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Free amino acids and volatile compounds in an ewe's milk cheese as affected by seasonal and cheese-making plant variations

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Abstract

Analysis of free amino acids (FAAs) and volatile compounds in Roncal cheese throughout the cheesemaking seasons was carried out. The possible relationships between them were studied. Also, the influence of two cheesemaking plants was analysed. Total FAAs was 30–50% higher in cheeses made in summer than in cheeses made in either winter or spring. The main amino acids, which accounted for about 69% of the total FAAs, were Glu (20%), Leu (14%), Val (10%), Lys (9%), Phe (6%), and Pro and Ile (about 5% each). Besides six miscellaneous components, the 64 volatile components identified comprised nine hydrocarbons, 16 alcohols, two sulfur-containing compounds, seven aldehydes, eight ketones, six acids and 10 esters. Season was the first factor affecting the concentration of FAAs, and dairy plant was the principal one influencing the level of volatile compounds. Significant interactions between both factors found for the volatile components made very difficult to correlate their formation with the precursor amino acids.

Keywords: Volatile compounds; Amino acids; Ewe's milk cheese; Season; GC-MS; HPLC

1. Introduction

There are numerous compounds involved in cheese aroma, which are derived mainly from three major metabolic pathways: lactose, lipid and protein catabolism. The activators of these pathways are endogenous enzymes of milk, clotting enzymes and from enzymes manufacturing and ripening microorganisms. The microbial flora of cheese plays a key role in flavour development. Two mains groups of bacteria are involved: starter bacteria and non-starter lactic acid bacteria (NSLAB). The main function of the starter bacteria is the production of lactic acid at an appropriate rate. In addition, these bacteria make important contributions to proteolysis during ripening and to the development of cheese flavour (Limsowtin et al., 1995; Lynch, McSweeney, Fox, Cogan, & Drinan, 1997). The formation of many compounds essential for cheese flavour is believed to result from the action of enzymes from the starter cultures (Broome & Limsowtin, 1998; Engels & Visser, 1996; Urbach, 1993). However, NSLAB have a wide range of hydrolytic enzymes, and, therefore, the potential to contribute to cheese maturation (Williams & Banks, 1997). NSLAB affect proteolysis and lipolysis during ripening (Lane & Fox, 1996; McSweeney, Fox, Lucey, Jordan, & Cogan, 1993), even though its significance in Cheddar cheese flavour is equivocal (Fox, McSweeney, & Lynch, 1998). It has been reported that NSLAB contribute to the formation of FAAs (Shakeel-Ur-Rehman, Banks, McSweeney, & Fox, 2000) and NSLAB adjunts accelerate the production of FAAs (Lane & Fox, 1996; Lynch et al., 1997; McSweeney, Walsh, Fox, Cogan, Drinan, & Vastelo-Gonzalez, 1994; Puchades, Lemeux, & Simard, 1989).

Based on sensory evaluation and analytical chemical analysis, various groups of volatiles have been identified as being important for the final taste and aroma of cheese. Such groups are: fatty acids, esters, aldehydes, alcohols, ketones and sulfur compounds (Bosset & Gauch, 1993; Engels, Dekker, de Jong, Neeter, & Visser, 1997; Izco, Irigoyen, Torre, & Barcina, 2000; Urbach, 1993). Short-chain fatty acids contribute

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directly to aroma in many aged cheeses, while methyl ketones and related secondary alcohols produced from free fatty acids (FFA) are the principal aroma components in blue cheese (McSweeney & Sousa, 2000). Most of the volatile flavour components detected are formed during ripening and some of them are derived from the catabolism of FAAs released during the proteolysis, e.g. 2-methylpropan-1-ol and 3-methylbutan-1-ol can be formed by reduction of the corresponding aldehydes formed by Strecker dregradation of Val and Leu, respectively (Smit, Verheul, Van Kranenburg, Ayad, Siezen, & Engels, 2000). Some authors (Fox & Wallace, 1997) have reported that the products of amino acid catabolism have a greater contribution to flavour than amino acids "per se". Much is now known of the enzyme systems involved in the conversion of caseins to FAAs, but attention has been paid only recently to the enzymes of starters involved in amino acid catabolism (Engels & Visser, 1996; Smit et al., 2000). Dias & Weiner (1998) reported the conversion of Met to thiols by lactococcal enzymes; the catabolism of Met and Cys to sulfur compounds is considered to be essential to the formation of Cheddar cheese flavour (Urbach, 1993).

Roncal cheese is made under an Appellation of Origin (AO) in northeastern Navarra in northern Spain between the months of December and July. It is an uncooked pressed-curd cheese made from raw ewes' milk that must be left to age for at least four months before marketing. Strains of *Lactococcus lactis* are the most important starters in the manufacture of this cheese, but it has been demonstrated that the NSLAB can play also an important role for the development of the flavour of Roncal cheese (Ortigosa, Torre, & Izco, 2001).

The objective of this work was to identify the FAAs and volatile compounds in Roncal cheese throughout the dairying seasons and define any relationship or interaction between them. Also, the influence of two cheesemaking plants on the FAAs and volatile compounds profile was analysed. This study is a part of a broader research project subsidized by the Instituto Nacional de Investigaciones Agroalimentarias (INIA) aimed at characterizing the odour and aroma of ewe's milk cheeses produced under the auspices of an AO.

2. Materials and methods

2.1. Cheese samples

Two of the five cheese makers registered with the Roncal Appellation of Origin (AO) were selected for this study. Raw ewe's milk was used for the production of Roncal cheese. Cheesemaking was carried out according to the method approved by the Regulatory Board of the Roncal Cheese AO (BOE, 1991).

From each of the two dairies and for each of the three seasons during which it is permited to make Roncal cheese (winter, spring and summer), two different manufacturing dates were chosen and cheeses ripened for 4 months (corresponding to the manufacturing dates selected) were taken in duplicate, making a total of 24 cheeses (2 dairies × 3 seasons × 2 dates × 2 duplicates). All the analyses were performed in duplicate.

2.2. Chemical analysis

2.2.1. Dry matter

Total dry matter was determined according to FIL-IDF Standard No.4 (International Dairy Federation, 1986).

2.2.2. Free amino acids

Extraction of FAAs from cheese samples and quantitation of FAAs by RP-HPLC was performed by the method used by Izco et al. (2000). The HPLC equipment (Waters Corporation, Mildford, MA) consisted of two M510 pumps connected to an ULTRA WISP 715 injector. The Pico-Tag column (Waters Corporation) was thermostated by a TCM temperature-controlled oven and the resolved peaks were detected with a Photo Diode Array M966 detector and a NEC PowerMate 486/33i computer with Millenium 2010 software was used for quantification.

2.2.3. Volatile compounds analysis

The extraction of the volatile compounds by Purge and Trap and their separation and identification by GC-MS was performed as described by Izco and Torre (2000). Briefly, 10 g of finely grated cheese were mixed with 10 g of anhydrous sodium sulfate and 100 μl of 13 mM L-Borneol (Sigma, St. Louis, MO, USA) as an internal standard (IS), and placed in an U vial. Volatile compounds were extracted with an automatic Purge and Trap Sample Concentrator 4460 A system (O.I. Analytical, College Station, TX, USA) with a non-cold trap (Tenax, O.I. Analytical). The Purge and Trap system was connected to a HP 6890 Series GC System coupled to a 5973 Mass Selective Detector (Hewlett Packard, Palo Alto, CA, USA). Peak identification was based upon MS spectra comparison with the HP Wiley 275 library and with spectra of injected standards and also on retention times of standards when available. The ratio peak area/ IS peak area was used as arbitrary units to calculate quantities for each volatile compound.

2.3. Statistical processing

SPSS computer program (version 8.0, SPSS Inc., Chicago, IL, USA) was used for statistical processing. A one-way analysis of variance was run on the volatile components analysed and on the FAAs to ascertain

whether or not the differences between seasons for each dairy independently considered. In addition to that, a two-ways analysis of variance was used to analyse simultaneously the effect of both factors (season and dairy) on the variables quantified, and whether the interactions between those two factors were significant.

3. Results and discussion

Table 1 shows the concentration of the FAAs quantified in Roncal cheese throughout the lactation period for both dairies and the results of the ANOVA for each dairy independently considered. Also, the level of significance obtained in the analysis of the variance considering both factors (season and dairy) and the interaction between them were calculated. In both dairy plants, the concentration of total FAAs (calculated as the sum of the amino acids individually considered) was 30 and 50% higher in cheeses made in summer than in cheeses made in either winter or spring, respectively. The higher concentration of FAAs in summer cheese might be due to the deeper proteolysis caused by quantitative and qualitative seasonal variations of the microbial composition of milk. According to Mendía, Ibañez, Torre, and Barcina (2000), the counts of microorganisms are higher in summer, while the number of microorganisms present in milk could be not enough for a proper fermentation in cheese made in winter. These authors found that the extensive proteolysis detected in Idiazabal cheese made in summer (a raw ewe's milk cheese very similar to Roncal cheese and produced in the same region) negatively affected the organoleptic characteristics of the cheese. Similar results were found for Serra (Macedo, Costa, & Malcata, 1996) and Cheddar (Fox & Wallace, 1997) cheeses.

The main amino acids, which accounted for about 69% of the total FAAs, were Glu (about 20%), Leu (about 14%), Val (about 10%), Lys (about 9%), Phe (about 6%), and Pro and Ile (about 5% each). Some of these amino acids have been considered indices of cheese ripening in other ewe's milk cheeses such as Ossau-Iraty (Izco et al., 2000) or Idiazabal (Mendia et al., 2000) cheeses. In accordance with the observation for total FAAs, the concentrations of the seven principal amino acids were higher in the cheeses made in summer, even though in some cases the differences were not significant from the values obtained in other sea-

Table 1
Amino acids (mg/100 g DM) in Roncal cheese (after 120 days of ripening) during winter, spring or summer at two dairies and significant levels for the effects season (S), dairy (D) and their interaction (S*D)[a]

	Dairy A			Dairy B			Effects (p)[b]		
	Winter	Spring	Summer	Winter	Spring	Summer	S	D	S*D
ASP	54.4a	64.1a	57.1a	69.1b	71.3b	110.6a	**	***	**
GLU	307.8a	364.9a	389.7a	393.1b	374.2b	561.1a	***	***	**
HYPRO	15.0c	17.3b	24.8a	15.7b	18.5b	27.8a	***	NS	NS
SER	19.6a	14.1a	27.2a	11.6a	5.9b	8.1ab	NS	**	NS
GLY	23.5b	22.7b	28.6a	18.5c	28.1b	32.8a	***	NS	***
GLN	55.9b	32.5b	101.7a	25.6b	49.3b	88.4a	***	NS	*
TAU	5.9a	3.8b	4.5ab	3.1a	1.9b	2.6ab	***	***	NS
HIS	45.5a	25.1b	41.8a	36.7b	35.0b	51.3a	***	*	***
GABA	91.4ab	41.8b	98.6a	27.1a	44.9a	48.6a	*	***	*
THR	51.8a	32.7b	50.7a	52.9b	48.1b	64.3a	***	***	*
ALA	58.1b	50.6b	77.9a	53.8b	52.0b	81.0a	***	NS	NS
ARG	26.6a	25.5a	19.6a	14.0a	5.4b	6.5b	NS	***	NS
PRO	102.6a	99.4a	121.9a	74.6c	101.7b	129.3a	***	NS	*
TYR	60.7a	54.5a	43.0a	7.9a	2.3b	11.3a	NS	***	*
VAL	184.3b	172.0b	219.0a	154.2b	169.7b	237.2a	***	NS	*
MET	70.2ab	62.7b	84.6a	50.0b	55.6b	69.9a	***	***	NS
ILE	103.4a	56.5b	117.7a	87.1b	67.1c	131.9a	***	NS	**
LEU	261.4b	254.8b	323.9a	228.7b	238.7b	340.9a	***	NS	NS
1H-LYS	1.9a	1.3a	2.0a	2.3ab	1.6b	3.1a	*	*	NS
2H-LYS	nq	nq	2.0	nq	Nq	0.4			
PHE	138.5b	73.6c	167.1	107.9b	67.2c	181.5a	***	NS	**
TRP	7.7a	5.7b	4.0c	1.42b	1.5b	8.4a	***	***	***
ORN	45.9b	42.2b	99.3a	40.0b	36.0b	63.0a	***	*	NS
LYS	184.4a	145.6b	221.8a	131.3b	146.4ab	164.7a	***	***	**
TOTAL	1916.1b	1665.1b	2328.4a	1606.5b	1622.3b	2424.8a	***	***	*

[a] a–c: for each dairy, different letters indicate significant differencesbetween seasons ($P < 0.05$).
[b] *** $P < 0.001$; ** $P < 0.01$; $P < 0.1$; NS: not significant; nq: not quantified.

sons. Similarly, the concentration of Asp and Thr, and their derived amino acids Ala and Gly respectively, are higher in the cheeses made in summer (Table 1).

Glu was the most abundant amino acid in Roncal cheese with 561.1±20.4 mg/100 g DM in summer cheeses made in Plant B (Table 1). Amino acid transamination is catalysed by aminotransferases and results in the formation of α-ketoacids while the α-ketoacid acceptor, often α-ketoglutarate, is transformed to the corresponding amino acid, Glu (Yvon & Rijnen, 2001). Also, its catabolism can produce γ-aminobutyric (GABA) by a decarboxylase (McSweeney & Sousa, 2000). However, we did not observe any clear correlation between the concentration of both Glu and GABA during the three seasons. While Glu has a low perception threshold (McSweeney & Sousa, 2000), GABA concentration has been correlated with an increasing number of eyes but has no direct or indirect impact on cheese flavour (Christensen, Dudley, Pederson, & Steele, 1999).

The concentration of Phe was significantly lower in cheeses made in spring. The catabolism of this amino acid by Strecker degradation can yield phenylethanol and phenylacetaldehyde, though we did not find these compounds in the cheeses; high concentrations of these products in Cheddar cheese contribute to astringent and bitter flavour sensations (Christensen et al., 1999). Tyr is another amino acid that, besides tyramine, can yield some phenyl-derived compounds by atypical Strecker degradation (McSweeney & Sousa, 2000). This amino acid accounted for 3.17, 3.28 and 1.85% of total FAAs in cheese from dairy A, while it represented only a 0.49, 0.14 and 0.46% in the case of dairy B. Besides Phe, the concentration of Tyr was lower in the cheeses made in spring, but only in the case of dairy B. This could affect the organoleptic characteristics of the cheese since usually, Roncal cheese made in spring have the most characteristics odor and flavour (Izco & Torre, 2000).

As shown in Table 1, the concentration of Orn in cheeses from both plants was about 50% higher in summer (99.3 and 63 mg/100 g DM) than in spring (42.2 and 36 mg/100 g DM) or winter (45.9 and 40 mg/100 g DM). According to Mendia et al. (2000), cheeses made in summer undergo deeper preoteolysis and were less appreciated by the panelists. The lower scores obtained for summer cheeses could be correlated with the higher concentration of amino acids derived from secondary metabolism, e.g. Orn.

The factor "season of the year" significantly affected the concentration of all of the FAAs, except that of Ser, Arg and Tyr, which were influenced only by the effect "dairy plant". Several amino acids were affected by both factors studied and the interaction between factors significantly affected the concentration of some of them (Asp, Glu, His, Gaba, Thr, Trp and Lys), indicating that the evolution of this compounds throughout the seasons changed in both dairies. The size of the "season" effect was higher (with F values of 46.66 and 26.301 respectively vs. 6.016 and 25.821, data not shown) than for the effect dairy only in the case of His and Thr. No differences were found between dairies for some of them (Table 1, Factor D). In fact, the relative proportions of individual amino acids appear to be very similar. This observation can be confirmed when comparing the percentages for each amino acid (calculated from Table 1) in winter, spring or summer in the cheeses from Plant A *versus* those percentages in the cheeses from dairy B, e.g. Hypro (0.78, 1.04 and 1.07% vs. 0.98, 1.14 and 1.14%), Gly (1.22, 1.36 and 1.23% vs. 1.15, 173 and 1.36%), Gin (2.92, 1.95 and 4.37% vs. 1.6, 3.04 and 3.65%) and Ala (3.03, 3.04 and 3.34% vs. 3.35, 3.2 and 3.34%), including some of the main amino acids,

Table 2
Hydrocarbons in Roncal cheese (after 120 days of ripening) during winter, spring or summer at two dairies and significant levels for the effects season (S), dairy (D) and their interaction (S*D)[a, b]

	Dairy A			Dairy B			Effects (P)[c]		
	Winter	Spring	Summer	Winter	Spring	Summer	S	D	S*D
Pentane	11.35a	5.72b	15.34a	7.44a	5.56a	9.38a	**	***	*
Hexane	72.81a	33.73b	52.60ab	47.10a	29.21a	39.97a	NS	*	NS
Methylcyclopentane(T) [d]	4.61a	0.70b	1.68b	2.77a	0.96b	1.28ab	*	***	*
Methylcyclohexane (T)	0.52a	0.14b	0.46a	0.22a	0.40a	0.32a	NS	NS	*
Octen-3-ene (T)	0.50b	0.60b	1.14a	0.48b	0.46b	0.75a	***	***	**
Ethylbenzene	3.87a		5.06a	1.85a	2.34a	3.22a			
p-Xylene	0.05b	0.05b	0.13a	0.07					
Styrene	0.04a	0.03a	0.05a		0.03	0.03			

[a] The ratio peak area/IS peak area was used to calculate the concentration of each volatile compound. Arbitrary units were used. Mean value of eight determinations (4 cheeses/each season × 2 duplicates).

[b] a–c: for each dairy, different letters indicate significant differences between seasons ($P<0.05$).

[c] Significance levels of two-way analysis of variance: *** $P<0.001$; ** $P<0.01$; $P<0.1$; NS: not significant.

[d] All compounds were confirmed by comparison of retention times and mass spectra of authentic substances except (T) tentatively identified only according to Wiley library and the literature.

Table 3
Alcohols in Roncal cheese (after 120 days of ripening) during winter, spring or summer at two dairies and significant levels for the effects season (S), dairy (D) and their interaction (S*D)[a]

	Dairy A			Dairy B			Effects (*P*)		
	Winter	Spring	Summer	Winter	Spring	Summer	S	D	S*D
Ethanol	42.91b	128.79a	62.04b	182.72a	192.09a	173.36a	***	***	**
Butan-2-ol	4.05c	60.71b	90.48a	100.10c	216.32a	153.94b	***	***	***
Propan-1-ol	3.92b	7.64a	5.97a	38.61a	27.38a	41.69a	*	***	**
2-Methylpropan-1-ol	2.23b	2.18b	8.94a	4.52a	6.11a	7.22a	***	NS	*
Prop-2-en-1-ol (T)	0.25b	1.77a	0.24b	21.38a	8.94b	5.34b	***	***	***
Pentan-2-ol	0.56b	0.91ab	1.75a	1.34b	2.31ab	3.30a	**	**	NS
1-Methoxy-2-propanol(T)	0.08	0.11							
Butan-1-ol	0.27a	0.27a	0.27a	1.35a	1.18a	1.30a	NS	***	NS
2-Methyl-3-buten-2-ol		0.06b	0.43a						
3-Methylbutan-1-ol	0.34b	0.07b	1.68a	5.48b	10.16a	7.44	**	**	
Hexan-2-ol	0.15b	0.13b	0.25a		0.20	0.12			
3-Methylbut-3-en-1-ol	0.16b	0.15b	0.23a	0.12b	0.15a	0.15	***	**	
Hexan-2-ol	0.50a	0.19b	0.43	0.12b	0.16ab	0.17a	***		
Heptan-2-ol	0.27a	0.11b	0.29a	0.08a	0.08a	0.08a	**	***	**
1-(2-Methoxypropoxy)-2-propanol(T)			0.06						

[a] See footnotes to Table 2.

Table 4
Sulfur-containing compounds in Roncal cheese (after 120 days of ripening) during winter, spring or summer at two dairies and significant levels for the effects season (S), dairy (D) and their interaction (S*D)[a]

	Dairy A			Dairy B			Effects (*P*)		
	Winter	Spring	Summer	Winter	Spring	Summer	S	D	S*D
Dimethyl sulfide	0.94a	0.47ab	0.45b	2.07a	1.17b	1.12b	***	***	NS
Dimethyl disulfide	0.56a	1.99a			0.87	0.77			

[a] See footnotes to Table 2.

Table 5
Aldehydes in Roncal cheese (after 120 days of ripening) during winter, spring or summer at two dairies and significant levels for the effects season (S), dairy (D) and their interaction (S*D)[a]

	Dairy A			Dairy B			Effects (*P*)		
	Winter	Spring	Summer	Winter	Spring	Summer	S	D	S*D
Butanal	0.12a	0.06a	0.10a	0.04b	0.05b	0.07a	NS	**	NS
3-Methyl-butanal	1.34ab	0.99b	2.33a	0.52a	0.72a	0.98a	**	***	NS
Nonanal				0.05					
Undecenal (T)						0.05			

[a] See footnotes to Table 2.

e.g. Pro (5.35, 5.97 and 5.24% vs. 4.64, 6.26 and 5.33%), Val (9.62, 10.33 and 9.4% vs. 9.6, 19.46 an 9.78%), Ile (5.4, 3.39 and 5.06 vs. 5.42, 4.14 and 5.44%), Leu (13.64, 15.30, 13.91% vs. 14.23, 14.72, 14.06%) or Phe (7.23, 4.42 an 7.18% vs. 6.72, 4.14 an 7.48%).

The volatile compounds present in Roncal cheese comprised eight hydrocarbons (Table 2), fifteen alcohols (Table 3), two sulfur-containing compounds (Table 4), four aldehydes (Table 5), eight ketones (Table 6), six acids (Table 7), nine esters (Table 8), and a miscellaneous group that included two chlorinated compounds, acetonitrile and hexamethyl cyclotrisiloxane (Table 9). There were also four unresolved chromatographic peaks, each peak comprising a further two substances (Table 10). Accordingly, a total of 64 volatile compounds were identified. Most of those compounds have already been reported in several types of cheeses, including those in the miscellaneous group (Table 9) (Bosset, Gauch, Mariaca, & Klein, 1995; Fernández-García, 1996) and some of the substances identified in the unresolved peaks (Table 10) (Izco and Torre, 2000; Ortigosa et al., 2001).

Table 6
Ketones in Roncal cheese (after 120 days of ripening) during winter, spring or summer at two dairies and significant levels for the effects season (S), dairy (D) and their interaction (S*D)[a]

	Dairy A			Dairy B			Effects (*P*)		
	Winter	Spring	Summer	Winter	Spring	Summer	S	D	S*D
Propan-2-one	29.57a	25.49a	35.00a	11.97a	21.08a	22.68a	NS	**	NS
Butan-2-one	141.01a	108.07a	147.74a	52.28a	67.60a	70.44a	NS	***	NS
Pentan-2-one	24.81a	10.27a	25.15a	2.29a	2.50a	7.05a	*	***	NS
Butan-2,3-dione	12.96a	6.47a	10.11a	3.07a	1.00b	1.17b	*	***	NS
Hexan-2-one	1.08b	3.43a	4.33a	0.06c	2.43b	3.97a	***	*	NS
Heptan-2-one	3.82a	1.08a	4.18a	0.38ab	0.30b	1.23a	NS	***	NS
3-Hydroxybutan-2-one	45.26a	10.64b	27.69b	1.60a	0.86a	1.01a	***	***	***
Nonan-2-one	3.29a	0.29b	1.03b	0.08a	0.08a	0.10a	**	***	**

[a] See footnotes to Table 2.

Table 7
Acids in Roncal cheese (after 120 days of ripening) during winter, spring or summer at two dairies and significant levels for the effects season (S), dairy (D) and their interaction (S*D)[a]

	Dairy A			Dairy B			Effects (*P*)		
	Winter	Spring	Summer	Winter	Spring	Summer	S	D	S*D
Acetic acid	2.76a	1.59a	2.80a	3.36a	0.68b	0.40b	*	NS	NS
Propanoicacid	0.20a	0.10a	0.20a	0.15a	0.11a	0.06a	NS	NS	NS
2-Methylpropanoicacid	0.25a	0.15a	0.16a	0.35a	0.14b	0.06b	***	NS	NS
Butanoicacid	3.37a	2.21a	3.14a	4.09a	1.88a	0.72a	NS	NS	NS
3-Methylbutanoicacid	0.44a	0.29a	0.33a	0.68a	0.36b	0.21b	**	NS	NS
Hexanoicacid	0.51a	0.43a	0.62a	0.25a	0.29a	0.18a	NS	*	NS

[a] See footnotes to Table 2.

Table 8
Esters in Roncal cheese (after 120 days of ripening) during winter, spring or summer at two dairies and significant levels for the effects season (S), dairy (D) and their interaction (S*D)[a]

	Dairy A			Dairy B			Effects (*P*)		
	Winter	Spring	Summer	Winter	Spring	Summer	S	D	S*D
Ethyl acetate	0.30b	1.41a	0.71b	2.85a	1.27b	2.88a	NS	***	***
Methylpropylacetate (T)						0.49			
Ethyl hexanoate	0.08b	0.24a	0.12b	0.64a	0.41b	0.49ab	NS	***	***
3-Methylbutylbutanoate(T)		0.08				0.04			
Propyl hexanoate (T)						0.03			
Ethyl heptanoate	0.06b	0.10a	0.06b	0.20a	0.13b	0.16ab	NS	***	**
2-Propyl hexanoate(T)	0.14a	0.06b	0.14ab						
Ethyl octanoate(T)		0.03		0.12a	0.05b	0.06b			
Ethyl decanoate	0.09a	0.13a	0.12a	0.21a	0.15ab	0.10b	NS	*	**

[a] See footnotes to Table 2.

A total of 10 hydrocarbons were detected (Tables 2 and 10). This family of secondary products of lipid autoxidation (Barbieri et al., 1994) does not make a major contribution to aroma, although these compounds may serve as precursors for the formation of other aromatic compounds (Arora, Cormier, & Lee, 1995). Styrene has been reported at trace levels in several cheeses (e.g. Camembert) and can be produced from Phe (Molimard & Spinnler, 1996). Nevertheless, we did not find a relationship between the concentration of styrene and Phe in Roncal cheese. In fact, styrene was not detected in some samples containing high levels of Phe or in samples when the concentration of Phe was the lowest, in spring (Tables 1 and 2).

Alcohols (Table 3) comprised the largest group of aroma components, accounting for 25% of all the volatile components identified. The season factor affected significantly all the alcohols quantified, except

Table 9
Miscellaneous in Roncal cheese (after 120 days of ripening) during winter, spring or summer at two dairies and significant levels for the effects season (S), dairy (D) and their interaction (S*D)[a]

	Dairy A			Dairy B			Effects (*P*)		
	Winter	Spring	Summer	Winter	Spring	Summer	S	D	S*D
Hexamethyl Cyclotrisiloxane	0.08b	0.09b	0.23a	0.05b	0.07b	0.15a	***	***	*
Acetonitrile	1.18b	0.98b	2.17a	0.76b	0.85b	1.51a	***	***	NS
Chloroform	18.12a	11.66ab	7.61b	19.81a	6.42b	5.71b	***	***	NS
2,2-Dichloroethanol	0.10b	0.07b	0.21a	0.02					

[a] See footnotes to Table 2.

Table 10
Unresolved compounds in Roncal cheese (after 120 days of ripening) during winter, spring or summer at two dairies and significant levels for the effects season (S), dairy (D) and their interaction (S*D)[a]

	Dairy A			Dairy B			Effects (*P*)		
	Winter	Spring	Summer	Winter	Spring	Summer	S	D	S*D
Propanal/Pentanal$^{(T)}$	0.30a	0.10b	0.17b	0.25a	0.15b	0.16b	***	NS	NS
Heptane/Acetaldehyde $^{(T)}$	3.15a	0.87c	1.78b	1.88a	1.34a	1.91a	***	NS	**
Propan-2-ol/Dichloroethane	2.35b	7.54a							
Toluene/Ethylbutanoate	1.52a		2.70a						

[a] See footnotes to Table 2.

butan-1-ol, in all samples. Also, all of them were affected by the effect dairy plant, except 2-methylpropan-1-ol. Although the concentration of alcohols in Roncal cheese is higher in summer than in spring (Izco & Torre, 2000), in the case of dairy B the concentration was the same in both seasons, though concentration of butan-2-ol was lower in summer.

Concentration of 2-methylpropan-1-ol and 3-methylbutan-1-ol were significantly higher in summer than in spring or winter, except for 2-methylpropan-1-ol in cheese from dairy B. Certain primary alcohols like 2-methylpropan-1-ol and 3-methylbutan-1-ol can be formed by reduction of aldehydes formed by Strecker degradation of the amino acids, e.g. Val and Leu, respectively (Larsen, 1998). As shown in Table 1, the concentration of these two amino acids was clearly higher in summer.

The level of propan-1-ol, an alcohol derived from Met (Collin, Osman, Delcambre, El Zayat, & Dufour, 1993), varied only in cheeses made in winter in dairy A, but remained constant during the rest of the year in this dairy, and also during the three seasons in dairy B. The concentration of Met detected in cheeses from both dairies was significantly higher in summer. Besides, the concentration of Met was just slightly higher in dairy A than in dairy B, while the concentration of propan-1-ol was about six fold higher in dairy B than in dairy A (Table 3). Therefore, the formation of propan-1-ol was faster in dairy B probably caused by faster catabolism of Met, although this observation could not be confirmed when comparing seasons. Met can be also transformed to sulfur compounds like dimethylsulphide (DMS) by *Lactobacillus* sp. (Thierry & Maillard, 2002) and dimethyldisulphide (DMDS). Breakdown of the sulfur-containing amino acids (Met and Cys) during cheese ripening produces such sulfuric components as hydrogen sulfide and methanethiol; oxidative reactions may then result in the formation of such other components as DMDS (Engels et al., 1997). However, sulfur compounds found in cheese must originate principally from Met, as there is higher concentration of Met in caseins than Cys (Cys residues in caseins are present at low levels only in αs_2- and k-caseins) (McSweeney & Sousa, 2000). In our case, in spite of having detected Cys, this amino acid could not be quantified because that peak co-eluted with an unknown compound, making its quantification very difficult. As shown in Table 4, concentration of DMS was higher in cheeses from dairy B. As happened in the case of propan-1-ol, this might have been caused by the fastest catabolism of Met in dairy B. In any case, only two sulphur compounds were detected, but only DMS was found in all samples. While sulfur-containing components are considered indispensable for the characteristic aroma of such cheeses as Cheddar, Emmental, Camembert, and Gruyère, we can speculate that they are not particularly significant in the development of Roncal cheese aroma, as mentioned by Izco and Torre (2000). According to these observations, a clear relationship between propan-1-ol and sulfur-containing compounds formed and the catabolism their

precursor amino acid (Met) can not be confirmed for Roncal cheese.

Ethanol, another primary alcohol, can be formed from Ala by Strecker degradation. In Roncal cheese, the concentration of Ala was higher in summer and there were not significant differences between both plants. On the other hand, concentration of ethanol was affected by both season and dairy, and also by the interaction between them. Also, its concentration remained the same in the cheeses from dairy B. The formation of ethanol did not correlate well with the catabolism of Ala and therefore, ethanol appears to be formed primarily by the fermentation of lactose. Lactose fermentation can be carried out by a number of homofermentative bacteria, such as enterococci, yielding formic acid, acetic acid and ethanol (Urdaneta, Raffe, Ferrer, Sulbarán de Ferrer, Cabrera, & Pérez, 1995), as well as by yeasts and *Leuconostoc* spp. (Fernandez-García, 1996).

Seven aldehydes were identified, but three of them were components of unresolved peaks (Table 10), and two appeared in only a few samples (Table 5). Branched aldehydes are produced from the metabolism of branched amino acids, but they do not accumulate in cheese because they are rapidly reduced to the correspondent alcohols. However, these aldehydes can be present at a high level in Emmental cheese; if it is not one of the compounds defining its typical flavour, it may play an important role suppressing the unpleasant, sweaty odor of butyric cheese (MacSweeney & Sousa, 2000). Only 3-methylbutanal, derived from Leu, has been quantified in the samples and its concentration varied similarly to that of the derived alcohol, 3-methylbutanol. Similarly, the other aldehydes present in the samples (propanal, butanal, nonanal and undecenal, which are formed by β-oxidation of unsaturated fatty acids) contribute to cheese flavour because of their low perception threshold and because are transformed rapidly to the corresponding alcohols.

As reported by Izco and Torre (2000), most of the ketones present in the Roncal cheeses were methyl ketones (Table 6). The formation of these compounds in cheese is mainly a result of the lipolytic action of the microflora in the cheeses, and they are typical of mould-ripened cheeses like blue cheeses, in which they contribute to the characteristic pungent aroma. Diacetyl is produced as a consequence of the lactose and citrate metabolism of the lactococci, in particular $citr^+$ *Lactococcus lactis* ssp. *lactis* (Crow, 1990). Diacetyl can be reduced to acetoin, which in turn can be reduced to butan-2,3-diol, then to butan-2-one, and finally to butan-2-ol. According to Urbach (1993), the production of diacetyl and acetoin and reduction of the latter to butan-2,3-diol can be attributed to the starter bacteria; however, subsequent reduction to butan-2-one and then to butan-2-ol is due to non-starter lactic acid bacteria (NSLAB). The concentration of diacetyl and acetoin were affected by both season and dairy plant, while concentration of butan-2-one varied only due to the factor dairy plant (Table 6). The concentrations of these three ketones were significantly lower in cheeses from the second dairy because these ketones are transformed rapidly. In fact, the concentration of the final product, butan-2-ol, was clearly higher in the cheeses made in this plant (Table 3). The other ketones found in Roncal cheese can originate because the free fatty acids formed by lipolysis are catabolized to methyl ketones by the microflora and these are afterwards reduced to their corresponding secondary alcohols by bacterial reductases as a defense mechanism of the microorganisms against toxicity (Molimard & Spinnler, 1996). As shown in Table 6, the other ketones were present at higher concentration in the cheeses from the dairy A. These observations again indicate quantitative and qualitative differences of microbial composition of the milk used to make Roncal cheese, which affects the formation of volatile compounds characteristic of this type of cheese. The importance of NSLAB for the development of the flavour compounds characteristic of Roncal cheese made from raw milk has been mentioned previously (Ortigosa et al., 2001).

Fatty acids are released on the lipolysis of the fat. While butanoic acid, propanoic acid and acetic acid may also be produced by the fermentation of lactose and lactic acid, the acids formed from the branched-chain 2-methylpropanoic (isobutyric) acid and 3-methylbutanoic (isovaleric) acid are produced by the catabolism of the amino acids Val and Leu, respectively (Molimard & Spinnler, 1996). The family of acids is the group of compounds less affected by any of the factors analysed in this study (Table 7), even no significant seasonal differences were found between the cheeses made in the plant A, probably because of the large standard deviation obtained for the duplicates when quantifying acids by this technique. This could be caused by the analytical technique used; as mentioned previously (Izco & Torre, 2000) quantification of free fatty acids using this analytical column is not very reliable.

Ten esters were identified, although only four (which were ethyl esters formed by esterification of the free fatty acids with ethanol) were quantified in all samples (Table 8). Again, the concentrations of the four ethyl esters were higher in the cheeses from dairy B, probably because these cheeses contained more ethanol available to esterify (Table 3). The interaction between both factors for these four compounds was significant because the concentrations of these esters varied distinctly during seasons in cheeses from both dairies. The important contribution of esters to cheese aroma is not in doubt, since esters containing few carbon atoms have a perception threshold ten times lower than the alcohols from which they are derived. Like Manchego cheese

(Martínez-Castro, Sanz, Amigo, Ramos, & Martín Alvarez, 1991), ethyl esters were the predominant esters in the Roncal cheeses, as previously reported (Izco & Torre, 2000; Ortigosa et al., 2001). Large quantities of ethanol and esters are associated with fruity flavours (Urbach, 1993), and the contribution of those compounds to the aroma of Roncal cheese would appear to be very important (Izco & Torre, 2000).

4. Conclusions

FAAs and volatile compounds of Roncal cheese were affected by both factors, season of the year and dairy plant. The season of production is the principal factor affecting the concentration of many FAAs present in Roncal cheese after four months of ripening. This is logical since the cheeses made in summer undergo deeper proteolysis than cheeses made in other seasons. In the case of the second factor, fewer significant differences were found when analysing the effect dairy plant; although the concentration of some FAAs was higher in dairy A (e.g. Tyr), the relative proportions of several individual amino acids appeared to be similar. In any case, the formation of some amino acids and further transformation of these to volatile compounds was faster in dairy B, as observed for Met or Tyr.

In contrast, fewer significant differences between seasons were found for the volatile compounds than for the FAAs. However, this factor (season) significantly affected the concentration of the volatile compounds derived from the catabolism of the branched chain amino acids, Val and Leu, such as the alcohols, 2-methypropan-1-ol or 3-methylbutan-1-ol, and the acids, 2-methylpropanoic and 3-methylbutanoic.

Significant interactions between season and dairy plant were found for all the volatile compounds except hexane, pentan-2-ol, butan-1-ol, DMS, and the families of aldehydes, ketones (except 3-hydroxybutan-2-one and nonan-2-one) and acids, which means that the concentration of those compounds in cheeses from both dairies plants distinctly varied with the seasons. This variation made it difficult to correlate the formation of volatile compounds and the amino acid precursor, as in the case of propan-1-ol or DMS with Met, or ethanol with Ala. Probably, these amino acids either were degraded by several different pathways, yielding different compounds, or those amino acids are not the only precursors for the volatile compounds mentioned.

In a previous study (Ortigosa et al., 2001) it was observed that the original microbial composition of raw milk (NSLAB) appeared to affect drastically the formation of butan-2-ol derived from diacetyl and acetoin (these previously transformed to butan-2-one). In the mentioned study, pasteurization affected the NSLAB counts and these reactions were slowed down while the characteristic aroma of Roncal cheese was affected negatively. As shown in Table 3, the concentration of that alcohol was clearly lower in dairy A and especially in winter, probably due again to the lowest NSLAB counts during that period. However, this should be confirmed by microbiological analysis.

Although some authors have suggested that its concentration of FAAs and flavour could not be correlated, we detected that the summer cheeses with higher concentrations of FAAs showed higher flavour intensity (data not shown), even though it was not more characteristic than the others. However, this was observed only in one of the two dairies.

New data to characterize Roncal cheese and to increase knowledge about the compounds responsible for the formation of characteristic aroma of this kind of cheese have been shown. The pattern of FAAs and volatile compounds is greatly affected by the season and dairy, owing firstly to the distinct microflora of the raw milk. Future work should be aimed at characterizing and evaluating the NSLAB containing the enzymes responsible for the formation of these compounds, in order to study their application as starter adjuncts to obtain a more homogeneous product.

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