Volatile Free Fatty Acids as Ripening Indicators for Serra da Estrela Cheese

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ABSTRACT

Serra da Estrela cheese is an artisanal cheese manufactured from raw ewe's milk coagulated with *Cynara cardunculus*; it is highly appreciated for its unique flavor and bouquet. This research effort focused on a search for the molecules responsible for those organoleptic characteristics. Eighty cheeses manufactured in 4 dairies located in the *Appellation d'Origine Protégée* region of Serra da Estrela, Portugal, were thus assayed for volatiles, in an attempt to characterize their odorous profile. Cheeses were analyzed from the time of manufacture up to 180 d of ripening. The volatile fraction was assayed by solid phase microextraction-gas chromatography/mass spectroscopy, and several compounds belonging to different chemical groups (e.g., fatty acids, esters, carbonyl compounds, pyrazines, and sulfur compounds) were detected in that traditional cheese. Among these, free fatty acids (FFA) were quantitatively the dominant family present. Furthermore, sensory descriptors for the typical aroma of this cheese included acidic, sweaty, and sheepy-like. Acetic, isobutyric, and isovaleric acids increased in concentration during the ripening process up to 90 d, and remained constant thereafter. Preliminary sensory analysis was performed by addition of the major FFA to an unripened cheese matrix; results showed that they could successfully be used as ripening indicators for this cheese. Such key molecules may thus be used to monitor ripening, and hence find the optimal consumption time for this gourmet dairy product.

(Key words: solid phase microextraction, dairy foods, gas chromatography-mass spectroscopy, sensory analysis)

Abbreviation key: GCMS = gas chromatography/mass spectroscopy, OAV = odor activity value, SPME = solid phase microextraction.

INTRODUCTION

Cheese is a complex matrix, the chemical features of which depend on the combined influence of several factors (e.g., pH, temperature, water activity, and microflora) throughout ripening. Furthermore, its sensory characteristics result from a dynamic interaction of chemical and microbiological parameters. It is well documented (Buchin et al., 1998) that cheeses manufactured from raw milk acquire a more intense flavor than those produced from pasteurized or heat-treated milks; such realization is mainly due to the high levels of native lactic acid bacteria present in raw milk (Grapin and Beuvier, 1997). As a consequence, variability is a major drawback in cheeses manufactured from raw milk, which is accounted for (among other causes) by seasonal variations in their indigenous microflora. This is the typical case of Serra da Estrela cheese, an artisanal raw (ewe's) milk cheese, coagulated with plant rennet without deliberate addition of any starter culture, which is produced in Portugal at approximately 1200 ton/yr, where it is by far the most famous traditional cheese.

Processes that minimize the aforementioned variability are urged, so as to not only improve microbiological safety but also to guarantee a final product bearing typical aroma attributes, on both artisanal and industrial levels. The addition of native cultures (Centeno et al., 1996, 1999; Menéndez et al., 1999, 2000) may be a way to reduce the heterogeneous quality of cheese. However, a priori knowledge on the molecules responsible for the unique organoleptic attributes of each particular cheese is in order; very few studies have meanwhile been published concerning the volatile compounds in Serra da Estrela cheese (Partidário et al., 1998), or other Portuguese ewe's cheeses (Pinho et al., 2001).

From a sensory perspective, odor descriptors associated with typical aroma of Serra da Estrela cheese are "acidic," "sweaty," and "sheepy-like" notes, as described in the Portuguese legal standard (NP-1922, 1985). These descriptors suggest that FFA play a major role in the aroma character of this cheese; hence, its FFA profile could be used to help establish the optimum ripening time for this cheese, defined as that providing

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the aroma attributes qualitatively and quantitatively mostly appreciated by consumers. A wide array of volatiles, for example, alcohols, esters, ketones, aldehydes, and FFA, are among the families of compounds reported to be present in cheeses manufactured from ewe's milk (Ortigosa et al., 2001; di Cagno et al., 2003; Muñoz et al., 2003). Among those, short-chain FFA are usually present to relatively high levels (Dirinck and de Winne, 1999; Akin et al., 2003), and are thus considered important owing to their aroma impact in the final product.

The primary aim of this research effort was to improve the extraction method of volatiles from the cheese matrix, and then characterize the volatile fraction of Serra da Estrela cheese throughout ripening. The second aim was to conduct preliminary sensory studies to determine which, among the most abundant molecules, exhibit the highest aroma impact.

MATERIALS AND METHODS

Cheese Manufacture

Serra da Estrela cheese was manufactured in 4 dairy farms scattered throughout the *Appellation d'Origine Protégée* (AOP) region from raw ewe's milk coagulated with *Cynara cardunculus* extracts, according to the traditional protocol described by Tavaría and Malcata (1998). Each dairy produced 20 cheeses from the same batch of milk and, at each ripening time (0, 3, 7, 18, 35, 60, 90, 120, 150, and 180 d), 2 cheeses at random were brought under refrigerated conditions (4 to 5°C) to our laboratory, where they were kept at -30°C until further chemical analyses.

Chemicals

Propionic, butyric, isovaleric, isobutyric, caproic, caprylic, capric, and lauric acids, as well as ethyl butanoate, hexanoate, octanoate, decanoate, and dodecanoate (purity: 99%) were purchased from Sigma Chemical Co. (St. Louis, MO); acetic acid (purity: 96%) was from Merck (Darmstadt, Germany).

Sample Preparation and Volatile Analysis

At each selected ripening time, 20 g of cheese from each dairy was macerated using a mortar and pestle and thoroughly mixed. From this mixture, a 10-g aliquot was then taken for volatile analysis. The cheese was homogenized using a sterile plastic bag in a stomacher laboratory blender 400 (from Seward Medical, London, UK) for 2 min. This sample was then put in a 100-mL flask capped with a gas-tight seal, at a constant temperature of 45°C. A volume of 100 μ L of an internal

standard, octan-3-ol, in a 1:1 (vol/vol) hydro-alcoholic solution at 461.97 mg/L was added, and the sample was gently stirred (100 rpm) using a stirring bar during volatile collection. After equilibration, the solid phase microextraction (SPME) fiber assembly of choice, containing a divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber coating (Supelco, Bellefonte, PA) was introduced, and left for 1 h to trap volatiles. After this period, the fiber was introduced in the injector port and left to desorb trapped volatiles for 10 min.

Method Development

As a solid matrix, cheese poses sampling problems owing to heterogeneity and geometrical constraints, which affect reproducibility of the quantitative results. To increase homogeneity, and hence maximize extraction, 2 temperatures were tested; the higher temperature (at which no artifact formation was observed) was then safely chosen. Furthermore, 3 types of fibers were tested, and the best exposure time was sought, as described in detail in the following sections.

Artifact Formation

It is well documented (Pérès et al., 2002) that high temperatures may produce artifacts because new compounds may form via chemical reactions induced by heating. To ascertain whether such degree of heating would produce artifacts, 3 samples were compared: (1) a sample of cheese was analyzed as described above, by equilibrating the sample at 25°C instead of 45°C; (2) the sample was then heated up to 45°C, allowed to equilibrate at that temperature and sampled after 1 h of exposure to the fiber; and (3) the sample was allowed to cool to 25°C and sampled again. These measurements were repeated 3 times.

Choice of Fiber

The fibers tested for volatile extraction were: polyacrylate (PA) 85 μ m, carboxen/polydimethylsiloxane (CAR/PDMS) 75 μ m, and divinylbenzene/carboxen/PDMS (DVB/CAR/PDMS) 50 to 30 μ m (from Supelco, Bellefonte, PA). Comparative performance of these fibers was assessed using ripened Serra da Estrela cheese. The volatile components were extracted via SPME by exposing the fibers for 60 min in the cheese headspace, inside a flask maintained at a constant temperature of 45°C.

Exposure Time

The adsorption kinetics was determined for 6 exposure times (15, 30, 45, 60, 90, and 120 min) of the fiber in

the headspace. Each measurement was repeated twice. The optimum exposure time was determined as the time at which the peaks were highest in area accounted for by the compounds of interest.

Reproducibility and Linearity

Volatiles from ripened Serra da Estrela cheese were extracted using the optimal conditions found for the several parameters tested (fiber, exposure time, and extraction temperature), and analyzed by gas chromatography/mass spectroscopy (GCMS). The coefficient of variation [$CV\% = (SD/mean) \times 100$] was determined for all compounds quantified. This was repeated 6 times. The effects of the various parameters upon extraction efficiency were determined using intensity and number of mass fragments obtained, as well as coefficient of variation of the measurements. Standard curves were prepared for each such compound by injecting increasing volumes (20, 40, 60, 80, 160, 320, and 1000 μL) of a mixture containing known amounts of those compounds, using unripened cheese as matrix. The values of K (partition coefficient) and r (correlation coefficient) were determined from these curves.

GCMS Conditions

Volatiles were thermally desorbed in the injector port (220°C) in splitless mode, and analyzed using a Thermo Quest Trace GC 2000 series gas chromatograph, equipped with a GCQ mass detector (Finnigan Mat, San Jose, CA). The split valve was opened 30 s after injection. Volatile compounds were separated in a 50-m FFAP (CP-Wax 58) column (0.25 mm $\times$ 0.39 mm $\times$ 0.20 μm) (Varian-Chrompack, Walnut Creek, CA). The oven temperature was held at 40°C for 1 min, and then increased at 2°C/min up to a final temperature of 220°C, which was held for 30 min. All mass spectra were acquired in electron impact mode. The carrier gas was helium C-60 (Gasin, Portugal), at a constant volumetric flow rate of 1 mL/min. The transfer line and source temperatures were 190 and 180°C, respectively. The emission current was 70 mV, and the electron multiplier was set according to the autotune procedure. The mass range was 29 to 350 m/z , with a scan rate of 7 scan/s. All analyses were carried out in full scan mode. Identification was achieved via comparison of the mass spectra obtained for a sample containing a mixture of pure standards injected under the same conditions to the mass spectra of the NIST 98 MS library database (or others available in the literature). The DM content of the cheese samples was determined according to the standard procedure of FIL-IDF (1982), and the results were expressed on a DM basis.

Sensory Analysis

Odor activity values (OAV) were calculated as concentration of compound/odor threshold for all short-chain FFA. The 2 FFA with the highest OAV (i.e., butyric and isovaleric acids), as well as a third compound believed to have no impact on the sensory perception of this cheese (i.e., ethyl hexanoate, an ethyl ester) were used in this analysis.

The aforementioned compounds were added independently, or in combination to 10 g of unripened cheese, at the amounts found in the reference sample (60-d-old cheese): 3.18 mg/kg ethyl hexanoate, and 442.58 and 601.03 mg/kg butyric and isovaleric acids, respectively. Samples were homogenized by stirring, and the individual flasks were covered with aluminum foil. Sets of 8 samples were presented to a panel of 12 people for odor assessment only. The control sample consisted of unripened curd, that is, curdled ewe's milk without any compound added. Three samples were added with each compound independently, 3 samples were added with pair combinations of the 3 compounds, and another sample was added with the combination of all 3 compounds. After the corresponding additions, samples were homogenized by stirring, and the individual flasks were covered with aluminum foil. The panel rated the odor of the samples by comparison to the reference sample of ripened cheese, using a 0 to 10 scale (where 0 means no similarity whatsoever, and 10 means equal to the matured sample). Experiments were carried out in individual booths at room temperature (~ 18 to 20°C). The experiment was repeated 4 times.

Statistical Analyses

Statistical analysis of the volatile data was performed using the SPSS statistical package (version 11.5 for Windows, from SPSS, Chicago, IL) via ANOVA, at a degree of significance of $\alpha = 0.05$. ANOVA was carried out so as to infer on the significance of the effect of ripening time on volatile formation; posthoc multiple comparisons were done using Tukey's test ($\alpha = 0.05$), after having performed tests that confirmed a normal distribution of the data. For the sensory data, the distribution was not normal even after simple data transformation, so these were analyzed using the nonparametric approach of Wilcoxon Signed Ranks test for pairwise comparisons and determination of homogeneous subsets. The appropriate correction for the Bonferroni's multiple comparison was carried out with $\alpha_{\beta} = 0.005$.

RESULTS AND DISCUSSION

Reproducibility and Linearity

The coefficient of variation (determined over 6 analytical cheese replicates) for each compound quantified

Table 1. Normalized peak area (of 6 replicates): average, standard deviation, and coefficient of variation, pertaining to major short-chain FFA and ethyl esters in Serra da Estrela cheese by 180 d of ripening, determined by the solid phase microextraction (SPME) method.

Compound	Average	SD	CV
Acetic acid (C ₂)	6.32	± 1.34	21.19
Butyric acid (C ₄)	17.25	± 2.47	14.33
Isovaleric acid (iC ₅)	5.85	± 0.64	10.95
Caproic acid (C ₆)	15.55	± 1.51	9.71
Caprylic acid (C ₈)	8.79	± 0.77	8.76
Ethyl hexanoate (C ₆ C ₂)	0.59	± 0.05	8.28
Ethyl octanoate (C ₈ C ₂)	0.98	± 0.09	9.47
Ethyl decanoate (C ₁₀ C ₂)	1.16	± 0.15	12.84
Ethyl dodecanoate (C ₁₂ C ₂)	0.37	± 0.04	10.77

ranged from 8 to 21%, as shown in Table 1. The method was linear for the compounds tested, with *r*-values ranging from 0.990 to 1.000.

Artifact Formation

Overlapping of the 3 chromatograms (Figure 1) indicates that, despite the 2-fold rise in some peaks (e.g., C₄ and C₆) caused by the temperature increase (Figure

1b), no new peaks are formed. Hence, it could be inferred that the temperature used was a good way to increase the extractability of the dominant compounds, without concomitant generation of unwanted artifacts.

Choice of Fiber

Comparison of the performance of the 3 fibers tested proved that the chromatographic information obtained was both qualitatively (number of compounds) and quantitatively (chromatogram peak area) higher after extraction using the DVB/CAR/PDMS fiber than using the other 2 alternative fibers. The polar characteristics of most volatile compounds likely contribute to make the DVB/CAR/PDMS fiber the best type of trapping support. Note that carboxen/PDMS has been shown (Lawlor et al., 2002) to be efficient in trapping short-chain FFA, whereas PDMS/DVB has a greater affinity for higher molecular weight FFA. Consequently, choice of the fiber containing the 3 types of support led to the best results.

Figure 1. Representative chromatograms of a given sample, assayed by gas chromatography-mass spectroscopy (GCMS) according to different temperature patterns for a given exposure time (60 min): A. Exposure to 25°C; B. Exposure to 45°C; and C. Exposure to 45°C (60 min), cooling down to 25°C, and exposure to 25°C (60 min).

Figure 2. Representative chromatograms of samples, assayed by gas chromatography-mass spectroscopy (GC-MS), for various exposure times of the divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber. Exposure for: A. 15 min; B. 30 min; C. 45 min; D. 60 min; E. 90 min; and F. 120 min.

Exposure Time

Inspection of Figure 2 points at an increase in peak intensity with increasing exposure time of the fiber assembly, as expected. After 60 min (Figure 2d), however, such an increase was no longer significant, so 60 min was chosen as the optimum exposure time for volatile extraction.

Volatile Analysis

Serra da Estrela cheese possesses a few unique features that render it quite different from other cheeses produced industrially using pasteurized or heat-treated cow's milk. First, it is manufactured from raw ewe's milk, which has almost twice as much fat as cow's milk (7.1 vs. 3.8%) (Biss, 1991). Similarly, the total fatty acid amount in sheep's milk is ~4.3 g/100 g, whereas in cow's milk it is 2.0 g/100 g. The microbial load is quite high by the time of manufacture, especially in terms of *Enterobacteriaceae*, which are known to produce lipases that breakdown milk fat, when compared with other cheeses manufactured from heat-treated milk. Second, the coagulant used in cheese making is a crude protease

mixture of plant origin, which acts only upon the proteinaceous fraction of milk (unlike commercial rennets, which usually bear a strong lipolytic capacity). Third, ripening takes place at a relatively low temperature (8 to 10°C), which does not favor lipolysis.

The volatile profile of Serra da Estrela cheese during ripening, as determined by SPME, is depicted in Table 2. Relative amounts are expressed as normalized peak areas (peak area/peak area of internal standard), on a DM basis. ANOVA of the profile of volatiles indicated that ripening time was a statistically significant ($P < 0.05$) parameter toward formation of most compounds—major families comprised ketones, pyrazines, alcohols, aldehydes, phenolic compounds, ethyl esters, and FFA. Among these, the significance and impact of FFA, owing to their relative amounts, upon the overall aroma is beyond doubt, similar to what happens in other artisanal cheeses (Gómez-Ruiz et al., 2002; Lawlor et al., 2002).

Free fatty acids were quantitatively and qualitatively the dominant volatile fraction in ripened Serra da Estrela cheese (Tables 2 and 3), as happens in Manchego cheese, especially when artisanal manufacturing prac-

Table 2. Compounds tentatively identified by solid phase microextraction-gas chromatography/mass spectroscopy analysis of the volatile fraction of Serra da Estrela cheese throughout ripening; normalized peak areas [(peak area/internal standard peak area)/DM] are averages of 2 replicates.

Compound	RT ¹ (min)	Ion	Ripening time (d)		
			0	60	180
Ethyl esters					
Ethyl butanoate	9.27	88	0.00	0.13	0.18
Ethyl pentanoate	9.30	85	0.00	0.07	0.11
Ethyl hexanoate	16.92	88	2.22	0.80	1.16
Ethyl octanoate	28.78	88	3.16	1.15	1.81
Ethyl decanoate	40.72	88	3.85	2.02	3.28
Ethyl dodecanoate	51.81	88	0.45	0.44	1.20
Ketones					
2-Heptanone	14.17	85	0.01	0.08	0.58
2-Nonanone	25.82	85	0.01	0.16	1.37
2-Undecanone	38.22	85	0.01	0.08	0.78
Phenolic compounds					
2,4-Dimethylbenzene	11.78	105	0.36	0.17	0.36
1,2,4-Trimethylbenzene	15.86	105	0.94	0.56	1.66
Benzoic acid	78.64	105	1.30	5.05	4.65
Alcohols					
1-Decanethiol	3.23	57	0.13	0.36	0.88
2-Phenylethanol	55.13	91	1.39	20.85	11.40
Phenol	59.92	94	0.18	0.43	0.73
4-Methyl phenol	63.56	108	0.14	0.47	0.59
Benzyl alcohol	63.89	108	0.08	0.31	0.60
4-Methyl-4-nonanol	80.85	57	0.49	0.35	1.99
Pyrazines					
2,5-Dimethylpyrazine	22.20	108	0.01	0.10	5.44
Trimethylpyrazine	26.83	122	0.01	0.04	3.30
Diethylpyrazine	30.90	136	0.00	0.00	2.27
Aldehydes					
2-Hydroxy-4-methylbenzaldehyde	29.19	136	0.00	0.00	1.20
2,4-Decadienal	30.74	81	0.20	0.22	0.21
2,4-Octadienal	32.35	81	0.67	0.78	0.48
2,4-Nonadienal	49.85	81	0.20	0.38	1.06
3,7-Dimethyl 7-octenal	50.22	81	0.27	0.13	0.26
2,4-Dodecadienal	55.40	81	0.06	0.03	0.14
Octadecanal	60.48	81	0.10	0.08	0.15
Sulfur derivatives					
Dimethyldisulfide	66.26	94	0.07	0.05	0.11

¹RT = Retention time.

tices were followed (Gómez-Ruiz et al., 2002). Replacement of whole milk with skim milk has proven that milk fat is an essential prerequisite for flavor develop-

ment (Fox et al., 1995). Free fatty acids may derive from lipid catabolism, be biosynthesized from acetyl-CoA, or appear as the result of metabolism of lactose

Table 3. Concentration of FFA (mg/kg of cheese DM) in the volatile fraction of Serra da Estrela cheese throughout ripening; concentrations are averages of 2 replicates.

Compound	Ripening time (d)									
	0	3	7	18	35	60	90	120	150	180
C ₂	3.27 ^a	30.69 ^{ab}	62.28 ^{ab}	114.98 ^{ab}	71.51 ^{ab}	64.58 ^{ab}	112.54 ^{ab}	68.38 ^{ab}	51.38 ^{ab}	105.52 ^b
C ₃	7.32 ^a	8.93 ^a	6.97 ^a	25.63 ^a	15.97 ^a	289.88 ^c	91.60 ^{ab}	46.86 ^{ab}	177.87 ^{bc}	174.34 ^{bc}
iC ₄	2.09 ^a	0.90 ^a	10.06 ^a	42.92 ^{ab}	74.68 ^{abc}	153.75 ^{bcd}	264.48 ^e	186.04 ^{de}	158.10 ^{cde}	189.22 ^{de}
C ₄	47.97 ^a	29.64 ^a	186.35 ^a	77.32 ^a	79.60 ^a	442.58 ^a	619.02 ^a	414.60 ^a	496.11 ^a	1766.45 ^b
iC ₅	4.67 ^a	3.08 ^a	25.55 ^a	101.58 ^a	269.96 ^a	601.03 ^b	744.07 ^b	572.98 ^b	562.19 ^b	606.29 ^b
C ₆	31.77 ^a	38.27 ^a	117.42 ^a	81.81 ^a	77.18 ^a	276.68 ^a	384.35 ^a	235.41 ^a	330.73 ^a	922.99 ^b
C ₈	49.10 ^a	60.48 ^a	173.89 ^a	82.74 ^a	94.82 ^a	166.14 ^a	361.22 ^a	191.04 ^a	299.93 ^a	760.80 ^b
C ₁₀	68.30 ^a	71.19 ^a	201.55 ^a	101.24 ^a	116.71 ^a	158.83 ^a	454.39 ^a	297.71 ^a	421.03 ^a	845.38 ^b
C ₁₂	15.45 ^a	18.43 ^a	23.75 ^a	11.46 ^a	21.09 ^a	21.75 ^a	82.90 ^a	152.60 ^a	169.05 ^a	167.74 ^a

^{a,b,c,d,e}Means in the same row without a common letter are significantly different ($P < 0.05$).

Table 4. Response factor and fractional recovery of each FFA obtained by solid phase microextraction-gas chromatography/mass spectroscopy from the volatile fraction of Serra da Estrela cheese.

Fatty acid	Response factor	Recovery (%)
Acetic acid (C ₂)	4.11	23.0
Propionic acid (C ₃)	114.52	76.0
Isobutyric acid (iC ₄)	33.63	64.6
Butyric acid (C ₄)	42.78	86.9
Isovaleric acid (iC ₅)	26.97	64.7
Caproic acid (C ₆)	20.53	81.9
Caprylic acid (C ₈)	19.55	85.1
Capric acid (C ₁₀)	22.58	81.9
Lauric acid (C ₁₂)	48.03	60.7

and lactic acid (e.g., butanoic, propanoic, and acetic acids) or amino acids, mainly leucine and valine, which together represent about 60% of the free amino acid pool in this cheese (Tavaria et al., 2003). Furthermore, the pH prevailing in cheese exerts a considerable influence on the flavor impact of FFA; by 60 d of ripening, pH is ~5.2 and a considerable portion of FFA is present in salt form (Fox et al., 1995), hence having little impact on aroma. At longer ripening times, pH decreases down to 4.8, so FFA are mainly in their protonated forms, thus causing a stronger impact on aroma.

The average amounts of FFA along the ripening process are tabulated in Table 3; letters represent the result of posthoc comparisons (Tukey HSD) between ripening times. Most FFA (i.e., C₂, C₄, C₆, C₈, and C₁₀) attained their highest concentrations by 180 d of ripening, hence indicating that their amounts increased with ripening. The amount found by this time was significantly different from those at all other times, thus suggesting that even-chain FFA are biosynthesized directly from acetyl-CoA or are the direct result of the metabolism of lactose, whereas iC₄ and iC₅ are probably formed from amino acid conversion (from valine and leucine, respectively). The concentration of such FFA in the cheese matrix tends to stabilize between 60 and

90 d of ripening; apparently, the presence of leucine and valine (Tavaria et al., 2003) is the rate-limiting step toward formation of FFA. From this table, it is also possible to infer the lipolytic index (amount released per day) associated with FFA. Acetic, caproic, caprylic, and capric acids attained the highest value for that index by 7 d of ripening (9.1, 20, 28, and 33 mg/kg per d, respectively). The other short-chain FFA (which are the major contributors to the volatile fraction) reached the highest values between 35 and 60 d of ripening (time at which this cheese is usually commercialized). Free fatty acids contribute directly to cheese flavor but also serve as precursors for a variety of other compounds, for example, alcohols, esters, aldehydes, ketones, and lactones (Martínez-Castro et al., 1991; Molimard and Spinnler, 1996; Urbach, 1997). Therefore, the origin of FFA in Serra da Estrela cheese is likely attributable to enzymatic biosynthesis or to enzymatic catabolism of free amino acids. Enzymes can exist inside microbial whole cells, or be released following bacterial lysis. The response factors for each FFA, as well as the percentage recovered are tabulated in Table 4.

Esters arise from esterification of FFA with ethanol. Ethanol may originate from lactose fermentation by several homofermentative bacteria, e.g., enterococci and lactobacilli, yeasts and *Leuconostoc* spp. Large quantities of ethanol and esters are normally associated with fruity flavors (Urbach, 1997). Odor activity values were determined for FFA (Table 5), and were highest for butyric and isovaleric acids by 180 and 90 d of ripening, respectively. Their OAV reached 88 and 232 by 90 d, and 143 and 95 by 180 d of ripening, for butyric and isovaleric acids, respectively. Interestingly, the OAV are maximum (Figure 3) for C₄, iC₅, and C₁₀. Although it has been shown elsewhere (Macedo and Malcata, 1996; Sousa et al., 1997) that long chain, saturated (C16:0 and C18:0), and unsaturated (C18:1, C18:2, and C18:3) FFA are the most abundant in this type of cheese, it is well known that the aroma impact of me-

Table 5. Odor activity values of FFA in the volatile fraction of Serra da Estrela cheese throughout ripening.

Compound	Odor threshold ¹ (ppm)	Odor activity values									
		0	3	7	18	35	60	90	120	150	180
C ₂	54.00 ^a	0.06	0.57	1.15	2.13	1.32	1.20	2.08	1.27	0.95	1.18
C ₃	40.30 ^a	0.18	0.22	0.17	0.64	0.40	7.19	2.27	1.16	4.41	2.53
iC ₄	5.30 ^a	0.40	0.17	1.90	8.10	14.09	29.01	49.90	35.10	29.83	20.91
C ₄	7.00 ^a	6.85	4.23	26.62	11.05	11.37	63.23	88.43	59.23	70.87	143.25
iC ₅	3.20 ^b	1.46	0.96	7.98	31.74	84.36	187.82	232.52	179.05	175.68	95.53
C ₆	15.00 ^a	2.12	2.55	7.83	5.45	5.15	18.45	25.62	15.69	22.05	33.04
C ₈	19.00 ^a	2.58	3.18	9.15	4.35	4.99	8.74	19.01	10.05	15.79	19.83
C ₁₀	4.00 ^a	17.07	17.80	50.39	25.31	29.18	39.71	113.60	74.43	105.26	152.30
C ₁₂	16.00 ^a	0.97	1.15	1.48	0.72	1.32	1.36	5.18	9.54	10.57	15.00

¹Values indicated with ^a are odor threshold values according to Molimard, Le Quére, and Spinnler (1997); values indicated with ^b are odor threshold values according to Thierry et al. (2004).

Figure 3. Odor activity values of FFA: acetic (*); propionic (□); isobutyric (▲); butyric (◆); isovaleric (●); caproic (◇); caprylic (△); capric (■); and lauric (○) acids in Serra da Estrela cheese throughout ripening. The dashed line represents the time at which the aroma impact is maximum.

dium chain FFA is limited due to their relatively low volatility. The time of ripening at which the OAV reach their maximum is represented in Figure 3; this falls between 60 and 90 d, which coincides with the typical consumption time of the cheese.

Sensory Analysis

To perform sensory tests, we have selected 2 short-chain fatty acids with the highest OAV (butyric and isovaleric acids) and a third compound with a negligible impact upon the sensory perception of this cheese (ethyl hexanoate). ANOVA proved that the panel was homogeneous, and that differences between the samples analyzed were statistically significant ($P < 0.05$). Afterwards, a pairwise comparison was performed involving each set of compounds, using the Kruskal-Wallis test. Results indicate that the panelists were able to detect differences between unripened (control) and reconstituted cheese (i.e., unripened cheese + butyric acid + isovaleric acid + ethyl hexanoate), as well as between the latter and ripened cheese. No significant differences were found between unripened cheese with added C_4 and unripened cheese with added iC_5 . This means that the aroma perception of both is similar, whereas the aroma perception of ethyl hexanoate alone is poor (the sample with added ethyl hexanoate received the same rating as the control sample, as expected). Samples containing one of the FFA plus ethyl hexanoate were also rated low.

The panelists' evaluation placed the samples in 3 different subsets (Table 6): one that included only the

reference sample; another that included the reconstituted sample, the one with both acids and the one with isovaleric acid only (closest to the reference sample); and the remaining one that comprised all other samples (closest to the control sample). Butyric and isovaleric acids can be related to the overall aroma of Serra da Estrela cheese, as they are the major FFA present (approximately 48% of the total) by 60 d of ripening; due to their OAV, they do indeed contribute to a great extent toward the overall aroma.

Based on the relative amounts of FFA present in Serra da Estrela cheese and on their flavor impact, it can be concluded that the appropriate ripening time for consumption of this cheese is at least 60 d following manufacture. Furthermore, our results suggest that FFA can be used as ripening indicators for this gourmet cheese, as well as quality indicators, owing to the importance ascribed to the lipolytic keynotes in the Portuguese standard for this cheese.

Table 6. Homogeneous subsets obtained by Tukey's Honestly Significantly Different test.

Control	2.09 ^a
$C_4 + C_6C_2$	3.82 ^{ab}
C_4	3.95 ^{ab}
C_6C_2	4.00 ^{ab}
$iC_5 + C_6C_2$	4.23 ^{ab}
$C_4 + iC_5 + C_6C_2$	4.50 ^b
iC_5	4.54 ^b
$C_4 + iC_5$	5.18 ^b
Reference	8.32 ^c

^{a,b,c} Means without a common letter are significantly different ($P < 0.05$).

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