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Changes in Terpene Composition from Pasture to Cheese*

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Introduction

Terpenes are secondary plant metabolites. They are important for the biological activity of plants and culinary herbs and are often key aroma compounds in essential oils. Some terpenes have been described as having antimicrobial activity (1, 2). For some years, terpenes have been used as "terroir" markers for the origin of dairy products such as milk and cheese. In particular, the terpene composition may allow the discrimination of highland and lowland produce and is therefore a valuable biomarker for products with protected designation of origin (PDO) (3, 4). The flora of highland pastures is highly diversified and comprises more dicotyledonous plants than lowland pasture. It is rich in terpenoids including monoterpenes such as β-myrcene, (E)-β-ocimene, limonene, γ-terpinene as well as α- and β-pinene (4). *Mariaca et al.* (5) showed that the lowland pastures contain more gramineae and are in general poor in terpenoids.

The aim of our study was to analyse the monoterpenoid composition in grass, milk, and in a Raclette-type cheese produced from the same milk, using purge and trap (P&T) gas chromatography-mass spectrometry (GC-MS). We were interested to investigate the impact of rumen fermentation on the monoterpenoid composition and the degradation products that are potentially transferred into milk and cheese. As a model we selected cow parsnip *(Heracleum sphondylium* L.), a plant belonging to the family of *Apiaceae,* which is rich in various terpenes and often found in pastures.

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Material and methods

Samples

Fresh grass of different botanical species (5) and cow parsnip was collected at different sites in Switzerland at altitudes between 1400 and 1920 m and at around 700 m, respectively. The samples were stored at –20 °C prior to use. Cow milk was from several farms located at the same sites and the Raclette-type cheese selected for analysis was produced from the same milk. Rumen fluid was obtained from 3 healthy cows aged 5–8 years, which had been fed with hay *ad libitum* for 14 days prior to the study. The cows had an artificial fistula connected to the rumen, which allows to take rumen fluid samples. These cows live, i.e. graze, and behave the same way as cows without artificial rumen fistula.

Analytical methods

Ground cow parsnip (2 g in 30 mL phosphate buffer pH 6.6 following (6)) was mixed with rumen fluid (15 mL) and incubated for 24 h at 39 °C in a shaking mixer under argon atmosphere to make sure that no oxygen impaired the fermentation.

The terpenes were extracted from the samples at 40 °C (water bath) employing a LSC 2000 P&T system (Tekmar, Cincinnati, USA) equipped with a trap no. 8 containing a mixture of Carbosieve SIII and Carbopack as described in (5). The conditions were as follows: purge gas nitrogen at 41 mL/min purge flow (vent); water bath 40 °C, purge 20 min; dry purge 11 min; cap cool down –150 °C; desorb 4 min at 240 °C; inject 1.5 min at 215 °C; bake 10 min at 260 °C; transfer line to GC 150 °C.

GC-MS was performed on a HP 5890 Series II instrument equipped with a 5971A MSD using a CP-Chirasil-Dex CB capillary column (Stehelin, CH-4003 Basel) with helium at 50 kPa, i.e. 1.2 mL/min flow at 35 °C. The temperature program was 35 °C (0.5 min hold) to 215 °C at 2.5 °C/min. The GC-MS transfer line was heated to 280 °C, the MSD operated in EI ionization mode at 70 eV and scanned from *m/z* 26 to 350 at 0.8 scans/s. The terpenes were identified on the basis of identical GC retention indices and mass spectra with the ones of authentic reference compounds. The terpene metabolites were tentatively identified by comparison of their mass spectra with the ones of mass spectra libraries. The ions listed in Table 1 were chosen for the comparison of the peak heights before and after fermentation with rumen fluid to follow the degradation of the terpenes. The ions indicated in Table 1 were selected to compare the peak heights before and after incubation with rumen fluid. The ion *m/z* 136 was chosen as qualifier ion for the monoterpenes.

The experiments were carried out over a period of several years. During this time, the chromatographic properties of the chiral capillary column were altered. The retention times and the elution order of some terpenes changed during the study, which led to co-elution of (Z)-β-ocimene and (+)-β-pinene, as well as (E)-β-ocimene and δ-3-carene, but to a separation of (+)-β-pinene and (+)-limonene and of (E)-β-ocimene and p-cymene in the rumen incubation study.

Results and discussion

Fresh grass was composed of plants of different botanical species, i. e. Apiaceae, Asteraceae, Campanulaceae, Fabaceae, Geraniaceae, Lamiaceae, Plantaginaceae, Poaceae, Polygonaceae, Ranunculaceae, Rosaceae, and Rubiaceae (5).

Dynamic headspace extraction (P&T) in combination with GC-MS using a chiral stationary phase was employed to analyse the monoterpene composition of the fresh grass sample. The most abundant monoterpenes detected in the grass sample were β-myrcene, (+)-limonene, p-cymene, (–)-limonene, (E)-β-ocimene, γ-terpinene, (Z)-β-ocimene, and (–)-α-pinene. α-Thujene, sabinene, (+)-α-pinene, α-phellandrene, (+)-β-pinene, (–)-β-pinene, and α-terpinolene were minor compounds (data not shown).

In addition to the terpenes found in fresh grass, milk from farms from the same sites revealed α-terpinene, and the tentatively identified 3,7-dimethyl-1,6-octadiene, 3,7-dimethyl-2-octene, 2,6-dimethyl-2,6-octadiene, and camphane, which were not present in the grass sample (Figure 1). On the other hand, (E)-β-ocimene was not detected in milk. The presence of the additional terpenoids pointed already to the possibility that these terpenes were generated from pasture to milk. *Buchin* and coworkers described the presence of these monoterpenoids in alpine milk (3).

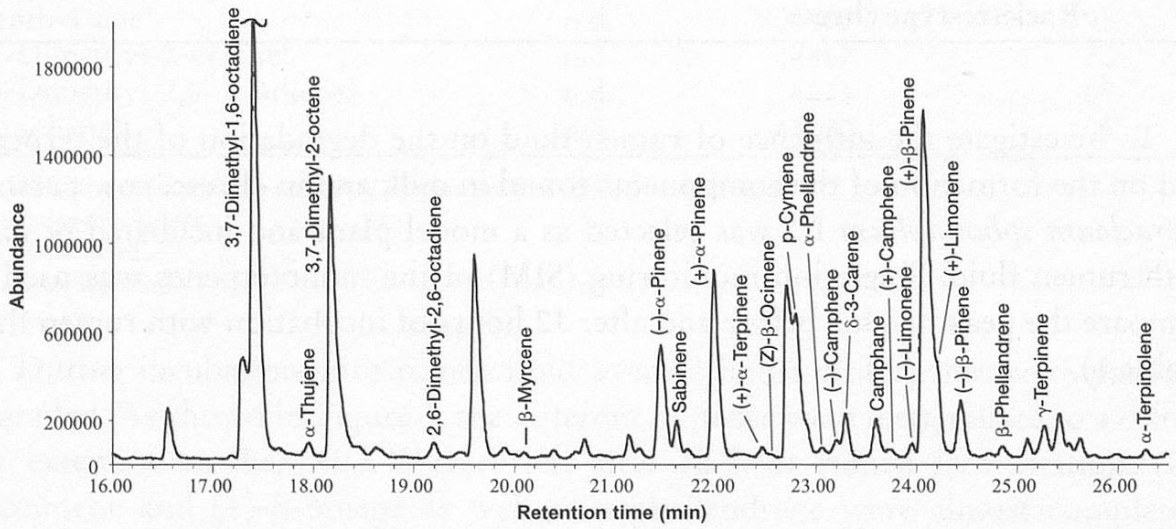

Figure 1 **GC-MS total ion chromatogram (TIC) depicting the monoterpene range of milk**

In addition to the monoterpenes in milk, 2-carene, menth-1-ene (tentatively identified), and (E)-β-ocimene were observed in the Raclette-type cheese, in contrast to the milk, however, no δ-3-carene was observed (Figure 2).

The ratios of (–)-α-pinene to (+)-α-pinene in milk and in cheese were different from the one analysed in grass. In the grass sample the (–)-enantiomer of α-pinene was prevalent, whereas (+)-α-pinene predominated in milk and, as expected, also in

the Raclette-type cheese. The most probable reason for these different enantiomeric ratios in grass and in milk and cheese is that the cows presumably grazed on a pasture containing plants of different species than the grass sample. (+)-limonene predominated in all three sample types over (–)-limonene.

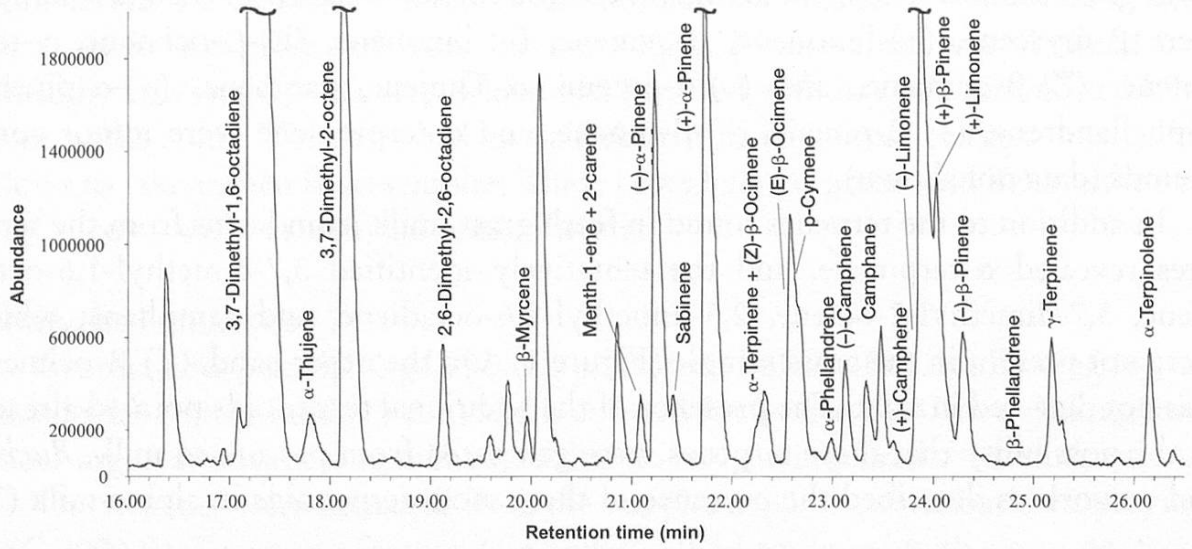

Figure 2 **GC-MS total ion chromatogram (TIC) depicting the monoterpene range of a Raclette-type cheese**

To investigate the influence of rumen fluid on the degradation of the terpenes and on the formation of the components found in milk and in cheese, cow parsnip, *Heracleum sphondylium* L., was selected as a model plant and incubated *in vitro* with rumen fluid. Single ion monitoring (SIM) of the monoterpenes was used to compare the peak heights before and after 12 hours of incubation with rumen fluid (Table 1).

Table 1
Terpene composition of cow parsnip (*Heracleum sphondylium* L.) before and 12 hours after fermentation with rumen fluid

Component	*Peak height (A.U.)*[1]		*Ion used for SIM*
	Before	*After 12 h fermentation*	*(m/z)*
α-Thujene	8640	3316	93
β-Myrcene	713100	1322	93
(–)-α-Pinene	103867	33003	93
Sabinene	22445	3416	93
(+)-α-Pinene	33019	7233	93
α-Terpinene	13236	2966	93
(Z)-β-Ocimene+(+)-β-Pinene[2]	93963	n.d.[3]	93
(E)-β-Ocimene+δ-3-Carene[2]	404397	257	93
p-Cymene	103314	39367	119
α-Phellandrene	5707	n.d.	93
(–)-Camphene	19094	4588	93
(+)-Camphene	3928	1958	93
(–)-Limonene	170667	51080	93
(+)-Limonene	264563	107440	93
(–)-β-Pinene	29890	7892	93
β-Phellandrene	203101	59540	93
γ-Terpinene	65072	22782	93
α-Terpinolene	5806	2240	93
3,7-Dimethyl-1,6-octadiene[4]	n.d.	95647	95
Menth-1-ene[4]	n.d.	291403	95
3,7-Dimethyl-2-octene[4]	n.d.	44807	70
2,6-Dimethyl-2,6-octadiene[4]	n.d.	4221	69
Camphane[4]	n.d.	41000	95

[1]Peak heights given in arbitrary units (A.U.) determined by single ion monitoring (SIM)
[2]Co-eluting components
[3]n.d.: not detected
[4]tentatively identified

During incubation with rumen fluid over 12 hours all terpenes were partially degraded. As shown in Figure 3, the different terpenes were metabolised to a different extent. Less than 40.6 % were left after 12 h of rumen fermentation. (Z)-β-ocimene and (+)-β-pinene as well as α-phellandrene were almost completely metabolised, on the other hand, the formation of 3,7-dimethyl-1,6-octadiene, 3,7-dimethyl-2-octene, 2,6-dimethyl-2,6-octadiene, and camphane, which were not present in the control samples without rumen fluid, could be observed during incubation.

The degradation of β-myrcene and of (E)-β-ocimene, both 3 times unsaturated, probably through hydrogenation by the rumen microflora (7), was correlated with the formation of the mono- and di-unsaturated 3,7-dimethyl-1,6-octadiene, 2,6-dimethyl-2,6-octadiene, 3,7-dimethyl-2-octene, respectively.

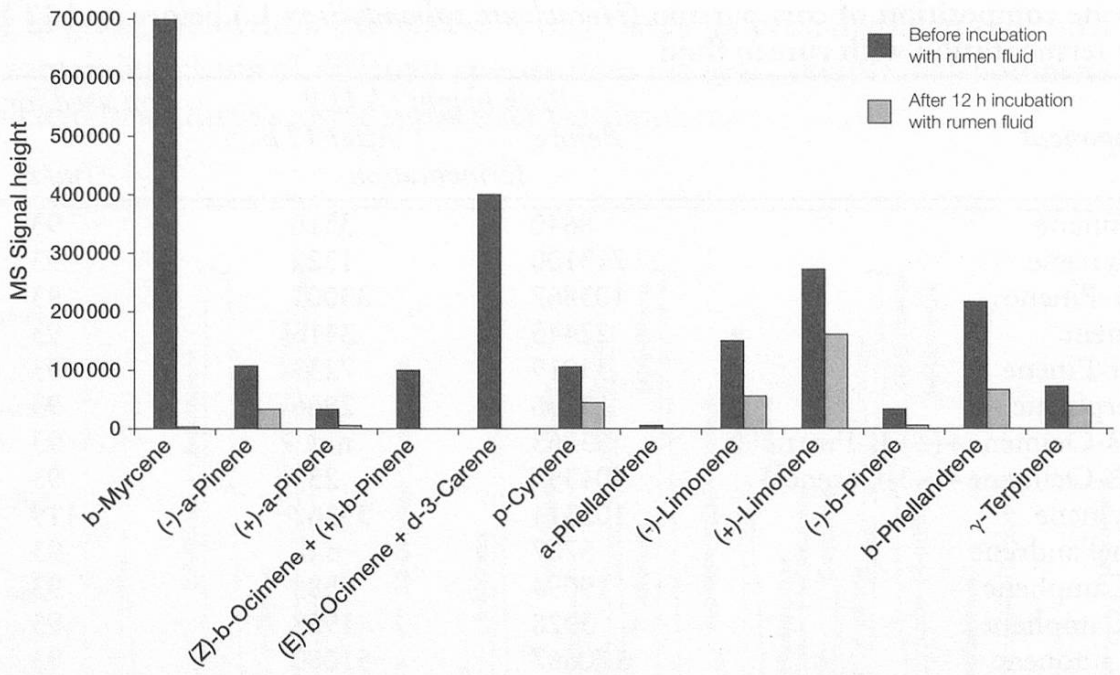

Figure 3 **Degradation of selected terpenes during incubation with rumen fluid**

Conclusion

The results demonstrate that the terpene composition of plants is transferred into milk and cheese, but rumen fermentation impacts the monoterpenoid profile considerably and leads to the formation of various metabolites, probably by hydrogenation reactions.

Acknowledgement

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Summary

Fresh grass collected between 1400 and 1920 m of altitude, milk from several farms from the same sites and a Raclette-type cheese produced from the same milk were analysed for their monoterpene composition by purge & trap GC-MS using a chiral stationary phase. The enantiomeric ratios of α-pinene were different between the milk and cheese samples and the grass samples analysed. In grass the (–)-α-pinene was predominant, whereas in the milk and, consequently, in the Raclette-type cheese the (+)-α-pinene prevailed. 3,7-Dimethyl-1,6-octadiene, 3,7-dimethyl-2-octene, 2,6-dimethyl-2,6-octadiene and camphane were tentatively identified in milk and in cheese, however not in the grass samples. The cheese furthermore revealed the presence of menth-1-ene. Cow parsnip *(Heracleum sphondylium* L.)

was selected as a model to study the impact of rumen fermentation on its monoterpene composition. After 12 hours of incubation with rumen fluid all terpenes were partially degraded. Camphane, 3,7-dimethyl-1,6-octadiene, 3,7-dimethyl-2-octene and 2,6-dimethyl-2,6-octadiene were generated during incubation. The formation of the latter three components was probably due to the hydrogenation of β-myrcene and (E)-β-ocimene by the rumen microflora.

Zusammenfassung

Frisches Gras, zwischen 1400 und 1920 m Meereshöhe gesammelt, Milch von Bauernhöfen der gleichen Lagen, und ein Käse vom Raclette-Typ, hergestellt aus der gleichen Milch, wurden auf ihre Monoterpenzusammensetzung hin mittels purge & trap GC-MS an einer chiralen stationären Phase untersucht. Das Enantiomerenverhältnis von α-Pinen in der Milch und im Käse war unterschiedlich von dem im Gras. Im Gras herrschte das (–)-α-Pinen vor, in der Milch und folglich im Käse dagegen dominierte das (+)-α-Pinen. 3,7-Dimethyl-1,6-octadien, 3,7-Dimethyl-2-octen und 2,6-Dimethyl-2,6-octadien wurden in Milch und in Käse, jedoch nicht im Gras vorläufig identifiziert. Des weiteren wurde im Käse Menth-1-en nachgewiesen. Bärenklau *(Heracleum sphondylium* L.) wurde als Modell gewählt, um den Einfluss der Fermentation im Pansen auf seine Monoterpenzusammensetzung zu untersuchen. Nach 12 Stunden Inkubation mit Pansensaft waren alle Monoterpene teilweise abgebaut. Camphan, 3,7-Dimethyl-1,6-octadien, 3,7-Dimethyl-2-octen und 2,6-Dimethyl-2,6-octadien wurden während der Inkubation mit Pansensaft gebildet. Die Bildung der drei letztgenannten Verbindungen erfolgte vermutlich durch Hydrogenierung von β-Myrcen und (E)-β-Ocimen durch die Pansenflora.

Résumé

Les compositions monoterpéniques de l'herbe fraîche, collectée entre 1400 et 1920 m d'altitude, du lait de plusieurs fermes des mêmes sites et d'un fromage du type Raclette, produit du même lait, ont été analysés par purge & trap GC-MS avec une phase stationaire chirale. Les rapports énantiomériques du α-pinène dans le lait et le fromage étaient différents de ceux trouvés dans l'herbe. Dans l'herbe, le (–)-α-pinène prédominait, alors que le (+)-α-pinène était prévalent dans le lait et dans le fromage du type Raclette. Le 3,7-diméthyl-1,6-octadiène, le 3,7-diméthyl-2-octène et le 2,6-diméthyl-2,6-octadiène ont été identifiés dans le lait et dans le fromage, mais non dans l'herbe. Dans le fromage on a en outre décelé la présence du menth-1-ène. La Grande berce *(Heracleum sphondylium* L.) était choisie comme modèle afin d'étudier l'influence de la fermentation ruminale sur sa composition monoterpénique. Après 12 heures d'incubation avec du jus de panse tous les monoterpènes étaient partiellement dégradés. Le camphane, le 3,7-diméthyl-1,6-octadiène, le 3,7-diméthyl-2-octène et le 2,6-diméthyl-2,6-octadiène ont été formés pendant la fermentation. La formation des trois derniers composés cités est probablement due à l'hydrogénation du β-myrcène et du (E)-β-ocimène par la microflore ruminale.

Key words

Monoterpenes, Rumen fermentation, Terpene degradation, Pasture, GC-MS

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