Study on the Influence of Pasture on Volatile Fraction of Ewes' Dairy Products by Solid-Phase Microextraction and Gas Chromatography-Mass Spectrometry

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ABSTRACT

The detection of markers of identification of the geographical origin of food is an attractive challenge and, as far as dairy products are concerned, this paper represents a contribution to this field. In this research the influence of feed on the volatile compound composition was investigated on milk, 2-mo-old cheese (Caciotta), and whey cheese (ricotta) obtained from the same flock of Sarda ewes, under standardized technological conditions. Three different types of pasture (mixture of *Lolium perenne* and *Trifolium squarrosum*; rough pasture; *Avena sativa*) were studied. Solid-phase microextraction combined with gas chromatography-mass spectrometry was used and principal component analysis was applied for statistical evaluation of the data set. The volatile composition was significantly affected by the type of pasture independently of the type of cheese and the ripening period. Moreover, a marker of rough pasture, tentatively identified as (E,E)-3,7,11-trimethyl-2,4,10-dodecatriene, was detected only in milk and cheeses produced when the ewe flock grazed on that pasture.

Key words: pasture, milk and cheese, solid-phase microextraction, gas chromatography-mass spectrometry

INTRODUCTION

Over the last few years, several studies have been conducted on the detection of markers that could be used to identify the geographical origin of milk and cheese. This subject is particularly important for Protected Designation of Origin cheeses because their link with the region of origin has to be traced. Terpenes and hydrocarbons, being transferred from forages into milk and cheese, were shown to be promising tools for this purpose (Bosset et al., 1994; Viallon et al., 2000; Buchin et al., 2002). Volatile fraction analysis is one of the most

important methods in quality evaluation of food and it was widely applied for this scope. Among the different extraction techniques, solid-phase microextraction (SPME), developed in 1990 (Arthur and Pawliszyn, 1990) to evaluate the volatile compounds from wastewaters, is a simple and effective sample preparation method that is now widely applied in food analysis (Kataoka et al., 2000). Flavor composition of dairy products has been evaluated in several studies by using different extraction techniques. Volatile compounds of milk have been studied concerning the effects of the type of feeding adopted in animal breeding (Bassette et al., 1966; Moio et al., 1996; Bendall, 2001; Bugaud et al., 2001a), heat treatment (Calvo and de la Hoz, 1992; Contarini and Povoło, 2002; Vasquez-Landaverde et al., 2005), storage conditions (Earley and Hansen, 1982; Marsili, 2000), and light exposure (Kim and Morr, 1996; Marsili, 1999). The quality of butter has been investigated by evaluating both the sulfur compounds, as affected by the type of cow feeding (Shooter et al., 1999), and the effects of the storage conditions (Christensen and Holmer, 1996; Povoło and Contarini, 2003). Volatiles of cheese have been widely analyzed to study the effects of the ripening process (Le Quéré et al., 1998; Carbonell et al., 2002), animal feeding (Buchin et al., 1999; Bugaud et al., 2001b), origin of defects (Kim et al., 2003), and influence of milk protein genetic variants (Verdier-Metz et al., 2000).

In our research the influence of 3 different types of pasture on the volatile compounds of ewe's milk and the derived 2-mo-old cheese (Caciotta) and whey cheese (ricotta) was investigated. The aim of our work was to provide a contribution to the detection of marker useful to link a dairy product to its region of origin. Analyses were made on samples for their chemical and volatile composition. Solid-phase microextraction combined with GC-MS was used and principal component analysis was applied for the statistical evaluation of the data set.

MATERIALS AND METHODS

Animals and Diet

A Sarda ewe flock, comprising 300 head and located in a lowland area of Livorno province (Tuscany, Italy),

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Table 1. Sampling plan

Botanical composition of the pasture (wt % on DM)	Sample code			
	Pasture	Milk	Cheese (ripening time)	Ricotta
Mixture of <i>Lolium perenne</i> and <i>Trifolium squarrosum</i>	P1	M1 (n = 3)	C1 t 0 (n = 3) 1 mo (n = 3) 2 mo (n = 3)	R1 (n = 3)
Rough pasture (15% Gramineae family; 8% Pea family; 77% other families, including 61% Crucifers; 5% Polygonaceae; 5% Ranunculaceae)	P2	M2 (n = 3)	C2 t 0 (n = 3) 1 mo (n = 3) 2 mo (n = 3)	R2 (n = 3)
Common oat pasture (<i>Avena sativa</i>)	P3	M3 (n = 3)	C3 t 0 (n = 3) 1 mo (n = 3) 2 mo (n = 3)	R3 (n = 3)

grazed on 3 different types of pasture. Table 1 reports the botanical composition (weight percentage on DM) of the pastures together with the codes adopted for labeling the dairy samples on the basis of the milk origin. Pastures 1 and 3 (**P1** and **P3**) were 2 fields under specific cultivation, whereas pasture 2 (**P2**) was a rough pasture. For each kind of pasture, grass was cut from 0.5-m² plots in 5 replications. Botanical composition was determined by hand separation. Air-dried herbage samples were divided into the main botanical fractions (grasses, legumes, and herbs) and DM content was determined after drying samples at 105°C for 24 h. Each grazing cycle lasted 1 mo: the first 3 wk were the adaptation period and the last week was the experimental period. The overall experimental period lasted 3 mo from April to June.

Cheese Making

Machine milking was adopted by the farmer. The milk obtained during the experimental periods was used to produce, in a small factory, a 2-mo-old cheese (Caciotta) and whey cheese (ricotta). Technological parameters adopted for the cheese production are described below. Milk was heated at 60°C for 15 min, inoculated with a mesophilic starter culture, and coagulated with liquid animal rennet at 35°C. After 15 min the curd was cut into small pieces (coffee-bean size), and allowed to stand for 2 min; then it was transferred into the molds, allowing the whey to drain off. The curd was maintained for 2 h at 25°C and during this time, the molds were turned upside down 5 times. A manual dry salting was done by spreading salt evenly over the curd. Cheeses were ripened at the dairy factory for 2 mo at 10°C and 85% relative humidity. Ricotta was produced by using the whey derived from the cheese production: whey was heated at 80°C to obtain the rising and coagulation of whey proteins; at the end of this

step, proteins were skimmed off manually and transferred into molds, allowing the residual whey to drain off for 2 d at refrigerating temperature (4°C).

Milk and Cheese Sampling

During the last week of each grazing cycle, 3 bulk milk samples (**M1**, **M2**, and **M3**; Table 1) were collected for analysis the day before the cheese making, divided into 50-mL vials, and frozen at −20°C. Three Caciotta cheeses (**C1**, **C2**, **C3**), from the same batch of production, were collected on the day after cheese making and after 1 and 2 mo of ripening. Three ricotta cheeses (**R1**, **R2**, and **R3**) were taken immediately after production. Caciotta and ricotta cheeses were divided into portions, vacuum-packed, and stored at −20°C until analysis, which was carried out within 1 mo of storage. Representative samples of the 3 pastures were collected, after having observed the behavior of the sheep flock during grazing, and stored at −20°C until the analysis.

Chemical Composition

Protein, fat, and ash content were determined on cheese samples according to AOAC (2000) methods.

Volatile Fraction Composition

A divinylbenzene/carboxen/polydimethylsiloxane, 50/30 μm, 2-cm-long fiber was used to collect volatile fractions by SPME. The fiber was conditioned at 270°C for 30 min in a GC split/splitless injector before analysis. Fifteen grams of milk, 6 g of grated cheese, 10 g of ricotta, or 5 g of pasture, roughly cut into small pieces shortly before the analysis, was weighed in a 20-mL crimp-top vial, and sealed with an aluminum cap provided with a pierceable septum (23 × 75 mm, Varian, Palo Alto, CA). Milk, cheese, and ricotta samples were

Table 2. Volatile compounds extracted from pasture

Compound ¹	Pasture			Retention index	Identification method ²
	P1	P2	P3		
Dimethyl sulfide	+ ³	+	— ⁴	751	PI
Acetone	—	—	+	812	PI
2-Butanone	—	—	+	893	PI
2-Methyl butanal	+	+	—	917	PI
3-Methyl butanal	++	+	—	922	MS/PI
Ethanol	++	++	+	938	MS/PI
2,2,4,6,6-Pentamethyl eptane	++	++	++	943	
2-Pentanone	—	—	++	981	MS/PI
α -Pinene	—	++	—	1,027	MS/PI
α -Thujene	—	++	—	1,033	PI
Toluene	—	—	++	1,051	MS/PI
Hexanal	++	++	+	1,090	MS/PI
β -Pinene	—	++	—	1,105	MS/PI
Sabinene	—	+	—	1,124	PI
Δ 3-Carene	—	+	—	1,154	PI
1-Penten-3-ol	—	—	++	1,166	PI
α -Phellandrene	—	+	—	1,170	PI
Myrcene	—	+	—	1,172	PI
Limonene	—	++	—	1,200	MS/PI
3-Methyl-1-butanol	—	++	—	1,212	MS/PI
t-2-Hexenal	++++	++	+	1,226	PI
β -Ocimene	—	+	—	1,246	PI
γ -Terpinene	—	+	—	1,253	PI
l-Pentanol	—	—	+	1,256	PI
3-Octanone	++	+	—	1,262	PI
1-Octen-3-one	+	—	—	1,309	PI
Cis-2-pentenol	+	+	+	1,325	
1-Hexanol	++	++	+	1,357	PI
c-3-Hexen-1-ol	++	+++	++	1,387	PI
t-2-Hexen-1-ol	++	++	+	1,410	PI
t,t-2,4-Hexadienal	++	+	—	1,411	
1-Octen-3-ol	++	++	+	1,454	PI
α -Cubebene	—	++	—	1,466	PI
α -Copaene	—	+	—	1,502	PI
Benzaldehyde	—	+	—	1,537	PI
β -Cubebene	—	+	—	1,548	PI
t2,c6-Nonadienal	+	—	—	1,596	PI
β -Caryophyllene	—	++	—	1,607	PI
t- β -Farnesene	—	+	—	1,669	PI
α -Humulene	—	+	—	1,684	PI
Germacrene D	—	+	—	1,722	PI
Δ -Cadinene	—	++	—	1,765	PI
γ -Cadinene	—	+	—	1,769	PI
Methyl salicylate	—	—	+	1,787	PI
Naphtalene	—	+	—	1,789	
Benzyl alcohol	—	+	—	1,879	PI
α -Bisabolol	—	+	—	2,222	PI

¹Identification by comparison with mass spectra of Wiley library (McLafferty and Stauffer, 1988).

²Identification methods: PI = retention indexes published (Jennings and Shibamoto, 1980; Acree and Arn, 2004); MS = mass spectra of authentic compounds.

³Range of area unit/100,000: + = 0 to 50; ++ = 50 to 500; +++ = 500 to 1,000; ++++ = more than 1,000.

⁴Not detected in pasture sample.

allowed to equilibrate to 45°C in a thermostatic bath for 5 min and the fiber was exposed to the headspace for 30 min. During the extraction, milk samples were kept under agitation by a microstir bar placed into the vial. The extraction of volatile compounds from pasture was performed in duplicate, maintaining the sample at room temperature, and exposing the fiber to the headspace for 15 min.

The gas chromatographic analysis of volatile compounds adsorbed on the SPME fiber was carried out with a CP-WAX 52CB capillary column (Varian; 60 m long, 0.32 mm i.d., 0.5 μ m film thickness). A Hewlett Packard (Palo Alto, CA) 5890 series II gas chromatograph coupled with a Hewlett Packard 5989A mass spectrometer was used. To achieve sharper peaks, the split/splitless injector was provided with a 0.75 mm i.d.

Figure 1. Solid-phase microextraction/GC-MS profiles of milk samples M1, M2, and M3 (peaks are numbered as in Table 3).

Table 3. Volatile compounds of milk and ricotta cheese (retention indexes and means of the area values/100,000 of the 3 replicates)

Peak	Compound ¹	Retention indices ²		Identification method ³	Milk			Ricotta		
		Milk	Ricotta		M1	M2	M3	R1	R2	R3
1	Dimethyl sulfide		753	PI	— ⁴	—	—	0.7	1.6	2.7
2	Acetone	811	823	MS/PI	19.6	20.6	23.5	12.1	36.1	25.2
3	3-Octene	840	841		—	—	—	3.2	9.7	—
4	2-Butanone	906	906	MS/PI	—	—	—	1.4	4.9	3.5
5	2-Methyl butanal	905		MS/PI	—	4.5	—	—	—	—
6	3-Methyl butanal	910		MS/PI	2.4	7.7	27.9	—	—	—
7	2-Pentanone	971	984	MS/PI	2.8	—	3.0	3.0	11.9	6.0
8	Diacetyl	976		MS/PI	—	—	12.8	—	—	—
9	Methyl butyrate	982		MS/PI	—	17.9	—	—	—	—
10	Pentanal		986	MS/PI	—	—	—	3.9	16.2	6.0
11	Toluene	1,043	1,051	MS/PI	29.8	8.5	—	0.8	15.6	—
12	Hexanal	1,087	1,089	MS/PI	4.9	3.4	—	1.2	3.8	1.6
13	2-Heptanone	1,182	1,189	MS/PI	2.6	3.5	1.8	4.5	18.4	7.3
14	Heptanal	1,186	1,192	PI	2.2	—	—	1.6	3.9	0.8
15	Methyl hexanoate	1,184	1,191	MS/PI	—	32.3	—	—	1.5	—
16	Limonene	1,195		MS/PI	—	—	2.6	—	—	—
17	3-Methyl-1-butanol	1,208	1,215	MS/PI	7.9	5.9	25.1	—	4.2	0.8
18	1-Pentanol	1,257	1,259	PI	3.6	6.4	—	1.1	5.5	1.8
19	Acetoin	1,292	1,295	PI	—	—	82.7	—	2.8	—
20	2-Nonanone		1,392	MS/PI	—	—	—	1.0	2.8	0.8
21	Methyl octanoate	1,390		MS/PI	—	7.0	—	—	—	—
22	Nonanal		1,396	PI	—	—	—	1.0	1.3	0.7
	RT 31.5	1,529	1,529		—	21.5	—	—	36.6	—
23	Butyric acid		1,636	MS/PI	—	—	—	—	2.2	2.5
24	Hexanoic acid		1,845	MS/PI	—	—	—	—	1.4	3.0
25	Dimethylsulphone		1,914		—	—	—	1.5	1.8	0.4
26	Octanoic acid		2,069	MS/PI	—	—	—	1.6	—	2.2
27	Decanoic acid		2,276	MS/PI	—	—	—	1.8	—	—

¹Identification by comparison with mass spectra of Wiley library (McLafferty and Stauffer, 1988).

²RI = Retention index measured in milk and ricotta cheese samples.

³PI = Retention indexes published (Jennings and Shibamoto, 1980; Acree and Arn, 2004); MS = mass spectra of authentic compounds.

⁴Not detected in any of the 3 milk or ricotta cheese samples.

inlet liner (Supelco, Bellefonte, PA). During the injection phase, a 3-min splitless mode was applied and the injector temperature was held at 250°C. Oven temperature was held at 40°C for 8 min, programmed to 220°C at a rate of 4°C/min, and held at 220°C for 20 min. Helium was used as a carrier gas at a flow rate of 1.0 mL/min. The MS temperatures adopted were as follows: interface 220°C, source 200°C, quadrupole 100°C; acquisition was performed in electron impact (EI) mode (70 eV) by 1.6 scans per second and the mass range used was m/z 35 to 270.

The identification of volatile compounds was performed with the following criteria: comparison with the mass spectra of the Wiley library (McLafferty and Stauffer, 1988), injection of authentic standards analyzed under the same GC-MS conditions, calculation of retention indices (RI) followed by comparison with those obtained from both authentic standards and literature (Jennings and Shibamoto, 1980; Acree and Arn, 2004).

The identification of the compound at RI 1,529 was performed by applying positive and negative chemical ionization. A Finnigan PolarisQ mass spectrometer (Thermo Electron, Milan, Italy) was used with the same GC column adopted in the EI analysis: the source temperature was at 150°C and reagent gases ammonia (1.5 mL/min), isobutane (1 mL/min), methane (1.5 mL/min) were tested.

Statistical Analysis

Data for cheese volatile compounds were subjected to principal components analysis (PCA) using the PARVUS package (Forina et al., 1988). Statistical evaluation was performed on the data obtained from SPME analysis. The data set consisted of the 27 samples (objects), reported in Table 1, and 15 volatile compounds (variables), with the following changes: area values of carboxylic acids (acetic, butyric, isobutyric, isopentanoic, hexanoic, octanoic, and decanoic acids) were

Figure 2. Solid-phase microextraction/GC-MS profiles of ricotta cheese samples R1, R2, and R3 (peaks are numbered as in Table 3).

Table 4. Caciotta cheese volatile compounds: mean values (area unit/100,000) of the 3 replicates of the samples collected at each ripening time

Peak	Compound ¹	RI ²	Identification method ³	C1			C2			C3		
				t 0	1 mo	2 mo	t 0	1 mo	2 mo	t 0	1 mo	2 mo
1	Dimethyl sulfide	748	PI	2.2	4.1	5.7	1.6	5.8	5.4	0.4	0.7	0.6
2	Acetone	820	MS/PI	20.8	16.7	19.1	18.1	19.5	20.8	17.2	31.0	17.3
3	3-Octene	840	PI	11.7	11.7	9.3	6.6	6.5	5.6	0.5	0.6	0.7
4	2-Butanone	906	MS/PI	5.9	11.4	92.1	4.8	7.8	6.2	5.3	7.7	75.0
5	Ethanol	938	MS/PI	7.4	12.4	22.0	6.5	13.6	16.8	4.4	13.0	53.9
6	2-Pentanone	982	MS/PI	2.5	45.2	30.7	2.1	16.3	32.3	1.9	21.9	114.7
7	Diacetyl	987	MS/PI	23.6	28.4	20.3	15.0	19.9	13.9	17.8	18.7	16.3
8	Toluene	1,051	MS/PI	5.5	6.3	3.2	14.5	11.4	9.3	0.4	0.2	0.3
9	2-Heptanone	1,188	MS/PI	4.1	146.0	55.9	6.0	33.0	63.5	3.0	13.7	100.4
10	3-Methyl-1-butanol	1,215	MS/PI	3.0	5.3	4.3	7.3	20.7	18.5	— ⁴	9.4	13.7
11	Acetoin	1,297	PI	475.0	316.6	189.7	329.3	254.0	166.9	365.4	360.9	184.0
12	2-Methyl-3-pentanol	1,353	PI	64.3	50.6	33.8	57.8	51.2	35.1	50.3	39.7	25.1
13	2-Nonanone	1,389	MS/PI	—	56.8	44.8	2.3	20.6	51.6	—	2.1	12.8
14	Acetic acid	1,462	MS/PI	54.0	53.5	139.2	87.6	102.0	188.9	19.6	33.5	49.5
	RT 31.5	1,529	—	—	—	—	63.6	64.4	63.3	—	—	—
15	Isobutyric acid	1,572	MS/PI	—	—	0.7	—	—	3.1	—	11.7	13.1
16	Butyric acid	1,634	MS/PI	74.5	192.5	469.3	208.6	584.3	844.9	52.8	163.1	245.3
17	Isopentanoic acid	1,672	MS/PI	—	—	2.2	1.8	2.2	6.7	—	20.3	29.4
18	Pentanoic acid	1,751	MS/PI	—	—	5.5	0.8	3.5	7.6	—	—	—
19	Hexanoic acid	1,846	MS/PI	57.8	180.9	438.5	171.5	468.5	633.5	55.9	132.6	216.9
20	Dimethylsulphone	1,911	PI	7.7	7.5	6.8	1.6	2.5	1.8	0.9	0.8	0.2
21	Octanoic acid	2,067	MS/PI	12.0	73.5	261.2	76.6	242.8	355.3	28.2	57.9	111.5
22	Decanoic acid	2,274	MS/PI	—	11.2	94.6	17.7	68.0	130.2	5.5	4.7	20.4

¹Identification by comparison with mass spectra of Wiley library (McLafferty and Stauffer, 1988).

²RI = Retention index measured in cheese samples.

³PI = Retention indexes published (Jennings and Shibamoto, 1980; Acree and Arn, 2004); MS = mass authentic compounds.

⁴Not detected in any of the 3 samples.

summed and treated as unique variables, labeled as “acids”; the compound having RI 1,529 was excluded from the data set. The PCA was applied using the correlation matrix (Pearson).

RESULTS AND DISCUSSION

Volatile Fraction of Pastures

The analysis of the volatile fraction of the 3 pastures by SPME/GC-MS analysis showed important differences among the samples (Table 2). Due to the variety of its botanical composition, the rough pasture (P2) was characterized by a very rich profile, especially when compared with P1. In the volatile fraction of P2, the most abundant compounds were alcohols and terpenes, the latter class being absent in the profiles of the other 2 pastures. Oat pasture (P3) had a volatile composition rich in alcohols, both saturated and unsaturated, and it was characterized by the presence of methyl salicylate.

Volatile Fraction of Milk

Differences in the volatile composition of milk samples were observed. Milk M2 provided the richest profile with respect to the milk samples deriving from the other

2 pastures (Figure 1). The presence of methyl esters (peaks 9, 15, and 21) and, in particular, of a compound at retention time 31.5, having RI 1,529, seemed to be characteristic of this milk. The peak at RI 1,529 was detected in all 3 M2 samples collected. On the other hand, M3 was characterized by the presence of diacetyl (peak 8) and acetoin (peak 19), substances deriving from the microbial metabolism of citrate. Moreover, toluene (peak 11) was detected only in milks M1 and M2 (Table 3). The presence of esters, aldehydes, ketones, alcohols, and toluene in ovine milk was previously detected by Moio et al. (1993a).

Volatile Fraction of Ricotta and Caciotta Cheeses

To characterize the cheeses produced with regard to their chemical composition, analyses of protein, fat, and ash content were performed. The mean values obtained for protein, fat, and ash content were 9.3, 8.8, and 1% for ricotta samples and 27.2, 28.5, and 1.9% for the 2-mo-old Caciotta cheese samples. The data obtained were in accordance with those reported for these types of products.

Volatile fractions of ricotta cheese varied for all 3 types of pastures. Figure 2 shows the GC-MS profiles

Figure 3. Solid-phase microextraction/GC-MS profiles of Caciotta cheese samples at 2 mo of ripening: A) total chromatogram of C2 sample; B) enlarged view of chromatograms of C1 and C3 samples. Peaks are numbered as in Table 4; C1, C2, and C3 represent cheeses made from milk from ewes pastured on each of 3 pastures.

of the 3 samples, and Table 3 reports the area values of the volatile compounds. The R2 sample showed the richest SPME/GC-MS profile under both a qualitative and quantitative point of view. Some correspondences with milk samples can be observed: particularly, toluenes were detected only in R1 and R2 (as was the case in M1 and M2). Moreover, 3-octene was detected only in R1 and R2 samples. The more interesting aspect was the presence of the compound at RI 1,529 in the GC-MS profile of R2.

The volatile fraction of the Caciotta cheese was analyzed at different ripening periods. Table 4 reports the data of area values obtained at the different ripening steps for each cheese production: mean values were calculated from the analyses performed on the 3 cheese samples produced on the same day from the same milk. Two sulfur compounds were detected: dimethyl sulfide, which is derived both from feed (Reddy et al., 1967) and by catabolism of sulfur amino acids, particularly methionine (McSweeney and Sousa, 2000), and dimeth-

Figure 4. Electron impact and chemical ionization (CI) spectra of the unknown compound eluting at 31.5 min.

Table 5. Loading (%) for volatile compounds on the first 3 eigenvec-tors¹

Compound	PC1	PC2	PC3
Dimethyl sulfide	3.45	14.97	4.98
Acetone	0.75	1.91	1.30
3-Octene	0.03	22.08	0.98
2-Butanone	9.97	0.58	5.17
Ethanol	13.37	4.47	1.94
2-Pentanone	12.70	2.15	5.21
Toluene	0.34	9.54	12.21
Diacetyl	0.09	7.03	14.95
2-Heptanone	9.38	2.75	7.52
3-Methyl-1-butanol	4.39	0.00	17.80
Acetoin	17.17	0.08	3.48
2-Methyl-3-pentanol	13.45	4.76	0.00
2-Nonanone	7.23	10.07	0.21
Dimethylsulfone	0.01	16.49	9.08
Acids	7.68	3.19	15.17
% of variance explained	32.17	25.42	17.55
% of total variance	32.17	57.59	75.14

¹PC = principal component.

ylsulfone, whose presence in milk has been related to animal metabolism (Williams et al., 1966). Dimethyl sulfide was higher in C2 cheese and it increased during the first month, whereas dimethylsulfone was more abundant in cheese C1 and did not change during ripening. Acetone was present in similar amounts in the 3 cheese categories and did not show changes during ripening; its presence in cows' milk was related to feeding (Gordon and Morgan, 1972). Diacetyl, acetoin, and 2-butanone are produced by the metabolic pathways of citrate (McSweeney and Sousa, 2000; Marilley and Casey, 2004). Acetoin was the most abundant volatile compound detected immediately after production in all cheese samples and it decreased during ripening. Diacetyl was higher in cheese C1 than in C2 and C3 and it showed the same behavior for the 3 pastures during ripening, having a slight increase at 1 mo and a decrease at 2 mo. 2-Butanone had the same area values for C1, C2, and C3 immediately after production, but showed a strong increase at 2 mo of ripening in samples C1 and C3. For alcohols, ethanol, 3-methyl-1-butanol, and 2-methyl-3-pentanol were detected. Ethanol, deriving from lactose microbial fermentation (Marilley and Casey, 2004), was found at the same level in all samples collected immediately after production and its increase during ripening showed the same rate in samples C1 and C2, whereas it was more intense in sample C3. 3-Methyl-1-butanol, which derives from the metabolism of leucine (Yvon and Rijnen, 2001), showed an increase only in samples C2 and C3. 2-Methyl-3-pentanol was found in the same amount in the 3 types of cheese and decreased during ripening. The amount of 2-pentanone, 2-heptanone, and 2-nonanone, which are produced from β -oxidation and decarboxylation of free fatty acids

β -farnesene
(E)-7,11-dimethyl-3-methylene-1,6,10-dodecatriene

(E,E)-3,7,11-trimethyl-2,4,10-dodecatriene

Figure 5. Molecular structures of β -farnesene and (E,E)-3,7,11-trimethyl-2,4,10-dodecatriene.

(Marilley and Casey, 2004), was comparable in all the samples analyzed immediately after production. During ripening, these compounds showed a progressive increase in samples C2 and C3, whereas in C1 they reached the highest value after 1 mo and then decreased or remained constant. Straight and branched-chain VFA, ranging from 2 to 10 carbon atoms, were detected. Low molecular weight fatty acids (C2 to C10) can be formed by both fat lipolysis and microbial metabolism (Yvon and Rijnen, 2001), whereas isobutyric and isopentanoic acids derive from the microbial catabolism of valine and leucine, respectively (Yvon and Rijnen, 2001). The C2 cheese samples showed (at first sampling and during ripening) the highest amount of straight-chain fatty acids. On the other hand, C3 samples were characterized by a higher amount of isobutyric and isopentanoic acids than C1 and C2. As regards hydrocarbons, 2 compounds were found: the monounsaturated 3-octene and the aromatic toluene. 3-Octene, which probably derived from oxidation of unsaturated fatty acids (Frankel, 1980), has previously been found in the volatile fraction of other cheeses, such as Roncal and Pecorino Sardo (Larráyoz et al., 2001). This compound, whose presence in our samples was not affected by the ripening period, was detected in C1 cheeses at a level twice that in C2, whereas in C3 it was very low. It is worth noting that, with regard to ricotta cheese, 3-oc-

Figure 6. Principal components analysis biplot on the first and second eigenvector (57.6% of the total variance explained) of 27 samples (objects) and 15 volatile compounds (variables) in C1 (○); C2 (△), C3 (□); C1, C2, and C3 represent cheeses made from milk from ewes pastured on each of 3 pastures. Numbers inside the symbols indicate the ripening time: immediately after production (0), 1 mo (1), and 2 mo (2).

tene was detected only in R1 and R2 samples. Toluene, which was detected in M1 and M2 milks, was found only in C1 and C2 cheeses. The reason of the presence of toluene in milk and cheese remains unclear. Moio et al. (1993b), referring to the research of Drawert et al. (1981), who described a natural origin of this compound as a degradation product of β -carotene, hypothesized a direct transfer from forage into milk. The recent researches of Spinhirne et al. (2003, 2004), who found toluene in the volatile profile of ruminal cultures (2003) and bovine breath (2004), seemed to support this assumption. On the other hand, regarding cheese, Bosset et al. (2000) observed that the freeze storage and packaging materials might be the origin of some benzene derivatives, including toluene; however, these researchers also put forward the hypothesis that these compounds could also have a microbial origin.

The most interesting difference among the cheeses was the presence of the same volatile substance appearing in M2 and R2 samples at RI 1,529 and detected (in all 3 sampling periods) in the GC-MS profile of cheese C2. Figure 3 reports the total SPME/GC-MS profile of the volatile fraction obtained from sample C2 at 2 mo of ripening together with the enlarged views of the time range 26 to 35 min of C1 and C3 at the same ripening time.

The EI spectrum of the compound at RI 1,529 (Figure 4) was compared with those of both Wiley library and

authentic standards of the most common volatiles but it did not match any compound satisfactorily. The type of fragmentation deriving from EI mode allowed some inferences about its structure to be made. This compound could have a molecular weight of 206, and the presence of fragments with sequential addition of methylene units (14 amu), together with the intensity of the ions C_nH_{2n-1} , lead to the hypothesis that this molecule could be an unsaturated hydrocarbon. Moreover, fragment ions with m/z 123 and 136, even though not particularly intense, suggested the presence of a terpenic structure. To obtain more information, chemical ionization (CI) with methane, isobutane, and ammonia was performed, in both positive- and negative-ion mode. The substance provided, in CI positive-ion mode, a very fragmented spectrum, as expected by an unsaturated hydrocarbon. By operating with CH_4 , the presence of ions m/z 205 ($M-H^+$) and 207 ($M+H^+$) confirmed the molecular weight of 206. This result was also demonstrated by the ions m/z 207 ($M+H^+$) and 224 ($M+NH_4^+$), obtained with NH_3 and isobutane. The negative chemical ionization using the 3 reagent gases did not produce other useful information. From the evaluation of these results, no complete identification of the compound at RI 1,529 was achieved, but it can be reasonably concluded that it was an unsaturated hydrocarbon (molecular weight of 206) with a terpenic structure. Moreover, a high degree of similarity was found between its EI

Figure 7. Principal components analysis biplot on the second and third eigenvector (43% of the total variance explained) of 27 samples (objects) and 15 volatile compounds (variables) in C1 (○); C2 (△); C3 (□); C1, C2, and C3 represent cheeses made from milk from ewes pastured on each of 3 pastures. Numbers inside the symbols indicate the ripening time: immediately after production (0), 1 mo (1), and 2 mo (2).

mass spectrum and that of some compounds reported in literature. Moio et al. (1996) found 2 compounds identified as sesquiterpenes and having a molecular weight of 206; the mass spectrum of one of them (peak 74) had strong similarity to that of our unknown molecule at RI 1,529. In addition, another affinity was observed with (E,E)-3,7,11-trimethyl-2,4,10-dodecatriene ($C_{15}H_{26}$), identified by Palomino et al. (1996), who reported for this compound the following EI fragmentation m/z (relative abundance): 206(10), 191(9), 177(5), 163(20), 149(8), 136(22), 121(43), 109(58), 95(75), 81(42), 69(100), 55(58). The compound at RI 1,529 could be an isomer of (E,E)-3,7,11-trimethyl-2,4,10-dodecatriene. By looking at the molecular structure of this substance and at the volatile compounds present in P2, a possible origin from β -farnesene can be assumed (Figure 5), according to the results showed by Schlichthaler-Cerny et al. (2004). Some unsaturated hydrocarbons, tentatively identified as 3,7-dimethyl-1,6-octadiene, 2,6-dimethyl-2,6-octadiene, and 3,7-dimethyl-2-octene, were found by these researchers in milk but not in grass. After studying the rumen reactions occurring on some components of the pastures, the authors observed a correlation between the formation of these compounds and the degradation of β -myrcene and (E)- β -ocimene. The same mechanism of reaction can explain the formation of (E,E)-3,7,11-trimethyl-2,4,10-dodecatriene from β -farnesene.

To get an overall evaluation of the influence of pasture on the evolution of volatile compounds during the ripening period, PCA was applied to the results obtained from the cheese samples (Figure 6). The compound having RI 1,529 was not included in the data set due to its obvious discriminating ability. The 3 ripening periods (labeled as 0, 1, and 2) are distinguished along the axis of the first eigenvector, where ketones and 2-methyl-3-pentanol had the highest loading (Table 5). The separation according to the type of pasture is really obtained only for C3 (in squares), along the axis of the second eigenvector. Good discrimination among the 3 types of pasture is obtained by taking into account the information contained in components 2 and 3 (Figure 7), explaining the 43% of the total variance. It is worth noting that the separation among the 3 pastures occurs along the axes of both components. The variables contributing more to the second eigenvector (3-octene, dimethylsulfone, and dimethyl sulfide) seemed to be able to distinguish P3 from the other 2 pastures, whereas those contributing more to the third eigenvector (toluene, diacetyl, 3-methyl-1-butanol, and acids) were able to distinguish pasture 1 from pasture 2.

The different composition of the volatile fraction of these cheeses, produced using milk from the same ewe flock, under the same standardized technological conditions, confirmed that the type of diet was responsible for the presence not only of a marker compound, but

also of other volatiles, showing differences in the animal metabolism, milk fat oxidation, and microbial development during ripening.

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