Development of New HS–SPME–GC–MS Technique to the Measurement of Volatile Terpenoid Profile of Milk

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Abstract
This study presented the development of such a HS–SPME–GC–MS technique, with the use of which, directly from the raw milk sample matrix, both qualitatively and quantitatively; the volatile terpenoids (α-pinene, sabinene, β-pinene, p-cymene, limonene, linalool, α-thujene, camphor, menthol, methyl chavicol, caryophyllene E, α-humulene) can be determined, derived from herbs eaten by the dairy animals by different feeding methods. Repeatability was less than 10% in the case of milk fat samples. The estimated limits of quantitation were between 2 and 16 ng/g. The lowest values were 2 ng/g for p-cymene and methyl chavicol; the highest value was 16 ng/g for caryophyllene E. In the case of goat milk, the repeatability was better than 8% except for α-thujene. The estimated limits of quantitation were between 1 and 8 ng/g. The lowest values were 1 ng/g for β-pinene, p-cymene and limonene, and the highest value was 8 ng/g for linalool. In milk fat, the highest concentration was identified in caryophyllene E (470 ng/g) and α-humulene (430 ng/g), while the lowest concentration was in p-cymene (2 ng/g) and camphor (2 ng/g). In goat milk, limonene was present in all samples, but its amount varied depending on the type of consumed herb. Methyl chavicol and caryophyllene E were detected in goat’s milk only in one case. The former was detected in sage milk at 2.09 ng/g and the latter in tarragon milk at 2.28 ng/g. We have also successfully demonstrated that the feed consumed by dairy animals also affects the quality of dairy products.

Keywords Volatile components · Terpenes · Gas chromatography · Mass spectrometry · SPME · Milk

Introduction
Solid-phase microextraction (SPME) is one of the fastest-growing extraction techniques, due to its many advantages. The first is simplicity. It is easy to automate and the SPME module can be easily integrated into the automatic sample feeder of all types of gas chromatographs. In addition to its automated version, there is a manual arrangement with a cheaper and recyclable “holder” in which the thread can be replaced. SPME sampling can therefore also be performed manually, unlike other techniques, which provide high reproducibility and do not differ much from the automated version. Another great advantage of the method is the variety of fibre material, which increases the selectivity of sampling (Ho et al. 2011).

The analysis of aroma components in dairy products poses a complex problem due to the complexity of the sample matrix and the complexity of the most commonly used sample preparation procedures, such as vacuum distillation and liquid–liquid extraction (De Frutos et al. 1991; Mariaca and Bosset 1997; Lubbers et al. 1998; Sides et al. 2000). Extraction tests are constantly being updated and new techniques are being invented to reduce extraction time, solvent and equipment requirements, increase sensitivity and ensure widespread applicability (Panseri et al. 2008).

Groups of chemical compounds that can be determined by SPME analysis contribute to the taste and smell of the products and act as an indicator if there is a change in quality or contamination in the product. The SPME process provides several uses for food volatile component testing.

SPME analysis of terpenoids in dairy products can determine the diet and region of origin of the animals (Abilleira et al. 2010; Cais-Sokolińska and Majcher, 2010; Erkaya and Şengül, 2011; Fernández-García et al. 2008; Majcher et al. 2010). Poulopoulou et al. (2012a, 2012b) studied sheep and goat milk and dairy products made from these milk by the
showed that the terpene profile of the studied cheeses was
from the milk of cows grazed in different areas. The results
(2001b) investigated terpene components from cheeses made
the dairy herd grazed on mountain pastures. Bugaud et al.
appeared only in summer-made Beaufort cheeses when
and Adda (1978) found that sesquiterpene compounds
and the place of production, animal feed and compounds
to the matrix effect of milk fat and the significantly different vapour pressures of each terpene
(Abilleira et al. 2010).

Terpenoids can be transferred to dairy products mainly
from digested plant food. Mono- and sesquiterpene com-
ponds can be derived from plants into milk in two ways,
via the digestive tract or via the respiratory tract. In the first
case, the molecules are transferred from the plants to the
rumen, where the terpenes may undergo a chemical trans-
f ormation. All of these molecules are obtained from the
plants into the rumen and are then absorbed from the rumen
into the bloodstream, and from there transferred into the
milk. Terpenes from plants, as well as those that arise in the
rumen, are well detectable (Schlichterle-Cerny et al. 2004).
In the second case, the components spread through the air
and enter the animal’s lungs and from there into the blood
(Viallon et al. 2000). Volatile components alter the intrinsic
values of milk, especially the composition of microbiologi-
cal and aromatic substances (Bugaud et al. 2001a; Viallon
et al. 2000; Scehovic et al. 1998; Buchin et al. 1999).

The results show that milk from different production
areas (highlands, lowlands) and seasons (winter, summer)
differ from each other (Bugaud et al. 2001a; Scehovic et al.
1998; Buchin et al. 1999).

Feeding dairy animals is also an important factor in shap-
ing the character of cheeses, as it influences milk-specific
parameters such as milk fat, protein, taste, microflora and
the function of lactic acid bacteria, which play a significant
role in cheese making (Collomb et al. 1999; Grandison et al.
1985). Numerous studies describe that the milk of animals
grazed on pastures overgrown with dicotyledons plant con-
tains significantly higher amounts of aroma components
than the milk of their counterparts fed hay, fodder or mono-
cotyledonous plants (Dumont and Adda, 1978; Viallon et al.
1999). This difference is due to the presence of significant
amounts of terpene compounds in dicotyledonous plants,
especially mono- and sesquiterpenes (Mariaca et al. 1997).

Several research groups have found correlations between
the organoleptic and physicochemical properties of cheeses
and the place of production, animal feed and compounds
derived from feed (Mariaca et al. 1997; Viallon et al. 1999,
2000; Bugaud et al. 2001a, b; Scehovic et al. 1998; Jeangros
et al. 1997; Cornu et al. 2001; Buchin et al. 2002). Dumont
and Adda (1978) found that sesquiterpene compounds
appeared only in summer-made Beaufort cheeses when
the dairy herd grazed on mountain pastures. Bugaud et al.
(2001b) investigated terpene components from cheeses made
from the milk of cows grazed in different areas. The results
showed that the terpene profile of the studied cheeses was
related to the botanical composition of the pastures. These
compounds were present in significant amounts in moun-
tain pastures where there are higher amounts of dicotyledons
than in the plains, where predominantly monocotyledonous
plants are present. A wide range of organic volatile com-
ponents is responsible for the aroma of the cheeses. These
components are very similar in different types of cheese, but
there may be differences in their proportions (Ziino et al.
2005).

Materials and Methods

Chemicals and Gases

Sabinene (75% purity), p-cimene (99% purity), 4-allylani-
sol (98% purity), α-pinene (98% purity), α-humulene (96%
purity), β-pinene (99% purity), cariofillene-E (98% purity)
and α-thujone (96% purity) were purchased from Sigma-
Aldrich (Budapest, Hungary). Limonene (94% purity), lin-
alool (98% purity), camphor (96% purity), menthol (99%
purity) and methanol (99.8% purity) were purchased from
Merck (Budapest, Hungary). Helium was of 99.999% purity
(Linde, Répcelak, Hungary).

Plant Materials

Several herbs and spices were chosen, such as milfoil (Achil-
lea millefolium L.), chamomile (Matricaria chamomilla
L.), woodruff (Galium odoratum L.), tarragon (Artemisia
dracunculus L.), ribgrass (Plantago lanceolata L.) and
sage (Salvia officinalis L.) which have favourable smell,
aroma and physiological effect based on literature and folk
observations.

Sampling Procedures

Investigations were carried out in different phases and the
samples used came from two different premises to the site
of analysis, Széchenyi István University, Faculty of Agricul-
tural and Food Sciences, Analytical Laboratory of Depart-
ment of Food Science. Samples from dairy cattle came from
the premises of Bicskei Mezőgazdasági Zrt. (Bicske, Hun-
gary) where the experimental animals were driven to pas-
tures with various herbs and by grazing herbs there, and the
herbal active ingredients to be tested were introduced to their
body. Milk and blood samples after 10 days of grazing were
derived from morning and evening milk of 2–2 experimental
animals in which the blood was taken as well.

The grazing herbal active ingredients were introduced
to the animal’s body and milk. Cheese samples analysed
in this study were made from the herbal milk of these
animals. The herbal and natural Trappist cheese samples were analysed in a 4-day fresh and 6-week mature ageing time.

The goat feeding experiments were carried out at the farm of József Csató farmer at Kerta (Hungary). In this experiment, we have already done the feeding with accurate amounts. Samples were obtained from 3–3 experimental animals, which were kept away from other animals and were fed for 10–10 days with 6 different herbs. The mass of plants mixed with the feed was gradually increased from 40 to 50 g during the 10 days, to avoid straining the rumen of small ruminants. The goat milk samples were taken from the evening milking of day 10 of the feeding experiment. After the feeding experiments, the samples of milk, blood and cheese were delivered to the laboratory and were processed and analysed.

**Sample Preparation Procedures**

The sample preparation of blood samples is based on Estell et al. (2010) method with some modifications. Because only a small amount of blood samples could be yielded from cows, therefore, to ensure the minimum 5 mL of plasma required for the examinations, the samples from two cows were combined then centrifuged for 1 h at 5500 g in a sealed sampling tube (3 K, Sigma). Supernatant plasma was stored at −20 °C until use.

During the sample preparation of cheese samples, 100 g of herbal and natural Trappist cheese was chopped for 10 min with a high-performance (750 W) electric kitchen grinder (Electrolux). After grinding, the cheese samples at 5–5 g were measured in 24-mL vials with a screw cap (Sigma-Aldrich) and stored at −20 °C until further use. To avoid the cross contaminations the vials were rinsed with methanol (Merck) and heated previously at 150 °C for 1 h.

Separate the milk fat from the milk the two-step centrifugation procedure was applied (Abilleira és mtsai; 2010). During the first step, 80 g of preserved milk was centrifuged with 3300 g at 5 °C for 15 min. Supernatant cream was further centrifuged (second step) in a sealed centrifugation tube at 35 °C with 5500 g for 2.5 h (3 K, Sigma) and then supernatant milk fat was stored at −20 °C until use.

The fat content of the butter sample from the retail trade was separated from the aqueous phase after thawing and centrifugation at 35 °C at 5500 g for 1 h (3 K, Sigma).

After receiving the goat milk samples, 5–5 g was measured in 24-mL vials with a screw cap and stored at −20 °C until further use. To avoid cross-contamination, the vials were flushed with methanol and heated previously at 150 °C for 1 h.

**SPME Sampling Procedures**

The extraction of volatile components was performed by SPME method and headspace (HS) analysis. For the sampling procedure, a Supelco handheld SPME sampler (57,330-U) was used. To analyse the volatile compounds of herbs and feeds, a 1-cm-long 100-μm PDMS-coated fibre was applied. For the HS analysis of blood plasma, milk, milk fat and cheese samples, a 2-cm-long 50-μm divinylbenzene 30-μm carboxen polydimethylsiloxane-coated (DVB/CAR/PDMS) StableFlex fibre was used, as recommended by the manufacturer (Supelco) for the trace analysis of volatile compounds. To remove the volatile components trapped in the SPME fibres from the air during storing, the fibres were heated at 260 °C for 15 min in our GC–MS injector. Samples are taken according to the data in Table 1.

**Gas Chromatography-Mass Spectrometry (GC–MS) System**

To analyse the volatile components of herbs, GCQ (Finnigan MAT) type ion trap was used, while for headspace analysis of plasma, milk and milk fat, QP-5000 (Shimadzu) type equipped with a quadruple analyser, gas chromatography–mass spectrometry systems were used. The test conditions used are listed in Table 2.

**Calibration of the Measuring Equipment**

The calibration of the developed GC–MS method with standard addition procedure was carried out by analysing spiked milk fats obtained from a sample of butter from retail trade and of spiked raw goat milk. The spiked samples were prepared as follows: in 24-mL vials, we weighted 5–5 g of milk fat obtained from butter from retail trade and 5–5 g

| Table 1 Conditions used during SPME sampling for herbs, blood plasma, cow’s milk and cheese |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| SPME fibre                     | Herb 1 cm, 100 μm PDMS          | Plasma 2 cm, 50/30 μm DVB/CAR/PDMS | Milk fat                          | Goat milk                        | Cheese                           |
| Volume of the vial              | 43 mL                           | 24 mL                           |                                   |                                 |                                  |
| Mass of the sample              | 2 g                             | 5 g                             |                                   |                                 |                                  |
| Pretreatment and sampling       | 55 °C                           | 40 °C, 80 °C, 60 °C, 60 °C      |                                   |                                 |                                  |
| temperature                     |                                 |                                 |                                   |                                 |                                  |
| Pretreatment time               | 50 min                          |                                 |                                   |                                 |                                  |
| Sampling time                   | 5 min                           | 60 min, 50 min, 60 min          | 600 l/min                         |                                 |                                  |
| Mixing                          | none                            | none                            | none                             | 600 l/min                        | none                             |

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of raw goat milk. I melted the fats in a 40 °C water bath, while directly after weighing, 50–50 µL methanol solution containing terpenes in 0.02–1.2 µg/g concentration was added to the raw goat milk. The vials were sealed and mixed throughout with vortex (VELP Scientifica Rx3, Italy). Samples were taken from the headspace of the spiked milk fat samples at 80 °C with a sampling time of 50 min, whereas from spiked raw goat milk samples at 60 °C with a sampling time of 60 min, and the sampling SPME fibres were taken into the injector of the GC–MS instrument. A straight line was drawn, with the method of least squares on the peak areas of ion chromatograms recorded in GC–MS selected ion monitoring (SIM) mode and on their respective terpene concentrations for value pairs. From the slope of the fitted lines, the sensitivity of the method and the lower limit of quantitation were determined (LOQ).

\[
\text{LOQ} = 10 \times \frac{SD}{m}
\]  

(1)

where SD is the standard deviation of the areas below the peak, and.

\( m \) is the slope of the fitted calibration line.

For qualitative determination of volatile terpenes in milk fat and raw goat milk samples, the retention times of volatile components and their mass spectrum recorded in the full-scan mode were compared with the retention time of the volatile components of each herb and with the mass spectra of NIST MS database. For quantitative determination of volatile terpene content of samples, the measuring equipment in SIM mode was calibrated, with standard addition method, in the case of milk fat for eleven and in the case of raw goat milk for twelve terpenes within the concentration range shown in Table 3, on 8 different concentrations. The standard terpene compounds used to calibrate the analysis of milk fat samples, the mass to charge ratio (m/z) of the ions used for their quantification, the retention time of the terpenes, and the linear ranges of analytical measurement curves, slopes and correlation coefficients (R) for each terpene, are shown in Table 3.

The repeatability of the measurements was given by the corrected empirical standard deviation of the concentration values obtained by three and three parallel analysis of the spiked milk fat and raw goat milk samples. The measured average concentration

| Table 2 | Test conditions for GC–MS analyses for herbs, blood plasma, cow’s milk and cheese |
|---------|-------------------------------------------------------------------------------------------------|
|         | GCQ (herb) | QP-5000 (blood plasma, milk fat, cheese) | QP-5000 (goat milk) |
| Injector | 280 °C, split, 1:30 | 260 °C, splitless, 5 min | 260 °C, splitless 4 min |
| Desorption time | 5 min | 5.5 min | 4.5 min |
| Liner | 2 mm ID, quartz | 0.75 mm ID, glass |
| Column | RTX-5 (Restek) 30 m, 0.25 mm ID, 0.25-µm film |
| Temperature programme | 50–200 °C, 3 °C/min | 50–185 °C, 3 °C/min | 40–160 °C, 3 °C/min |
| Carrier gas | He (5.0, Linde), 35 cm/s |
| Ion source | EI, 70 eV, 200 °C |

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| Table 3 | Mass to charge ratio (m/z), retention times (tR), linear ranges of analytical measurement curves, average slope and standard deviation (±SD), and correlation coefficients of ions used for GC–MS quantitation of terpenes in case of milk fat samples. Analytical performance data for headspace analysis of dairy milk fat samples in SPME (Cad, additive concentration (ng/g); RSD (%), relative corrected empirical standard deviation of peak areas measured in parallel analyses (n=3); LOQ, the estimated limit of quantitation of the method) |
|---------|-------------------------------------------------------------------------------------------------|
| No | Volatile component | m/z Th | tR (min) | Linear range (ng/g) | Slope ± SD (g/ng) | R | Cadm (ng/g) | RSD (%) | LOQ (ng/g) |
| 1 | α-Pinene | 93 | 7.63 | 10–1000 | 4550 ± 64 | 0.999 | 46 | 3.2 | 3 |
| 2 | Sabinene | 93 | 9.02 | 5–770 | 3472 ± 38 | 0.999 | 23 | 6.6 | 3 |
| 3 | β-Pinene | 93 | 9.13 | 10–1230 | 2857 ± 74 | 0.997 | 141 | 3.6 | 4 |
| 4 | p-Cymene | 119 | 11.00 | 10–1000 | 6216 ± 86 | 0.999 | 65 | 9.5 | 2 |
| 5 | Limonene | 93 | 11.14 | 10–1000 | 2007 ± 36 | 0.998 | 143 | 9.8 | 4 |
| 6 | Linalool | 93 | 14.25 | 10–1000 | 945 ± 12 | 0.999 | 98 | 6.1 | 8 |
| 7 | α-Thujene | 110 | 14.48 | 10–1000 | 797 ± 14 | 0.998 | 66 | 8.6 | 6 |
| 8 | Camphor | 95 | 16.19 | 10–1000 | 1418 ± 26 | 0.998 | 47 | 6.7 | 4 |
| 9 | Methyl chavicol | 148 | 18.61 | 10–1000 | 6640 ± 188 | 0.994 | 16 | 12.9 | 2 |
| 10 | Caryophyllene E | 133 | 28.06 | 20–2000 | 276 ± 8 | 0.994 | 388 | 2.5 | 16 |
| 11 | α-Humulene | 93 | 29.50 | 20–2000 | 1576 ± 42 | 0.995 | 136 | 3.4 | 6 |
of terpenes in the milk fat with repeatability and the estimated limits of quantitation for the 10:1 signal–noise ratio are shown in Table 3. Repeatability was less than 10% except for methyl chavicol. The estimated limits of quantitation were between 2 and 16 ng/g. The lowest values were 2 ng/g for p-cymene and methyl chavicol, and the highest value was 16 ng/g for caryophyllene.

The standard terpene compounds used to calibrate the analysis of raw goat milk samples, the mass/charge of the ions used for their quantitation, the retention time of the terpenes, and the linear ranges of analytical measurement curves, slopes and correlation coefficients (R) for each terpene, are shown in Table 4.

During the calibration with goat milk, the highest sensitivity was for p-cymene (10,817 ± 96 g/ng) and methyl chavicol (9969 ± 161 g/ng), and the lowest for caryophyllene (1733 ± 35 g/ng) and camphor (1875 ± 34 g/ng). Low uncertainties in the slope of analytical curves fitted to measurement points during calibration and the correlation coefficients greater than 0.996 for fitted lines all prove the adequate accuracy of my calibration.

The measured average concentration of terpenes in the raw goat milk with repeatability and the estimated limits of quantitation for the 10:1 signal–noise ratio are shown in Table 4. In the case of goat milk, the repeatability was better than 8% except for α-thujene. The estimated limits of quantitation were between 1 and 8 ng/g. The lowest values were 1 ng/g for β-pinene, p-cymene and limonene, and the highest value was 8 ng/g for linalool.

### Results and discussion

#### Optimisation of SPME Sampling Conditions

A quantitative study of terpene-like volatile components was done with 2-cm-long 50/30-µm DVB/CAR/PDM fibre by SPME sampling of the headspace of the butter fat obtained from each milk sample and of the raw goat milk samples. The dependence of sampling temperature and sampling time of fibre-dissolved terpenes and terpene derivatives was investigated for optimal SPME sampling conditions.

For buttermilk sample, the optimal headspace sampling temperature was determined by the headspace analysis of 5–5 g of butter spiked with terpenes at 40, 60 and 80 °C temperatures with 60-min sampling period.

The determination of optimal sampling time from headspace of spiked butter samples took place at 80 °C and for 1, 2, 5, 10, 20, 30 and 50 min, while from headspace of spiked raw goat milk at 60 °C and for 1, 2, 5, 10, 15, 20, 40 and 60 min.

When analysing milk fat samples, the largest of the three different HS-SPME samples taken at 40, 60 and 80 °C provided 80 °C with the best sensitivity of my measurements and the fastest equilibrium state. The optimal sampling time was determined by sampling 1, 2, 5, 10, 20, 30, 40 and 50 min of terpene-added milk fat headspace and plotting of each terpene normed peak area’s dependence from sampling time (Fig. 1).

In the case of spiked raw goat milk, out of the three different 40, 60 and 80 °C SPME sampling temperatures, 40 °C has not yet provided sufficient sensitivity for less volatile components while at 80 °C the “skin” of the surface of the

| No | Volatile component | m/z (Th) | tR (min) | Linear range (ng/g) | Slope (± SD) (g/ng) | R | Cad (ng/g) | RSD (%) | LOQ (ng/g) |
|----|-------------------|----------|----------|---------------------|---------------------|---|------------|----------|-------------|
| 1  | α-Pinene          | 93.10    | 9.70     | 5–1040              | 3115 ± 71           | 0.996 | 29         | 7.5      | 3           |
| 2  | Sabinene          | 93.05    | 11.38    | 10–1320             | 2610 ± 45           | 0.997 | 43         | 7.8      | 3           |
| 3  | β-Pinene          | 93.15    | 11.48    | 5–1370              | 3282 ± 83           | 0.996 | 31         | 6.7      | 1           |
| 4  | p-Cymene          | 119.15   | 13.67    | 15–1300             | 10,817 ± 96         | 0.997 | 56         | 7.5      | 1           |
| 5  | Limonene          | 68.10    | 13.83    | 10–1270             | 5421 ± 32           | 0.998 | 38         | 5.7      | 1           |
| 6  | Linalool          | 71.10    | 17.23    | 10–1300             | 3437 ± 15           | 0.998 | 51         | 7.4      | 8           |
| 7  | α-Thujene         | 81.15    | 17.49    | 15–1530             | 2235 ± 12           | 0.998 | 71         | 9.3      | 6           |
| 8  | Camphor           | 95.15    | 19.29    | 10–1400             | 1875 ± 34           | 0.999 | 40         | 7.8      | 2           |
| 9  | Menthol           | 71.10    | 20.66    | 10–1425             | 2788 ± 81           | 0.999 | 43         | 7.8      | 2           |
| 10 | Methyl chavicol   | 148.2    | 21.84    | 10–1370             | 9969 ± 161          | 0.998 | 44         | 7.2      | 2           |
| 11 | Caryophyllene E   | 69.15    | 31.42    | 5–1470              | 1733 ± 35           | 0.999 | 34         | 7.7      | 2           |
| 12 | α-Humulene        | 93.15    | 32.85    | 5–1350              | 5196 ± 69           | 0.998 | 29         | 7.0      | 2           |
sample had already reduced the sensitivity; therefore, for my analysis, the SPME headspace sampling was carried out at 60 °C while mixing the sample. The optimal sampling time was determined by sampling 1, 2, 5, 10, 20, 30, 40 and 50 min of terpene-spiked goat milk headspace and by plotting each terpene normed peak area’s dependence from sampling time (Fig. 2).

In the case of milk fat samples, it can be well seen (Fig. 1) that volatile β-pinene reached its peak, that is, its equilibrium state after 5 min, camphor after 10, α-thujene after 20 and caryophyllene after 40 min. Surprisingly, the equilibrium state of p-cymene, which is more volatile than caryophyllene, reached the equilibrium state only after 50 min. Although α-humulene did not reach full equilibrium state even after 50 min, longer sampling time did not significantly increase sensitivity, but it has significantly reduced the productivity of the measurements. Considering the above, the 50-min sampling time was chosen for milk fat samples.

In the case of spiked raw goat milk, it can be well seen on Fig. 2 that more volatile β-pinene, camphor, α-thujene, and p-cymene reached their peaks after 5, 10, 20, and 50 min, respectively.

Fig. 1 Dependence of GC–MS normed peak area of volatile components on sampling time (T = 80 °C) in cattle milk fat SPME headspace sampling

Fig. 2 Dependence of GC–MS peak area of volatile components on sampling time (T = 60 °C) in goat milk SPME headspace sampling
α-pinene, sabinene, p-cymene, limonene, linalool and menthol already reached the maximum of its peak area after 20 min that is its equilibrium state. Not surprisingly, the less volatile methyl chavicol, caryophyllene and humulene reached its equilibrium state only after 40 min. Nevertheless, the 60-min sampling time was chosen in the case of goat milk samples, which ensured adequate repeatability only with the used sampling time, which was probably due to the sensitivity of a small amount of terpene compound found in milk samples to environmental parameters.

### Terpenes in the Milk Fat of Cows Fed with a Mixture of Different Herbs

During the analysis of the milk fat content of the milk samples, among the volatile components of herbs, α-pinene and α-humulene from 28, limonene from 26, p-cymene from 16, caryophyllene E from 13, β-pinene from 11, sabinene from 9, methyl chavicol from 8, and camphor from 4 samples were quantified. In milk fat, the highest concentration was identified in caryophyllene E (470 ng/g) and α-humulene (430 ng/g), while the lowest concentration was in p-cymene (2 ng/g) and camphor (2 ng/g) (Table 5). The presence of most of the identified components has already been reported in the case of milk or cheeses (Dumont and Adda, 1978; Guichard et al. 1987; Buchin et al. 1999; Viallon et al. 1999, 2000; Fernandez et al. 2003). Borge et al. (2016) compared the terpene content of creams made from the milk of grazed and barn-fed cows. Samples from barn-fed and grazed cows also showed α-pinene and limonene; however, the amount of α-pinene was significantly higher in the samples of grazed individuals, while the amount of limonene was significantly higher in the samples of individuals fed in the barn. Based on the results in the literature (Buchin et al. 1999) and ours, it can be stated that limonene is one of the most characteristic monoterpene in milk and dairy products as it is present in most plants.

### Volatile Terpenes in Goats’ Milk Fed with Various Herbs

The proportion of mono- and sesquiterpene compounds in milk depends on the composition of the same compounds in the animal feed (Viallon et al. 2000; Bugaud et al. 2001a). However, the quantitative and qualitative analyses of the terpene components until now could only be made from the fat fraction separated from the raw milk. However, the new HS–SPME–GC–MS analytical method was developed to allow the quantitative and qualitative quantitation of terpenoids directly from the raw milk sample matrices; thus, the analysis does not require any preliminary sample preparation procedure. Terpene concentrations and standard deviation (ng/g) of goat milk samples from goat feeding experiments are shown in Table 6.

De Noni and Battelli (2008) also found that the botanical composition of different pastures causes changes in the terpene profile of milk and cheeses. This result is consistent with the results presented that the terpene composition of milk is changed when cows and goats are fed different herbs.

### Volatile Terpene Content in Blood Plasma of Dairy Cattle Fed with Herbs Spiked Feed and in Cheese Made from Herbal Milk

In the case of blood plasma samples, there is a high probability of precipitation of plasma proteins above 40 °C that is why were performed headspace sampling at the highest temperature which is still considered safe at 40 °C. The terpenes identified and defined in the plasma of cows fed with herbs and their retention times are shown in Table 7.

Boyle et al. (2002) examined the amount of cineole in the blood of brushtail possums which consuming eucalyptus using the SPME sampling procedure.
Malecky et al. (2012) examined the terpenoid content of goat blood plasma by dynamic SPME sampling. In both studies, it was found that the consumed food has an effect on the quantity and quality of terpenes in the blood of animals.

### Terpenes in Cheese Samples

The chemical analysis by the HS-SPME method results of fresh and matured natural and herbal Trappist cheese of 2 cows fed with herbal feed are given in Table 8, where the compounds are listed in order of their elution from the RTX-5 column. HS–SPME–GC–MS analysis led to the identification of 6 terpenes components (alpha-pinene, myrcene, limonene, gamma-terpinene, linalool and alpha-humulene) from the fresh natural, and 12 terpenes from fresh herbal Trappist cheese. Compared to the control sample (fresh natural cheese), the fresh herbal Trappist cheese contained the previous terpenes and also six more as alpha-thujene, alpha-pinene, beta-pinene, p-cymene, limonene, 1,8-cineole, alpha-thujone, beta-thujone, camphor, trans-pinocamphone, cis-pinocamphone and alpha-terpinene.

### Table 6 Terpene concentration of herbal goat milk samples and standard deviation of results (ng/g), (n = 3)

| No | Volatile component | Concentration ± standard deviation (n = 3), µg/g |
|----|--------------------|--------------------------------------------------|
|    |                    | Control  | Milfoil  | Sage    | Woodruff | Camomile | Tarragon | Plantain |
| 1  | alpha-Pinene       | 2.05 ± 0.06 | n.d       | 15.06 ± 0.06 | 1.47 ± 0.09 | 13.14 ± 0.07 | 3.35 ± 0.03 | 7.23 ± 0.05 |
| 2  | Sabinene           | 2.02 ± 0.09 | n.d       | 5.19 ± 0.09 | n.d       | 6.32 ± 0.06 | n.d       | 3.47 ± 0.04 |
| 3  | beta-Pinene        | n.d       | n.d       | 7.23 ± 0.05 | 1.32 ± 0.08 | 7.19 ± 0.04 | 1.28 ± 0.09 | 2.41 ± 0.03 |
| 4  | p-Cymene           | 2.11 ± 0.09 | n.d       | 34.42 ± 0.09 | 1.15 ± 0.09 | 40.35 ± 0.09 | 1.12 ± 0.05 | 23.39 ± 0.14 |
| 5  | Limonene           | 13.15 ± 0.07 | 1.32 ± 0.08 | 53.36 ± 0.07 | 2.31 ± 0.06 | 57.25 ± 0.02 | 1.07 ± 0.02 | 32.49 ± 0.10 |
| 6  | Linalool           | n.d       | n.d       | n.d       | n.d       | n.d       | n.d       | n.d       |
| 7  | alpha-Thujene      | n.d       | 18.47 ± 0.08 | 11.29 ± 0.06 | 13.28 ± 0.03 | 15.33 ± 0.04 | 13.26 ± 0.09 | 20.17 ± 0.01 |
| 8  | Camphor            | n.d       | 2.32 ± 0.08 | n.d       | 2.22 ± 0.03 | n.d       | n.d       | n.d       |
| 9  | Menthol            | n.d       | n.d       | n.d       | n.d       | n.d       | n.d       | n.d       |
| 10 | Methyl chavicol    | n.d       | 2.09 ± 0.06 | n.d       | n.d       | n.d       | n.d       | n.d       |
| 11 | Caryophyllene E    | n.d       | n.d       | n.d       | n.d       | 2.28 ± 0.10 | n.d       | n.d       |
| 12 | alpha-Humulene     | 1.24 ± 0.09 | n.d       | 3.17 ± 0.09 | n.d       | 2.48 ± 0.08 | n.d       | n.d       |

n.d., not detected

### Table 7 Terpenes (+) and retention time of terpenes identified by HS–SPME–GC–MS technique in the blood plasma of cows fed with various herbs

| No | Volatile component | t_R (min) | Control | Milfoil | Hyssop, sage, woodruff | Camomile | Goat’s rue | Ribgrass | Hay with wild thyme |
|----|--------------------|-----------|---------|---------|------------------------|----------|------------|----------|-------------------|
| 1  | alpha-Thujene      | 7.42      | -       | +       | -                      | -        | +          | -        | -                |
| 2  | alpha-Pinene       | 7.65      | +       | +       | +                      | +        | +          | +        | +                |
| 3  | Camphene           | 8.15      | +       | -       | +                      | -        | -          | -        | -                |
| 4  | Sabinene           | 9.00      | -       | -       | +                      | -        | +          | -        | +                |
| 5  | beta-Pinene        | 9.10      | -       | -       | +                      | -        | -          | +        | +                |
| 6  | p-Cymene           | 10.97     | -       | +       | -                      | +        | +          | -        | +                |
| 7  | Limonene           | 11.10     | +       | +       | +                      | -        | +          | +        | +                |
| 8  | 1,8-Cineole        | 11.23     | -       | +       | -                      | -        | -          | -        | -                |
| 9  | alpha-Thujone      | 15.22     | -       | +       | -                      | -        | -          | -        | -                |
| 10 | beta-Thujone       | 15.69     | -       | +       | -                      | -        | -          | -        | -                |
| 11 | Camphor            | 16.18     | -       | +       | -                      | -        | -          | -        | -                |
| 12 | Trans-pinocamphone | 16.87     | -       | +       | -                      | -        | -          | -        | -                |
| 13 | Cis-pinocamphone   | 17.50     | -       | +       | -                      | -        | -          | -        | -                |
| 14 | alpha-Terpinol     | 18.32     | -       | +       | -                      | -        | -          | -        | -                |
| 15 | Bornil-acetate     | 22.50     | -       | +       | -                      | -        | -          | -        | -                |
| 16 | Caryophyllene E    | 28.22     | -       | +       | -                      | -        | -          | -        | +                |
| 17 | alpha-Humulene     | 29.67     | +       | -       | +                      | -        | +          | +        | -                |
sabinene, beta-pinene, alpha-phellandrene, para-cymene and caryophyllene E (Figs. 3 and 4).

Comparing the area proportions of the chromatography peaks of fresh natural and herbal Trappist cheese, it was realised that six compounds had a significant increase in the peak area of the alpha-pinene, myrcene, limonene, gamma-terpinene, linalool and alpha-humulene in the case of fresh herbal Trappist cheese. The results prove that the amount of the identified volatile components in the test cheese (herbal Trappist cheese) increased by approximately two to three times compared to the control sample (natural Trappist cheese).

In the case of ripened cheeses, it was also observed that the control samples contained twice or three times less terpene than the herbal cheese samples. Comparing the content of terpenes in fresh and matured Trappist cheeses, it was determined that the content of terpenes in matured cheeses has increased significantly, because cheeses lose water during ripening, resulting in a more concentrated texture and a higher concentration of analytes. Therefore, previously undetectable terpene compounds appeared in matured cheeses (e.g. bornyl-acetate). In some cases, terpenes disappeared during the ripening, probably because they were...

Table 8 Peak areas of the volatile terpenes identified in the SIM ion chromatogram (m/z 93 + 119) recorded by HS–SPME–GC–MS technique of headspace of fresh and 6-week maturation of natural and herbal Trappist cheeses, as well as peak area ratios of herbal and natural cheeses (RSd 10%)

| No | Volatile component | Natural Trappist (×10^5) | Herbal Trappist (×10^5) | Area proportions (Gy/N) | Mature natural Trappist (×10^5) | Mature herbal Trappist (×10^5) | Area proportions (Gy/N) |
|----|--------------------|--------------------------|-------------------------|-------------------------|-------------------------------|-------------------------------|-------------------------|
| 1  | α-Thujene           | n.d                      | 0.57                    | -                       | n.d                           | n.d                           | -                       |
| 2  | α-Pinene            | 0.67                     | 9.49                    | 14.16                   | 2.96                          | 6.49                          | 2.19                    |
| 3  | Sabinene            | n.d                      | 1.46                    | -                       | 0.49                          | 0.75                          | 1.53                    |
| 4  | β-Pinene            | n.d                      | 3.34                    | -                       | 1.50                          | 4.71                          | 3.14                    |
| 5  | Myrcene             | 1.95                     | 0.56                    | 0.29                    | 10.2                          | 20.7                          | 2.03                    |
| 6  | α-Phellandrene      | n.d                      | 0.99                    | -                       | n.d                           | n.d                           | -                       |
| 7  | p-Cymene            | n.d                      | 0.99                    | -                       | 11.9                          | 30.2                          | 2.54                    |
| 8  | Limonene            | 40.3                     | 11.5                    | 0.29                    | 255                           | 750                           | 2.94                    |
| 9  | γ-Terpinene         | 1.78                     | 1.01                    | 0.57                    | 2.69                          | 8.75                          | 3.25                    |
| 10 | Linalool            | 0.94                     | 0.48                    | 0.51                    | 1.60                          | 3.72                          | 2.33                    |
| 11 | t-Pinocamphone      | n.d                      | n.d                     | -                       | 6.08                          | 15.4                          | 2.53                    |
| 12 | c-Pinocamphone      | n.d                      | n.d                     | -                       | 9.10                          | 29.6                          | 3.25                    |
| 13 | Bornyl-acetate      | n.d                      | n.d                     | -                       | 0.32                          | 3.75                          | 11.7                   |
| 14 | Caryophyllene E     | n.d                      | 5.55                    | 8.72                    | 1.20                          | 1.33                          | 1.11                    |

n.d., not detected
decayed (e.g. alpha-thujene, alpha-phellandrene and caryophyllene E).

Delgado et al. (2011) investigated the terpene composition of goat cheeses by the HS–SPME–GC–MS method. The DIV/CAR/PDMS fibre recommended by the manufacturer was used for sampling. Among the terpenes we examined, they were able to detect limonene and cymol. Ziino et al. (2005) examined “Provola dei Nebrodi” cheeses by the SPME–GC–MS method and identified limonene as a typical terpene compound in the cheeses. According to Viallon et al. (1999), hydrocarbons, especially limonene, are derived directly from cow feed into cheeses. This statement also supports the conclusion that the said monoterpenic is also present in cheeses made from the milk of control individuals. Chion et al. (2010) examined the terpene profile of “Toma piemontese” cheeses depending on the seasons. The terpene composition of cheeses made in different seasons showed a difference, and the terpene profile of the cheeses reflected the terpene profile of the cheese milk, thus proving that terpenes can be transferred from milk to cheeses.

Conclusions

Based on this study, it can be clearly stated that terpene solutions of herbal origin may pass from the dairy animals’ blood not only into the animals’ milk and their milk fat but also into the cheese prepared from their milk.

The developed GC–MS method is suitable for the separation and identification of volatile terpenes in milk and milk products by the SIM method. We examined the dependence of the amount of terpenes dissolved in the SPME film on the sampling temperature and its time during SPME sampling. We determined the optimal sampling temperature and time for the SPME procedure, and the sensitivity, repeatability and limit of detection of our developed SPME–GC–MS method for each terpene. Our results demonstrate that there has been a significant increase in the number of terpene compounds in milk derived from cows or goats with consuming herbs. In the study, we successfully determined the concentrations of terpene compounds from the milk fat of cows as well as directly from goat milk by the SPME–GC–MS method.

It also verifies that during herbal nutrition, many active ingredients pass through the blood plasma into the milk of the dairy animals and the dairy products made from them. That is how it was proven as well that by feeding with the inclusion of herbs, it is possible to produce such raw materials for processing plants, by the developed and applied analytical procedure, which favourably affects the characteristics of dairy products, particularly of cheeses.

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B. Sik: data processing.
E. Hanczné Lakatos: project manager, head of department.
Zs. Ajtony: supervisor.

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Declarations

Conflict of Interest Rita Székelyhidi declares that she has no conflict of interest. Erika Hanczé Lakatos declares that she has no conflict of interest. Beatrix Sik declares that she has no conflict of interest. Zsolt Ajtony declares that he has no conflict of interest.

Informed Consent Not applicable.

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Food Analytical Methods (2021) 14:2585–2596 2595
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