Use of Headspace Sampling in the Quantitative Analysis of Artisanal Spanish Cheese Aroma

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An automatic headspace injector connected to a GC−MS instrument was used for the quantitative analysis of artisanal Afuega'l Pitu cheese aroma throughout ripening. Ethyl propionate and camphor were added as internal standards. The standard addition method was used to quantify 17 of the 40 compounds identified in the cheese headspace profile. Good linearity ($r > 0.99$) was exhibited by most of the volatile compounds within the concentrations used. Cheese aroma components changed qualitatively and quantitatively during a 30 day ripening period. Compounds found in the highest concentration during the first two ripening weeks were ethanol, diacetyl, acetoin, 2-methyl-1-propanol, 3-methyl-1-butanol, and acetic acid. The concentration of ethyl esters of fatty acids, acetaldehyde, and sulfur compounds increased during the following 2 weeks.

INTRODUCTION

The volatile constituents of food are usually considered as the fraction most related to the food flavor which can be subjected to instrumental analysis (Reineccius and Anandaraman, 1984), even though some nonvolatile water soluble compounds can also make a significant contribution (Urbach, 1993). Gas chromatography coupled to mass spectrometry (GC−MS) has been the selected technique in the investigation of the aroma of many cheeses (Bosset and Liardon, 1985; Gallois and Langlois, 1990; Vandeweghe and Reineccius, 1993). Among the methodologies used to extract the volatile organic compounds from the cheese matrix, headspace sampling (HS), involving the collection of the surrounding vapor phase, is a discriminating technique for components with the highest volatility. Theoretically at least, the fraction extracted by HS should be closer to the genuine aroma sensed by the nose than that obtained by means of other extraction procedures like high vacuum (Vandeweghe and Reineccius, 1993; Jollivet et al., 1993) or steam distillation (Liebich et al., 1970), followed by solvent extraction, or simultaneous distillation extraction (SDE) (González de Llano et al., 1990; de Frutos et al., 1991; Martínez-Castro et al., 1991; Careri et al., 1994). Dynamic HS has been the method of choice in recent investigations on cheese flavor (Horwood, 1989; Preininger and Grosch, 1994) and metabolic products of dairy starter cultures (Imhof et al., 1995). Static HS has not been used as widely as other methods mainly due to its lack of sensitivity; however, it has the advantage of minimal manipulation of the sample, which avoids undesirable contaminants and artifacts. Few quantitative approaches for headspace flavor compounds have been reported. Marsili (1981) and Ulberth (1991) used static HS and standard addition quantification in the study of buttermilk and yogurt aroma, respectively. Gallois and Langlois (1990) used static HS for the extraction of sulfur compounds

from blue cheeses. Bosset and Gauch (1993) studied the volatile fraction of six European cheeses by means of dynamic HS, but no quantitative data were reported. Imhof and Bosset (1994) and Imhof et al. (1995) used dynamic HS for the extraction of volatile compounds from milk and fermented milks, quantifying by the standard addition method without internal standard, which can lead to a lack of repeatability. Lin and Jeon (1985) used a combination of steam distillation with HS for the analysis of volatile compounds in cheese, applying the standard addition method for the quantitative analysis, also without internal standard. Careri et al. (1994) compared SDE and dynamic HS methods for the qualitative analysis of Parmesan cheese aroma, identifying as many as 110 organic compounds in the headspace.

This paper presents a method for the quantitative analysis of cheese headspace, by using an automatic headspace sampler connected to a gas liquid chromatograph with mass selective detection. The method was applied in the characterization of Afuega'l Pitu cheese aroma at various stages during ripening. Afuega'l Pitu is a very aromatic, acid-coagulated cheese, homemade from bovine raw milk in Asturias (Northern Spain), and consumed both fresh and mature. Its characterization has been part of a more comprehensive project focused on the development of a starter culture and an Origin Appellation for this product (Cuesta et al., 1996).

MATERIALS AND METHODS

Standard Solutions. Aqueous solutions of volatile compounds were prepared from high-purity chemicals (usually higher than 98%) purchased from Sigma (St. Louis, MO), Aldrich Chemie (Steinheim, Germany), Jansen Chimica (Geel, Belgium), and Chemservice (West Chester, PA). Four calibration standard solutions with increasing concentrations of 2-propanone, ethyl acetate, 2-butanone, 3-methylbutanal, ethanol, 2-pentanone, diacetyl, 2-butanol, ethyl butanoate, 1-propanol, 2-methyl-1-propanol, isoamyl acetate, 2-heptanone, 3-methyl-1-butanol, ethyl hexanoate, acetoin, and 2-heptanol were prepared and stored in hermetically sealed headspace vials at −20 °C until they were used.

Samples. Three raw milk Afuega'l Pitu cheeses of the white troncoconical variety (Cuesta et al., 1996) were pur-

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chased for each age analyzed: 3, 7, 15, and 30 days, all of them from one dairy farm considered to produce regular quality cheeses from raw milk and representative among regional artisans. Cheese samples were analyzed as soon as received or after frozen storage (-20 °C, vacuum-packaged in plastic bags) for less than 1 month.

Sample Preparation. A 10 g sample of finely grated cheese with 100 μL camphor (100 μg/mL) and ethyl propionate (150 μg/mL) aqueous solutions, added to it as internal standards, was prepared. The sample was thoroughly mixed, and a variable amount of Na_2SO_4 was added, depending on the age of the cheese sample (the same amount used for calibration, see below), and mixed with a spatula. A 20 mL vial was filled with the resulting mixture up to the top, care being taken not to press the cheese, and was hermetically sealed by a hand crimper with a PTFE-coated rubber septum and an aluminum cap (Symta, Madrid, Spain). During sample equilibration, the cheese powder softens and compresses, leaving enough space at the top for the injector needle. Duplicate analyses were conducted.

Apparatus and Chromatographic Conditions. An Automatic Headspace Sampler 19395 A, connected to a 5890 Series II Gas Chromatograph coupled to a 5972 Mass Selective Detector (Hewlett-Packard, Palo Alto, CA), was used. Data were recorded and analyzed with the HP G1034C MS Chemstation Software installed in a Vectra 486/33VL Personal Computer.

The headspace sampler was equipped with a 3 mL injection loop and a transfer line directly attached to the gas chromatograph injector. Sample vials were equilibrated during 60 min at 70 °C, and the injection loop was heated at 90 °C. Injection conditions were as follows: 1 s pressurization time, 5 s equilibration, 2 s vent time, and 2 min injection. High-purity helium (Carburos Metálicos, Barcelona, Spain), filtered through moisture and oxygen traps (Hewlett-Packard), was used for vial pressurization as auxiliary gas with pressure set at 1.4 bar and as HS sample carrier gas at a flow rate of 20 mL/min measured at the splitter outlet. The injector temperature was set at 150 °C.

The separation of volatile compounds was performed in an HP INNOWax capillary column (60 m × 0.25 mm inside diameter) coated with cross-linked polyethylene glycol (0.25 μm film thickness). Helium pressure at the column head was set at 2.2 bar, which means approximately a column flow rate of 2.4 mL/min at 32 °C and a split ratio of 8:1. Oven temperature programming was as follows: 32 °C for 4 min, then a temperature increase at a rate of 1 °C/min up to 38 °C, followed by a second heating ramp of 25 °C/min up to 210 °C, and finally 210 °C for 4 min, for a total cycle time of 21 min. The total run time between injections was 30 min.

The quadrupole mass selective detector, with electron-impact ionization and an electron multiplier voltage of 1650 V, operated in the scan mode, with a mass range of 33–250 amu, at 2.0 scans/s, and a solvent delay of 3.2 min was utilized. The detector interface temperature was set at 280 °C, with the actual temperature in the MS source reaching approximately 180 °C.

Quantification of Volatile Compounds. The standard addition method was used for quantification. Calibration curves were calculated for cheeses of each ripening time under study. For the calibration curves construction, cheese samples (10 g) of each ripening time (3, 7, 15, and 30 days) were spiked with 100 μL of the calibration standard solutions and 100 μL of each internal standard solution. A standardized amount of Na_2SO_4 was added for each ripening time: 13 g to the 3-day-old, 11 g to the 7-day-old, 10 g to the 15-day-old, and 7 g to the 30-day-old cheese. Peaks were quantified by their area. As MS data were recorded in the scan mode, quantification of compounds on the basis of selected ions was performed automatically by the calibration software on the reconstructed ion chromatogram. Linear regression curves were calculated for each compound by subtracting the area response found in the control sample (cheese without standard addition) from the area responses given by the spiked samples, always relative to the internal standards. Peaks with lower retention times, from 2-propanone to diacetyl, were quantified with ethyl

propionate as internal standard, which elutes at 9 min, and the remaining peaks with camphor, which elutes at 18.3 min. Two replicates of each curve point were carried out, and curves were forced to pass through the origin. Peak identification was based upon MS spectra comparison with the HP Wiley 138 library and with spectra of injected standards, and also on retention time of standards when available. Detection limits were calculated approximately from the amount of added compound that caused a response increase of more than 3 times the background peaks signal.

RESULTS AND DISCUSSION

Chromatographic Method. The first objective of the method setup was to improve HS sensitivity (Rothweiler, 1994). For that purpose, Na_2SO_4 was mixed with the cheese sample to displace the volatile compound's equilibrium toward the vapor phase. Different amounts of Na_2SO_4 were added to the cheeses with different maturation times, basically due to the different water content of cheeses. Increasing concentrations of the salt were assayed for each maturation time, before standardizing the procedure with the amount that provided the maximum concentration of volatile compounds in the headspace. The maximum cheese surface was exposed to equilibration by using the highest amount of prepared sample (cheese plus standards plus Na_2SO_4) that could be introduced without pressing into a 20 mL vial. This maximum amount was standardized for each ripening time. In addition, a 3 mL injection loop was used and the splitter:column flow ratio was optimized (8:1) to allow the highest amount of sample to be injected (low splitter flow) without excessive broadening of early peaks. Injection was performed with minimum vent and vial pressurization times, 2 and 1 s, respectively. Finally, the mass detector was tuned at maximum sensitivity. Two internal standards, ethyl propionate for the first and camphor for the second part of the chromatogram, were included to obviate the response variability due to slight uncontrollable oscillations in the splitter flow rate (not electronically controlled), which can cause small fluctuations in the amount of the higher-volatility compounds that enter the column.

As many as 40 compounds were separated and identified by retention time and MS in Afuega'l Pitu cheese. Table 1 lists the volatile organic compounds identified throughout ripening. On the basis of preliminary HS injections, 17 compounds were selected for quantification. Figure 1 shows a total ion chromatogram (TIC) corresponding to the headspace profile of a 7-day-old Afuega'l Pitu cheese spiked with different concentrations of the 17 quantified compounds. Headspace injection at low split ratio values causes early eluting peaks to broaden, while peaks eluting later focus and appear much sharper. The use of higher splitter flow rates up to 25 mL/min improved peak width, although it decreased sensitivity. The use of a higher initial temperature (40 °C) improved peak width too but failed in resolving 2-pentanone from diacetyl and ethyl butanoate from 1-propanol. In 15- and 30-day-old cheeses, 2-methylpropanal and probably butane coeluted with acetone. A lower initial temperature could have possibly resolved the three peaks, but that was not feasible in our conditions.

Although present in appreciable amounts, acetic, butanoic, and 3-methylbutanoic acids could not be quantified due to carryover problems observed for this substance as well as for 2-ethyl-1-hexanol. Two isomers of 2,3-butanediol (I and II in Table 1) were identified,

Table 1. Volatile Organic Compounds Identified in Afuega'l Pitu Cheese during Ripening

| peak no.	RT (min)	volatile organic compound	identification RT	identification MS	peak no.	RT (min)	volatile organic compound	identification RT	identification MS
1	3.49	methanethiol		+	21	13.75	isoamyl acetate	+	+
2	3.65	acetaldehyde	+	+	22	14.05	2-pentanol	+	+
3	3.90	carbon disulfide		+	23	14.45	1-butanol	+	+
4	4.02	dimethyl sulfide		+	24	14.75	2-heptanone	+	+
5	4.86	2-propanone	+	+	25	15.25	3-methyl-1-butanol	+	+
6	6.46	ethyl acetate	+	+	26	15.39	ethyl caproate	+	+
7	6.79	2-butanone	+	+	27	15.75	styrene	+	+
8	7.12	2-methylbutanal	+	+	28	16.29	acetoin	+	+
9	7.33	3-methylbutanal	+	+	29	16.40	2-heptanol	+	+
10	8.20	2-propanol	+	+	30	16.81	3-hydroxy-2-pentanone[a]		+
11	8.22	ethanol	+	+	31	16.91	3-penten-2-ol[a]		+
12	9.81	2-pentanone	+	+	32	17.04	2-nonanone	+	+
13	10.23	diacetyl	+	+	33	17.15	dimethyl trisulfide	+	+
14	11.28	isobutyl acetate		+	34	17.36	ethyl octanoate	+	+
15	11.78	2-butanol	+	+	35	17.59	acetic acid	+	+
16	11.94	ethyl butanoate	+	+	36	17.76	2-ethyl-1-hexanol	+	+
17	12.08	1-propanol	+	+	37	18.20	2,3-butanediol (I)	+	+
						18.38	2,3-butanediol (II)		
18	12.65	2,3-pentanedione[a]		+	38	18.78	butanoic acid	+	+
19	12.80	dimethyl disulfide	+	+	39	19.03	3-methylbutanoic acid	+	+
20	13.46	2-methyl-1-propanol	+	+	40	20.30	caproic acid	+	+

[a] Tentatively identified.
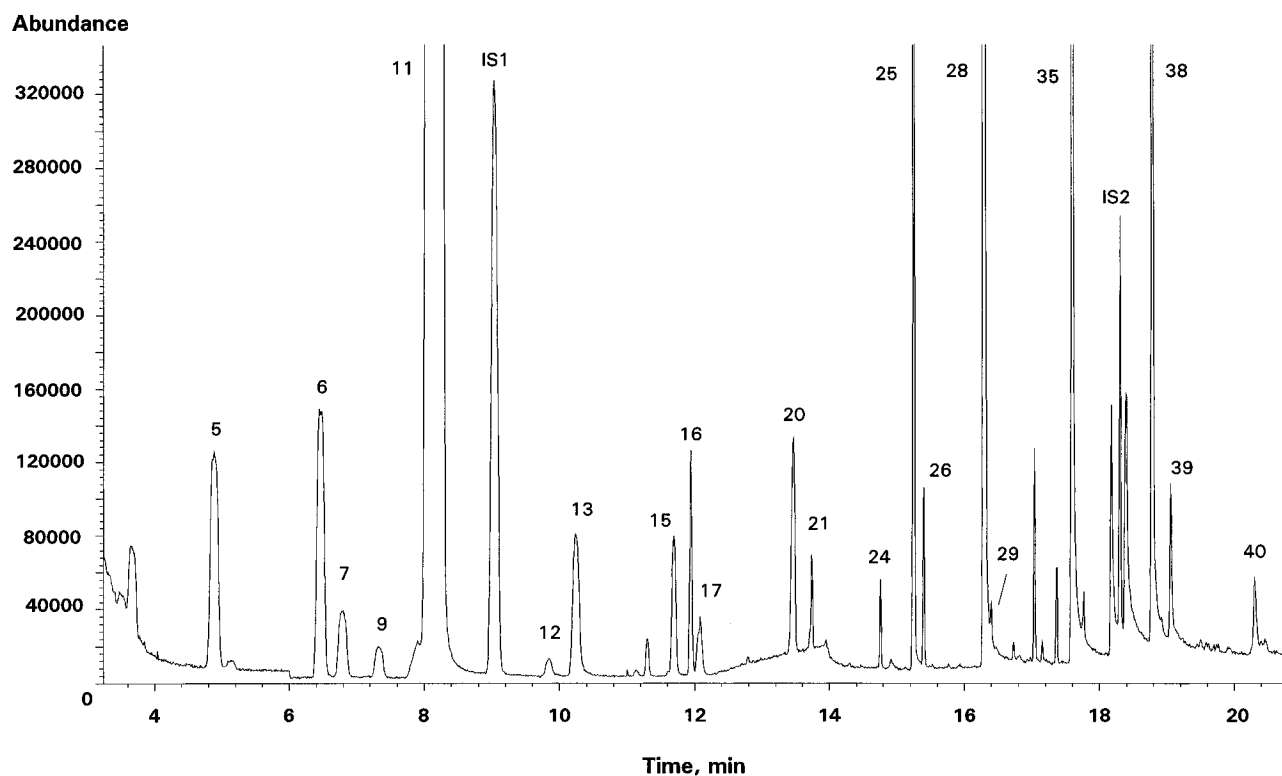
Figure 1. Total ion chromatogram corresponding to the headspace fraction of a 7-day-old cheese spiked with the following standard concentrations (expressed as micrograms of compound per gram of cheese): 2.16 μg/g of acetone (5), 1.14 μg/g of ethyl acetate (6), 1.02 μg/g of 2-butanone (7), 0.524 μg/g of 3-methylbutanal (9), 0.218 μg/g of 2-pentanone (12), 1.31 μg/g of diacetyl (13), 1.02 μg/g of 2-butanol (15), 0.567 μg/g of ethyl butanoate (16), 1.03 μg/g of 1-propanol (17), 1.01 μg/g of 2-methylpropanol (20), 0.235 μg/g of isoamyl acetate (21), 0.217 μg/g of 2-heptanone (24), 1.02 μg/g of 2-methylbutanol (25), 0.236 μg/g of ethyl caproate (26), 5.74 μg/g of acetoin (28), and 0.204 μg/g of 2-heptanol. IS1 represents internal standard ethyl propanoate. IS2 represents internal standard camphor.

but this compound could not be quantified because of its low volatility, which produced inaccurate concentration:response ratios. Other compounds were not selected for quantification, though they may be very important, because their presence was not consistent in all the analyzed samples of the same age, and when observed, peaks were sometimes very small and difficult to measure with accuracy.

The repeatability of the compounds' retention time (RT) was very good, with individual coefficients of variation lower than 0.1% for within-day injections (eight samples) and ranging from 0.1 to 0.7% for injections on different days (the higher the RT, the lower the coefficient). A matrix effect on the compounds' retention times was not observed.

Quantification of Volatile Compounds. As expected, different absolute responses were observed among the different matrices studied. The same concentration of a volatile compound gave a higher response in a 3-day-old than in a 30-day-old cheese, the internal

Table 2. Amount of the Standard Volatile Compounds Spiked to a 30-Day-Old Cheese for Quantitative Analysis, Ions Used for Peak Quantitation, Detection Limits, Slope Values, and Correlation Coefficients of the Linear Regresion Curve Obtained (Relative Response = b × Relative Concentration)

peak no.		calibration level				curve slope (b)	correl coeff (r)	detection limit (μg/g)	quantitation ions[a]
		1	2	3	4				
5	2-propanone	1.62[c]	6.46	12.92	25.84	3.62	0.995	<0.1	TIC
6	ethyl acetate	0.85	3.41	6.82	13.65	2.94	0.998	<0.1	TIC
7	2-butanone	0.77	3.07	6.14	12.28	3.43	0.999	<0.1	TIC
9	3-methylbutanal	0.39	1.57	3.14	6.29	2.64	0.997	<0.05	TIC
11	ethanol[b]	3.91	15.63	31.27	62.53	0.25	0.980	~2	45
12	2-pentanone	0.16	0.65	1.31	2.62	3.0	0.999	0.03	TIC
13	diacetyl	0.98	3.92	7.84	15.68	1.56	0.999	<0.2	43
15	2-butanol	0.76	3.06	6.11	12.22	5.45	0.996	0.05	45
16	ethyl butanoate	0.43	1.70	3.4	6.8	1.2	0.998	<0.08	71
17	1-propanol	0.77	3.10	6.19	12.38	0.26	0.993	0.1	59
20	2-methyl-1-propanol	0.76	3.02	6.05	12.10	7.27	0.999	0.1	TIC
21	isoamyl acetate	0.18	0.71	1.41	–	3.53	0.997	0.04	TIC
24	2-heptanone	0.16	0.65	1.30	2.6	5.28	0.990	0.04	TIC
25	3-methyl-1-butanol	0.77	3.07	6.14	12.29	1.31	0.999	<0.3	55
26	ethyl caproate	0.18	0.71	1.42	2.83	2.23	0.998	0.04	TIC
28	acetoin	4.3	17.2	34.43	68.86	0.79	0.993	<0.9	43
29	2-heptanol	0.15	0.61	1.22	2.45	1.77	0.997	0.03	45

[a] TIC represents total ion chromatogram. [b] Calibration for ethanol was performed only in the 3-day-old cheese. [c] Amounts in micrograms per gram of cheese.

Table 3. Mean Concentration ($n = 3$) ± Standard Deviation of the Quantified Volatile Organic Compounds in the Afuega'l Pitu Cheese Headspace at Different Stages of Ripening

peak no.	volatile compound	cheese-ripening			
		3 days	7 days	15 days	30 days
5	2-propanone	4.35 ± 0.72[a]	3.08 ± 1.35	2.29 ± 0.84	0.97 ± 0.19
6	ethyl acetate	0.15 ± 0.07	1.02 ± 0.59	2.93 ± 0.98	1.33 ± 0.47
7	2-butanone	0.15 ± 0.06	0.14 ± 0.09	0.1 ± 0.052	0.17 ± 0.05
9	3-methylbutanal	NF[b]	0.48 ± 0.23	0.93 ± 0.45	0.62 ± 0.12
11	ethanol	57 ± 35	1892 ± 623	1968 ± 195	844 ± 332
13	diacetyl	2.60 ± 1.24	4.53 ± 1.23	1.66 ± 0.16	0.51 ± 0.18
16	ethyl butanoate	NQ[c]	NQ	0.2 ± 0.01	0.83 ± 0.43
17	1-propanol	NQ	2.15 ± 1.92	0.83 ± 0.59	0.71 ± 0.01
20	2-methyl-1-propanol	NQ	4.47 ± 1.65	3.42 ± 0.35	2.21 ± 0.35
21	isoamyl acetate	NF	NF	0.35 ± 0.19	0.54 ± 0.28
24	2-heptanone	NF	0.024 ± 0.01	0.077 ± 0.015	0.27 ± 0.10
25	3-methyl-1-butanol	0.1	7.09 ± 2.76	12.99 ± 2.39	9.21 ± 1.35
26	ethyl caproate	NF	NF	0.062 ± 0.051	1.11 ± 0.33
28	acetoin	248.1 ± 2.06	146.9 ± 44.18	65.15 ± 28.94	27.16 ± 18.14
29	2-heptanol	NF	NF	0.18 ± 0.00	0.54 ± 0.20

[a] Amounts in micrograms per gram of cheese. [b] NF represents not found. [c] NQ represents not quantified, below the detection limit.

standards also being affected. The use of internal standards corrected the matrix effect to some extent, but, in spite of it, calibrations were performed for the four cheese ages.

Table 2 shows the concentration of standards spiked to a 30-day-old cheese sample, the slope (b) of the calculated regression curve (relative response = b × relative concentration) with the internal standard correction, correlation coefficients (r), the base ions used for peak quantification, and approximate detection limits. Peaks of internal standards ethyl propionate and camphor were quantified on the basis of ion 57 and TIC, respectively. Good linearity ($r > 0.99$) was exhibited by most of the volatile compounds within the concentrations used. In spite of the frequently reported lack of sensitivity of HS extraction, detection limits were rather low in our conditions, less than 0.05 μg/g for some compounds. Ethanol could only be quantified in the 3-day-old cheese because its concentration was very high at later stages of ripening and caused column saturation. The curve calculated in the 3-day-old cheese for ethanol was used for its quantification in the older cheeses. Quantitative results shown for ethanol have thus relative value, due to matrix effect and column saturation, but were useful to determine its evolution in the cheese. The high level of ethanol found in all the samples has been a drawback for the chromatographic method to detect other possible aroma components; because ethanol represents more than 90% of the headspace profile, it composes most of the vapor that enters the column in the GC, displacing other less abundant compounds. The area response repeatability was acceptable with coefficients of variation between duplicates lower than 10% for the less abundant components and lower than 5% for the more abundant compounds.

Volatile Components in Afuega'l Pitu Cheese. Figure 2A–C shows the headspace profile of Afuega'l Pitu cheese at 3, 15, and 30 d of ripening, respectively. Table 3 summarizes the means and standard deviations for 15 quantified components in cheese. 2-Pentanone and 2-butanol were only found in 2 of the 13 samples analyzed, with a concentration of 0.38 and 0.26 μg/g, respectively. All the volatile compounds found have been previously reported for other cheeses, although in different concentrations. Afuega'l Pitu cheese presents a singular and characteristic aroma. Due to its high moisture and low salt content at the early stages of maturation, the growth rate of microorganisms is enhanced in this cheese variety, and because of the small size of the cheese pieces, evaporation takes place at a fast rate. These are the reasons why deep changes
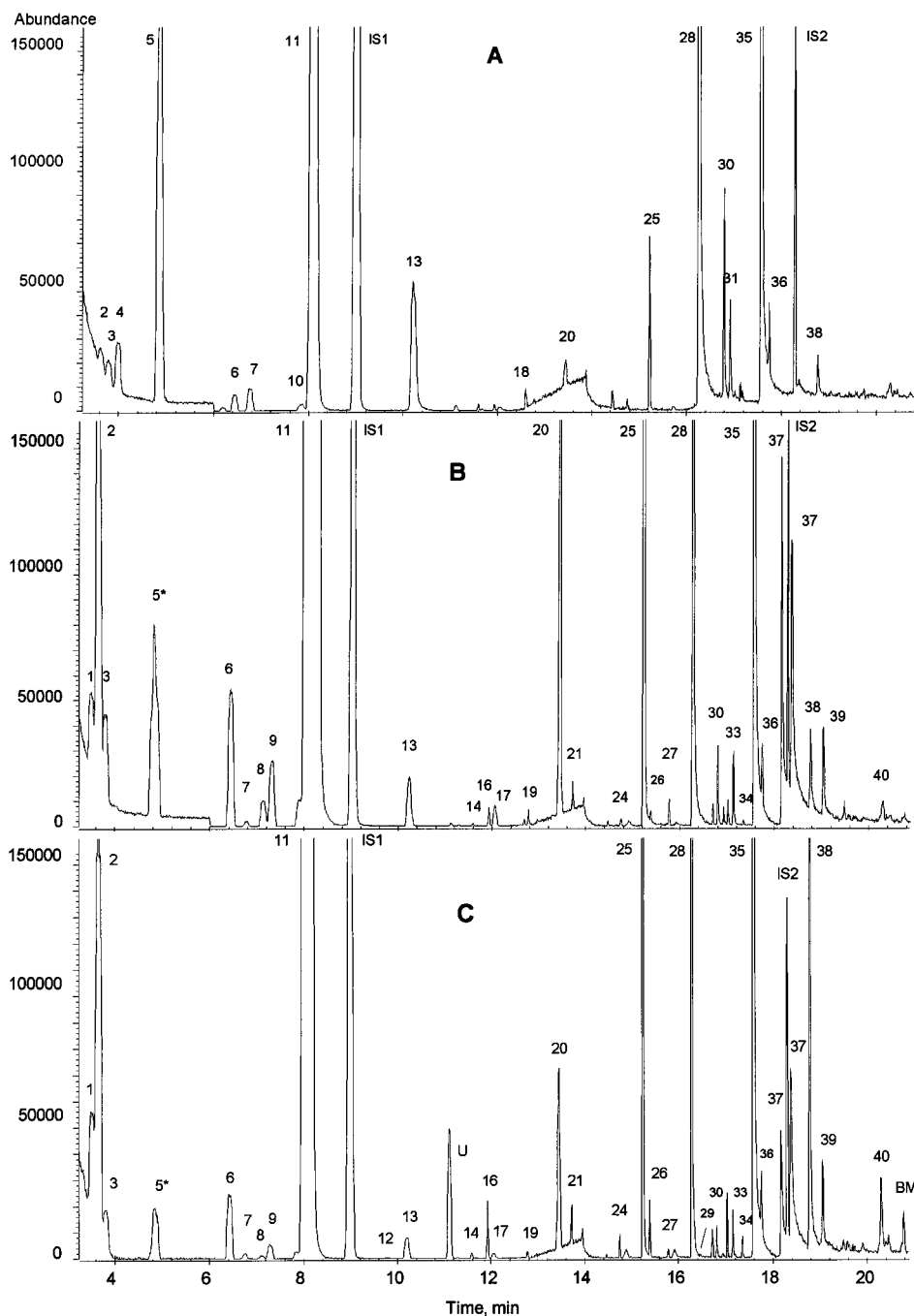
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Figure 2. Total ion chromatograms of Afuega'l Pitu cheese headspace: (A) 3-day-old cheese, (B) 15-day-old cheese, and (C) 30-day-old cheese. For peak identification, see Table 1. 5* is acetone, 2-methylpropanal, and butane. IS1 represents internal standard ethyl propanoate. IS2 represents internal standard camphor. BM represents benzyl alcohol.

in flavor and texture occur in a relatively short ripening period. Fresh Afuega'l Pitu cheese aroma seems more related to fermented milks, resting mostly on the presence of diacetyl, acetoin, ethanol, and other alcohols. The older cheese aroma becomes richer though still mild, more cheeselike, with the production of free fatty acids, branched alcohols, and esters from reaction of free fatty acids with ethanol and other alcohols. In a comparative study carried out on the volatile fraction collected by SDE of several artisanal Spanish cheeses, de Frutos et al. (1991) did not observe a very rich aroma profile for Afuega'l Pitu cheese, with reported concentrations below 0.1 μg/g for most of the extracted volatile compounds, only low-carbon number ketones, ethyl esters, and fatty acids being found in higher concentrations. The method used in the present study permitted the extraction of volatile compounds that might have

been lost with the SDE procedure or that coeluted with the solvent, like 2-propanone, ethyl acetate, 3-methylbutanal, diacetyl, ethanol, and some other alcohols with higher molecular weights (1-propanol, 2-methyl-1-propanol, and 3-methyl-1-butanol) not detected in that previous work.

Diacetyl and acetoin may originate from the reduction of pyruvate by citrate utilizing lactic acid bacteria, which have actually been identified in this cheese variety (Cuesta et al., 1995). The high levels of diacetyl and acetoin found in 3-day-old and 7-day-old cheeses decrease during ripening, giving rise to the low-volatility compound 2,3-butanediol. Odor threshold perception values, calculated in Emmentaler cheese by Preininger and Grosch (1994) for diacetyl (0.001 μg/g), 3-methylbutanal (0.01 μg/g), and ethyl butanoate (0.004 μg/g),

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were well below the concentrations found in Afuega'l Pitu cheese.

Ethanol was the most abundant compound in the headspace and was probably produced through fermentation by *Leuconostoc* and yeasts, microorganisms that grow on the cheese surface creating a kind of smear (Cuesta et al., 1996). Quite important concentrations of branched chain alcohols were found from the second week of ripening. 3-Methylbutanol and 2-methylpropanol may come from the catabolism of leucine and valine by *Penicillium camemberti* (Jollivet et al., 1993), which in fact grows on these cheese surfaces. Virgili et al. (1994) related sweet and fragrant notes of Parmegiano-Reggiano cheese with 2-methylpropanol and 3-methylbutanol, which are among the most abundant volatile compounds of Afuega'l Pitu cheese headspace. Bosset and Gauch (1993) reported high levels of ethanol, 3-methylbutanol, and 2-methylpropanol in Spanish Mahón cheese; the other European cheeses studied contained lower amounts of these branched alcohols, although no quantitative data were reported.

Regarding the carbonyl compounds, five ketones and three aldehydes were found. 2-Heptanone amounts increased steadily during ripening, whereas 2-pentanone and 2-nonanone were only observed in some of the samples. Higher amounts of 2-pentanone and 2-heptanone were found in ewes Manchego cheese using SDE (Martínez-Castro et al., 1991). The amounts of 2-methylbutanal and 3-methylbutanal increased at the beginning of ripening, and then decreased, while acetaldehyde appeared already in some of the 3-day-old samples and the amount increased continuously during ripening.

Low concentrations of methanethiol have been found, although not quantified, in our cheese samples together with other sulfur compounds, carbon disulfide, dimethyl sulfide, and dimethyl and trimethyl disulfides. Methanethiol, dimethyl disulfide, and dimethyl trisulfide appeared in the cheeses during the second week, and their amounts increased throughout ripening. Dimethyl sulfide and carbon disulfide occurred already in 3-day-old cheeses, probably coming from the milk; the first one disappears, while the amount of the latter increases during ripening. Due to the low detection threshold of the human smell sense to these substances (Shankaranarayana et al., 1974; Urbach, 1993), it would be rather reasonable to attribute to the sulfur compounds some role in the Afuega'l Pitu cheese flavor at the later stages of ripening.

Among the volatile compounds found but not quantified in Afuega'l Pitu cheese, acetic acid was present in all samples, presumably in high concentrations and its amount increasing with ripening time. Butanoic, 3-methylbutanoic, and caproic acid concentrations also increased during ripening. 3-Methylbutanoic acid has sweatlike and slightly sweet flavor notes in the range of 25–50 ppm (Brennand et al., 1989). Unfortunately, acids could not be quantified due to carryover problems. The presence of styrene could be explained as an environmental contamination, although as it appears in mature samples (15- and 30-day-old cheeses), it can also be related to *P. camemberti* starvation metabolism (Spinnler et al., 1992).

At present, we can only speculate that Afuega'l Pitu cheese owes its characteristic aroma to a balanced number of volatile compounds, mainly fatty acids, esters, sulfur and carbonyl compounds, and alcohols on top of it, originating from the metabolism of different microorganisms among which yeast and *P. camemberti*, producing alcohols, and lactic acid bacteria, producing diacetyl, could play a key role.

CONCLUSIONS

The present method is a relatively simple, reliable quantitative approach to the analysis of volatile compounds in cheese and other dairy products. The use of internal standards and the standard addition calibration compensates for the matrix effect, although this procedure is a little time-consuming. In spite of static headspace lack of sensitivity, and of the mild aroma of Afuega'l Pitu cheese, 40 volatile compounds were found.

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