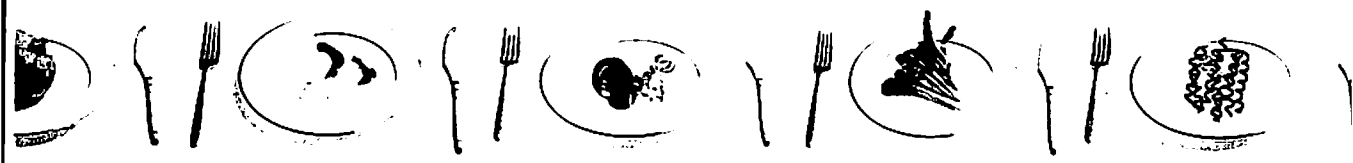

EuroFoodChem XV

FOOD FOR THE FUTURE

- the contribution of chemistry
to improvement of food quality

Proceeding 1

Copenhagen, Denmark
5-8 July 2009

M148:
Terpene and Conjugated Linoleic Acid Composition of Mountain Dairy Products

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KEYWORDS
terpenes; CLA; milk; cheese; mountain pastures

SUMMARY
Terpenes and conjugated fatty acids (CLA) are two classes of compounds, occurring in dairy products that can issue from animal feeding. In particular, terpenes can be transferred from the ingested plants, above all dicotyledons typical of highland pastures, to cow milk and consequently to cheese. Most of CLA are synthesized in the mammary gland but part of it is an intermediate of ruminal biohydrogenation of unsaturated fatty acids. Thus, as already demonstrated, a pasture feeding causes a higher biosynthesis of CLA in milk. For these reasons both terpenes and CLA have been proposed as feed tracers of mountain dairy products. Even if terpene content of dairy products might be influenced by many factors (vegetation, climate, length of the grazing season, etc...) and that of CLA depends mainly on feed, few experimental works dealing with both diversified highland pasture and grazing period effects on terpenes and CLA have been carried out.
In this work we try to deeply investigate these effects collecting milk and cheese samples from two different Italian alpine sites, each characterized by diversified vegetation types, on summer 2007, during two grazing periods. For terpene determination samples were distilled under vacuum and cooled in liquid nitrogen. Distilled aqueous solutions were analyzed by headspace solid-phase micro extraction–gas chromatography/mass spectrometry (HS-SPME–GC/MS) technique. For CLA determination, lipids were extracted with a petroleum ether/hexane mixture, then fatty acids were methylated and analyzed by gas chromatography.
Results showed that milk and cheeses from the diversified alpine areas can be differentiated on the basis of their terpene fingerprint. Monoterpenes were more abundant than sesquiterpenes both in milk and cheeses. The amount of both mono- and sesquiterpenes depended on both the vegetation type and the grazing period. The CLA content of milk and cheese was not generally influenced by the vegetation type. Instead a significant effect on CLA content of dairy products was exerted by the grazing period.

INTRODUCTION
Terpenes are secondary plant metabolites, particularly abundant in herbaceous species as those spread over mountain pastures (Mariaca *et al.* 1997). These molecules can occur in dairy products after the ingestion of plants by cows fed on highlands (Mariaca *et al.* 1997, Dumond and Adda

1978, Viallon *et al.* 2000). As consequence many authors have proposed terpenes as suitable tracers of feeding and a lot of work has been focused on determining the terpene profile of a wide variety of cheeses in order to delineate their traceability (De Noni and Battelli 2008, Favaro *et al.* 2005, Fernández García *et al.* 2008, Cornu *et al.* 2005). However cheese terpene content might be influenced by many factors as grazed vegetation, climate, length of the grazing season, grazing management, etc. (Tornambè *et al.* 2006). Conjugated fatty acid (CLA) has been considered by many authors another tracer of dairy products. In fact milk and meat of the ruminants are major source of CLA. Among these the most abundant isomer is the *cis*-9, *trans*-11 linoleic acid (C18:2) or rumenic acid and it has been demonstrated that its content arose when a feeding based on green forage was adopted (Collomb *et al.* 2002). CLA content in milk depends mainly on the biosynthesis that takes place in the mammary gland but also on the ruminal biohydrogenation of unsaturated fatty acids, particularly abundant in fresh green forage. The *cis*-9-*trans*-11 C18:2 is the most known also for its potential beneficial effects on the human health. It acts as reducer of lipid accumulation in adipose tissues (Pariza *et al.* 1996) and it exerts positive effects on the immune and cardiovascular systems (Tricon *et al.* 2004). From a healthy point of view also terpenes have demonstrated properties as antimicrobial, antifungal, antiviral, anti-inflammatory and chemotherapeutic (Ajikumar *et al.* 2008). As few experimental works dealing with both diversified highland pasture and grazing period effects on terpenes and CLA have been carried out, in this work we try to deeply investigate these effects planning during summer 2007 a collection of dairy samples from two Italian alpine pastures in two different grazing periods.

MATERIALS AND METHODS

Raw milk and cheese samples were collected from cows grazing two alpine sites, Stura Valley and Asiago; the first is located in Piedmont and the second in Veneto (North Italy). For each alpine site two different vegetation types were compared: alpine clover vegetation type and red fescue vegetation type in Stura Valley and poor-nutrient red fescue vegetation type and rich-nutrient red fescue vegetation type in Asiago. Two periods of grazing were planned for each site: in Stura Valley the 1° period ranged from July 18 to July 23 and the 2° from July 29 to August 3; in Asiago the 1° period ranged from July 11 to July 17 and the 2° from July 23 to July 29. Milk and cheese samples were collected daily and analyses of terpenes and *cis*-9, *trans*-11 linoleic acid were performed on samples of 1°, 3° and 6° day of each period.

Terpenes analysis

This analysis was carried out using the HS-SPME–GC/MS technique. Milk samples were acidified using HCl 1N; then 12 g acid curd were weighted and added of 400 µl of 10 ppm 1,3,5-triisopropylbenzene solution as internal standard. For cheese samples 15 g were weighted and added of the same internal standard. Then samples were distilled under high vacuum and cooled in liquid nitrogen. Three ml distilled sample were put into 10 ml vial and extracted in a thermostatic aluminium block at 45°C for 30', after 5' equilibration time, with a StableFlex 2cm-50/30µm divinilbenzene/carboxen/polydimethylsiloxane fiber. Analyses were carried out on a Shimadzu GC-17A gas chromatograph coupled with a Shimadzu QP-5000 quadrupole mass spectrometer, using a DB-WAX fused silica capillary column, 30m×0.25mm i.d., 0.25µm film thickness. Column temperature: 35°C for 5 min, increased to 173°C at 2°C/min for 1 min then raised to 210°C at 15°C/min and maintained for 5 min. Splitless injector: 270°C, desorption for 4'. Carrier gas: helium; flow rate 1.0 ml/min. Mass spectra were recorded in electron ionization mode (70 eV) in the 33–300 amu range. Ion source and interface: 230°C. Scan rate: 500amu/s, start time 1.0 min. Terpenes were detected in SIM. A qualitative analysis was performed integrating 93 ion peaks for

monoterpenes, 161 for sesquiterpenes, the more abundant or characteristic ion peaks for other biomarkers and results were expressed as normalized areas with respect to the internal standard (arbitrary area unit). Compound identification was achieved by using standard compounds, NIST12, NIST62 and Adams mass spectral databases and LRI (C5-C25 *n*-alkanes).

CLA analysis

For CLA analysis 20 g milk and 10 g cheese were weighted and extracted using petroleum ether/*n*-hexane (70:30). One hundred mg lipid extract were added of 200 µl of a 5000 ppm solution of *cis*-11, *cis*-14 methyl eicosadienoate as internal standard. Then fatty acids were trans-esterified according to ISO 5509:2000E procedure. Fatty acid methyl esters were analysed by a Varian 3400 gas chromatograph equipped with a DB-WAX capillary column, 30m×0.25mm i.d., 0.25µm film thickness and a flame ionization detector. Injector and detector temperature was 250°C. Column temperature 150°C for 10', then to 230°C at 2,5 °C/min and at 230°C for 15'. Compounds were identified by the injection of a mix standard. Peaks were integrated by using the Chrom Card software, and results were expressed as mg/g fat.

Statistical analysis

Statistica 7.0 Software was used to process data. A full factorial analysis of variance was used to study the effect of grazing period, vegetation types and sampling day on terpenes and *cis*-9, *trans*-11 linoleic acid.

RESULTS AND DISCUSSION

Data on the terpenic composition showed the presence of a "pool" of terpenes (α-pinene, β-pinene, limonene, cineol, *p*-cymene, menthone isomer, dihydromyrcenol, linalool, β-caryophyllene, menthol, α-terpineol, carvone) in all milk samples independently from the vegetation type or the grazing period. In cheese samples the same group of compounds was found but, additionally, sabinene, β-myrcene, γ-terpinene were also detected. Some monoterpenes, α-terpinene, terpinolene and myrtenol, were identified only in dairy samples of Asiago. Thus they could be used as potential biomarkers of dairy products of Asiago area. The full factorial analysis of variance showed that grazing period significantly influenced the abundance of terpenic components. A general increase of monoterpenes and sesquiterpenes in dairy samples collected from cows grazing the red fescue vegetation types from the 1° to the 2° grazing period was highlighted. Instead in the same time interval a general decrease of these components was encountered in dairy samples collected from the alpine clover vegetation type.

The full factorial analysis of variance also showed an effect of the grazing period on the CLA content. Data for Asiago area showed an increase of milk CLA from the 1° to the 2° grazing period; in particular inside each period in correspondence to the 3° sampling day the highest CLA mean amount was ascertained (17.3 mg/g fat). Also CLA cheese content increased from the 1° to the 2° period and a higher mean amount of CLA was found in cheese from poor-nutrient red fescue vegetation type (17.2 mg/g fat).

Data for Stura Valley showed a decrease of the average content of milk CLA from the 1° to the 2° grazing period, and a decrease inside each period between the 3° and the 6° day of sampling (from 17.3 to 14.6 mg/g fat). Generally milk samples from red fescue contained a higher mean amount of CLA (17.1 mg/g fat) than those from alpine clover (15.6 mg/g fat).

CONCLUSIONS

Results showed that diversified alpine pastures could give a different fingerprint to associated dairy products. Although it was possible to delineate from a qualitative point of view a pool of terpenic biomarkers for the investigated areas, it must be taken into account that the grazing period and also the day of sampling could affect the presence and the abundance of these compounds. But to establish a specific and precise pool of terpenic biomarkers these results must be confirmed with experimental data from different years. According with data reported in literature dairy products obtained from cows fed with fresh forage contained a greater amount of CLA, but in this study also a general dependence of CLA content on the vegetation type and of grazing period was showed. In addition to represent a suitable tracer of mountain dairy products together with terpenes, CLA contribute also to add high nutritional and healthy value to dairy products from raw whole milk, towards which the demand for high quality is increasing.

ACKNOWLEDGEMENTS

This work has been financed within the Italian research project FISR 'Pro Alpe' by the financial interdepartmental support of MEF, MIUR, MiPAAF and MATT. Manuscript n. 24.

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