UNIVERSITÀ DEGLI STUDI DI TORINO

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4   **EFFICACY OF FATTY ACIDS AND TERPENOIDS AND**

5   **WEAKNESS OF ELECTRONIC NOSE RESPONSE AS**

6   **TRACERS OF ASIAGO D'ALLEVO PDO CHEESE**

7   **PRODUCED IN DIFFERENT SEASONS**

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Abstract – The goal of this study was to verify the suitability of fatty acids, terpenoids and electronic nose response as potential tracers of the seasonal variations of Asiago d'Allevo PDO cheese. Cheese samples produced during the early and late summer grazing and the autumn/winter indoor seasons were compared. Based on their FA composition, the early and late summer cheeses were almost completely undistinguishable from each other. However, they both demonstrated higher quality if compared to the autumn/winter ones, showing lower levels of hypercholesterolemic saturated FAs (C12, C14, and C16) and higher levels of total mono- and polyunsaturated FAs, oleic (C18:1 *c*9), *trans*-vaccenic (C18:1 *t*11), rumenic (C18:2 *c*9*t*11), and α-linolenic (C18:3 *c*9*c*12*c*15) acids. Among terpenoids, camphene, cedrane, and total sesquiterpenoids were able to differentiate the cheeses manufactured during the three periods of production. Moreover, α-pinene, α-thujene, β-pinene, δ-3-carene, myrtenol, dihydrocarveol isomer 1, and unidentified sesquiterpene 1 distinguished the late summer cheeses from those obtained in the other two seasons. Principal component analysis of electronic nose data showed that the Asiago cheese samples were widely dispersed in the score plot and no data clusters appeared evident. Furthermore, cross-validated linear discriminant analysis of electronic nose data showed unsatisfactory classification performance (53.8%) regarding the period of production. Our results showed that coupling FAs and terpenoids information could be suitable for tracing Asiago d'Allevo PDO cheeses according to their season of production. However, no reliable information at this level seemed to be obtainable from electronic nose response.

1   **Key words:** Asiago PDO cheese, fatty acids, terpenoids, electronic nose, seasonal

2   changes

3   **1 Introduction**

4   In the Italian Alpine regions, milk from ruminants is mainly used for dairy

5   transformation into traditional cheeses. Among these is Asiago, a typical semi-

6   hard, half-cooked cheese made with bovine milk. Since 1996 Asiago cheese has

7   been awarded Protected Designation of Origin (PDO) status by the European

8   Union Commission (Commission Regulation 1996). This cheese has considerable

9   commercial relevance, with an annual production of about two million forms,

10   corresponding to more than 24,000 tons.

11   Milk used to produce Asiago PDO cheese is usually obtained from cows fed

12   pasture with limited concentrate supplementations during the grazing season,

13   while during the indoor season the feeding is mainly based on conserved forages

14   and notably higher amounts of concentrates. These seasonal variations in the

15   feeding regimen applied at farm level have been widely reported to affect the

16   chemical and nutritional characteristics of dairy products.

17   The possibility of using milk constituents deriving from the ruminant metabolism

18   (fatty acids) and secondary plant metabolites (terpenoids) as specific molecular

19   biomarkers to trace the changes in the management conditions of herds has

20   recently gained increasing attention in the dairy industry (Engel et al. 2007).

21   The fatty acid composition of milk and dairy products has been reported to be

22   particularly affected by dietary changes. Concentrations of omega 3 (n-3) fatty

1   acids (FAs), *trans*-vaccenic (TVA) and rumenic (RA) acids were found to

2   increase as grass (particularly fresh grass) replaces non-grass constituents in the

3   diet of ruminants. For this reason, these constituents of the lipid fraction have

4   been proposed as potential tracers of grass feeding in dairy products (Monahan et

5   al. 2010).

6   Since terpenoids can be transferred from plant to milk (Viallon et al. 2000), they

7   have also been widely reported as suitable tracers of production zone (Bugaud et

8   al. 2001; Zeppa et al. 2005) and feeding regimen (De Noni and Battelli 2008;

9   Revello Chion et al. 2010). Moreover, as the terpenoidic content in plants is

10   known to increase with maturity, dairy products obtained from pasture-fed

11   ruminants could also be distinguished on the basis of the phenological phase of

12   the grazed swards (Revello Chion et al. 2010; Tornambè et al. 2006).

13   The aromatic response obtainable from electronic nose (EN) technology has also

14   been reported as being suitable for use for traceability of dairy products (Ampuero

15   and Bosset 2003). Compared to fatty acids and terpenoids analysis, the EN

16   technology is more rapid and cheaper. Previous studies showed successful

17   applications of EN in discriminating cheeses according to variety (Contarini et al.

18   2001), geographical origin (Pillonel et al. 2003), ripening (Trihaas et al. 2005),

19   and shelf life (Benedetti et al. 2005). However, contradictory results were

20   obtained in detecting seasonal variations in cheese aroma (Benedetti and Mannino

21   2007; O'Riordan and Delahunty 2003; Schaller et al. 1999).

22   Previous studies conducted on the traceability of Asiago PDO cheese showed that

23   sesquiterpenes can be successfully used as biomarkers of cheeses produced with

1 milk from cows grazing on different mountain pastures (Favaro et al. 2005) and

2 that fatty acids are able to differentiate cheeses produced in alpine farms from

3 those produced in lowland and mountain industrialized cheese factories

4 (Schievano et al. 2008). No studies are currently available on the possibility of

5 using the fatty acid and terpenoidic compositions of Asiago PDO cheese and the

6 response obtainable from EN analysis to trace the seasonal variations of this

7 cheese, associated with changes in the management system of the herds.

8 The aim of this study was therefore to assess the potential of fatty acids,

9 terpenoids and electronic nose response to discriminate Asiago d'Allevo PDO

10 cheeses produced in different seasons. This *on-farm* research reflected the real

11 commercial production systems of Asiago PDO cheese, since it was performed

12 under actual herd management conditions rather than under the typical tight

13 control of experimental conditions.

14 **2 Materials and methods**

15 2.1 Experimental site, animals and management conditions

16 Ten alpine farms were chosen in the mountain territory of the 'Altopiano dei Sette

17 Comuni' (Veneto Region, NE Italy), located within the officially recognized

18 geographical area of Asiago PDO cheese manufacturing. The alpine farms had a

19 herd size ranging between 30 and 65 lactating dairy cows. The majority of the

20 farm-raised dairy cattle (64% of total cows) belonged to high-producing mono-

21 purpose Italian Brown and Holstein Friesian breeds. The remaining cows

22 belonged to local dual-purpose breeds (Simmental, Rendena, Alpine Grey, and

1   Burlina) characterized by medium milk production levels but good functional and

2   conformation traits which make them highly adaptable to the mountain

3   environment. All selected alpine farms regularly operate during the grazing

4   season only, (in accordance with an extensive management regime) by making

5   rational use of natural resources (local forages from pasture lands). In these farms,

6   variable amounts of concentrates (never exceeding 20% of total dry matter intake)

7   are usually provided as dietary supplementation for lactating cows at pasture.

8   In the autumn and winter months, the ten selected herds were housed indoors and

9   fed common hay-and concentrate-based diets, according to the latest Asiago PDO

10   Cheese Production Regulations (Gazzetta Ufficiale 2006). The herds were raised

11   in farms also located within the 'Altopiano dei Sette Comuni' territory, but in

12   lower lands. While two of these farms processed their milk at farm level (as

13   occurring during the grazing season), the other eight farms delivered their milk to

14   a cooperative cheese factory.

15   2.2 Cheese-making procedure

16   All Asiago d'Allevo cheese samples were produced according to the official

17   Asiago PDO Cheese Production Regulations (Gazzetta Ufficiale 2006). Briefly,

18   milk from one or two milkings was collected, partially skimmed after overnight

19   creaming at about 20°C, added to termophilic starter cultures, and subsequently

20   coagulated with bovine rennet at 35±2°C (coagulation time: 15-30 min). The curd

21   was cut into hazelnut-size particles and half-cooked until a temperature of 47±2°C

22   was reached. The curd was then extracted and molded in typical Asiago forms.

1 Asiago d'Allevo PDO cheese has a flattened cylindrical form, measuring 30-36

2 cm diameter, 9-12 cm height and 8-12 kg weight. The forms were turned various

3 times while the whey was completely removed. Salting was performed either by

4 surface dry-salting technique or by washing with brine. Maturing occurred for

5 four months in storage bays at 10-15°C temperature and 80-85% relative

6 humidity.

7 2.3 Cheese sampling

8 Samples collection was carried out in such a way that the different periods of

9 production were covered. Ten Asiago cheeses produced in June and July 2007

10 (corresponding to the early alpine grazing season) and ten cheeses produced in

11 August and September 2007 (corresponding to the late alpine grazing season)

12 were collected directly *in situ* from the ten selected alpine farms. Between

13 October 2007 and February 2008 (corresponding to the autumn/winter indoor

14 season), a total of nine cheeses were collected from the two farms and the

15 cooperative cheese factory that operated during the autumn and winter months.

16 All cheeses were collected at four months of ripening. Nine representative slices

17 (three for fatty acids, three for terpenoids, and three for electronic nose analyses)

18 were cut from each sampled wheel. The rind was removed, as it is not generally

19 consumed. All samples were vacuum-packed and subsequently frozen at −20°C

20 until analyses.

1 2.4 Fatty acids analysis

2 A composite sample was obtained from the three slices previously grounded and

3 homogenized in a blender. Cheese total lipids were extracted according to IDF

4 (1986). Fatty acid methyl esters (FAMEs) were prepared according to

5 Christopherson and Glass (1969) and were separated and quantified by gas

6 chromatography (Shimadzu GC17A, Shimadzu Corporation, Kyoto, Japan) using

7 a CP-Sil 88 capillary column (100 m × 0.25 mm ID, 0.20 μm film thickness;

8 Varian Inc., Lake Forest, CA, USA). The column temperature was held at 45°C

9 for 5 min and then raised 20°C·min^{-1} to a final temperature of 195°C, where it

10 remained for 50 min. The temperatures of the injector and the flame-ionization

11 detector were maintained at 250 and 280°C, respectively. The injection volume

12 was 0.1 μL, with a split ratio of 21:1. The nitrogen constant linear flow rate and

13 its average velocity were set at 1.4 mL·min^{-1} and 22 cm·s^{-1}, respectively. Peaks

14 were identified by comparing their retention times with pure FAME standards

15 (Matreya Inc., Pleasant Gap, PA, USA and Restek Corporation, Bellefonte, PA,

16 USA). A butter oil reference standard (CRM 164; Commission of the European

17 Communities, Community Bureau of Reference, Brussels, Belgium) was

18 periodically analyzed as a control to verify column performance and was used to

19 estimate correction factors for short chain (C4:0 to C10:0) fatty acids. Results

20 were expressed as g·100 g^{-1} FAMEs. Fatty acid analyses were performed in

21 duplicate.

1   2.5 Terpenoids analysis

2   A representative sample was obtained from the three slices previously ground and

3   homogenized in a blender. Then 15 g were weighed, added to 400 μL of 10 ppm

4   1,3,5-triisopropylbenzene solution as internal standard, distilled under high

5   vacuum and cooled in liquid nitrogen.

6   Three mL distilled aqueous samples were put into 10 mL vial and extracted with a

7   2-cm (50/30 μm divinylbenzene/carboxen/polydimethylsiloxane) solid-phase

8   microextraction (SPME) fiber (Supelco Analytical, Bellefonte, PA, USA)

9   according to Zeppa et al. (2005) with modifications. Briefly, distillates were

10   equilibrated in a thermostatic aluminium block at 45°C for 5 min and extracted at

11   the same temperature for 30 min under stirring. After extraction, the fiber was

12   introduced into the injector of a gas chromatograph coupled with a mass

13   spectrometer (GC–MS) and maintained at 270°C for 4 min for thermal desorption

14   of terpenoids.

15   GC–MS analyses were carried out on a Shimadzu GC-17A gas chromatograph

16   equipped with a Shimadzu QP-5000 quadrupole mass spectrometer detector

17   (Shimadzu Corporation, Kyoto, Japan). Separation was achieved using a DB-

18   WAX capillary column (30 m × 0.25 mm ID, 0.25 μm film thickness; J&W

19   Scientific Inc., Folsom, CA, USA). The oven temperature was programmed at

20   35°C and held for 5 min, increased to 173°C at 2°C·min^{-1} for 1 min, then raised to

21   210°C at 15°C·min^{-1} and maintained for 5 min. Injector temperature was 270°C

22   and splitless injection mode was adopted. Helium was used as the carrier gas at

23   1.0 mL·min^{-1}. The detector operated in electron impact ionization mode at 70 eV

1 with the GC–MS interface at 230°C, and an *m/z* scan range of 33 to 300 was

2 collected. Compound identification was achieved by comparing mass spectra and

3 linear retention indices (LRI) with those of authentic standards and/or with those

4 recorded in NIST12, NIST62 (National Institute of Standards and Technology,

5 Gaithersburg, MD, USA), and other published mass spectral databases (Adams

6 2001). LRI were calculated by linear interpolation relative to retention times of

7 C5–C25 n-alkanes as external references. For each terpene, the area (arbitrary

8 unit) of the most abundant or characteristic ion *m/z* was extracted from the total

9 ion current (TIC) and integrated with a Class-5000 Data Station ver. 2.0 software

10 (Shimadzu Corporation, Kyoto, Japan). A semi-quantitative analysis was

11 performed assuming that the terpenoids have the same response factor to that of

12 the internal standard. Analyses were performed in triplicate.

13 2.6 Electronic nose analysis

14 The gas-sensor array instrument used was a portable EN PEN2 (Airsense

15 Analytics GmbH, Schwerin, Germany) equipped with 10 metal oxide

16 semiconductor (MOS) sensors. For the gas-sensor measurements, three samples

17 (one from each slice) were divided into two aliquots (approximately 2 g of

18 cheese) and transferred to 40 mL glass vials. These were sealed with preheated

19 (105°C) Teflon/silicon septa and open screw caps and were allowed to equilibrate

20 at room temperature for 10 min before analysis. Thereafter the samples were

21 incubated at 40°C for 20 min before headspace gas was pumped into the sensor

22 chamber for 20 s at a flow rate of 150 mL·min^{-1}. Recovery time for the sensors

1   was 120 s (flushing with charcoal-filtered ambient air). Samples were analyzed in

2   random order. All analytical procedures took place in an air-conditioned

3   laboratory.

4   The response of the sensors during sample measurement is a curve representing

5   the sensors' conductivity against time. The sensor signal, in fact, is expressed by

6   the ratio G/G0 where G is the conductance of the sensor in presence of the

7   sample, and G0 is the conductance of the sensors in reference air. The feature

8   extracted from the sensor signal (response) was determined by identifying the

9   curve's peak (absolute response) and then by subtracting the baseline from that

10   value. The ten responses represent the variables in the data matrix considered for

11   the statistical analysis.

12   2.7 Statistical analysis

13   All statistical analyses were performed using the SPSS software (version 16.0 for

14   Windows, SPSS Inc., Chicago, Illinois, USA).

15   Fatty acids data were submitted to one-way analysis of variance according to the

16   following model: $Y_{ij} = \mu + \alpha_i + \varepsilon_{ij}$, where: Y_{ij} = mean of response variable; μ =

17   overall mean; α_i = effect of sampling period (1 = early alpine grazing; 2 = late

18   alpine grazing; 3 = autumn/winter indoor); ε_{ij} = residual error. The assumption of

19   equal variances was assessed by Levene's homogeneity of variance test. If such

20   assumption did not hold, the Brown-Forsythe statistic was performed to test for

21   the equality of group means instead of the F one. Pairwise multiple comparisons

22   were performed to test the difference between each pair of means (Tukey's HSD

1 test and Tamhane's T2 in the cases of equal variances assumed or not assumed,

2 respectively). Significance was declared at P≤0.05. Data were expressed as mean

3 ± standard error (SE).

4 Terpenoids data were submitted to a Kruskal-Wallis non-parametric independent

5 group comparison. A significant Kruskal-Wallis test was followed by a Mann-

6 Whitney U test to compare each pair of groups. The *P*-values obtained with the

7 latter test were adjusted according to a Bonferroni correction based on the Holm-

8 Bonferroni method. Data were expressed as mean ± SE.

9 Principal Component Analysis (PCA) was applied to EN data to explore and

10 visualize hidden patterns in the data set. PCA involves a mathematical procedure

11 that transforms a number of possibly correlated variables into a smaller number of

12 uncorrelated variables called principal components. The first principal component

13 accounts for as much of the variability in the data as possible, and each

14 succeeding component accounts for as much of the remaining variability as

15 possible, and so on. All the variables were mean-centered and scaled to equal

16 variance prior to PCA. Furthermore, a stepwise linear discriminant analysis

17 (LDA) was also applied to EN data. This analysis is a supervised technique of

18 classification and assignation of a sample to a previously defined group. The

19 classification model is built by maximizing the variance between groups and

20 minimizing the variance within groups. The model performance is then assessed

21 on the basis of the number of samples correctly predicted as belonging to an

22 assigned group.

1   **3 Results**

2   3.1 Fatty acid composition

3   The FA profiles of Asiago d'Allevo PDO cheese manufactured during summer

4   months (early and late alpine grazing seasons) and autumn/winter ones (indoor

5   season) are presented in Table 1.

6   The most remarkable differences in fatty acids were detected between the grazing

7   season (both early and late summer) and the indoor season. Considering the main

8   FA groups, total saturated FAs were significantly higher (P≤0.001) while total

9   mono- and polyunsaturated FAs were significantly lower (P≤0.001) in the

10   samples produced during the autumn/winter months than those produced in

11   summer. Among saturated FAs, notable variations were detected in the majority

12   of short- and medium-chain FAs, while no significant differences were found for

13   margaric (C17) and stearic (C18) acids. Considering unsaturated FAs, remarkable

14   seasonal differences were observed in the contents of *trans*-vaccenic (C18:1 *t*11,

15   TVA) and rumenic (C18:2 *c*9*t*11, RA) acids. Cheeses produced during both the

16   early and late summer grazing seasons showed approximately doubled levels of

17   both TVA and RA in comparison to the cheeses produced during the indoor

18   season (P≤0.001). Conspicuous differences were also observed between the

19   summer and autumn/winter cheeses in the levels of oleic (C18:1 *c*9) and α-

20   linolenic (C18:3 *c*9*c*12*c*15, ALA) acids, both significantly higher in the summer

21   samples (P≤0.001 and P≤0.01, respectively).

1 The cheeses produced during the early and late summer grazing seasons were

2 instead almost completely undistinguishable from each other. They only differed

3 in three minor detected FAs (13-methyltetradecanoic acid -C15 *iso*-, myristoleic -

4 C14:1 *c*9- and arachidic -C20- acids) which were significantly higher in the

5 cheeses produced during the late alpine grazing season.

6 3.2 Terpenoidic composition

7 The terpenoidic compositions of Asiago d'Allevo PDO cheese manufactured

8 during the early and late alpine summer grazing season<u>s</u> and during the

9 autumn/winter indoor season are presented in Table 2.

10 All cheese samples showed a prevalence of monoterpenoids, whose total amount

11 was basically (P≤0.10) more abundant (on average 2.4 times) during the grazing

12 season<u>s</u> than during the indoor season. The total sesquiterpenoid content showed

13 highly significant differences among all three periods of production in question

14 (P≤0.001). Total sesquiterpenoids were about 3- and 17-fold higher in the early

15 and late grazing seasons as compared to the indoor season, respectively.

16 Some compounds (α-thujene, δ-3-carene, dihydrocarveol isomer 1, the

17 unidentified monoterpene 1, the unidentified sesquiterpene 1, and β-ionone) were

18 detected only in early and/or late summer cheeses, while others, such as the two

19 menthone isomers, were found only in cheeses produced in the autumn/winter

20 months.

21 Considering the terpenoids that were detected in all three periods of production,

22 camphene, terpinen-4-ol, β-caryophyllene, and cedrane showed significantly

1 higher amounts in cheeses produced during both the early and late summer than in

2 cheeses produced in the indoor period. Moreover, other compounds such as α-

3 pinene, β-pinene, linalool, carvone, and myrtenol, thus not being significantly

4 different between the cheeses produced in the early grazing and indoor seasons,

5 showed significantly higher amounts in samples produced in late summer as

6 opposed to those produced in autumn and winter.

7 Many compounds showed significant differences between cheeses produced

8 during the early and the late summer grazing seasons. In particular, some

9 monoterpenoids (α-pinene, camphene, β-pinene, δ-3-carene, and myrtenol) and

10 the sesquiterpenoid cedrane significantly increased with the advance of the

11 grazing season, reaching their maximum content in cheeses produced in August

12 and September. This trend was then followed by a general decrease in

13 correspondence with the indoor season. The monoterpenoid γ-terpinene was the

14 only compound that showed, on the contrary, a significantly higher content in the

15 early rather than late summer cheese samples ($P \leq 0.05$).

16 Some compounds (β-myrcene, limonene, cineol, terpinolene, camphor, bornyl

17 acetate, α-terpineol, unidentified sesquiterpene 2, methyl salicylate, and methyl

18 dihydrojasmonate) as well as the total "miscellaneous" were detected in all the

19 periods of production in question, without showing any significant difference

20 among them.

1   3.3 Electronic nose response

2   Figure 1 shows the PCA score plot of gas sensors responses. This graph represents

3   the original samples projected on the plane formed with the two principal

4   components (PCs). The first PC described 46.12% of sample variance; the second

5   one described 31.89%. The sum of the first three PCs accounted for more than

6   90% of the total explained variance.

7   Among the ten MOS sensors that equipped the EN used in the present study, three

8   sensors resulted more sensitive to the headspace of the samples. They were

9   reported to have broad-range sensitivity, especially to aromatic compounds,

10 hydrocarbons, and nitrogen oxides. The same three sensors were the most

11 significant in defining PCs.

12 Samples were thinly dispersed in the plot area defined by the first two PCs and no

13 data clusters appeared evident. The three Asiago groups (early alpine grazing

14 season, late alpine grazing season, and indoor season) were widely overlapping.

15 The results of the LDA confirmed the graphical overview obtained with the PCA.

16 Since the number of samples was limited to allow a separation into two subsets

17 (*training* and *test* set), classification of LDA was performed using the same data

18 that were used to derive the discriminant function. To reduce the bias deriving

19 from this procedure, each observation was classified using a discriminant function

20 computed from all the other observations (full cross-validation). Even with these

21 technical adjustments, the number of misclassifications into groups was quite

22 high. The percent of erroneously classified samples was 29.5% for "early grazing"

1   group, 45.9% for the "indoor" group, and as high as 65.8% in the case of "late

2   grazing".

3   **4 Discussion**

4   During both the early and late alpine grazing seasons, the herds were managed

5   according to extensive farming methods, with fresh grass from pasture being the

6   main feeding resource coupled with only limited concentrate supplementations.

7   On the contrary, in autumn and winter the herds were mainly fed with conserved

8   forages (hay) and remarkable higher amounts of concentrates if compared to those

9   supplied in the summer months. Such differences in the feeding regimen applied

10   at farm level in the periods of production under analysis explain the observed

11   significant variations in the FA compositions between the two summer grazing

12   seasons on one hand and the indoor months on the other hand. Fresh grass,

13   particularly from alpine pastures, has been reported to consistently ameliorate the

14   FA profile of milk and dairy products from ruminants, by lowering the levels of

15   medium chain hypercholesterolemic saturated lauric (C12), myristic (C14), and

16   palmitic (C16) acids, and by contemporarily raising the amounts of peculiarly

17   beneficial FAs such as oleic, *trans*-vaccenic, rumenic, and α-linolenic acids (van

18   Dorland et al. 2006). Fresh grass is an important source of lipids in the ruminants'

19   diet and, if compared to hay, has been reported to contain higher amounts of

20   ALA, the latter usually representing more than 50% of total FAs (Clapham et al.

21   2005). Alpha-linolenic acid is extensively biohydrogenated within the rumen,

22   leading to the formation of intermediate products (TVA and RA) characterized by

1   putative beneficial effects for human health (Bauman and Lock 2010). Similar

2   results to those obtained in the current study have been previously obtained by

3   other authors, who reported significant seasonal (summer *versus* winter) changes

4   in the FA compositions of milk and dairy products from ruminants, mainly as a

5   consequence of the nature and composition of the feedstuffs fed to the animals

6   (among others, Revello Chion et al. 2010; Abilleira et al. 2009). Our results show

7   that many detected fatty acids (including ALA, TVA, and RA) could be

8   particularly useful for the discrimination of Asiago d'Allevo PDO cheeses

9   produced during the grazing and indoor seasons. However, the same fatty acids do

10   not prove useful for the discrimination of Asiago cheeses produced during the

11   early and late summer grazing seasons.

12   The results obtained showed that terpenoids were very effective in differentiating

13   Asiago PDO cheeses not only between the summer grazing and the indoor seasons

14   but also between the early and late summer grazing periods. It is known that, as

15   with green grass, hay prepared from natural grasslands can also provide

16   terpenoids (Viallon et al. 1999). The differences in terpenoids observed between

17   the summer and autumn/winter Asiago cheese samples could be consequently

18   ascribed to a diverse terpenoidic composition of hay and grazed grass vegetation

19   types. Moreover, it has also been reported that even though the haymaking

20   process does not significantly affect the terpene profile of milk, the amount of

21   terpenoids in milk can be strongly reduced (Fedele et al. 2007).

22   The observed increase of the content of some terpenoids during the grazing season

23   (early *versus* late summer) could probably be related to the accumulation of plant

secondary metabolites. The sole presence of dihydrocarveol isomer 1 and unidentified sesquiterpene 1 in the late summer could be explained in the same way, as well as the evidence that a lot of monoterpenoids (α- and β-pinene, γ-terpinene, linalool, carvone, and myrtenol) did not show significantly different amounts between the early summer and the indoor seasons.

In order to trace Asiago d'Allevo PDO cheese according to its season of production, the most effective terpenoids were found to be camphene, δ-3-carene, and cedrane. In fact, they all significantly differed among the three production periods analysed, showing their maximum levels in cheeses produced during the late summer grazing season. Among other detected terpenoids, α-thujene and β-ionone were found to be discriminative of early summer cheeses. A thick group of compounds (α-pinene, β-pinene, myrtenol, dihydrocarveol isomer 1, and unidentified sesquiterpene 1) allowed a good discrimination of late summer cheeses. Finally, the two menthone isomers, terpinen-4-ol, and β-caryophyllene allowed a good discrimination of cheeses produced during the indoor season.

Sesquiterpenoids have been reported as being preferable to monoterpenoids for traceability purposes (Engel et al. 2007). If considering the main terpenoid groups, the results obtained in the current study seem to corroborate such a finding. In fact, even though the total monoterpenoids were by far more abundant in the Asiago cheese samples than the total sesquiterpenoids in all three periods in question, the total sesquiterpenoids were found to better discriminate the samples according to their season of production.

1   Some monoterpenoid compounds, such as β-myrcene, limonene, cineol,

2   terpinolene, camphor, bornyl acetate, and α-terpineol, did not differ among the

3   three periods of production. They were consequently ineffective in the attempt to

4   trace the Asiago PDO cheese produced in different seasons.

5   The current study provided the first investigation into the possible role of a

6   chemical class of volatiles, denominated "miscellaneous" category, as

7   biochemical markers to be used for dairy products. These plant-derived

8   compounds are C13-norisoprenoidic volatiles such as β-ionone and methyl

9   dihydrojasmonate, or benzene derivatives such as methyl salicylate. The latter is

10   biosynthesized in plants by carboxyl methyltransferase from salicylic acid, which

11   in turn derives from phenylalanine (Effmert et al. 2005). Methyl

12   dihydrojasmonate is responsible for flowery aroma and it has been reported as a

13   linoleic acid derived molecule (Barreto et al. 2011), while β-ionone is a

14   ubiquitous constituent of many vegetables and fruits (Winterhalter and Rouseff

15   2001). The results obtained in this study showed that, even though the total

16   miscellaneous compounds did not vary significantly among the three periods of

17   production, β-ionone was detected only in the Asiago cheese samples produced in

18   the early summer grazing period. β-ionone has already been detected in dairy

19   products, and it has recently been found useful in differentiating milk and cheeses

20   produced under extensive (with pasture-fed dairy cows) or intensive (with cows

21   fed total mixed rations) farming conditions (Belviso et al. *in press*). This

22   norisoprenoidic volatile could be consequently considered a promising compound

23   to be used for traceability purposes in the dairy sector.

1   Contrary to both fatty acid and terpenoidic compositions, the response obtained by

2   the gas sensors equipment used in this study did not appear to be useful in

3   classifying Asiago d'Allevo PDO cheeses according to their season of production.

4   The same EN used in the present study was successfully applied in order to

5   differentiate milk collected from dairy cows grazing on two different alpine

6   vegetation types (Falchero et al. 2009). In strictly defined and controlled

7   experimental conditions, these authors reported good discrimination ability of the

8   model (LDA classification rate: >90%) highlighting actual applicability of this

9   instrument to milk samples.

10   Concerning Asiago PDO cheese, Benedetti and Mannino (2007) first analyzed it

11   with an EN. These authors submitted samples of "Pressato" (20 days of ripening)

12   and "d'Allevo" (3-12 months of ripening) Asiago PDO cheeses to gas sensors

13   measurements. Both fresh and ripened samples were produced in winter and

14   summer months. The EN was able to identify some outliers (identically

15   recognized as anomalous samples with sensory analysis by experts) probably due

16   to unusual fermentations. However, the instrument detected similar headspace

17   compositions for the ordinary samples and no discrimination according to the

18   season of production (summer *versus* winter) was then possible, neither for the

19   "Pressato" nor for the "d'Allevo" Asiago cheeses.

20   Although several studies demonstrated the ability of ENs to discriminate aged

21   cheeses with different geographical origin at different maturation stages (Pillonel

22   et al. 2003) and even at the same ripening age (Schaller et al. 1999), O'Riordan

23   and Delahunty (2003) also suggested that the MOS sensors-based EN used in their

1 study gradually deteriorated in its accuracy in classifying Cheddar cheeses with

2 the progression of cheese maturation. The results obtained in the present study

3 support those reported by Benedetti and Mannino (2007) and O'Riordan and

4 Delahunty (2003). Differences detected by EN analysis in flavor and volatile

5 compounds of fresh cheeses due to animal diet and milk microflora could be

6 progressively attenuated due to complex microbiological and enzymatic processes

7 that occur during ripening.

8 **CONCLUSIONS**

9 The results obtained in the present *on-farm* research showed that both fatty acids

10 and terpenoids can be considered suitable biomarkers to be used as tracers in

11 discriminating Asiago d'Allevo PDO cheeses according to their season of

12 production. Fatty acids and terpenoids, in fact, were found to be valuable as

13 chemical fingerprint for the characterization of the dairy cows' feeding regimen. It

14 is worth mentioning that information obtained from both fatty acids and

15 terpenoidic data provided more comprehensive and precise information. Fatty

16 acids data were in fact very useful in discriminating summer from autumn/winter

17 cheeses, while terpenoids proved to be very useful also in discriminating cheeses

18 produced during the early and late summer grazing seasons. On the contrary, the

19 use of a MOS sensors-based electronic nose on Asiago d'Allevo cheese did not

20 seem to provide helpful information, producing an unsatisfactory classification

21 rate of the collected samples regarding their period of production.

1   The obtained results suggest the usefulness of fatty acids and terpenoids as

2   biomarkers for the traceability of cheeses originating from PDO areas, due to the

3   strong link existing between the animals' diet and the chemical composition of

4   ruminant-derived dairy products.

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1 **LIST OF TABLES AND FIGURES**

2

3 **Table 1** Fatty acid composition (g·100g^{-1} FAMEs) of Asiago d'Allevo PDO

4 cheese manufactured during the early and late summer alpine grazing seasons and

5 during the autumn/winter indoor season (mean±SE)

6

7 **Table 2** Terpenoidic composition (normalized arbitrary area units × 10^{4}) of

8 Asiago d'Allevo PDO cheese manufactured during the early and late summer

9 alpine grazing seasons and during the autumn/winter indoor season (mean±SE)

10

11 **Fig. 1** PCA score plot based on gas sensors responses of Asiago d'Allevo PDO

12 cheese according to the season of production: early alpine grazing season (Jun-

13 Jul) (■), late alpine grazing season (Aug-Sep) (○), and autumn/winter indoor

14 season (Oct-Feb) (▲)

1 **Table 1** Fatty acid composition (g·100g^{-1} FAMEs) of Asiago d'Allevo PDO

2 cheese manufactured during the early and late summer alpine grazing seasons and

3 during the autumn/winter indoor season.

	Early alpine grazing season (Jun-Jul)	Late alpine grazing season (Aug-Sep)	Indoor season (Oct-Feb)	SEM	*P*
C4	2.14	1.96	2.13		ns
C6	1.72[b]	1.64[b]	1.88[a]		**
C8	1.13[b]	1.06[b]	1.28[a]		***
C10	2.27[b]	2.14[b]	2.78[a]		***
C10:1 *c*9	0.25[b]	0.26[b]	0.29[a]		***
C12	2.49[b]	2.43[b]	3.12[a]		***
C13	0.14[b]	0.15[b]	0.18[a]		***
C14 *iso*	0.15	0.18	0.18		ns
C14	9.39[b]	9.46[b]	10.78[a]		***
C15 *iso*	0.31[b]	0.37[a]	0.32[ab]		*
C15 *aiso*	0.60	0.66	0.58		ns
C14:1 *c*9	0.75[c]	0.83[b]	0.92[a]		***
C15	1.07	1.16	1.13		ns
C16 *aiso*	0.28	0.33	0.32		ns
C16	25.88[b]	25.92[b]	29.54[a]		***
C17 *iso*	0.13	0.11	0.13		ns
C17 *aiso*	0.61[a]	0.60[a]	0.47[b]		***
C16:1 *c*7	0.30	0.29	0.25		ns
C16:1 *c*9	1.78	1.70	1.84		ns
C17	0.76	0.74	0.70		ns
C17:1	0.27[a]	0.24[a]	0.18[b]		***
C18	12.18	12.39	11.61		ns
C18:1 *t*11 (TVA)	4.46[a]	4.52[a]	2.38[b]		***
C18:1 *t*9	0.55	0.47	0.68		ns
C18:1 *c*9	24.32[a]	24.12[a]	21.43[b]		***
C18:1 *c*11	0.57[a]	0.57[a]	0.46[b]		**
C18:1 *c*12	0.18	0.18	0.25		ns
C18:1 *c*13	0.44[a]	0.45[a]	0.35[b]		***
C18:2 *c*9*c*12 (LA)	2.37	2.49	2.29		ns
C20	0.17[b]	0.20[a]	0.17[b]		***
C20:1	0.92[a]	0.94[a]	0.68[b]		***
C18:3 *c*9*c*12*c*15 (ALA)	0.02[a]	0.02[a]	0.01[b]		**
C18:2 *c*9*t*11 (RA)	1.37[a]	1.42[a]	0.67[b]		***
Σ SFA	61.44[b]	61.52[b]	67.34[a]		***

Σ MUFA	34.80[a]	34.56[a]	29.70[b]	***
Σ PUFA	3.77[a]	3.92[a]	2.97[b]	***
HSFA[x]	37.77[b]	37.81[b]	43.44[a]	***

1

2 Abbreviations: FAME, fatty acid methyl ester; *c*, *cis*; *t*, *trans*; TVA, *trans*-vaccenic acid; LA,

3 linoleic acid; ALA, α-linolenic acid; RA, rumenic acid; SFA, saturated fatty acids; MUFA,

4 monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; HSFA, hypercholesterolemic

5 saturated fatty acids.

6 Probability: * P≤0.05; ** P≤0.01; *** P≤0.001; ns, not significant (P>0.05). Different letters

7 within rows indicate statistically significant differences between groups.

8 [x] Calculated as C12:0+C14:0+C16:0.

1   **Table 2** Terpenoidic composition (normalized arbitrary area units) of Asiago

2   d'Allevo PDO cheese manufactured during the early and late summer alpine

3   grazing seasons and during the autumn/winter indoor season (mean±SE).

	Early alpine grazing season (Jun-Jul)	Late alpine grazing season (Aug-Sep)	Indoor season (Oct-Feb)	*P*
Monoterpenoids				
α-Pinene	0.282 ± 0.140 [b]	0.952 ± 0.372 [a]	0.090 ± 0.024 [b]	***
α-Thujene	0.070 ± 0.054	0.002 ± 0.002	nd	**
Camphene	0.161 ± 0.104 [b]	0.455 ± 0.213 [a]	0.011 ± 0.007 [c]	***
β-Pinene	0.589 ± 0.239 [b]	1.174 ± 0.426 [a]	0.108 ± 0.024 [b]	***
Sabinene	0.179 ± 0.103 [a]	0.096 ± 0.042 [ab]	0.008 ± 0.004 [b]	*
δ-3-Carene	0.015 ± 0.006	0.025 ± 0.017	nd	*
β-Myrcene	0.067 ± 0.039	0.038 ± 0.017	0.027 ± 0.014	ns
Limonene	1.299 ± 0.389	0.738 ± 0.119	0.509 ± 0.117	ns
Cineol	0.019 ± 0.005	0.138 ± 0.037	0.131 ± 0.049	0.08
γ-Terpinene	0.233 ± 0.141 [a]	0.023 ± 0.015 [b]	0.024 ± 0.010 [ab]	*
p-Cymene	1.087 ± 0.451 [a]	0.820 ± 0.179 [ab]	0.414 ± 0.123 [b]	*
Terpinolene	0.109 ± 0.075	0.003 ± 0.002	0.011 ± 0.005	ns
Menthone1	nd	nd	0.476 ± 0.190	-
Menthone2	nd	nd	0.093 ± 0.059	-
Camphor	0.757 ± 0.283	0.833 ± 0.205	0.361 ± 0.112	ns
Linalool	1.565 ± 0.439 [ab]	2.058 ± 0.547 [a]	0.749 ± 0.178 [b]	*
Bornyl acetate	0.053 ± 0.017	0.060 ± 0.021	0.190 ± 0.090	ns
α-Terpineol	2.396 ± 0.543	3.660 ± 0.918	2.030 ± 0.490	ns
Terpinen-4-ol	0.480 ± 0.184 [a]	0.457 ± 0.100 [a]	0.084 ± 0.027 [b]	***
Carvone	0.277 ± 0.130 [ab]	0.366 ± 0.117 [a]	0.029 ± 0.013 [b]	**
Myrtenol	0.316 ± 0.194 [b]	1.534 ± 0.423 [a]	0.041 ± 0.011 [b]	***
Geranyl acetone	5.135 ± 1.332 [a]	4.948 ± 1.007 [ab]	1.515 ± 0.216 [b]	*
Dihydrocarveol is.1	nd	0.120 ± 0.071	nd	-
Monoterpene ni1	0.007 ± 0.004	0.006 ± 0.004	nd	ns
Sesquiterpenoids				
β-Caryophyllene	0.122 ± 0.035 [a]	0.442 ± 0.182 [a]	0.036 ± 0.011 [b]	*
Cedrane	0.073 ± 0.030 [b]	0.584 ± 0.156 [a]	0.022 ± 0.014 [c]	***
Sesquiterpene ni1	nd	0.006 ± 0.004	nd	-
Sesquiterpene ni2	0.021 ± 0.010	0.095 ± 0.051	0.007 ± 0.005	ns
Miscellaneous				
Methyl salicylate	0.114 ± 0.064	0.135 ± 0.059	0.077 ± 0.036	ns
β-Ionone	0.015 ± 0.011	nd	nd	-

Methyl dihydrojasmonate	0.876 ± 0.234	0.831 ± 0.166	0.750 ± 0.180	ns
Σ Monoterpenoids	15.276 ± 3.508	18.507 ± 3.950	6.900 ± 0.984	0.10
Σ Sesquiterpenoids	0.216 ± 0.063 [b]	1.127 ± 0.337 [a]	0.065 ± 0.025 [c]	***
Σ Miscellaneous	1.005 ± 0.250	0.967 ± 0.185	0.826 ± 0.178	ns

1

2 Abbreviations: nd, not detected; is., isomer; ni, not identified.

3 Probability: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; the P-value is shown if, although being not

4 significant, it shows a tendency ($P \leq 0.10$); ns, not significant ($P > 0.10$). Different letters within rows

5 indicate statistically significant differences between groups. The statistical analysis was not

6 performed for menthone 1, menthone 2, dihydrocarveol is.1, sesquiterpene ni1, and β-ionone since

7 these variables were not detected in the samples belonging to two of the three groups.

1   **Fig. 1** PCA score plot based on gas sensors responses of Asiago d'Allevo PDO cheese according to the season of production: early

2   alpine grazing season (Jun-Jul) (■), late alpine grazing season (Aug-Sep) (O), and autumn/winter indoor season (Oct-Feb) (▲)

3