THESES OF DOCTORAL (PhD) DISSERTATION

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MOSONMAGYARÓVÁR

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VOLATILE HERBAL INGREDIENTS IN FORAGE OF RUMINANTS, BLOOD PLASMA, MILK, MILK FAT AND CHEESES OF DOMESTIC ANIMALS

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1. INTRODUCTION AND AIMS

Grazing dairy herd have access to herbs and spices by feeding, under controlled circumstances, which make positive changes in animal health, and, on the other hand, proven by laboratory tests, we can expect changes in the milk's nutrition values and taste.

In this work, I followed from herbal nutrition to cheese making, the presence of active ingredients from the active ingredient substance of the spiked plants, through the analysis of extract milk, until the examination of test products made from the milk. I chose several herbs and spices which have favourable smell, aroma and physiological effect based on literature and folk observations.

I set a goal to develop 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 can be determined, derived from herbs eaten by the dairy animals by different feeding methods. I also wanted to verify, that during herbal nutrition many active ingredients pass through the plasma into the milk of the dairy animals and the dairy products made from them. That is how I proved as well, that by feeding with the inclusion of herbs, it is possible to produce such raw materials for processing plants, proven by the developed and applied analytical procedure, which favourably affects the characteristics of dairy products – particularly of cheeses.

2. EXAMINATIONS

2.2. Sampling and sample preparation procedures

My 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 Agricultural and Food Sciences, Analytical Laboratory of Department of Food Science. Samples from dairy cattle came from the premises of Bicskei Mezőgazdasági Zrt. where the experimental animals were driven to pastures with various herbs and by grazing herbs there, 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 and that's when the blood was taken as well. Cheese samples analyzed in this dissertation were made from the herbal milk of these animals by the staff of Bicskei Mezőgazdasági Zrt. The goat feeding experiments were carried out at the farm of József Csató farmer at Kerta. In this experiment, I 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 g 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 at the laboratory and were processed and analysed.

The sample preparation of blood samples 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 hour at 5500 g in a sealed sampling tube (3K, Sigma). Supernatant plasma was stored at -20 °C until use.

To store the milk samples for a shorter time (2-3 days), they were preserved with adding sodium azide in 1g/L concentration at the time of arrival. For longer storage (>3 days) samples were stored at -20 °C until use.

Separate the milk fat from the milk the two-step centrifugation procedure was applied (Abilleira és mtsai; 2010). During the first step, 80 grams of preserved milk was centrifuged with 3 300 g at 5 °C for 15 minutes. Supernatant cream was further centrifuged (second step) in a sealed centrifugation tube at 35 °C with 5 500 g for 2.5 hours (3K, Sigma) and then supernatant milk fat was stored at -20 °C until use.

The fat content of the butter sample from retail trade was separated from the aqueous phase after thawing and centrifugation at 35 °C at 5 500g for 1 hour (3K, Sigma).

After received the goat milk samples 5-5 g were measured in a 24 mL vials with screw cap and stored at -20 °C until further use. To avoid the cross contaminations the vials were heated previously at 150 °C for 1 hours.

2.3. Solid-phase microextraction 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 (57330-U) was used. To analyse the volatile compounds of herbs and feeds a 1 cm long and 100 µm PDMS-coated fiber 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 PDMS-coated (DVB/CAR/PDMS) StableFlex fiber was used, as recommended by the manufacturer (Supelco) for the trace analysis of

volatile compounds. To remove the volatile components trapped in the SPME fibers from the air during storing, the fibers were heated at 260 °C for 15 minutes in our GC-MS injector. Samples were taken according to the data of Table 1.

Table 1 Conditions used during SPME sampling for herbs, blood plasma, cow's milk and cheese.

	Herb	**Plasma**	**Milk fat**	**Goat milk**	**Cheese**
SPME fiber:	1 cm, 100 μm PDMS	2 cm, 50/30μm DVB/CAR/PDMS			
Volume of the vial:	43 mL	24 mL			
Mass of the sample:	2 g	5 g			
Pretreatment and sampling temperature:	55 °C	40 °C	80 °C	60 °C	60 °C
Pretreatment time:	50 min				
Sampling time:	5 min	60 min	50 min	60 min	60 min
Mixing:	none	none	none	600 1/min	none

2.4. Gas chromatography-mass spectrometry (GC-MS) system

To analyse the volatile components of herbs, GCQ (Finnigan MAT) type, ion trap, 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.

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 program	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		

2.5. Optimization of SPME sampling conditions

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

For butter-milk sample the optimal headspace sampling temperature was determined by the headspace analysis of 5-5g 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 minutes, while from headspace of spiked raw goat milk at 60 °C and for 1, 2, 5, 10, 15, 20, 40, and 60 minutes.

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 minutes of terpene-added milk fat headspace and plotting of each terpene normed peak area's dependence from sampling time (Figure 1). It can be well seen here, that volatile *β*-pinene reached its peak, that is its equilibrium state after 5 minutes, camphor after 10, *α*-thujene after 20, and caryophyllene after 40 minutes. Surprisingly, the equilibrium state of *p*-cymene, which is more volatile than caryophyllene reached equilibrium state only after 50 minutes. Although *α*-humulene did not reach full equilibrium state even after 50 minutes, however, longer sampling time did not significantly increase sensitivity, but it has significantly reduced the productivity of my measurements. Considering the above, I choose the 50 minutes sampling time for milk fat samples.

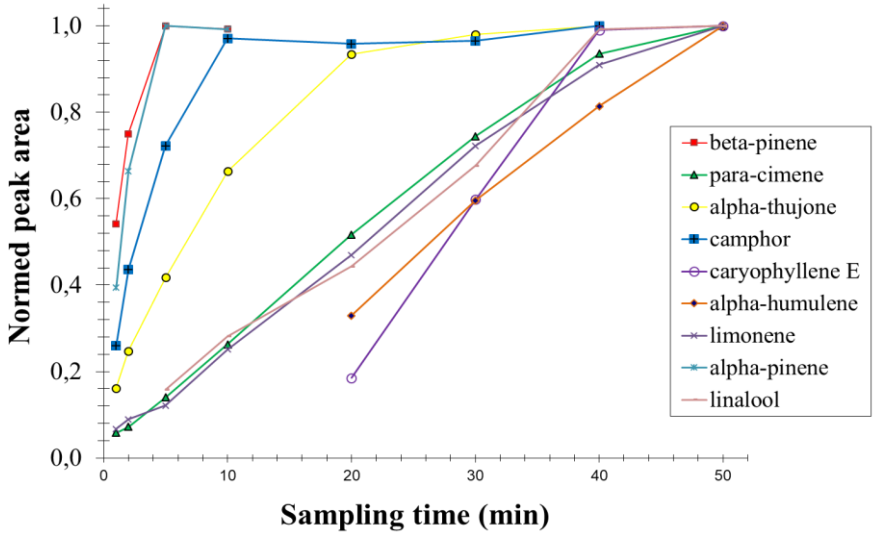

Figure 1 Dependence of GC-MS normed peak area of volatile components on sampling time (T=80°C) in cattle milk fat SPME headspace sampling.

In 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 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 minutes of terpene-spiked goat milk headspace and by plotting of each terpene normed peak area's dependence from sampling time (Figure 2). It can be well seen on the figure, that more volatile *β*-pinene, camphor, *α*-thujene, *α*-pinene, sabinene, *p*-cymene, limonene, linalool and menthol already reached the maximum of its peak area after 20 minutes that is its equilibrium state. Not surprisingly, the less volatile methyl

chavicol, caryophyllene and humulene reached its equilibrium state only after 40 minutes. Nevertheless, I chose the 60 minute sampling time in case of goat milk samples, since I could ensure the adequate repeatability only with this sampling time, which was probably due to sensitivity of small amount of terpene compound found in milk samples to environmental parameters.

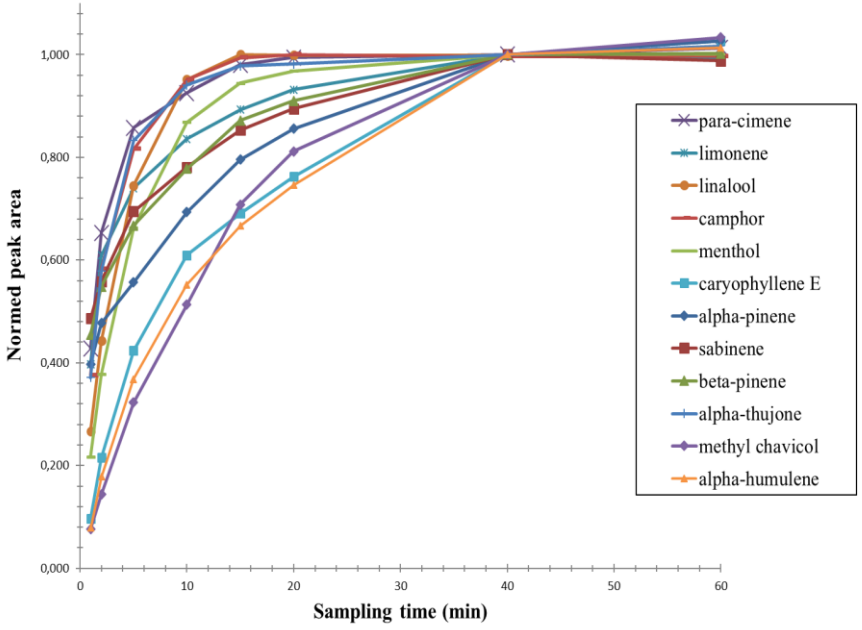

Figure 2 Dependence of GC-MS peak area of volatile components on sampling time (T=60°C) in goat milk SPME headspace sampling.

2.6. Calibration of the measuring equipment

The calibration of the developed GC-MS method with standard addition procedure was carried out by analysing of 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 I weighted 5-5 g of milk fat obtained from butter from retail trade and 5-5 g of raw goat milk. I melted the fats in a 40 °C water bath, while directly after weighting 50-50 µl of 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 Rx^3, Italy). I took samples from the headspace of the spiked milk fat samples at 80 °C with a sampling time of 50 minutes, whereas from spiked raw goat milk samples at 60 °C with sampling time of 60 minutes, and I took the sampling SPME fiber into the injector of the GC-MS instrument. I draw a straight line 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 I determined the sensitivity of the method and the lower limit of quantitation (LOQ).

$$LOQ = 10 \times SD/m \qquad (1)$$

Where SD is the standard deviation of the areas below the peak,

m is the slope of the fitted calibration line.

For qualitative determination of volatile terpenes in milk fat and raw goat milk samples I compared the retention time of volatile components and their mass spectrum recorded in full-scan mode 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 I calibrated the measuring equipment in SIM mode, with standard addition method, in case of milk fat for eleven and in 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.

Table 3 Mass to charge ratio (m/z), retention times (t_R), 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.

N°	Volatile component	m/z Th	t_R (min)	Linear range (ng/g)	Slope ±SD (g/ng)	R
1.	***α*-pinene**	93	7.63	10-1000	4550 ± 64	0,999
2.	**sabinene**	93	9.02	5-770	3472 ± 38	0,999
3.	***β*-pinene**	93	9.13	10-1230	2857 ± 74	0,997
4.	***p*-cymene**	119	11.00	10-1000	6216 ± 86	0,999
5.	**limonene**	93	11.14	10-1000	2007 ± 36	0,998
6.	**linalool**	93	14.25	10-1000	945 ± 12	0,999
7.	***α*-thujene**	110	14.48	10-1000	797 ± 14	0,998
8.	**camphor**	95	16.19	10-1000	1418 ± 26	0,998
9.	**methyl chavicol**	148	18.61	10-1000	6640 ± 188	0,994
10.	**caryophyllene E**	133	28.06	20-2000	276 ± 8	0,994
11.	***α*-humulene**	93	29.50	20-2000	1576 ± 42	0,995

The repeatability of my 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 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 4. Repeatability was less than 10% with the except for methyl chavicol. The estimated limits of quantitation were between 2-16 ng/g. The lowest values were 2 ng/g for *p*-cymene and methyl chavicol, the highest value was 16 ng/g for caryophyllene.

Table 4 Analytical performance data for headspace analysis of dairy milk fat samples in SPME (C_{ad} – 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).

N°	Volatile component	C_{ad} (ng/g)	RSD (%)	LOQ (ng/g)
1.	***α*-pinene**	46	3.2	3
2.	**sabinene**	23	6.6	3
3.	***β*-pinene**	141	3.6	4
4.	***p*-cymene**	65	9.5	2
5.	**limonene**	143	9.8	4
6.	**linalool**	98	6.1	8
7.	***α*-thujene**	66	8.6	6
8.	**camphor**	47	6.7	4
9.	**methyl chavicol**	16	12.9	2
10.	**caryophyllene E**	388	2.5	16
11.	***α*-humulene**	136	3.4	6

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 5.

During the calibration with goat milk the highest sensitivity was for *p*-cymene (10817 ± 96 g/ng) and methyl chavicol (9969 ± 161g/ng), 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.

Table 5 Mass to charge ratio (m/z) , retention times (t_R), linear ranges of analytical measurement curves, average slope and standard deviation (±SD), and correlation coefficients (R) of ions used for GC-MS quantitation of terpenes in case of goat milk samples.

N°	Volatile component	m/z (Th)	t_R (min)	Linear range (ng/g)	Slope (±SD) (g/ng)	R
1	***α*-pinene**	93.10	9.70	5-1040	3115 ± 71	0.996
2	**sabinene**	93.05	11.38	10-1320	2610 ± 45	0.997
3	***β-pinene***	93.15	11.48	5-1370	3282 ± 83	0.996
4	***p-cymene***	119.15	13.67	15-1300	10817 ± 96	0.997
5	**limonene**	68.10	13.83	10-1270	5421 ± 32	0.998
6	**linalool**	71.10	17.23	10-1300	3437 ± 15	0.998
7	***α*-thujene**	81.15	17.49	15-1530	2235 ± 12	0.998
8	**camphor**	95.15	19.29	10-1400	1875 ± 34	0.999
9	**menthol**	71.10	20.66	10-1425	2788 ± 81	0.999
10	**methyl chavicol**	148.2	21.84	10-1370	9969 ± 161	0.998
11	**caryophyllene E**	69.15	31.42	5-1470	1733 ± 35	0.999
12	***α*-humulene**	93.15	32.85	5-1350	5196 ± 69	0.998

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 6. In case of goat milk the repeatability was better than 8% with the except for *α*-thujene. The estimated limits of quantitation were between 1-8 ng/g. The lowest values were 1 ng/g for *β*-pinene, *p*-cymene and limonene, the highest value was 8 ng/g for linalool.

Table 6 Analytical performance data for headspace analysis of spiked dairy milk fat samples in SPME (C_{ad} – 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).

N°	Volatile component	C_{ad} (ng/g)	RSD (%)	LOQ (ng/g)
1	***α*-pinene**	29	7.5	3
2	**sabinene**	43	7.8	3
3	***β-pinene***	31	6.7	1
4	***p-cymene***	56	7.5	1
5	**limonene**	38	5.7	1
6	**linalool**	51	7.4	8
7	***α*-thujene**	71	9.3	6
8	**camphor**	40	7.8	2
9	**menthol**	43	7.8	2
10	**methyl chavicol**	44	7.2	2
11	**caryophyllene E**	34	7.7	2
12	***α*-humulene**	29	7.0	2

2.7. 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, 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 7).

Table 7 Number of samples containing the tested terpenes in concentrations greater than the limit of detection (N), minimum, maximum, mean, standard deviation, and median of terpene concentrations in milk fat

	Terpene concentrations (ng/g)					
	N	**Min**	**Max**	**Mean**	**Standard deviation**	**Median**
α-pinene	28	4	79	18	16	17
sabinene	9	2	28	11	10	10
β-pinene	11	3	104	36	31	33
p-cymene	16	2	9	4	2	3
limonene	26	3	114	38	28	33
camphor	4	3	7	4	2	3
metil-chavicol	8	2	39	8	13	10
caryophyllene E	13	39	470	127	116	121
α-humulene	28	3	430	46	83	64

2.8. 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., 2001). However, the quantitative and qualitative analysis 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 I have developed allows 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 8.

Table 8. Terpene concentration of herbal goat milk samples and standard deviation of results (ng/g), (n=3).

N	Volatile component	Concentration ± standard deviation (n=3), μg/g						
		Control	Milfoil	Sage	Woodruff	Camomile	Tarragon	Plantain
1.	α-pinene	2.05 ± 0.06	–	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	–	5.19 ± 0.09	–	6.32 ± 0.06	–	3.47 ± 0.04
3.	β-pinene	–	–	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	–	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	–	–	–	–	–	–	–
7.	α-thujene	–	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	–	2.32 ± 0.08	–	2.22 ± 0.03	–	–	–
9.	menthol	–	–	–	–	–	–	–
10.	methyl chavicol	–	–	2.09 ± 0.06	–	–	–	–
11.	caryophyllene E	–	–	–	–	–	2.28 ± 0.10	–
12.	α-humulene	1.24 ± 0.09	–	3.17 ± 0.09	–	2.48 ± 0.08	–	–

2.9. Volatile terpene content in blood plasma of dairy cattle fed with herbs spiked feed and in cheese made from herbal milk

2.9.1. Blood plasma samples

In case of blood plasma samples there is a high probability of precipitation of plasma proteins above 40 °C that is why I performed the 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 9.

Table 9 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	Rib-grass	Hay with wild thyme
1.	**α-thujene**	7.42	–	+	–	–	+	–	–
2.	**α-pinene**	7.65	+	+	+	+	+	+	+
3.	**camphene**	8.15	+	–	+	–	–	–	–
4.	**sabinene**	9.00	–	–	+	–	+	–	+
5.	**β-pinene**	9.10	–	–	+	–	+	–	+
6.	**p-cymene**	10.97	–	+	–	+	+	–	+
7.	**limonene**	11.10	+	+	+	–	+	+	+
8.	**1,8-cineole**	11.23	–	+	–	–	–	–	–
9.	**α-thujone**	15.22	–	+	–	–	–	–	–
10.	**β-thujone**	15.69	–	+	–	–	–	–	–
11.	**camphor**	16.18	–	+	–	–	–	–	–
12.	**trans-pinocamphone**	16.87	–	+	–	–	–	–	–
13.	**cis-pinocamhone**	17.50	–	+	+	–	–	–	–
14.	**α-terpineol**	18.32	–	+	+	–	–	–	–
15.	**bornil-acetate**	22.50	–	+	+	–	–	–	–
16.	**caryophyllene E**	28.22	–	–	+	–	–	–	+
17.	**α-humulene**	29.67	+	–	+	–	+	+	–

2.9.2. Cheese samples

During the qualitative analysis of the volatile terpene content of cheeses, the number of volatile terpenes increased in fresh cheeses made from milk of animals consuming herbal feed, from 3 to 7 in case of curd cheese, from 5 to 11 in case of Port Salut cheese, as a result of herbal nutrition. The number of volatile terpenes found in six-week matured herbal and control (natural) Port Salut cheese increased to 12-12 from the previous 5 and 11 respectively, probably due to increased concentration due to water loss during ripening. The results prove that the herbal feed supplement increased the concentration of the identified volatile components in the test cheese (herbal Port Salut cheese) by approximately two to three times compared to the control sample (natural Port Salut cheese).

Table 10 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 six-week maturation of natural and herbal Port Salut cheeses, as well as peak area ratios of herbal and natural cheeses (RSd 10%).

Nº	Volatile component	Natural Port Salut ($x10^5$)	Herbal Port Salut ($x10^5$)	Area proportions (H/N)	Mature natural Port Salut ($x10^5$)	Mature herbal Port Salut ($x10^5$)	Area proportions (H/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.	**bornil-acetate**	n.d.	n.d.	-	0.32	3.75	11.7
14.	**caryophyllene E**	n.d.	5.55	-	n.d.	n.d.	-
15.	**α-humulene**	0.50	4.36	8.72	1.20	1.33	1.11

n.d. – not detected.

3 . NEW SCIENTIFIC RESULTS

1. I developed a gas chromatographic-mass spectrometry (GC-MS) procedure to separate the 12 terpene components that can be found in food matrices, with the right choice and optimization of injection temperature of our gas chromatograph for their highly sensitive and selective identification, of its desorption time, of carrier gas flow rate, of column temperature program, furthermore of the temperature of the ion trap connected to the gas chromatograph and later on of the temperature of the ion source mass spectrometer equipped with quadruple analyser, of scan speed, of mass and time of ions scanned.
2. I developed a headspace (HS) sampling solid-state microextraction (SPME) method for the determination of the mono- and sesquiterpene content of milk fat. Measured by the previously developed GC-MS technique, using HS-SPME sampling, I determined from the headspace of milk fat spiked with 12 mono- and sesquiterpenes the dependence of sampling time and temperature of the quantity of terpenes absorbed in the SPME fiber coating, furthermore, the optimal temperature and time of HS-SPME sampling of milk fat.
3. I validated the applicability of my developed analytical HS-SPME-GC-MS technique for terpene concentration of milk fat by determination of performance characteristics (limit of detection, linear range, sensitivity, repeatability) obtained by analysing milk fat spiked in various concentrations with methanol standard solution of total 12 mono- and sesquiterpenes.
4. I determined the terpene concentration in the milk fat of the 28 milk samples originated from cattle fed with herbal feed by the developed HS-SPME-GC-MS method. Compared to the control

sample, I showed α-pinene in 28, sabinene in 9, β-pinene in 11, *p*-cymene in 16, limonene in 26, camphor in 4, methyl chavicol in 8 samples of the cows' milk fat, thus, proving, that certain terpene components of the cows' feed pass into the milk of the animals during lactation.

5. For determining the mono- and sesquiterpene concentration of goats' milk fed with herbal feed I developed an additionally new HS-SPME sampling technique. I placed the SPME fiber into the sealed headspace of raw goat milk spiked with terpene solutions and during continuous mixing of milk samples I measured the dependence of terpene concentration dissolved in the coating of the SPME fiber on the sampling time and temperature with the help of the developed GC-MS procedure. I determined the optimal temperature and time of HS-SPME sampling of raw goat milk.
6. In order to verify that the HS-SPME-GC-MS analytical technique for analysis of goat milk samples is proper, I determined the linear range, sensitivity, repeatability and limit of detection of my analytical method from the HS-SPME-GC-MS analysis of goat milk samples spiked in various concentrations of methanol standard solution of total 12 mono- and sesquiterpene compound.
7. I determined the terpene concentration in the milk of 6 goats fed with herbal feed with the HS-SPME-GC-MS technique developed for goat milk sample matrix. Compared to the control sample I showed α-pinene in 5, sabinene in 3, β-pinene in 5, *p*-cymene in 5, limonene in 6, α-thujone in 6, camphor in 2, methyl chavicol in 1, caryophyllene E in 1, α-humulene in 2 cases. I identified the most

terpene (8) in goat milk with sage, while the least (3) in goat milk with milfoil.

8. From the blood plasma of cows fed with herbal feed, with a new HS-SPME-GC-MS technique I was successful to show several terpene solutions originating from herbs, thus proving, that the terpenes may pass to the blood of dairy animal during herbal feeding. I was able to detect terpenes from cheese samples from the milk of cows fed with herbal feed with another, but also HS-SPME-GC-MS procedure. Based on these, it can be clearly stated, that terpene solutions of herbal origin may pass from the dairy animals' blood not only to the animals' milk and their milk fat but also into the cheese prepared from their milk.

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