Terpene profiles in Cantal and Saint-Nectaire-type cheese made from raw or pasteurised milk†

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Abstract: Terpene profiles in cheese can be considered a ‘terroir’ fingerprint as the information contained in it should enable the pastures on which the animals were fed to be recognised. Yet a certain elasticity of the signature must be taken into account when determining authentication strategies, since products acknowledged as containing a common signature may have undergone certain procedures, such as cheese making and milk pasteurisation, that could have potentially altered their terpene profiles. In this study, Cantal and Saint-Nectaire-type cheeses were made from both raw and pasteurised milk from the same herd of dairy cows that had been grazed on natural grassland. Cheeses from raw and pasteurised milk were made from the same milking on the same days. Cantal and Saint-Nectaire-type cheeses were made on 4 different days, alternatively over four weeks. The terpenes in the cheese fat were analysed by dynamic headspace/gas chromatography/mass spectrometry. A great diversity of monoterpenes, sesquiterpenes and oxygen-containing derivatives were identified. The major terpenes identified in most cheeses were β-caryophyllene, α- and β-pinene and limonene. Milk pasteurisation did not induce changes in the terpene profile of the cheese. Significant differences (p < 0.001) were observed between Cantal and Saint-Nectaire cheeses: α-pinene, β-myrcene and β-phellandrene were, respectively, three, five and five times more abundant in Cantal cheese, while tricyclene, α-phellandrene and geraniol were found exclusively in Cantal cheese. In contrast, unidentified sesquiterpenes with retention indices (KI) = 1342 and 1511, α-cubebene, longifolene and γ-elemene were more abundant or exclusively found in Saint-Nectaire cheese. A significant relationship with the date of milking (p < 0.01) was observed for α-pinene and tricyclene in Cantal, for β-myrcene, δ-3-carene, p-cymene and α-terpinene in Saint-Nectaire cheese.

Keywords: terpene; animal food; milk; cheese; traceability; pasture; pasteurisation

INTRODUCTION

Terpenes are plant secondary metabolites that are ingested by herbivores and subsequently found in associated milk and meat. Forage terpene content varies greatly according to the plant species. It is either absent or very scarce in cultivated grassland plants such as Poaceae or legumes, and greater amounts and range of monoterpenes, sesquiterpenes and oxygen-containing derivatives are found in some dicots of permanent grassland.1–3 The terpene fingerprint of a given grassland results from the range of plant species and is also dependent on the soil, climate, geographical localisation and grassland management practices. Back in 1978, Dumont and Adda4 underlined that seasonal or geographical variations of dairy cow diets could be traced through the sesquiterpenes found in cheese. Milk and cheese produced on pastures with highly diversified botanical composition have been shown to differ significantly in their terpene content from those obtained on monospecific grassland.3–7 Likewise, milk obtained from natural pasture contained approximately ten times more terpenes than milk obtained from the corresponding hay.8 Terpene analysis enabled the discrimination between milk obtained from pastures located in different geographical areas, in both milk9,10 and meat.11,12

As a measurable component of the terroir-to-product linkage, the terpene fingerprint could help in delimiting areas for Protected Denomination of Origin
(PDO) products, and to verify that specifications concerning animal feed have been fulfilled. The information contained in terpene profiles, however, is far from being completely understood. Most of the observations collected until now have to be confirmed under experimental conditions, to allow the separate factors that potentially affect terpene profiles to be controlled: eg pasture management and location, specific herd of cows, cattle management, cheese-making technology, profile of plants.

The objective of the work presented here was to study the separate effects of two technological factors that can affect the terpene fingerprints transmitted from the grassland pasture to the mature cheese: milk pasteurisation and cheese-making technology. Two varieties of PDO-labelled cheeses originating from Auvergne (France), Cantal and Saint-Nectaire cheeses, were produced with experimental facilities from either raw or pasteurised milk of the same milkings.

EXPERIMENTAL
Cheese making
Sixteen Montbéliarde dairy cows were used; these had been grazed on natural grassland at the INRA Experimental Center of Marcenat (Cantal, France). The milk was collected in the evening and stored overnight at +4°C, mixed with un-refrigerated morning milk and immediately transported to the INRA experimental dairy plant at Aurillac (Cantal, France) within approximately 1.5 h. Half of the milk was left raw whilst the other half was pasteurised for 30 s at 72°C. Raw milk cheese and pasteurised-milk cheese were processed on the same cheese-making days, whereas the two varieties of cheese were made on different days: Cantal cheese on June, 5, 14, 18 and 21; and Saint-Nectaire cheeses on June 7, 19, 26 and 29. Overall, 16 cheeses were used for this study: they were 2 varieties of cheese × 2 milk treatments × 4 replications.

For the experiment requirements, smaller Cantal cheeses (10 instead of 40 kg), ‘Cantalets’ (also PDO authorised), were manufactured from 110 l of milk. Once heated to 33°C, both raw and pasteurised milk were inoculated with 0.2 g of a lyophilised mesophilic mesenteroides spp cremoris 5%; Monilev: Monilevella 2 × 1011 l−1; Candida famata 6.25 × 1011 l−1; Penbac: Brevibacterium 4 × 1013 l−1, Penicillium nalgiovenensis 4 × 1010 l−1, Micrococcus. Forty-five minutes later, the curd was cut for 5 min to produce grains 5–6 mm in diameter. The curd–whey mixture was then mixed for 12 min and left to stand for 7 min. After extraction of the whey, the curd was placed into a pressing tray where it was pressed, cut into 15-cm cubes, and turned 12 times for approximately 3 h in order to reach 50% dry matter. After pressing, the curd cubes were left to drain for 24 h at 20°C and were poured into grains 20 mm in diameter. The mixture was salted with 20 g kg−1 dry salt and left to stand for 6 h at 20°C before one cheese per vat was formed in a cloth mould and pressed for 24 h at 13°C. The cheeses were placed in a ripening cellar at 10°C and 95% minimum relative humidity.

Raw and pasteurised Saint-Nectaire cheeses were manufactured in two vats, each containing 32 kg of milk heated to 33°C. The vats of milk were inoculated with a lyophilised mesophilic and thermophilic starter culture (respectively 0.1 g kg−1 of MA400 and 2.35 g kg−1 of MY800, Texel, Dangé-Saint-Romain, France) reconstituted in sterile skimmed milk (100 g l−1), with a ripening starter (7 × 106 germ l−1), Groupement d’Intérêt Economique, Laboratoire Interprofessionnel de Production, Aurillac, France) and 33 g kg−1 of a 520 mg active chymosin l−1 rennet (Gand-Gassiot, France). The composition of the starters were as follows: MY 800: Streptococcus salivarius spp thermophilus, Lactobacillus delbrueckii spp lactis and Lactobacillus delbrueckii spp bulgaricus; MA 400: Lactococcus lactis spp lactis, Lactococcus lactis spp cremoris, Lactococcus lactis spp lactis biovar diacetylactis and Streptococcus salivarius spp thermophilus. The clotting time was assessed visually and lasted for around 1 h, after which the curd was cut for 3 min, stirred for 6 min and left to stand for 4 min. The curd was separated by using a griddle and the whey was drained off. The curd was cut into 24 cubes to further extract the whey. These cubes were then placed into two moulds to shape in a moulding machine (Duprat, France). Each cheese was rolled in a cheese cloth and hoop, salted on the surface (40 g NaCl a side) and placed in polypropylene moulds. These cheeses were pressed for 24 h under 3 bars, then ripened in a cellar at 10°C and 95% minimum relative humidity.

Samples of Saint-Nectaire cheese were taken after 44 days maturation and Cantal cheese samples after 123 days. These samples (about 100 g) were wrapped in aluminium foils, sealed in polyethylene bags under reduced pressure with a vacuum seal machine (Multivac, F77462 Lagny sur Marne) and stored at −20°C.

Terpene analysis
Fat was recovered as a supernatant after centrifuging 40 g of cheese for 2 h at 75 600 × g at 25°C in a Beckman Avanti J-301 centrifuge (Fullerton, CA 92834-3100, USA). Terpenes were extracted from 0.2 g of fat by dynamic headspace (DHS) using an automatic Tekmar LSC 2000 system (Cincinnati, OH 45234, USA). The fat was deposited on 0.15 g of
glass wool in a 40-ml cylindrical glass extractor (Ets Maillères Frères, Aubière 63 170 France) maintained at 110 °C. Volatile compounds were purged for 30 min by a helium flow of 90 ml min⁻¹, trapped on Tenax at 28 °C, desorbed by heating the trap for 5 min at 220 °C and cryofocused in the GC column head at −150 °C with liquid nitrogen. Molecules were separated and identified by GC-MS using a SPB5 Supelco capillary column as previously described, except that the GC program was continued until the oven temperature reached 230 °C. Terpene peaks were located on the chromatograms by the means of their specific mass fragments: 93 and 136 for monoterpenoids, 93, 136, 161, 204 for sesquiterpenoids. They were identified by comparing the experimental mass spectra and retention indices (Kᵢ) with those contained in published databases. Semi-quantification was achieved by integrating the ion current of the mass fragment 93 for monoterpenoids and 161 for sesquiterpenoids. The results were expressed in arbitrary area units.

Median terpene values were used in preference to their means in accordance with Feinberg and Ducauze who stated that they were better adapted for analytical data with potentially dissymmetric distributions. The median, rather than the mean, represents the central value of a data set and is less sensitive to aberrant values. Variation ranges over groups of four cheeses were calculated as follows:

\[ \text{Var\%} = 100 \times \left( \frac{\text{max value} - \text{min value}}{\text{median}} \right) \]

Where terpenes were found in only one of the four cheeses they had no median and their variation range could not be calculated (×/0).

**Statistical analyses**

Data were analysed using Statistica 5.5 (Statsoft, Paris, France) software. The effects of milk treatment and the variety of cheese on the amount desorbed for each terpene were assessed using the ANCOVA test. The model was:

\[ X_i = \mu + P_i + C_j + (\text{covar date}) + P_i \times C_j \]

where \( P_i \) was the milk treatment (raw or pasteurised), \( i = 2 \), and \( C_j \) was the variety of cheese (Cantal or Saint-Nectaire), \( j = 2 \). The date of milking was included as a covariable in the model to take into account the stage of grassland plant maturity. The value for the date was between 5 and 29. Correlation coefficients for the amounts of terpene desorbed with the date of cheese making were calculated for groups of eight cheeses using Excel software and compared with the significant thresholds given by Snedecor and Cochran.

**RESULTS**

Examples of extracted ion chromatograms are presented in the Fig. 1. A total of 51 terpenes were detected in the 16 samples: 23 monoterpenes of which 4 contained oxygen (Table 1); 28 sesquiterpenes of which one was esterified and one contained oxygen (Table 2). In most cheeses, the major terpenes were \( \beta \)-caryophyllene, \( \alpha \)-pinene, \( \beta \)-pinene and limonene in which \( \beta \)-phellandrene co-eluted in several samples.

When available, variation ranges were between 100 and 500% in most cases and over 1000% in five cases, all of them observed for Cantal cheese. The highest variation ranges were not necessarily associated with the terpenes detected in trace amounts, as would be expected if variations originated in the analysis method.

No significant difference in the amount of individual terpenes desorbed from the cheese fat was found between cheeses made from raw or pasteurised milk. The most significant probability found was 0.06 for an unidentified ester at Kᵢ = 1386.

Differences were observed between Cantal and Saint-Nectaire cheeses. Higher quantities and a greater diversity of terpene compounds were found in Cantal cheese. The major terpene was \( \alpha \)-pinene in all the Cantal cheese samples, whereas in all the Saint-Nectaire cheese samples it was \( \beta \)-caryophyllene. Several terpenes were found to be significantly different (\( p < 0.001 \)) in the two varieties of cheese: tricyclog, \( \alpha \)-pinene, \( \beta \)-myrcene, limonene + \( \beta \)-phellandrene, geraniol, the unidentified sesquiterpene at Kᵢ = 1342, \( \alpha \)-cubebene, longifolene and \( \gamma \)-elemene.

**Figure 1.** Profiles of the extracted ion chromatograms of m/z = 93 for the retention times between 25 and 48 min (monoterpenes) and m/z = 161 from 48 to 65 min (sesquiterpenes). (a) Raw-milk Cantal cheese made on June 21 and (b) raw-milk Saint-Nectaire-type cheese made on June 26. 1: tricyclog, 2: \( \alpha \)-thujene, 3: \( \alpha \)-pinene, 4: camphene, 5: \( \beta \)-pinene, 6: \( \beta \)-myrcene, 7: \( \alpha \)-3-carene, 8: limonene, 9: \( \beta \)-phellandrene, 10: (E)-\( \alpha \)-ocimene, 11: \( \gamma \)-terpinene, 12: \( \beta \)-caryophyllene.
The covariable ‘date’ was found significant at

\[ p < 0.005 \] for \( \alpha \)-terpinene, \( \beta \)-cymene and geraniol and at

\[ p < 0.05 \] for \( \gamma \)-terpinene, iso-caryophyllene, longi-

folene, \( \gamma \)-elemene, \( \beta \)-selinene and three unidentified

esesquiterpenes at KI = 1342, 1426 and 1560.

The terpene profiles varied greatly between cheeses of the same variety made on different days. In Cantal cheese, a significant correlation (\( p < 0.01 \)) linking the day of milking (ie the date of cheese making) was observed for \( \alpha \)-pinene (\( R = 0.98 \), Fig 2) and tricyclene (\( R = 0.81 \)). In Saint-Nectaire cheese, \( \beta \)-myrcene (\( R = 0.82 \)), \( \delta \)-3-carene (\( R = 0.82 \)), \( \beta \)-cymene (\( R = 0.81 \)) and \( \alpha \)-terpinene (\( R = 0.80 \)) were correlated (\( p < 0.01 \)) with the day of milking.

**DISCUSSION**

Milk from dairy cows grazed on diversified permanent grasslands is known to contain a higher diversity and abundance of terpenoid compounds than can be obtained with any other type of diet. A great diversity of terpenes was found in both Cantal and Saint-Nectaire cheeses, regardless of whether the milk had been pasteurised or not. The most abundant monoterpenes such as \( \alpha \)-pinene and limonene, have long been found in milk volatile compounds and are considered common and ubiquitous. Many terpenes however, are present in trace amounts in milk and in cheese and need powerful analytical tools to be detected. Terpene extraction from a ‘terpene rich’ milk has been evaluated as approximately equivalent to that observed in milk low in terpenes after adding \( 0 \).1 \( \mu L \) of essential oil per litre.

Therefore, only recent papers report large diversities of terpene molecules. The major terpenes found in the cheeses were previously observed as major or abundant in the milk from approximately the same herd grazing on the same pasture. Twelve of the monoterpenes found, five of the sesquiterpenes and none of the oxygen-containing molecules were observed in milk produced from pastures in the Alps.

Milk pasteurisation could have affected cheese ter-

pene profiles directly through the thermal treatment, or indirectly via the suppression of the indigenous microflora. In fact, the relatively low temperature applied (72°C), the very short duration and the absence of air oxygen, did not constitute a drastic treatment, considering that terpenes, as the main
Table 2. Sesquiterpenoid compounds desorbed (arbitrary area unit \( \times 10^{-5} \)) from the cheese fat

<table>
<thead>
<tr>
<th></th>
<th>Cantal cheese</th>
<th>Saint-Nectaire cheese</th>
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<th>Milking date effect</th>
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<td>Raw milk</td>
<td>Pasteurised milk</td>
<td>Median Var%</td>
<td>Median Var%</td>
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<tr>
<td></td>
<td></td>
<td></td>
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<td>(a)</td>
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<tr>
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<td>Median</td>
<td>Var%d</td>
<td>Median Var%</td>
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<td>African-2(6)-ene</td>
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<td>0.2 3119</td>
<td>0.2 2226</td>
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<tr>
<td>S</td>
<td>1342</td>
<td>0.0 0.0</td>
<td>3.8 141</td>
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<td>14.2 396</td>
<td>20.8 123</td>
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<tr>
<td>Oxygen-containing S</td>
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<td>0.4 268</td>
<td>0.0 0.0</td>
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<td>β-Bourbonene</td>
<td>1418</td>
<td>1.2 1635</td>
<td>0.0 0.0</td>
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<tr>
<td>S</td>
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<td>0.0 0.0</td>
<td>0.4 553</td>
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<td>1.5 123</td>
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<td>S</td>
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<tr>
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<td>0.0 0.0</td>
<td>2.1 125</td>
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</table>

a–e See Table 1 for footnotes. S: unidentified sesquiterpene.

Figure 2. α-Pine desorbed from the cheese fat (arbitrary area unit). Cantal cheese: full symbols, Saint-Nectaire cheese: empty symbols. Raw milk cheeses: losanges, pasteurised milk cheeses: triangles. Dotted lines: adjusted curves among groups of eight (Cantal or Saint-Nectaire) cheeses. \( R^2 \): correlation coefficients with the milking date (days) and their significance (\( **: p < 0.01, *: p < 0.05 \)).

constituents of essential oils, resist hydrodistillation. Milk indigenous microflora has been shown to play an important role in Swiss-type cheese aroma during maturation. Buchin et al. found volatile compound differences between raw and pasteurised milk in semi-hard cheese. The first one contained higher amounts of alcohols, fatty acids and sulphur compounds, the second one higher amounts of ketones, but none of the seven terpenes detected was affected by milk pasteurisation.

Terpene profile differences between Cantal and Saint-Nectaire cheese varieties were significant. These differences may have originated from a combination of physical, chemical, biochemical or microbial mechanisms during the cheese making. For example, in both cheese making procedures, the milk had been initially heated to 33°C. This might have induced some terpenes to evaporate, at rates that depended on the volume of milk being heated, the method of stirring, the porosity of the curd, etc. In addition to the variation in cheese making technology, the microbial flora may have induced different chemical reactions on terpene molecules. The many differences between the two cheese making processes may be important...
with regard to microbial activity. The main differences are the microbial flora present in the starters added to the milk, and the length of the ripening period, which was 79 days longer in Cantal than in Saint-Nectaire cheese. With the except of the observations made by Larsen that cheese associated Penicillium fungi were able to produce terpene, to our knowledge, microbial activity on terpenes has never been studied in cheese.

With a very low number of observations, the experimental procedure was not designed to detect changes in the terpene profiles over time. However, several significant correlations were observed between the amount of terpene desorbed by the cheese fat and the date of milking. This was particularly clear for α-pinene in Cantal cheese (Fig 2). In fact, the dairy cows from which the milk was obtained were grazed on natural grassland, whose botanical flora matured progressively as the time elapsed. It is well known that plant terpene profiles also change as plants mature. Although additional confirmation is needed, those results confirm that the terpene fingerprints found in cheese contains information related to the dairy cows pasture feeding, and to the pasture plant maturity stage.

**CONCLUSION**

As the result of the botanical composition of the pasture grazed by dairy cows, terpene profiles of dairy products are a potential source of valuable information concerning their geographical origin. This work provides at least a partial answer to three questions concerning factors that could be instrumental in hampering the message contained in the termite profiles from being clarified. Cheese-making induced the most important changes in terpene profiles, possibly through the physicochemical conditions applied, through the microbial populations and through the duration of the cheese maturation period. Moreover, since no significant difference was observed between pasteurised and raw milk cheeses, the added micro-organisms were more likely to be involved than the endogenous bacteria. For several terpenes, the significant relation with the date of milk collection reflected the maturity stage of the grassland plants ingested by the dairy cows. The terpene molecules representative of the local flora that can play a key role in grassland fingerprinting need to be found amongst those influenced by neither cheese-making technology nor pasteurisation.

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