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SPME/GC-MS Characterization of the Volatile Fraction of an Italian PDO Sheep Cheese to Prevalent Lypolitic Ripening: the Case of Fiore Sardo

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Abstract A specifically aimed SPME/GC-MS method has been assessed in order to describe the volatile fraction of intense flavoring cheeses like Fiore Sardo PDO, a prevalent lipolytic ripening sheep cheese from Sardinia, Italy. A DVB/CAR/PDMS 50/30 μm fiber and a 3-min exposure time showed to be the best compromise between the possibility to extract compounds with a wide range of polarity and molecular mass and the need to avoid competition and displacement effects among analytes. The volatile compound profile of Fiore Sardo PDO sheep cheese was largely characterized by carboxylic acids (about 68% of the total area of recognized peaks), whereas esters (14%), ketones (9%), and alcohols (8%) represented other abundant classes of low molecular weight species. A number of low-smelling threshold trace compounds were also identified as likely contributors of aroma of the Fiore Sardo PDO cheese.

Keywords Fiore Sardo PDO · Sheep cheese · Lipolytic ripening · SPME/GC-MS

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Introduction

Fiore Sardo PDO (Commission Regulation (EC) 1996) is the most ancient and traditional hard cheese made from raw whole Sardinian sheep's milk. The use of raw milk is a key feature in its manufacturing process because it is the only way for the product to express all the intense sensory characteristics of the pastures of the production area. Another crucial point in the production of Fiore Sardo PDO is the use of lamb rennet paste. This rennet is used also for the production of some varieties of PDO sheep milk cheeses from southern Europe, such as Idiazabal and Roncal in Spain, Feta in Greece, and Canestrato Pugliese Pecorino Romano, Pecorino Siciliano, and Pecorino di Filiano in Italy. The presence of lipolytic enzymes in lamb rennet paste causes a pronounced hydrolysis of milk fat triglycerides yielding free fatty acids. Both free fatty acids and their degradation products can impart a peculiar (and often very intense) sensory characteristic to cheese (Addis et al. 2008).

The volatile fraction of Fiore Sardo PDO cheese has been evaluated using two different extraction techniques: dynamic headspace (Larráyoz et al. 2001) and simultaneous steam distillation–extraction (Larráyoz et al. 2001; Di Cagno et al. 2003), but results are often not comparable as consequence of very deep differences in the performances of the extraction method used, hence a reliable characterization of the volatile fraction of Fiore Sardo PDO is still lacking. In addition, these works have involved only a small number of

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samples and therefore a systematic study on volatile fraction of Fiore Sardo PDO has not been performed yet.

Among possible analytical approaches used to characterize the volatile fraction of cheese, SPME/GC-MS is one of the most appealing, because it is solvent free, cheap, easy to use, relatively fast, and highly sensitive (Chin et al. 1996; Peres et al. 2001; Pinho et al. 2003; Verzera et al. 2004; Frank et al. 2004; Mallia et al. 2005; Ziino et al. 2005; Januszkiewicz et al. 2008; Wolf et al. 2010, 2011). Previous contributions reported the use of single phase such as polyacrylate (Chin et al. 1996; Peres et al. 2001; Pinho et al. 2003; Januszkiewicz et al. 2008) and polydimethylsiloxane (PDMS, Chin et al. 1996; Peres et al. 2001; Pinho et al. 2003) or mixed coating fibers such as Carboxen/polydimethylsiloxane (CAR/PDMS, Peres et al. 2001; Pinho et al. 2003; Frank et al. 2004; Januszkiewicz et al. 2008), Carbowax/divinylbenzene (CW/DVB, Peres et al. 2001; Pinho et al. 2003; Januszkiewicz et al. 2008), polydimethylsiloxane/divinylbenzene (PDMS/DVB, Pinho et al. 2003), divinylbenzene/Carboxen/polydimethylsiloxane (DVB/CAR/PDMS, Pinho et al. 2003; Verzera et al. 2004; Mallia et al. 2005; Ziino et al. 2005; Januszkiewicz et al. 2008; Wolf et al. 2010, 2011). These types of fibers were used for the characterization of light/medium flavor cheeses like Provola dei Nebrodi (Verzera et al. 2004; Ziino et al. 2005); blue cheese (Frank et al. 2004; Wolf et al. 2011); Grana Padano and Parmesan (Frank et al. 2004); Reggianito (Wolf et al. 2010), Gruyere, Manchego, and Ragusano (Mallia et al. 2005); Terrincho (Pinho et al. 2003); Camembert (Peres et al. 2001); cheddar (Chin et al. 1996; Januszkiewicz et al. 2008); and Romano (Chin et al. 1996). However, the performance of SPME techniques strongly depends on experimental conditions. In particular, biases in the determination can derive from the complexity of the sample matrix and factors such as competitions and displacement phenomena among compounds due to the use of adsorbent fibers and the presence of some analytes at high concentrations or with high affinity to the fiber (Frank et al. 2004; Roberts et al. 2000).

These drawbacks are emphasised when analyzing cheeses characterized by an intense flavor like those obtained by using lamb rennet paste. Hence, aims of this contribution are the optimization of an SPME/GC-MS method useful to describe the volatile fraction of prevalent lipolytic ripening cheeses and the identification of volatile species in Fiore Sardo PDO sheep cheese.

Materials and Methods

Samples

One hundred eighty-day ripened Fiore Sardo PDO cheese samples were provided by 18 artisanal producers distributed throughout Sardinia, Italy chosen in collaboration with the Fiore Sardo PDO Cheese Consortium (consisting of about 50 farms). Samples were prepared according to Larráyoz et al. (2001). Just before analysis 3 g of finely grated cheese was weighted into a 10-ml vial and left 15 min at 40 °C to equilibrate until the fiber was exposed on the headspace. Each sample was analyzed twice.

SPME Extraction

Extraction was carried out with a DVB/CAR/PDMS 50/30 μm fiber of 1 cm of length (Supelco, Milan, Italy), using exposure times between 3 and 60 min. The fiber was conditioned prior to use according to the supplier's instructions in order to remove any possible contaminants. After the extraction step, the analytes were thermally desorbed from the fiber into an injector port (model 1079, Varian, Milan, Italy) operating at 250 °C in splitless mode for the first 5 min and then with a 1:60 split ratio for the upcoming 5 min. Also three additional mixed coating fibers (CAR/PDMS 85 μm, CW/DVB 65 μm, and PDMS/DVB 85 μm) were tested during the phases of method assessment.

GC-MS Analysis

A Varian 3800 gas chromatograph directly coupled with a Saturn 2000 ion trap mass detector (Varian, Milan, Italy) was used for the separation and identification of analytes. Chromatographic separation was performed on a DB-WAXetr (J&W, Agilent), 60 m×0.32 mm i.d., 0.5 μm film thickness, with the following temperature program: 45 °C for 4 min then increased to 150 °C at a rate of 5.0 °C min^{-1}, held for 3 min then increased to 250 °C at a rate of 10 °C min^{-1}. Helium was used as carrier gas at a constant flow of 1 ml/min. MS detector was programmed as follows: EI ion source operating at 70 eV, acquisition range between 20 *m*/*z* and 300 *m*/*z*, scan rate of 1 scan/s. The trap, manifold, and transfer line temperature were set to 200, 80, and 200 °C, respectively.

Each compound was identified using its mass spectral data (NIST98 library, NIST, USA), its linear retention indexes (Van den Dool and Kratz 1963), the comparison with authentic standard (when available) and the molecular weight obtained by mass spectra acquired in CI mode (using acetonitrile as reagent gas).

Results and Discussion

Preliminary Evaluation

Despite the extensive application of the SPME/GC-MS technique to the evaluation of the volatile fraction of

different dairy products, it was important to consider that almost none of them have been characterized by a very intense flavor, and this fact has certainly lowered the risk of displacement/competition phenomena (Frank et al. 2004). For this reason the problem of studying the volatile fraction of a very strong smelling cheese like Fiore Sardo PDO surely could not be reduced to the mere application of one of the previously published methods, but a careful optimization work of experimental conditions needed to be done. The key parameters affecting SPME technique are (1) type of fiber, (2) extraction temperature, (3) extraction time, (4) salt concentration, and (5) sample volume (Zhang and Pawliszyn 1993; Wardencki et al. 2004).

On the basis of our instrumental equipment and the nature of the sample, we could optimize only the first three parameters, because the salt concentration could not be modified (the sample was solid) and the sample volume was imposed by suitability with the GC-MS autosampler. Although the effect of sampling temperature on volatile analytes desorption peak area was well known (Verzera et al. 2004), we chose to operate at a temperature close to that of physiological smell perceiving, aiming to a correlation with data obtained by sensory analysis (Scintu et al. 2010). Moreover, the abundance of the volatile compounds in Fiore Sardo PDO cheese suggested that it was not necessary to increase the extraction temperature in order to enhance the sensitivity. The choice of the fiber type was not an easy task due to the need to analyze a very complex mixture of compounds from different chemical groups.

It has been observed that single-phase fibers produced lower signal intensities than mixed coating fibers, which were also capable to detect a wider range of compounds (Pinho et al. 2003). For these reasons a preliminary evaluation of the performances of a number of fibers on Fiore Sardo PDO cheese has been done only on four mixed coating fibers (i.e., CAR/PDMS, CW/DVB, PDMS/DVB, and DVB/CAR/PDMS). Among all, the DVB/CAR/PDMS fiber provided best results both from qualitative and quantitative viewpoint, which was in agreement with the results of Mallia et al. (2005). Indeed, this fiber was able to extract up to 64 compounds at the temperature of 40 °C belonging to many different functional classes (e.g., short- and medium-chain linear carboxylic acids, aldehydes, alcohols and phenols, esters, ketones, sulphur, and aromatic compounds) with a wide range of polarity and molecular mass.

Optimization of the Extraction Time

Figure 1 shows the chromatogram of the volatile fraction of Fiore Sardo PDO cheese extracted by a DVB/CAR/PDMS fiber (30 min of exposition at 40 °C). The chromatogram clearly reveals that the volatile compound profile of Fiore Sardo PDO cheese obtained with 30 min extraction time (i.e., a quite short length of the extraction step in comparison to those previously used for many cheeses) was characterized for intense signals (up to 24 megacounts) corresponding to short-chain acids (mainly butanoic and hexanoic acid) from fat lipolysis. Although the prevalent presence of these compounds on sheep cheeses had been already observed by other authors (Pinho et al. 2003; Mallia et al. 2005; Barron et al. 2005), the intensity of these signals suggested us the need to optimize the duration of the exposition step in order to avoid both saturation and competition events on the fiber surface (Roberts et al. 2000). Hence, extraction tests devoted to better clarify the time-dependent adsorption mechanism of most representative analytes (six linear carboxylic acids, six among alcohols and phenols, seven esters, and four 2-methylketones) onto the fiber have been accomplished. Extractions were performed three times by exposing the fiber in the sample headspace for 3, 5, 15, 30, and 60 min, respectively. Results are reported in Fig. 2.

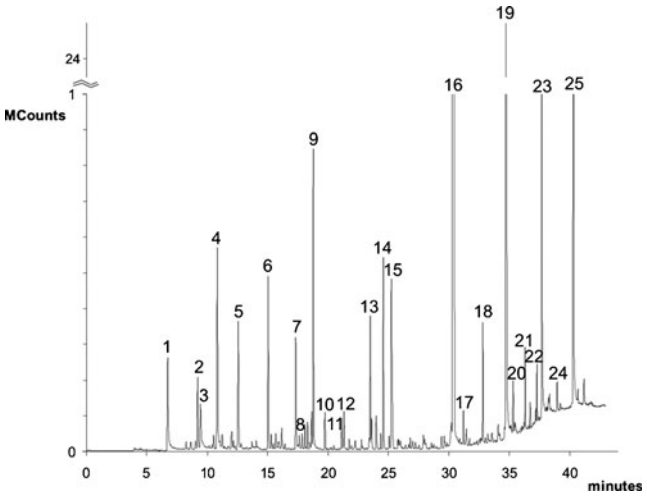

Fig. 1 Main components (ID number, name, LRI) in the typical chromatogram of the volatile fraction of Fiore Sardo PDO cheese extracted by a 1-cm DVB/CAR/PDMS fiber (30 min of exposition at 40 °C). *1* 2-propanone, 841; *2* 2-propanol, 948; *3* ethanol, 958; *4* 2-pentanone + diacetyl, 1006; *5* ethyl butanoate, 1061; *6* 2-pentanol, 1139; *7* 2-heptanone, 1214; *8* 3-methyl-1-butanol, 1229; *9* ethyl hexanoate, 1259; *10* 3-methylbutyl butanoate, 1291; *11*, 2-heptanol, 1339; *12* propyl hexanoate, 1346; *13* 2-nonanone, 1423; *14* ethyl octanoate, 1459; *15* acetic acid, 1493; *16* butanoic acid, 1650, *17* 2-furanmethanol, 1671 *18* pentanoic acid, 1761; *19* hexanoic acid, 1892; *20* 2-methoxyphenol, 1934; *21* heptanoic acid, 1993; *22* phenol, 2038; *23* octanoic acid, 2106; *24* nonanoic acid, 2207; *25* decanoic acid, 2310

Even using low extraction times, it was evident a pronounced competition effect between two major analytes: butanoic and hexanoic acids [Fig. 2 (1)]. Indeed, the ratio between the relevant chromatographic areas changed significantly passing from 3 to 5 min and an inversion of the relative proportions between the two compounds was observed for extraction times higher than 15 min, confirming a behavior elsewhere observed (Frank et al. 2004). The high affinity of acids for the DVB/CAR/PDMS fiber was

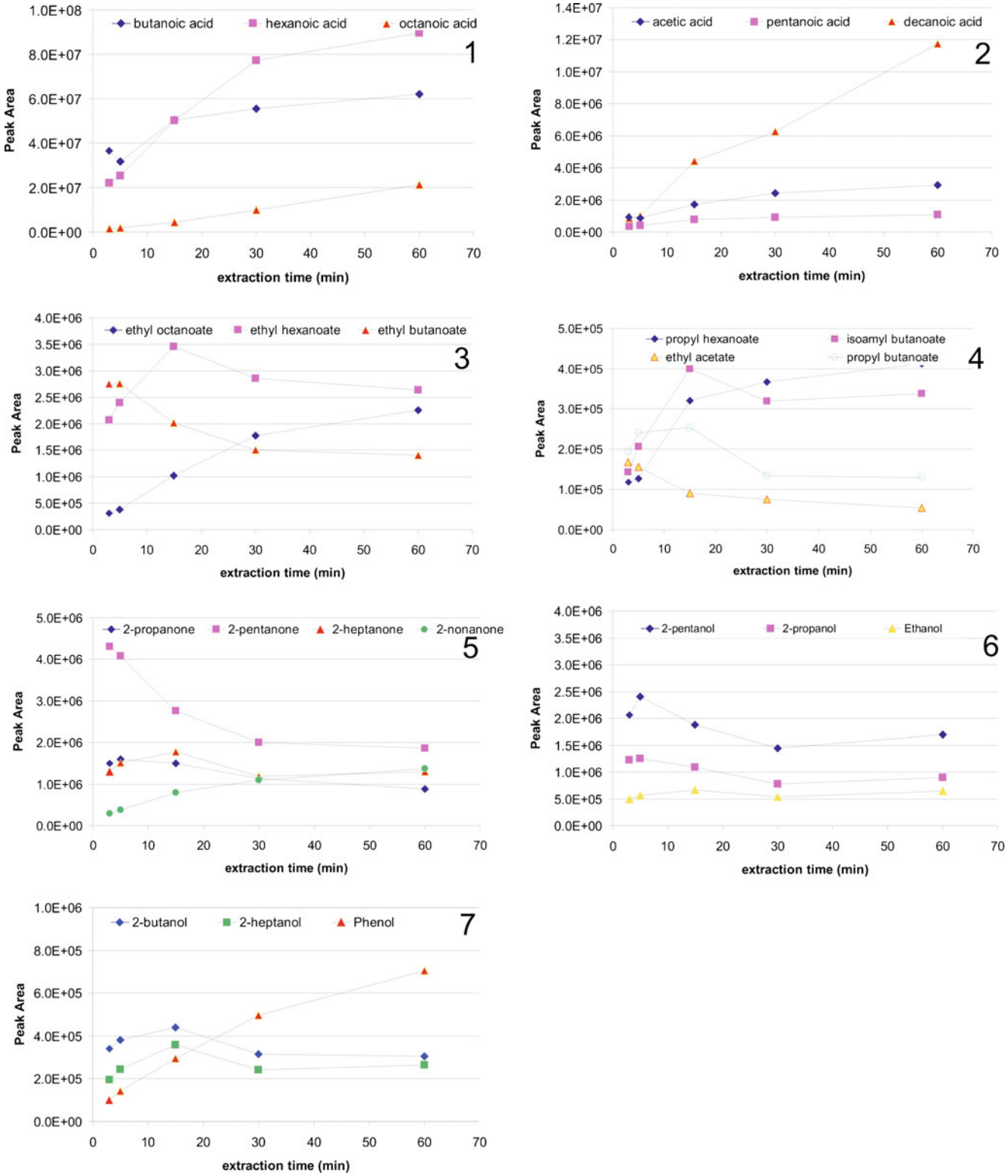

Fig. 2 Dependence of on-fiber concentration of selected volatile analytes (integrate area counts) over time (3–60 min) for the Fiore Sardo PDO cheese. *1* major acids, *2* minor acids, *3* major esters, *4* minor esters, *5* 2-methylketones, *6* major alcohols, *7* minor alcohols and phenol. Extraction conditions: 1 cm DVB/CAR/PDMS fiber, 40 °C

supported by the general increase of the response at increasing extraction times. Figure 2 (2) shows the behavior of some minor acids. A slight increment in the area values was detected for acetic and pentanoic acids when the

extraction time increased, whereas a more pronounced area increment was observed for the decanoic acid.

The most representative ethyl esters, ethyl butanoate, and ethyl hexanoate showed an opposite trend regarding relative sensitivity [Fig. 2 (3)] by increasing the extraction time between 5 and 15 min. In particular, the response for ethyl butanoate decreased for extraction times longer than 5 min, while the response for ethyl hexanoate became maximum at 15 min. Higher molecular weight ethyl esters like ethyl octanate exhibited a roughly linear increase of their chromatographic area when increasing extraction times. Since the behavior of minor esters was variable, a general trend could not be identified for this subclass of analytes [Fig. 2 (4)].

2-Methylketones [Fig. 2 (5)] did not show a homogeneous trend: the relevant sensitivities of 2-pentanone and 2-nonanone did not parallel (the former decreased by increasing the extraction times, whereas the latter slowly increased under the same conditions). On the other hand, the responses of 2-propanone and 2-heptanone were substantially invariant for extraction times between 3 and 15 min whereas a decrease of the analytical signal was evident for longer extraction times.

In general the area of the alcohols underwent a low increase when the extraction time was increased between 5 and 15 min, whereas further increments caused often a decrease of the chromatographic area, probably due to competition/saturation effects on the active sites [Fig. 2 (6 and 7)]. Phenol was a particular case. Area values for this compound were substantially increased at higher extraction times.

According to the results obtained, a general trend was observed for high molecular weight acids, esters, and ketones: their sensitivity generally increased by increasing extraction times, and the effect was more evident for long extraction times. However, long extraction times led to competition and displacement effects among analytes. Therefore, as a consequence of our findings, the optimized extraction time length for the SMPE/GC-MS evaluation of volatile compounds in Fiore Sardo PDO cheese was fixed in 3 min.

SPME/GC-MS Profile of Fiore Sardo PDO Cheese

The optimized SPME/GC-MS method was applied in order to characterize the volatile profile of Fiore Sardo PDO cheese. Figure 3 summarizes the results obtained.

Figure 3a gives account for the presence of carboxylic acids (about 68% of the area of identified peaks), which largely prevailed all volatile compounds. Moreover esters (14%), carbonyl compounds (9%), and alcohols (8%) represented other valuable classes of low molecular weight species. In addition, several minor components (1% in all) were identified, such as phenols, pyrazines, sulphur compounds, substituted furans, and other aromatic compounds. The characteristic volatile profile of artisanal cheeses was affected by a marked variability, probably as a consequence of both the variability in the native microflora of raw milk and the specific technological procedures adopted by each farmer. Our results appeared to be only scarcely comparable with those previously reported (Larráyoz et al. 2001; Di Cagno et al. 2003), therefore the key role in detection of volatile compounds in cheeses was confirmed (Curioni and Bosset 2002). Entering in the specificity of different classes of compounds, short-chain linear fatty acids (SCFAs), especially butanoic (60.78%), hexanoic (31.64%), and octanoic acid (2.62%), were the most representative compounds within carboxylic acids (Fig. 3b). This was mainly due to the activity of pregastric lipase from the rennet paste, which is very high on triglycerides containing SCFAs, especially butyric acid, which is almost always bound on the sn-3 position of triglycerides in sheep milk fat (Addis et al. 2005). The abundant presence of SCFAs is thought responsible for the characteristic piquant taste of this cheese (Addis et al. 2008; Larráyoz et al. 2001).

The ratio observed between butanoic and hexanoic acids (ca. 2:1) was similar to those reported by Larráyoz et al. (2001) using dynamic headspace, which is a technique more efficient for extracting highly volatile components. On the contrary, by using the simultaneous distillation extraction technique, the yield of hexanoic acid was higher than butanoic acid (Larráyoz et al. 2001; Di Cagno et al. 2003).

Acetic acid, accounted for 2.58%, originates from several processes, e.g., the catabolism of residual lactose and citrate by lactic and citrate-positive bacteria (McSweeney and Sousa 2000). Branched chain acids, such as 2-methylpropanoic and 3-methylbutanoic acid, were also found in Fiore Sardo only at low levels (0.20% and 0.35% of their class, respectively); their presence is mainly related to the valine and leucine catabolism, respectively (Yvon and Rijnen 2001).

The presence of esters in cheese is related to the esterification of free fatty acids with alcohols both by chemical and enzymatic reaction (Curioni and Bosset 2002), although more recently, alternative mechanisms of biosynthesis have been proposed (Liu et al. 2004). A total of 15 esters were identified in the analyzed cheeses (Fig. 3c), mainly ethyl esters. Branched chain alcohols were found to be esterified with only butanoic acid. Ethyl butanoate was largely the most abundant ester, giving account for about 55% of the class. Also ethyl hexanoate was a very representative ester, by constituting around 25% of the total amount of this family. Either ethyl butanoate (Larráyoz et al. 2001) and ethyl hexanoate (Di Cagno et al. 2003) has been reported among the most abundant esters in Fiore Sardo PDO cheese. Ethyl esters (especially ethyl butanoate and ethyl hexanoate) are considered key odorants in some cheese varieties such as blue cheeses (Moio et al. 2000) and grana-type cheeses (Moio and Addeo 1998; Qian

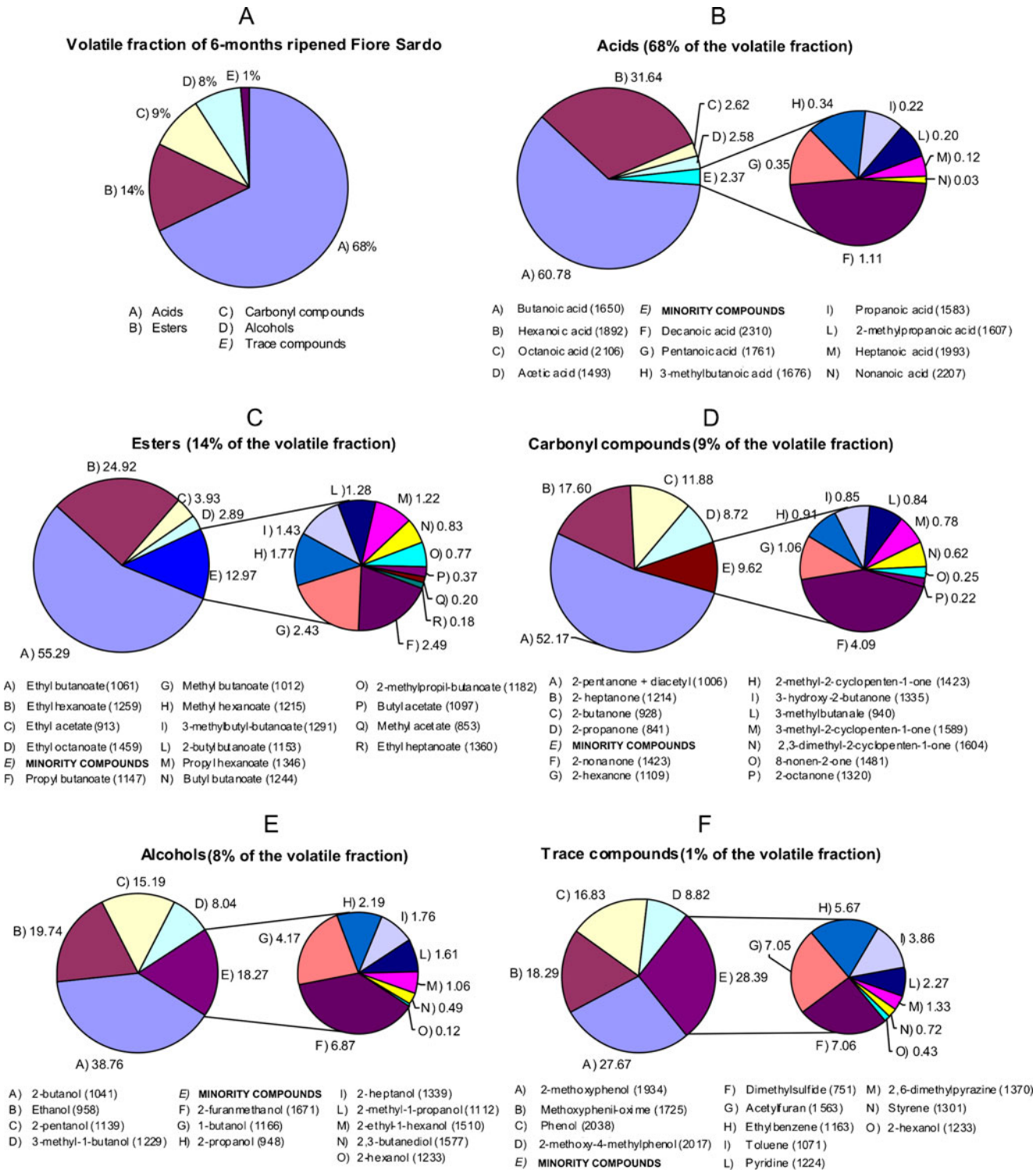

Fig. 3 SPME/GC-MS profile of Fiore Sardo PDO cheese headspace. **a** Percentage repartition among different functional classes, **b** relative composition within the class of acids, **c** relative composition within the class of esters, **d** relative composition within the class of carbonyl compounds, **e** relative composition within the class of alcohols, and **f** relative composition within the class of trace compounds. The LRI of each analyte has been reported in *brackets*. Extraction conditions: 1-cm DVB/CAR/PDMS fiber, 40 °C, 3 min

and Reineccius 2002, 2003). They have low perception thresholds and can contribute to the cheese aroma with typical fruity notes (Liu et al. 2004).

Other twelve esters were identified, but the proportions of each of them were always under 5% inside the class. Among the most representative minor esters, we can

mention ethyl acetate, ethyl octanoate, and a number of linear and branched C1–C4 butanoates. The presence of these esters reflected in principle the concentration of the correspondent free fatty acids in the Fiore Sardo PDO samples. In addition, raw milk provides a wide diversity of microorganisms able to synthesize these compounds (Liu et al. 2004).

Methyl ketones were the most abundant carbonyl compounds in Fiore Sardo PDO cheese (Fig. 3d). They derived from the correspondent $C_{(n+1)}$ acids by β-oxidation and subsequent decarboxylation (Collins et al. 2003). Among these, 2-pentanone, which partially coeluted with diacetyl, was the most abundant one (52.17%), followed by 2-heptanone (17.60%), 2-butanone (11.88%), and 2-propanone (8.72%), respectively.

Despite the relative low concentration of 3-hydroxy-2-butanone (acetoin) and 2,3-butanedione (diacetyl), we found a considerable amount of 2-butanone. This was consistent with the progressive degradation during ripening of acetoin by the nonstarter lactic acid bacteria enzymes, a process that results favorable in raw milk-made cheeses (Barron et al. 2005; Smit et al. 2005; Urbach 1993). Furthermore, some other cyclic or branched ketones were present in the volatile fraction of Fiore Sardo PDO, although the abundance of each of them was almost always less than 1% of the class.

Among the aldehydes, we could identify only 3-methylbutanal, which was related to the branched-chain amino acid catabolism. This compound is recognized as key odorant in a number of cheeses, like Camambert, aged Cheddar, Emmental, and Gruyére (Curioni and Bosset 2002). The low number of aldehydes in the volatile fraction of cheeses was not surprising, since they tend to not accumulate in cheese being rapidly converted to alcohols or acids (Ziino et al. 2005).

Many different metabolic pathways are responsible of the presence of alcohols in cheese (Curioni and Bosset 2002). Compared with the other alcohols, 2-butanol, which is the final reduction product of the above-mentioned acetoin, showed the highest peak area (38.76%, Fig. 3e). In addition, high levels of unesterified ethanol (19.74%) were found, which might explain the high representativity of ethyl esters inside their class, as it is the limiting factor for ester formation in hard cheeses (Liu et al. 2004). Other secondary alcohols (i.e., the species obtained from reduction of the correspondent ketones) altogether represented about 22% of the chemical class, the most important of them being the 2-pentanol (18.27% of the class abundance). Among the minor alcohols, we quantified also 2-furanmethanol (6.87% in the class) that was previously identified in the volatile profile of other cheeses like Parmigiano Reggiano (Qian and Reineccius 2002) and two branched alcohols (i.e., 2-methyl-propanol and 3-methylbutanol), both deriving from the reduction of the correspondent aldehydes.

Despite their low abundance, also trace compounds could contribute to the aroma of Fiore Sardo because of their low sensory threshold (Fig. 3f). They derived from the degradation of aromatic amino acids (Seitz 1990) such as phenol and methoxyphenols that have been identified in strongly flavored cheeses or could be present in this cheese as a result of the smoking process (Guillèn et al. 2004).

Conclusions

A specifically aimed SPME/GC-MS method has been assessed in order to describe the volatile fraction of intense flavoring cheeses. The key features of this method are related to the proper choice of the type of fiber used for the extraction and mainly the optimization of the extraction time. A DVB/CAR/PDMS fiber and 3-min extraction time have showed to be the best compromise between the possibility of extract compounds with a wide range of polarity and molecular mass and the need of avoid competition and inversion effects among analytes.

The method has been tested in order to determine the volatile compounds profile of prevalent lipolytic ripening cheese like Fiore Sardo PDO sheep cheese. As expected, data obtained appeared to be only scarcely comparable with those elsewhere published due to the differences in the extraction conditions used. Carboxylic acids from C2 to C10 largely prevailed (about 68%) on all volatile compounds, whereas esters (14%), ketones (9%), and alcohols (8%) represented other abundant classes of low molecular weight species. In addition, a number of low-smelling threshold trace compounds were identified as likely responsible of aroma of Fiore Sardo PDO cheese. By a closer look inside each class, the effect of lipolysis was reflected on the nature of identified compounds. SCFAs (C4–C10) represented almost two thirds of the area of the peaks ascribed to acids. Also significant amounts of SCFAs were present in the volatile fraction in form of ethyl esters. Furthermore, degradation products of SCFAs, like C3–C9 methyl ketones, were found in meaningful amounts. Alcohols were mainly represented by 2-butanol and 2-pentanol (i.e., products resulting from completion of SCFAs degradation/reduction pathways) and ethanol. Finally, the method was capable to reveal also the presence of trace compounds (e.g., phenol and methoxyphenols) that could affect the aroma of Fiore Sardo PDO.

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