbering gives the order of elution.
n.d, not detected.
[a,b,c] Analysis of variance ($P < 0.05$). Means followed by different letters within the same row (for the same sampling date) indicate significant differences between lots. Values are means of nine determinations (three cheeses per ripening date × three replicate samples). With very few exceptions standard error values ranged between 1% and 30% on average.

Alonso (1993) has reported the influence of ripening period (until 60 days), on TG composition of Afuega'l Pitu cows' milk cheese. The TG group ranged from CN26 to CN34, and declined during ripening, which was correlated with an increase in FFA concentration (from 1528 to 5661 mg/kg). Partidário, Barbosa, and Vilas Boas (1998) studied variations in TG composition of "Serra da Estella" ewes' milk cheese and observed that LMWTGs (C24–CN38) in samples of 45 days were somewhat lower in concentration than those values corresponding to the milk samples. This fact suggest, as previously has been reported by other authors (Christie, 1995), that lipolysis occurs predominantly on LMWTG, releasing short chain FA, preferentially in the *sn*3 position of the TG molecule. Contarini and Toppino (1995) also analyzed the MG, DG and TG fractions to determine the lipolysis level in Gorgonzola cows' milk cheese, not finding any effect on MG and DG amounts during maturation. These authors suggested that, due to the great rate of lipolysis characteristic of this cheese, the intermediate compounds (MGs, DGs) are rapidly hydrolyzed to FFAs.

3.2. Analysis of volatile compounds

3.2.1. General

General volatile compounds of cheeses were analysed at 7, 15, 30 and 60 ripening days by GC–MS, employing the head-space method for the extraction process.

Twenty-eight volatile compounds were identified (Table 2), which can be placed into the following categories: alcohols, ketones, aldehydes, esters and volatile acids. Concentration (μg/100 g) of most compounds in the volatile

fraction increases gradually during ripening in all samples. Nevertheless, the exceptions to this general trend were 2,3-butanedione (diacetyl), 3-methyl-butan-1-ol and 3-hydroxy-butan-2-one (acetoin), in agreement with other authors (Larráyoz et al., 2001; Ortigosa, Torre, & Izco, 2001). Thus, the amounts of these compounds decreased in IL and AL from the early ripening times; in EL they increased until 30 maturation days and then slightly decreased. Differences between the different cheese lots as regards volatile compounds, were principally quantitative, EL and AL showing the greatest contents from the beginning of the ripening (Table 2).

3.2.2. Ketones

The concentration of ketones in cheese depends on the amount and composition of fat in the original milk (Banks, Brechany, & Christie, 1992), and their appearance is mainly a result of the lypolytic action of microflora in the cheese. Methylketones are formed in a metabolic pathway connected to partial β-oxidation of fatty acids. The FFA arising from the lipolysis are then catabolised to methylketones by the microorganisms. The methylketones are afterwards reduced to their corresponding secondary alcohols by bacterial reductases as a defence mechanism of the microorganism against toxicity (Molimard & Spinnler, 1996). Methyl ketones, together with their reduction products, are considered the most important compounds for the aroma of soft and mold-ripened cheeses, but they are also present in most hard and semi-hard varieties (Gómez-Ruiz, Ballesteros, González Viñas, Cabezas, & Martinez-Castro, 2002).

Most of the ketones in Majorero cheese were methylketones (Table 2), as occurred in other cheese varieties (Carbonell et al., 2002; Izco & Torre, 2000; Ortigosa et al., 2001). The relative proportion of these compounds in Majorero cheese differs from certain other varieties such as mold-ripened cheeses, which show higher levels of heptan-2-one and nonan-2-one. This fact could probably be explained by milkfat composition, which is an essential prerequisite to flavour development (Marilley & Casey, 2004), as well as by the presence of microorganisms with different lipolytic activities. Propan-2-one was present in larger amounts than any other methyl ketone, at all ripening times analysed. This compound is usually formed from butyric acid oxidation, although other routes are also possible. Thus, propan-2-one can be biosynthesized in mammary gland from, where it would pass to the milk.

Ketones, such as 2,3-butanodione (diacetyl) and 3-hydroxy-butan-2-one (acetoin), were also present at high concentrations, especially in EL cheeses ($P < 0.05$). Thus, the amounts of these compounds decreased in IL and AL from the early ripening times, contrary to EL, where they increased until 30 maturation days and then slightly decreased. As the content of these ketones decreased throughout the ripening, it seems probable that both were involved in cheese flavour development in early maturation. Acetoin is produced by reduction of diacetyl (Urbach, 1993) but can also be synthesised from pyruvate, lactose or citrate, which depend on the lactic acid bacteria, especially *L. lactis* subsp. *lactis* (Crow, 1990). Important levels of acetoin appeared and, given its low perception threshold (0.12 ppm), its effect on the aroma of Majorero cheese should be considered very important.

3.2.3. Aldehydes

Carbonylic compounds, namely ketones and aldehydes, play a key role in developing of goat's cheeses flavour. Aldehydes, intermediate and unstable compounds that are usually reduced to alcohols, appear at low concentration in the volatile fraction of most cheeses.

The straight-chain aldehydes (C4–C10) are formed during β-oxidation of unsaturated FA (Collin, Osman, Delcambre, El-Zayat, & Dufour, 1993), while the branched chain 3-methylbutanal probably originates from Leu degradation by enzymatic pathways, as well as non-enzymatic processes (Larráyoz et al., 2001). The aroma compounds identified in this group included 3-methylbutanal, hexanal and heptanal, which is hardly detectable in AL and IL. Although 3-methylbutanal was the major aldehyde in the three lots, the other aldehydes found can also play a important role in flavour development (Izco & Torre, 2000) because of their low perception thresholds. Hence, 3 methyl butanal, hexanal and heptanal would provide malty, green aroma notes to Majorero cheese.

3.2.4. Alcohols

Overall, the strong reducing conditions in cheese may favour the rapid reduction of aldehydes and ketones to primary and secondary alcohols. Alcohols, although indirectly, can also be responsible for cheese flavour because of their ability to form esters with FFA (Gripon, Monnet, Lamberet, & Desmazeaud, 1991).

Large quantities of ethanol were detected in cheeses from the three lots. This primary alcohol, besides being a direct product of lactose fermentation, can be derived from the reduction of the aldehydes formed via Strecker degradation from the amino acid Ala. The appearance of high concentrations of ethanol has been related to the presence of *Leuconostoc* and some facultative heterofermentative lactobacilli able to metabolise lactose (Axelsson, 1993). Over the ripening period, ethanol levels held steady in EL whereas, in IL and AL ($P < 0.05$), they increased, especially until 30 days of maturation. According to microbial analysis (data not shown), it seems unlikely that *Leuconostocs* are responsible for the ethanol formation, since these microorganisms were not detected in IL and their counts declined sharply in EL cheeses.

Other primary alcohols, such as 2-methyl propan-1-ol and 3 methylbutan-1-ol, can form by reduction of the aldehydes formed by Strecker degradation of the amino acids Val and Leu, respectively (Larsen, 1998). A pathway initiated by an aminotransferase can also produce these alcohols. Enzyme-catalyzed transamination of amino acids results in the formation of an intermediate imide that is

subsequently decarboxylated, forming an aldehyde. This aldehyde can then be reduced to alcohols by an alcohol dehydrogenase (McSweeney & Sousa, 2000; Marilley & Casey, 2004). 3-Methyl-1-butanol is responsible for the pleasant aroma of fresh cheese and in general, is very abundant in rennet-curd cheeses during early ripening (Moio, Dekimpe, Etievant, & Addeo, 1993). Concentrations of these two alcohols were lower in cheeses from IL. Surprisingly, the highest amounts of both 2-methyl-1-propanol and 3-methyl-1-butanol were detected in EL ($P < 0.05$), which was also made using pasteurized milk as IL (Table 2). It has been reported that the starter culture composition influences relative abundance of some volatile compounds, such as 2-methyl-1-propanol and 3 methyl-1-butanol and 3-methyl-butyl butanoate, especially when high levels of *L. lactis* are included (Centeno, Tomillo, Fernández-García, Gaya, & Nuñez, 2002). Therefore, our results could be explained to some extent by the use of IFPL starters (including *L. lactis*) which replace, in EL, the beneficial microorganisms destroyed by pasteurization.

Secondary alcohols, such as 2-propanol, 2-pentanol and 2-heptanol, which have also been found in some Italian Cheeses (Di Cagno et al., 2003), may be derived by the reduction of their corresponding methyl ketones (Molimard & Spinnler, 1996).

3.2.5. Volatile acids

During cheese ripening, aliphatic FFAs with four or more carbons rise from triglyceride lipolysis. Lipolysis may be due to the action of the native milk lipase (in cheese made from raw milk), to the action of microbial lipases (even though lactic bacteria present in starter cultures only have a weakly lipolytic activity), or to the action of the lipases present in the rennet paste used in cheesemaking (Larráyoz et al., 2001). The high level of lipolysis detected at the beginning of the ripening in AL cheeses could be related to the action of native lipase present in milk, which was inactivated by pasteurization in the other lots.

Volatile FFA, including aliphatic and branched-chain and other minor fatty acids hydrolyzed from milk fat by lipases, provide characteristic flavour to many cheeses (Ha & Lindsay, 1993). Volatile acids in the three lots were qualitatively similar, butanoic, hexanoic and acetic being the major acids. However, significant differences ($P < 0.05$) in the acid concentrations between lots were found during ripening. Thus, the total concentration of volatile acids in IL at the end of ripening, was only about 26% of that found in those lots manufactured with hygienized rennet paste (AL and EL) which reached similar contents (~23,000 ppm).

Butanoic was the most abundant acid in all cheese lots, and constituted, at the end of maturation, 55%, 68% and 74% of the volatile acids found in IL, AL and EL, respectively. Butanoic levels were distinctly lower in IL over the ripening period ($P < 0.05$). It is well known that butanoic acid is an important flavour compound, which imparts a desirable sharp, "piquant" taste to Majorero cheese. The high concentration of this acid in EL, could be attributed, not only to the lipolytic action of the HRP (Calvo & Fontecha, 2004), but also to the presence of *L. casei* subsp. *casei* IFPL 731, added along with starter, and characterized by a marked esterase activity (Castillo et al., 1999). Butanoic acid may also be produced by fermentation of lactose and lactic acid.

Acetic acid is produced mainly by citrate and sugar metabolism in milk and dairy products (Cogan, 1995). In cheese it can also be produced by oxidative deamination of Gly, Ala and Ser (Partidário et al., 1998). This volatile compound was also present at high concentrations in cheese samples, especially in EL and AL cheeses. Probably, most of the acetic acid found in both lots, originated from the degradation of lactose by heterofermentative lactic bacteria such as *Leuconostoc* (Di Cagno et al., 2003), which were not present in IL, as shown by the microbial analysis of cheeses (data not shown).

The branched-chain fatty acids (BCFA), 2-methylpropanoic (isobutyric) and 3-methylbutanoic (isovaleric) are derived from the metabolism of Val and Leu, respectively (Molimard & Spinnler, 1996). Both compounds were found at higher concentration in AL and EL ($P < 0.05$) over the ripening period. These results may be explained by the presence of diversified flora, in contrast to IL made with pasteurized milk and simplified starters. Although the presence of 4-methyl and 4-ethyl octanoic acids has been considered to be a distinctive characteristic of goat cheese (Salles et al., 2002), in this Majorero cheese, the main BCFA were 2-methyl propanoic and 3-methyl butanoic acids. Perhaps, this fact could be attributable to the composition of the milk as result of either the goat breed or the feed, as has been shown by Guillén et al. (2004) in Palmero cheese. Likewise, the presence of microorganisms with different amino acid catabolic activities might also play a key role.

The presence of straight-chain fatty acids with odd numbers of carbon atoms, such as propanoic and pentanoic, may also suggest their partial fermentative origin (Moio & Addeo, 1998).

3.2.6. Esters

Formation of esters and methylketones is the most important transformation suffered by FFA during cheese ripening. Esters are formed by esterification of short-and medium-chain acids with alcohols. Although yeasts are the microorganisms involved in ester formation in cheese (Molimard & Spinnler, 1996), some lactic acid bacteria and *Micrococcaceae*, together with chemical reaction, may also be responsible (Gripon et al., 1991).

The predominant esters in Majorero cheese were ethyl esters. Similar results have been described in Spanish ewe's milk cheeses, such as Manchego, Roncal and Zamorano (Barron et al., 2005; Fernández-García et al., 2004; Izco & Torre, 2000; Martínez-Castro et al., 1991).

Ethyl esters were less abundant in IL, in agreement with the lower abundance of acids detected in this lot. Thus, an

explanation for our results could be the lesser microbial activity of IL cheeses made with simplified starter and pasteurized milk. Besides, EL or AL cheeses manufactured with HRP and IFPL starter or with HRP and raw milk, showed a greater lipolytic activity and hence a larger concentration of volatile FFA was generated which could esterify with ethanol.

Ethyl acetate was detected in cheeses from the three lots, always in higher amounts in EL ($P < 0.05$). This compound, that possesses a fruity sweet flavour, is considered to be one of the major esters contributing to the aromatic profile in goat cheeses. Certain components, such as ethyl octanoate (ethyl caprylate), were not detected in the 30-days old cheeses but were found to be present after 60 days of ripening. Ethyl hexanoate and ethyl butanoate were the esters found at the highest level, especially in EL and AL ($P < 0.05$) where the largest concentrations of carboxylic acid were also detected (see Table 2).

It should be pointed out that 3 methyl butyl butanoate (isoamyl butyrate), although at low levels, is present in all cheeses and its concentration increased slightly through the ripening. The level of this ester was higher in EL which could be attributed to the action of *L. lactis* subsp. *lactis* IFPL 359 included in the IFPL starter, as has been proposed by Centeno et al. (2002).

Esters, especially those containing few carbon atoms, contribute in a synergistic way to the aroma of cheese since they have a low perception threshold concentration which is 10-fold lower than their alcohol precursors (Preininger & Grosch, 1994). Hence, esters could be considered as key constituents of the aroma of this cheese variety, providing fruity notes that minimise the strong aroma produced by FFA, in agreement with Collin et al. (1993).

4. Conclusions

The HRP was certainly responsible for the greater level of lipolysis in AL and EL cheeses which is directly related to increase in both LMW-TG (MG) and short-chain compounds, such as butanoic acid, which imparts a desirable "piquant" taste to Majorero cheese. However, examination of the results suggests that the high concentration of volatile compounds (including VFFA) in EL could be attributed not only to the lipolytic action of the HRP, but also to the starter culture composition. Thus, the employment of a defined strain starter in EL serves to successfully overcome the negative effect of pasteurization on the levels of certain volatile compounds, in particular esters and fatty acids. These results may be of possible concern for the dairy industry in imitating the peculiarities of artisanal cheeses made from raw milk.

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