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ORIGINAL ARTICLE

Volatile compounds in Nanos cheese: their formation during ripening and sesonal variation

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Abstract The objective of this study was to evaluate the formation of volatile compounds (VCs) during ripening time in different seasons (summer, winter) of Nanos cheese with Protected Designation of Origin (PDO). The study was also undertaken to compare the aroma profile of feed and milk sampled in two different seasons from the protected area. The VCs analysis was performed by solid-phase micro extraction-gas chromatography–mass spectrometry. Altogether, 62 different VCs were detected throughout cheese ripening where their quantity varied with the time of ripening and season. In cheeses from winter season, there was a higher concentration of some fatty acids and esters. Besides, the evaluated differences of cheese samples by season indicated the transfers of some VCs from feed and/or milk into cheese. The most important possible links between cheese with feed and/or milk from different season were found in case of 9 VCs (acetic acid, butanoic acid, 3-methyl butanoic acid, hexanoic acid; ethyl ester of hexanoic acid, -octanoic acid, and -decanoic acid-; 2-butanone, pinane, sabinene) which also define the season. The changes in the amount of some VCs through ripening were significant (e.g. content of some fatty acids and esters increased while content of acetoin and diacetyl decreased). This is the first report which deals with the changes in cheese during ripening influenced by season and feeding system and the transfers of VCs from feed and milk to cheese in the case of traditional Nanos cheese, which was chosen as a model system for other hard type cheeses.

Keywords Cheese · Volatile compounds · Ripening · Season · Feed

Introduction

Cheese quality - chemical, technological and microbiological – depends mainly on the characteristics of milk and cheese making process (Stefanon and Procida 2004; Amenu and Deeth 2007). One of the key cheese quality components is its flavour (Ross et al. 2000; Delgado et al. 2010). Cheeses, especially traditional cheeses, have unique flavour because of the various local characteristics of the specific area (Carpino et al. 2004). Today, however the predominant trend in agro-industrial markets reveals a growing interest among consumers in traditional products that are closely linked to a specific place of origin, like Nanos cheeses from the Vipava valley in Slovenia, which Protected Designation of Origin (PDO) status was implemented by EU 987/2011 regulation.

In fact, the flavour of cheese is defined by the complex environment of volatile compounds (VCs) and non-volatile compounds (Delgado et al. 2010). The analysis of VCs in foods, including dairy products, is one of the most common method for quality determination. VCs in cheeses are of different chemical classes including fatty acids, alcohols, ketones, esters, aldehydes, etc. (Milosavljević et al. 2012).

Various factors affect the final cheese aroma and its aromatic profile, where the feed, season and ripening are important factors of interest (Centeno et al. 2004; Stefanon and

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Procida 2004; Gioacchini et al. 2010; Verzera et al. 2010). Season can alter milk composition and consequently affect the final cheese flavour (Fernández-García et al. 2002). The difference between seasons comes mainly due to different forage. The impacts of feed on VCs in milk and further in cheese have been proven in various studies (Buchin et al. 1998; Carpino et al. 2004). The most studied VCs are undoubtly terpenes with their impact being published in different researches (Buchin et al. 1998; Bugaud et al. 2001; Cornu et al. 2005). However, recent study indicates that is impossible to clearly define the origin of many VCs, whether they originate from feed or if they are formed during cheese ripening (Kalač 2011). Some VCs in milk are produced in mammary glands while others are transferred from feed. The latter ones can be transferred to milk by different pathways: from inhaled air, from rumen gases or the digestive tract through blood and from there to milk (Toso et al. 2002). Compounds inhaled by cows during the consumption of the forage could pass very quickly through bloodstream into milk (Carpino et al. 2004). During cheese ripening VCs could be formed as a result of various biochemical processes such as lipolysis, proteolysis, metabolism of lactate and citrate, metabolism of fatty acids and amino acids (McSweeney and Sousa 2000; Alewijn 2006).

The analytical method for determinations of VCs profiles in cheese have been performed by employing gas chromatography (GC) – mass spectrometry (MS) (Verzera et al. 2004). During the last 20 years the improvement of GC - MS analysis took place by introduction and development of new extraction method named solid phase micro extraction (SPME) (Pinho et al. 2003; Milosavljević et al. 2012) that was efficiently implemented also in cheese (Chin et al. 1996; Delgado et al. 2010; Milosavljević et al. 2012) and milk (Panseri et al. 2009) aroma studies during the last decade. Although the chromatographic analysis (instrumental analysis) of VCs is one of the most important methods in determining the quality of the food (Abilleira et al. 2010) it cannot replace the sensory analysis but it complements it. This is also important as sensory analysis belongs to the criteria that influence the consumer's choice (Perotti et al. 2009). Instrumental determination of flavour has always been of interest to both researchers and food manufacturers because it could serve as an alternative or complement to sensory evaluation. The focus of our study was the instrumental determination of VCs important for the aroma of Nanos cheese. The aromatic notes defining particular VC were found in the literature. The aim of our work was thus to evaluate changes during ripening time and seasons (summer/pasture and winter/non pasture) on the VCs profiles in Nanos cheese, the first Slovenian cheese with the PDO status recognized at the European Union level. To examine changes of the amount of single VC identified in Nanos cheese we define time of ripening as the effect from the statistical point of view. The study was oriented in the examination of VCs transfer from feed and milk to cheese in order to elucidate some important facts in the production of Nanos cheese and other traditional cheeses of the EU and wide world. In our study we were particularly interested in differences between seasons (summer, winter), including the facts that the feed in both seasons is from the same region, milk is heat-treated (thermised) and the technological process of cheese making is always the same.

Material and methods

Experimental design

Slovenian traditional Nanos cheese is a hard type cheese and is made from the milk of cows which are pastured from May to October. During the pasture season (summer season) the basis of feed is grazing (fresh grass) whereas in the non pasture season (winter season) cows are fed with grass silage. In the protected area there are more than 20 dairy farms supplying milk for Nanos cheese production. Nanos cheese milk is standardized at 3.05 to 3.15 % milk fat content, thermised, inoculated with thermophilic starter culture (*Streptococcus thermophilus* and *Lactobacillus helveticus*) and rennet. Cheese ripening lasts for at least 60 days at 9–15 °C and 80–90 % humidity. The cheese wheels weigh between 8 and 10 kg.

Two batches of cheese from winter and summer seasons, respectively, were sampled throughout the ripening time as follows: 0^{th} (curd), 7^{th}, 21^{st}, 35^{th}, 49^{th}, 63^{th}, 77^{th} and 99^{th} day. In each batch three different cheese wheels were randomly sampled.

Basic feed and milk samples were collected in each season from the same six farms. In the summer season we sampled milk and fresh grass, while in the winter season we sampled milk and grass silage.

Analyse-determination of VCs in samples

Analytical standards

Analytical standards of VCs were purchased from several suppliers: hexanal, octanal, 1-hexanol, 2-octanone from Alfa Aeser; 2-ethyl 1-hexanol, 3-methyl 1-butanol, acetic acid, α-pinene, 2-pentanone, 2-butanol, acetic acid ethyl ester, hexanoic acid ethyl ester, 2-pentanone from Fluka; δ-decalactone, 1-octanol (internal standard) from SAFC, FCC KOSHER; 2,3-butanediol, 1-butanol, 3-methyl butanoic acid, butanoic acid, β-carryophyllene, sabinene, p-cymene, heptanoic acids, 2-butanone, benzaldehyde, octanoic acid, 3-methyl butanal from Sigma-Aldrich; 2-methyl butanoic acid, decanoic acid, 2-pentanol, nonanal, 2-heptanone, 2-nonanone, limonene from Merck KGaA and 3-hydroxy-2-butanon from

Supelco. The chromatogram purity was in all cases greater than 95 %.

Sample preparation

Cheeses were sampled by cutting the cheese wheel with conical cheese borer from the lateral surface of the cheese to the centre. The outer 10 mm of cheese samples were discarded while the rest was grounded in a blender and the amount of 4 g was immediately transferred into 20 mL headspace vial.

Samples of feed (fresh grass and grass silage) were first chopped with a manual blade and 4 g of sample were immediately placed into 100 mL headspace vial. For the milk analyses 100 mL headspace vial was first filled with 5 g Na_2SO_4 and then topped up with 50 mL of milk. Each sampling of feed, milk and cheese was performed in triplicates.

Extraction of the VCs

VCs present in feed, milk and cheese samples were analysed using solid phase micro-extraction – SPME kit (Supelco, Bellefonte, PA, USA). The detailed description follows.

Cheese: On the SPME device the 20 mm 50/30 μm DVB/CAR/PDMS fiber (divinylbenzene / carboxen / polydimethylsiloxane)-grey fiber and 85 μ CAR/PDMS-black fiber was used. The individual VC was then evaluated according the best performance of either black or grey fiber. Samples of cheese were exposed for 24 h extraction at 25±1 °C. The relatively long time was applied because of semi-volatile compounds and with low temperature we achieved a non-altered sample matrix compounds. Besides, we performed preliminary test employing different times of exposure of the fibre (45 min, 16 h (Frank et al. 2004), 24 h), resulting in 24 h the best.

Feed and milk: Samples of feed and milk were thermised for 45 min at 37±1 °C and then exposed for 45 min extraction at 37±1 °C to the DVB/CAR/PDMS SPME fibre. This fiber was chosen, because it showed better response to terpenes (e.g. α-pinene, p-cymene and limonene) according to CAR/PDMS fiber. These terpenes are known substances coming from feed.

Gas chromatography

Afterwards the SPME device was manually introduced in a gas chromatograph with a mass selective detector (GC-MS - Agilent 6890 Series GC System with Agilent 5973 Mass Selective Detector) in the splitless injector 270 °C for 10 min (tested before for complete desorption). Daily prior to analysis, the fibre was conditioned and activated by inserting it into the GC injector at 270 °C for 30 min. Volatiles were separated on Rtx-20 column (60 m, 0.25 mmID, 1 μm, Restek, USA). The temperature program was as follows: initial temperature 50 °C (2 min) - 10 °C min^{-1} - 150 °C (for 3 min) - 10 °C min^{-1} - 250 °C (for 5 min). Total run time was 30 min. The mass spectrometer was operated in the electron ionisation mode at a voltage of 70 eV, the temperature of the MS Quad was set at 150 °C and the ion source at 230 °C.

Identification of VCs

Compounds were identified in two ways. Some of them were identified by comparison with standards (in the Results and disussion, notified in the Table 1 under column ID-identification, as ST-standard), the others were identified on the basis of their retention times using the searchable EI-MS spectra library (NIST02) (in the Results and disussion, notified in the Table 1 under column ID-identification, as MS-mass spectra library). The peak area for quantification was measured in TIC chromatogram. The quantification was done using the internal standard 1-octanol ($6{,}85 \cdot 10^{-3}$ mg), but the statistical analysis was completely done employing peak areas.

Statistical analyses

Statistical analysis of data was performed with Statistica for Windows software. The evaluation the effect of season on VCs and changes of VCs profile throughout the ripening time were assessed using General Linear Model (GLM) with nested analysis of variance, where baches were nested within seasons and wheels were nested within baches and seasons. The nested design was carried out with the model:

$$y_{ijkl} = \mu + S_i + B_{ij} + w_{ijk} + R_l + e_{ijkl}$$

It includes effects of S_i season (winter, summer), B_{ij} - baches (j=1,2), w_{ijk} - wheels (w=1-12), and R_l - stages of ripening time (l=0 (curd), 7, 21, 35, 49, 63, 77 and 99-day). The model had three fixed factors (S, B and R) and one random effect (w).

Tukey's multiple comparison tests were carried out to determine the differences between means of individual VC in different stages of ripening time within winter and summer season. To test the difference in quantity of VCs in milk and feed samples in different seasons the nonparametric Wilcoxon matched pair test was used.

Principal component analysis (PCA) was applied to determine the differences among samples of cheese from summer and winter season on the 63th day of ripening. Though PCA is not a classification method, the program gives the possibility of making a group assignment by Euclidean distances in the

Table 1 Volatile compounds (VCs) identified in Nanos cheese with statistical parameters for each effect (Season, Ripening time, Baches, Wheels) from general linear model (GLM) used and VCs' flavour notes

n VC	VCs by functional groups	Flavour notes	Rt	ID	Season		Ripening time		Baches		Wheels	
	Fatty acids				F	P	F	P	F	P	F	P
V1	Acetic acid	Vinegar sour, sharp[1*]	6.87, 6.95	ST	3.34	ns	4.71	***	11.33	***	3.45	***
V2	Butanoic acid	Cheesy, rotten, sharp[1]	11.09, 11.18	ST	10.68	**	9.73	***	1.86	ns	10.56	***
V3	Butanoic acid, 3-methyl-	Rotten cheesy[1]	12.09	ST	9.63	**	46.91	***	4.58	**	2.98	**
V4	Butanoic acid, 2-methyl-	Fruity[10]	12.30	ST	13.97	**	64.17	***	5.11	*	2.74	**
V5	Pentanoic acid	Rain, wood, vegetable, spicy, nutty, grain, swiss cheese[2]	13.10	MS	85.05	***	34.50	***	0.00	ns	2.53	**
V6	Hexanoic acid	Sharp-goaty[1]	15.58	MS	139.44	***	62.21	***	18.88	***	1.66	ns
V7	Heptanoic acid	Goaty, cheesy[1]	17.85	ST	4.19	ns	22.87	***	0.82	ns	2.46	**
V8	Octanoic acid	Body odour, sweat, fatty, rancid, cheese, pungent[2]	20.04	ST	212.85	***	14.38	***	17.13	***	5.46	***
V9	Decanoic acid	Warm, stale, butter, sour fruit, grassy, fatty, goat[2]	23.73	ST	52.26	***	3.44	**	3.78	*	4.94	***
	Alcoholes											
V10	2-butanol	Alcoholic odor[13]	7.51	ST	7.30	*	6.36	***	1.58	ns	1.29	ns
V11	1-butanol	Fruity, nutty[4]	8.81	ST	14.72	***	7.70	***	5.07	*	2.28	*
V12	2-Pentanol	Fresh[2]	9.43, 9.47	ST	9.95	**	49.28	***	0.68	ns	4.29	***
V13	1-Butanol, 3-methyl-	Harsh, dull[5]	10.29	ST	1.37	ns	8.38	***	1.61	ns	1.14	ns
V14	2,3-Butanediol		11.93	ST	3.44	ns	33.20	***	0.23	ns	4.60	***
V15	3-Pentanol, 2-methyl-		12.38	MS	18.97	***	153.77	***	0.28	ns	3.44	***
V16	1-Hexanol	Green[2]	13.17	ST	96.17	***	80.45	***	1.26	ns	3.34	***
V17	2-Heptanol	Herbaceous, oily, green, earthy, fruity[2]	13.76	MS	0.00	ns	54.04	***	0.92	ns	1.69	ns
V18	1-Hexanol, 2-ethyl-		17.09	ST	6.44	*	3.10	**	1.85	ns	8.33	***
V19	2-nonanol	Fatty, green[2]	18.55	MS	0.15	ns	7.14	***	0.25	ns	0.85	ns
	Aldehydes											
V20	Butanal, 3-methyl-	Green, malty, herbaceous[2]	8.89	ST	4.13	ns	7.11	***	3.01	ns	5.40	***
V21	Hexanal	Green[2]	11.99	ST	2.42	ns	72.28	***	3.13	ns	2.07	*
V22	Heptanal	Soapy, harbaceous[2]	14.22	MS	0.24	ns	3.78	***	0.02	ns	0.60	ns
V23	Octanal	Fatty, green[2]	16.76	ST	208.52	***	1.94	ns	1.72	ns	3.68	***
V24	Benzaldehyde	Almond[6]	17.11	ST	1.15	ns	216.62	***	0.47	ns	3.82	***
V25	Nonanal	Green, fatty, soapy[2]	19.03	ST	8.89	**	12.15	***	0.36	ns	5.05	***
V26	Benzeneacetaldehyde	Toasted[7]	19.13	MS	5.01	*	7.40	***	5.97	**	0.25	ns
V27	2-nonenal	Fatty, cucumber[1]	20.56	MS	3.82	ns	5.22	***	0.31	ns	1.75	ns
V28	Decanal	Green[2]	21.22	MS	16.86	***	15.90	***	0.30	ns	0.78	ns
	Esters											
V29	Acetic acid, ethyl ester	Fruity, pinapple, juicy fruit gum, apples[2]	7.87	ST	7.02	*	8.31	***	0.02	ns	1.41	ns
V30	Butanoic acid, ethyl ester	Sweet, fruity, apple, green[2]	11.70	MS	0.57	ns	4.66	***	0.72	ns	4.97	***
V31	1-Butanol, 3-methyl-, acetate	Fruit, banana, caramel, peanuts[2]	13.37	MS	6.81	*	43.90	***	1.20	ns	0.83	ns
V32	Hexanoic acid, ethyl ester	Fruity, grape melon[1]	16.29	ST	35.88	***	22.92	***	0.76	ns	1.70	ns
V33	Octanoic acid, methyl ester		19.22	MS	87.34	***	12.17	***	0.27	ns	8.28	***
V34	Octanoic acid, ethyl ester	Fruit, pear, banana, pineapple, wine, flowers[2]	20.69	MS	180.19	***	5.82	***	0.85	ns	0.92	ns
V35	Decanoic acid, ethyl ester	Fruity[7]	24.17	MS	56.26	***	3.54	**	5.90	*	7.56	***

Table 1 (continued)

n VC	VCs by functional groups	Flavour notes	Rt	ID	Season		Ripening time		Baches		Wheels	
	Fatty acids				F	P	F	P	F	P	F	P
	Ketones											
V36	Diacetyl	Buttery-sweet[1]	7.58	MS	1.10	ns	89.64	***	1.70	ns	10.49	***
V37	2-butanone	Sap-acetone[1]	7.76	ST	1.12	ns	4.22	***	2.72	ns	0.67	ns
V38	2-pentanone	Orange peel[9]	9.58, 9.89	ST	2.82	ns	13.64	***	0.43	ns	4.86	***
V39	2,3-Pentanedione		9.73	MS	12.81	**	120.91	***	1.26	ns	14.77	***
V40	Acetoin	Sour milk[2]	10.43	ST	1.71	ns	80.29	***	0.62	ns	4.94	***
V41	2-Hydroxy-3-pentanone	Truffle, earth-nut[2]	12.51	MS	21.41	***	148.56	***	0.24	ns	4.03	***
V42	2,3-Heptanedione		12.57	MS	13.94	**	0.00	ns	0.71	ns	47.96	***
V43	2-Heptanone	Musty, varnish, sweet[1]	13.93	ST	1.52	ns	11.27	***	0.34	ns	0.23	ns
V44	2-octanone	Fruity, green[1]	16.42	ST	0.12	ns	5.92	***	0.23	ns	1.33	ns
V45	5-Hepten-2-one, 6-methyl-	Woody-moss[10]	16.54	MS	13.10	**	0.00	ns	1.17	ns	64.25	***
V46	2-nonanone	Floral, fruity, peachy[1]	18.73	ST	0.15	ns	6.90	***	0.14	ns	2.21	*
V47	8-Nonen-2-one	Animal/stinky[7]	19.42	MS	87.34	***	12.17	***	0.27	ns	8.28	***
V48	2-Undecanone	Fruity, floral, stuffy[2]	22.74	MS	93.84	***	29.58	***	0.76	ns	7.62	***
	Terpenes											
V49	Pinane	Pungent alcohol[11]	14.37	MS	77.84	***	17.07	***	8.74	**	11.47	***
V50	α-pinene	Pinegreen[2]	14.77	ST	74.92	***	33.47	***	0.18	ns	6.00	***
V51	Sabinen	Fragrant, woody, resinous[12]	15.94	ST	139.44	***	62.21	***	18.88	***	1.66	ns
V52	Limonene	Mild, citrus, sweet, orange lemon[9]	17.25	ST	0.09	ns	7.55	***	0.71	ns	1.63	ns
V53	p-Cymene	Weak, spicy harbecous, citrus-like, fresh[12]	17.43	ST	8.24	*	2.15	*	0.09	ns	8.35	***
V54	β-Caryophyllene	Sickly sweet, wallflowers, woody-spicy[12]	25.63	ST	21.49	***	2.76	*	1.74	ns	13.16	***
	Miscellaneous											
V55	2-Octene		11.2	MS	8.76	*	8.62	***	3.26	ns	17.63	***
V56	Toluene	Sweet, pungent, caramel, ethereal, fruity,rubbery[9]	11.35	MS	100.60	***	16.23	***	2.07	ns	2.80	**
V57	Ethylbenzene		13.55	MS	2.95	ns	13.15	***	3.22	ns	1.46	ns
V58	Dimethylsulfone	Sulfurous, hot milk, burnt[2]	16.89	MS	23.29	***	28.14	***	0.69	ns	2.30	*
V59	Undecane		17.64	MS	208.84	***	3.36	**	0.00	ns	1.05	ns
V60	p-Cresol	Barnyard, phenolic[1]	19.07	MS	12.46	**	9.13	***	2.17	ns	2.43	**
V61	δ-octalactone	Coconut-like, peach-like, fruity[2]	24.35	MS	56.49	***	26.49	***	1.35	ns	3.31	***
V62	δ-decalactone	Coconut[2,3], peachy[2], sweet[2]	27.74	ST	22.33	***	1.39	ns	0.05	ns	9.25	***

nVC, identification number of volatile compounds; [1-13] index of authors from the references (e.g. [11] Langler (1966); [9] Jung et al. 2013; [13] Chemicalland2 (2013)); *ID*, identification of VCs used; *ST*, identification with standards of VCs; *MS*, identification of VCs with spectra library; *Rt*, retention time for VCs in cheese; *F*, F-value from GLM; *P*, *P*-value from GLM; *ns*, not significant at $P>0.05$; * $0.01 < P<0.05$; **$0.001 < P<0.01$; *** $P<0.001$

multidimensional space created by the PCA. For each separation pattern, a new set of parameters was chosen to calculate the principal component scores (Pillonel et al. 2003). PCA was applied to the VCs data using the correlation matrix and Varimax rotation. In our case the data of mean of each VC by wheels were used, consequently the eigenvalues and eigenvectors of the matrix were then calculated.

Results and discussion

Results obtained from the study could be useful for the application in the characterization of cheese from the region with PDO. The main emphasis of the research was to enhance product quality with indicator as cheese aroma profile and elucidate the parameters (season, ripening time) influencing

the aroma of PDO cheese. For these purpose milk and feed from winter and summer season was analysed in order to highlight the components, which were later identified in the cheese elucidating the essential differences (qualitative and quantitative) of the aromatic profile between seasons. Further we focused on the VCs in Nanos cheese, their formation during ripening and the main differences from summer and winter season. Lastly, we present some VCs, which most strongly characterized samples of cheese wheels from summer or winter seasons on the 63th day of ripening, defined by PCA and were also present in feed and/or milk.

Feed and milk

In the summer season fodder of cows based on outdoor fresh pasture of average 15 h/day grazing. The content of nutrients in fresh grass was: 235 g dray matter (DM), 97.02 g/kg DM crude proteins, 17.87 g/kg DM crude fat, 394.48 g/kg DM crude fiber and 62.98 g/kg DM ash. Whereas in winter the basic fodder was 18 kg of grass silage. Average grass silage composition was 504.3 g DM, 120.76 g/kg DM crude proteins, 25.38 g/kg DM crude fat and 83.28 g/kg DM ash.

The statistical difference of amount of VCs in milk and feed samples according to different seasons was evaluated using the nonparametric Wilcoxon matched pair test (data collected in Table 2). The results of VCs content (Mean) are expressed in arbitrary area units, whereas the statistical difference among seasons is expressed as *P*-value (Table 2, column *P*-value). Although analysis of feed and milk from both seasons revealed 146 and 54 VCs (data not shown), respectively, only those present also in cheese samples were evaluated.

Cows' feeding diets could impact on sensory characteristics of cheese in various ways (Coulon et al. 2004) and it is known that certain milk components are directly derived from feed (carotene, terpene) (Coulon et al. 2004; Martin et al. 2005) or are produced during silage fermentation (Kalač 2011). On the other hand, different feeding system could also have indirect impact on cheese sensory properties by modifying the dynamics of the microbial ecosystem (Coulon et al. 2004). Moreover, Martin et al. (2005) claimed that some molecules are produced by animals (plasmin, fatty acids) as consequence of specific feed. But it is known, that some microbial effect due to diet origin are eliminated if the milk is pasteurised (Coulon et al. 2004). Heat treatment inactivates enzymes and microorganisms present in milk which are related to the formation of VCs which could be responsible for aroma of cheese (Delgado et al. 2011). Also heating of milk at lower temperature, which is used in cheese making process of Nanos cheese - thermisation regime (63–65 °C, 30 min), slightly modifies the characteristics of microbiota (Desmazeaud 2000) (inactivates enzymes which lead to certain changes in biochemical and microbiological processes during ripening). All of these changes have influenced also on VC and flavour of cheese (Ozcan and Kurdal 2012). However some enzyme present in milk are resistant to heat treatment and could play an important role during cheese ripening. One of such enzymes is plasmin, endogenous enzyme which originates from blood (Martin et al. 2005). Plasmin with its proteolytic action (Sousa et al. 2001) (cleaves casein into longer peptides) is involved in the maturation of cheeses (Choisy et al. 2000; Sousa et al. 2001).

Many studies proved the carry-over of terpenes from feed to milk and cheese. Due to the effect of pasture Carpino et al. (2004) found in cheese eight unique aroma-active compounds and only three of them were terpenoid compounds (other were aldehydes, estres and one sulfur compound). But data of carry-over of other compounds (alcohols, acids, esters, aldehydes and ketones) to milk are insufficient (Kalač 2011).

In our case hexanoic acid ethyl ester was identified only in samples of feed and milk from the winter season. Ethyl acetate marked the milk samples from the summer season, whereas 2-butanone those from the winter season (Table 2). Also Mounchili et al. (2005) found that feeding with the grass silage influenced on the higher amount of 2-butanone. Aldehydes (hexanal and pentanal) were predominant in milk from grass silage (Kalač 2011), but in our case hexanal was identified only in fresh grass and pentanal only in samples of grass silage. However the milk of both seasons contained pentanal. Acetone, on the other hand reached higher levels in the milk of the winter season. Its content is however affected by the introduction of ethanol feed (grass silage) (Kalač 2011). Toluene was present in both seasons but resulting higher amounts in the summer season. All the terpenes identified in the cheese were present also in the feed and milk. Terpenes like pinane, α-pinene and p-cymene were higher in feed and milk from the summer season.

Some VCs (e.g. 3-methyl 1-butanol, 3-methyl butanal, hexanal), which were predominant in feed or milk of the summer or winter season, were not selective for the differentiation of the cheeses from the winter or summer season. According to the obtained results presented in Table 2 it can be concluded that some VCs (e.g. butanoic acid) identified in milk and feed originate not only from feed, but have also other sources (synthesis in the mammary gland) (Becker and Kumar 1965).

All other components identified in the feed and / or milk which are possibly linked to cheese, are pointed out in the further subsections (VCs in Nanos cheese, PCA of Nanos cheese from different season (pasture and non-pasture) on the 63th day of ripening).

VCs in Nanos cheese

Throughout the ripening time (including 99th day) 62 VCs were detected in cheese samples (Table 1) which were not present at all times in both seasons (Online resource 1).

Table 2 Some VCs (arbitrary area units) identified in feed and milk with statistical parameters

VCs by functional groups	Feed			Milk		
	Mean		*P*-value	Mean		*P*-value
Fatty acids	Summer	Winter		Summer	Winter	
Acetic acid	$5.49\ 10^{6}$	$1.90\ 10^{8}$	*			/
Butanoic acid		$2.08\ 10^{9}$	*	$1.44\ 10^{7}$	$1.22\ 10^{7}$	ns
Butanoic acid, 3-methyl-		$6.53\ 10^{7}$	*			/
Butanoic acid, 2-methyl-		$4.93\ 10^{7}$	*			/
Pentanoic acid		$1.56\ 10^{8}$	ns			/
Hexanoic acid		$1.47\ 10^{8}$	*	$7.86\ 10^{5}$	$9.36\ 10^{5}$	ns
Alcoholes						
2-butanol			/		$1.82\ 10^{6}$	ns
1-butanol		$2.67\ 10^{7}$	ns		$4.52\ 10^{5}$	ns
1-Butanol, 3-methyl-	$3.68\ 10^{6}$	$5.47\ 10^{7}$	*	$1.43\ 10^{6}$	$7.45\ 10^{4}$	ns
2,3-Butanediol		$1.84\ 10^{6}$	ns			/
1-Hexanol, 2-ethyl-			/	$4.69\ 10^{5}$	$5.43\ 10^{5}$	ns
Aldehydes						
Butanal, 3-methyl-		$6.12\ 10^{7}$	*	$3.38\ 10^{6}$	$2.03\ 10^{5}$	ns
Hexanal	$2.21\ 10^{6}$		*	$5.94\ 10^{5}$	$3.77\ 10^{6}$	*
Heptanal		$7.99\ 10^{5}$	ns	$2.43\ 10^{5}$	$7.05\ 10^{5}$	*
Octanal		$2.21\ 10^{7}$	*	$1.37\ 10^{6}$	$1.71\ 10^{6}$	ns
Benzaldehyde		$3.76\ 10^{8}$	*			/
Nonanal		$1.37\ 10^{7}$	ns	$2.35\ 10^{5}$	$3.88\ 10^{5}$	ns
Benzeneacetaldehyde		$3.26\ 10^{7}$	*			/
Esters						
Acetic acid, ethyl ester	$2.81\ 10^{8}$	$1.49\ 10^{8}$	ns	$2.88\ 10^{6}$		*
Butanoic acid, ethyl ester		$7.53\ 10^{8}$	*	$9.68\ 10^{6}$	$1.61\ 10^{7}$	ns
Hexanoic acid, ethyl ester		$6.74\ 10^{7}$	*		$3.16\ 10^{6}$	*
Octanoic acid, methyl ester		$6.84\ 10^{6}$	ns			/
Octanoic acid, ethyl ester			/	$4.84\ 10^{5}$	$8.16\ 10^{5}$	ns
Decanoic acid, ethyl ester		$1.28\ 10^{7}$	ns			/
Ketones						
Diacetyl			/	$2.04\ 10^{5}$		ns
2-butanone	$1.02\ 10^{6}$	$5.98\ 10^{6}$	ns	$5.53\ 10^{6}$	$1.76\ 10^{8}$	*

Table 2 (continued)

VCs by functional groups	Feed			Milk		
	Mean		*P*-value	Mean		*P*-value
Fatty acids	Summer	Winter		Summer	Winter	
2-pentanone	2.54 10^6	1.18 10^6	ns	3.01 10^5	1.97 10^6	*
Acetoin	7.99 10^6	1.08 10^7	ns	7.88 10^5	2.69 10^6	ns
5-Hepten-2-one, 6-methyl-	8.70 10^5	1.63 10^7	*			/
2-nonanone			/	2.42 10^5	1.10 10^5	ns
Terpenes						
Pinane	1.51 10^8	4.85 10^6	*	3.50 10^6	3.56 10^5	*
α-pinen	1.50 10^8	2.21 10^7	*	1.03 10^7	1.75 10^6	*
sabinen	3.18 10^8	2.61 10^7	*	6.74 10^5	3.89 10^5	ns
Limonene	1.31 10^8	2.21 10^7	*	2.62 10^6	1.65 10^6	ns
p-Cymene	1.60 10^8	2.07 10^7	*	1.81 10^6	3.06 10^5	*
β-Caryophyllene	3.59 10^8	3.32 10^8	ns	8.44 10^5	2.72 10^5	*
Miscellaneous						
2-Octene	2.20 10^7		/	6.50 10^5		*
Toluene	5.04 10^6	1.28 10^6	*	8.77 10^6	1.62 10^6	*
Undecane			/	6.98 10^5	5.36 10^4	*

NS, not significant at $P>0.05$; * $0.01 < P<0.05$; ** $0.001 < P<0.01$; *** $P<0.001$

Different groups of VCs were identified in cheese including fatty acids, alcohols, aldehydes, esters, ketones, terpenes and other compounds (miscellaneous) all collected in that order in Table 1.

Nine different fatty acids (Table 1, V1-V9) were identified in Nanos cheese. It is interesting that the effect of ripening time was statistical significant for all fatty acid identified in Nanos cheese (Table 1, column Ripening time). Also Fernández-García et al. (2004) found for all identified fatty acids in Zamorano cheese (hard type of cheese) that age of cheese significantly affected them. But throughout all the stages of ripening time (from 0 to 99th day) only four fatty acids were present in Nanos cheese: acetic, butanoic, hexanoic acid and octanoic acid (Online resource 1). Fatty acids as butanoic, hexanoic and octanoic acids are found in cheeses as the most abundant fatty acids (Aminifar et al. 2012). Pentanoic acid was only present in the summer season while other fatty acids were present in both seasons. Especially the content of 3-methyl butanoic acid (isovaleric acid), 2-methyl butanoic acid and hexanoic acid (Fig. 1a) in both seasons increased along with ripening. The total concentration of all fatty acids (Online Resource 2) was higher at the beginning (in the curd) and at the end of ripening (99th day) in the winter season. This suggests that the final content of fatty acids is however affected by the initial concentration in the curd. Alewijn (2006) showed that fatty acids found in curd mostly originated from milk. But in our case the milk for Nanos cheese was standardized at 3.05 to 3.15 % milk fat content so the reason for higher fatty acids in crud from winter season must be other. For Spanish "Idiazábal" cheese, Chávarri et al. (1999) reported that the content of short fatty acids (up to C 12) was higher in the winter season due to the action of lipoprotein lipase. Important effect on the amount of fatty acid because of microbal lipase found also Aminifar and Emam-Djomeh (2014). As we have already indicated the extent of lipolysis in cheese is also influenced by other factors, including cows' feed (Park 2001), lipolytic enzymes which can be derived from milk (Collins et al. 2003), due to enzymes of different microorganisms (lactic acid bacteria, moulds) (Curioni and Bosset 2002) and also due to rennet (Aminifar and Emam-Djomeh 2014). But Urbach (1990) argues that due to sufficiently low plane of nutrition, cows can produce spontaneously lipolyzing milk. In our case the higher amount in the winter have also branched-chain fatty acids (3-methyl butanoic acid, 2-methyl butanoic acid) which originate from free amino acids (leucin and izoleucin respectively) (Curioni and Bosset 2002; Delgado et al. 2011). In contrast with our results, the study of Perotti et al. (2009) revealed, that the content of fatty acids was higher in the summer season due to the higher numbers of psychrotrophic bacteria present in raw milk. Psychrotrophic bacteria develop with prolonged storage of milk at low temperature (2–4 °C). It is known their lipolytic and proteolytic enzymes (Bergère and Lenoir 2000) are not destroyed by the temperature of pasteurization (Hermier and Cerf 2004). Probably this is also one reason for the higher amount of fatty acids in the winter season in our case. But to prove it further microbiological analysis would be required. Most of the fatty acids, having 4–20 C-atoms, come from the lipolysis of triglycerides by moulds (Curioni and Bosset 2002). In our case it could be one of the reasons of higher amount of fatty acids in winter. In winter there was higher humidity in ripening room as a result of weather condition. Higher humidity is more suitable for the growth of moulds.

Among fatty acids detected in Nanos cheese butanoic acid and hexanoic acid belong to components with high-concentration, on the 99th day $1.76 \cdot 10^8$, $7.61 \cdot 10^7$ respectively (Online resource 1). Also Stuknyte et al. (2014) found for butanoic acid as a major compound in hard type Bitto cheese. It is known that butanoic acid significantly contribute to the flavour formation in cheese (Curioni and Bosset 2002) and usually represents one of the most extensive aromatically active compounds in cheese (Cornu et al. 2009). Considering the high content of butanoic acid (in comparison with the other fatty acids) we could conclude that this acid regulated the trend of the total concentration of fatty acids in the course of maturation. The high content of hexanoic acid was already reported by Delgado et al. (2011) who classified this component as one of the major contributors in final flavour formation in hard type cheese. Moreover, in Nanos cheese this component was detected in the winter season, what is in accordance with the study of Carpino et al. (2004), where they confirmed the absence of hexanoic acid in cheeses made from milk from pasture season.

Among identified 13 ketones (Table 1, V37-V49), two of them (2,3 heptadione, 6-methyl 5-hepten-2-one) were present only in Nanos cheese samples from the summer season.

According to the changing trend of ketones during ripening period, it could be summarized that the ketones are mainly precursors for other components, since the oscillating trend was observed also by Delgado et al. (2010). It is known that methyl ketones result from oxidation of the fatty acids and they can be converted into the secondary alcohols (Brulè et al. 2000). Diacetyl, derived from lactose and citric acid (Zeppa and Rolie 2008), had a pronounced downward trend (Fig. 1b) which was also found by Mallia et al. (2005). It is known that diacetyl (V36) can be converted into acetoin, 2,3 butanediol and 2-butanone (McSweeney and Sousa 2000), by the action of non-starter bacteria (Engels et al. 1997). Downward trend of acetoin was probably due to the known fact that acetoin could also be precursor for 2,3-butanediol (McSweeney and Sousa 2000). Decreasing trend of acetoin was found also in some other studies cited by Cakmakci et al. (2012).

We noticed very similar oscillating trends for 2-octanone and 2-nonanone of their concentration during ripening (Fig. 1c and 1d). As methyl ketones result from the oxidation of fatty acids (McSweeney and Sousa 2000) the large quantity of them are

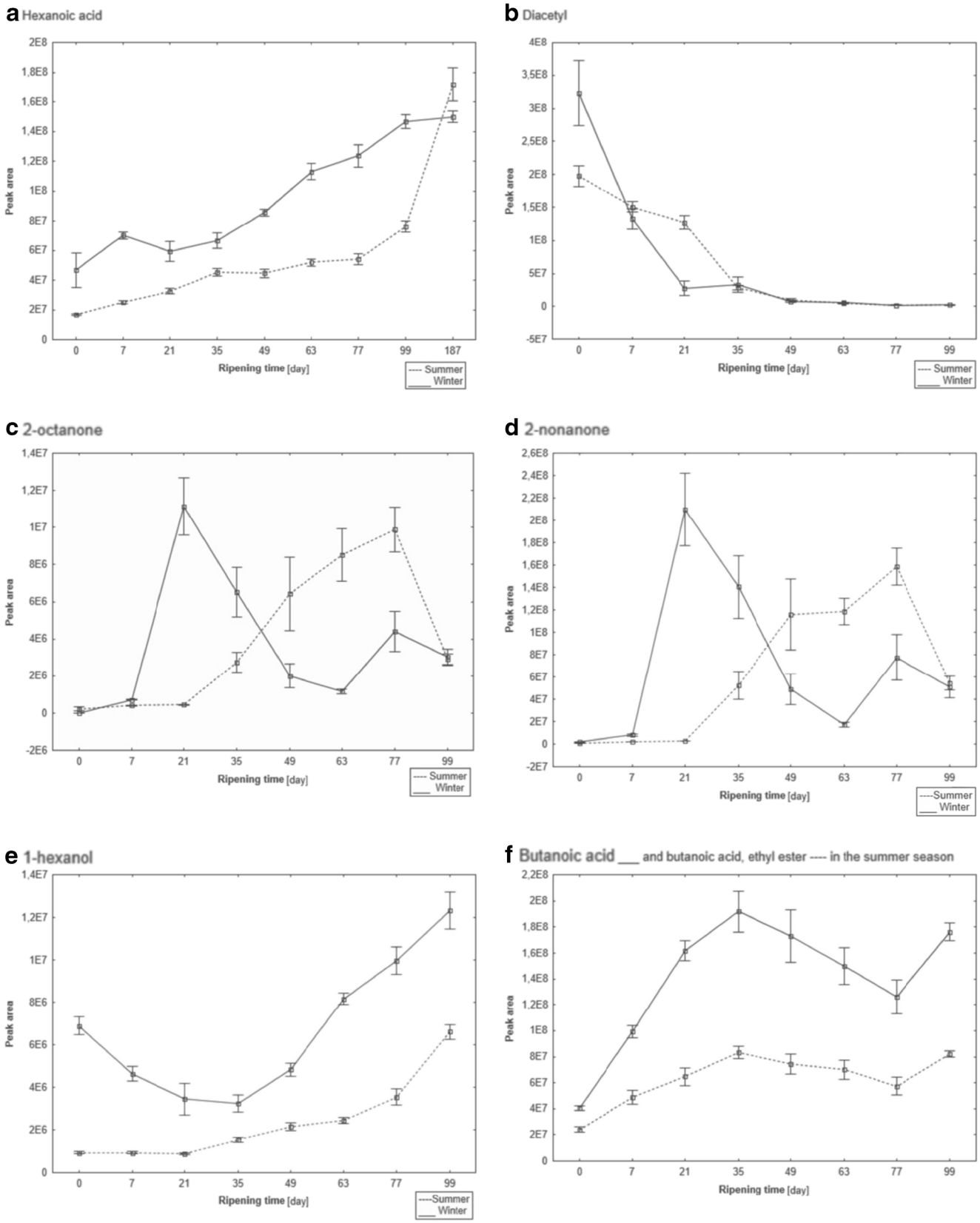

Fig. 1 Trends of some volatile compounds (mean ± standard error) in cheese during ripening time

expected in case of cheeses with molds and hard type cheeses (Verzera et al. 2004), which have a longer time of ripening. This fact explains the rising trend, whereas the lately decreasing trend could be explained by the fact, that they are known precursors of some secondary alcohols (Delgado et al. 2011).

In Nanos cheese in both seasons 10 different alcohols (Table 2, V10-V19) were identified, whereas only 1-hexanol (Fig. 1e) and 2-ethyl hexanol (Online resource 1) were present in the summer and winter seasons throughout the ripening. Maximum values of all alcohols were observed in the summer season additional data are given in Online Resource 2.

In both seasons, 2-heptanol reached slightly higher maximum concentration in comparison with 2-nonanol, $5.91\ 10^7$, $3.86\ 10^6$ respectively in the summer and $5.91\ 10^7$, $2.89\ 10^6$ respectively in the winter season (Online resource 1). This could be explained by a higher concentration of 2-heptanone (precursor of 2-heptanol) compared with the 2-nonanone (precursor of 2-nonanol), $3.92\ 10^8$ for 2-heptanone, $1.59\ 10^8$ for 2-nonanon in the summer and $4.09\ 10^8$ for 2-heptanone, $2.10\ 10^8$ for 2-nonanone in the winter season (Online resource 1).

The concentration of primary alcohol 1-hexanol increased with ripening (Fig. 1e), which can be explained by the fact that it has origin in fatty acids (Alewijn 2006). Fernández-García (2002) also indicated that 1-hexanol significantly increased during the cheese ripening. Petersen et al. (2008) found out that some strains of *Lb. helveticus*, which was also added to Nanos cheese as starter culture, increased the content of 1-hexanol in cheese. The amount of 1-hexanol was higher in our case over the winter season (Fig. 1e). Besides, primary alcohols can also be formed in the reduction of aldehydes (McSweeney and Sousa 2000). This is in accordance with our observation, since higher amounts of hexanal were present in the milk of the winter season stimulating higher conversion into 1-hexanol.

Nine esters (Table 2, V29-V36) were identified in Nanos cheese, with the majority of ethyl esters which are responsible for the floral note of other cheeses (Grana Padano, Minas, Castelmagno) (Cakmakci et al. 2012). Among the most important aromatic compounds in cheese are acetic acid ethyl ester (ethyl acetate), butanoic acid ethyl esters, octanoic acid ethyl ester and hexanoic acid ethyl ester (Singh et al. 2003). These esters were also identified in Nanos cheese, with highest concentrations of butanoic acid ethyl ester and hexanoic acid ethyl ester. Ethyl hexanoat was also found as the most significant ester in Montasio cheese (Innocente et al. 2013). While both esters (ethyl butanoat, ethyl hexanoat) were predominant esters with an important role in the flavour formation of Grana Padano cheese (Wolf et al. 2010). Octanoic acid methyl ester and decanoic acid ethyl ester were present only in the winter season (Online resource 1).

It is known that esters can be formed during ripening (McSweeney 2004) among various esterase activities of lactic acid bacteria also due to esterase activity of *Lb. helveticus* (Aminifar et al. 2012). The raising trend was also evident in our case, since the total amount of esters increased (Online Resource 2). By the 99 day of ripening it was higher in the winter season.

In both seasons the predominant ester was butanoic acid ethyl ester. In addition, also its precursor - butanoic acid – was the predominant fatty acids in Nanos cheese. This finding is supported by the known fact that esters are formed by the esterification of free fatty acids and alcohols (Gioacchini et al. 2010). *Str. thermophilus*, which was also added to Nanos cheese as starter culture, greatly contribute to the formation of butanoic acid ethyl ester (Liu et al. 1998).

The fact that the content of ethyl ester could be dependent on the amount of fatty acids (Liu et al. 1998; Mallia et al. 2005) was also found in our case for some esters (butanoic ester ethyl ester, hexanoic acid ethyl ester, octanoic acid ethyl ester) and their precursors. For example the trend of butanoic acid followed the trend of its ethyl ester, which is evident especially in the summer season (Fig. 1f). On the other hand it was noticed for hexanoic acid ethyl ester and octanoic acid ethyl ester as well as for their precursors hexanoic acid (Fig. 1a), octanoic acid (Online resource 1) that they reached higher values during the winter season.

There was a similar trend in the total aldehyde content noticed in both seasons. In fact, they firstly increased (up to the 7^{th} day of ripening) and after that a fast decrease was observed (Online Resource 2). It is known, that aldehydes can be rapidly reduced to primary alcohols or oxidized to certain acids (Curioni and Bosset 2002). In Nanos cheese the overall content of aldehydes was higher in the winter season. But only for octanal, nonanal, benzacetaldehyde and decanal effect of season was significant different (Table 2, column Season). In contrast ripening time had significant effect for all aldehydes except for octanal (Table 2, column Ripening time). The proportion of the total content of aldehydes was very low (0.84 to 4.58 % in summer and 1.18 to 4.71 % in the winter season) (Online Resource 2). Gioacchini et al. (2010) found out that in hard type cheese "Fossa" aldehydes achieved very low proportion (8 %) in comparison with the other components.

In Nanos cheese there were nine aldehydes (Table 2, V20-V28), which were not present at all times of ripening. Among nine aldehydes benzaldehyde, heptanal, nonanal and octanal were the most abundant ones. It is known, that a diet has more influence on the content and composition of the milk fat rather than the protein content (Cornu et al. 2009). Most of identified aldehydes (hexanal, heptanal, octanal, nonanal, 2-nonenal, decanal) originate from fatty acids (Alewijn 2006). Regarding the higher content of some fatty acids (one of the known precursors of aldehydes) in the winter season, the increased content of aldehydes in the winter season was thus expected. Mallia et al. (2005) also argue that various concentrations of

alkenes in cheese could be due to different diets of the animals. While Cifuni et al. (2007) found that ray-grass silage influenced on the higher content of decanal and octanal in cheese in comparing with cheese made from milk of hay.

Octanal and nonanal also reached higher concentrations in Nanos cheese from the winter season. It is interesting that both aldehydes were present only in the feed of winter season and their concentration was also higher in milk from winter season (Table 2).

In Nanos cheese five monoterpenes (pinane, α-pinene, sabinene, limonene, p-cymene) and a sesquiterpene (β-caryophyllene) were identified. Only sabinene was not detected during the winter season. Terpenes have been already identified in the cheeses made from the milk of animals of different feed intake and were not present only with grazing animals (Zeppa et al. 2004). But also if the animals consumed hay, grass silage and maize silage (Stefanon and Procida 2004). In our case, the impact of season was statistically significant for all terpenes identified in cheese except for limonene (Table 2, column Season). As expected, higher amounts of terpens were noted in feed, milk and cheese samples from the summer season compare to the winter season (Table 1). In winter, the amounts were lower probably due to the fact that drying of feed could lower the content of terpenes in plants as indicated by Tompa (2005).

In Nanos cheese also seven not classified VCs (Table 2, V56-V63: 2-octene, toluene, ethylbenzene, dimethyl sulfone, undecane, p-cresol, δ-octalactone and δ-decalactone) were identified. The amount of phenolic component toluene (Curioni and Bosset 2002; Alewijn 2006) which comes from carotene degradation in fresh grass, was higher in the summer season and in cheese from the pasture season (Abilleira et al. 2010) in addition, the effect of season was statistically significant (Table 2, column Season). Similar observations were noted in our study where the presence of toluene was identified in fresh grass and in the summer milk (Table 1). This fact suggests the possible passage of this component from feed to cheese. Frank et al. (2004) reported that toluene had no contribution to the cheese aroma, while Curioni and Bosset (2002) and Delgado et al. (2011) confirmed a nutty aroma which is also characteristic of Nanos cheese.

PCA of Nanos cheese from different season (pasture and non-pasture) on the 63th day of ripening

To analyze the amount of each VC in different seasons PCA was also used because it can show natural group tendencies as the system does not know how to build the groups (untrained). With this method, a linear combination of n parameters is calculated to maximize the distance between the points or samples in the n-dimensional space created (Pillonel et al. 2003). In our case the PCA was done on the 63th day of ripening (minimal ripening period for commercial uses of Nanos cheese), discriminating VCs in ripened cheese wheels from two different seasons (summer, winter). The PCA plots of VCs are illustrated on Fig. 2. With the first component (F1) we explained approximately 20 % of the total variance and with second component (F2) approximately 8 % of the total variance (Fig. 2). The component F1 separates summer wheels (K1-6) from the winter wheels (K7-12) (Fig. 2). VCs which contribute to first and second component are shown in Table 3.

Some VCs which most strongly characterize samples of cheese wheels from summer or winter seasons on the 63th day of ripening by PCA, were also identified in feed and/or milk. For these VCs we found some differences in the amount between seasons (Table 1) which could indicate the possible effect of feed on VCs in cheese. Compared to summer season, cheese from winter season contain higher amounts of acetic acid (V1), butanoic acid (V2), 3-methyl butanoic acid (V3), hexanoic acid (V6), octanoic acid ethyl ester (V30), hexanoic acid ethyl ester (V32), decanoic acid ethyl ester (V35) and 2-butanone (V37).

Acetic acid (V1) which has vinegar sour and sharp odour (Frank et al. 2004), also had higher amount in grass silage, but it was not identified in milk sample. Compared with other identified VCs, acetic acid reached higher level. Even in other hard type cheeses (Gioacchini et al. 2010) it was of higher content compared to the other VCs. Acetic acid is formed due to the activity of different microorganisms (e.g. among LAB also from genus *Steptococcus*) in the metabolism of lactose (Delgado et al. 2011). Carpino et al. (2004) claim that this acid

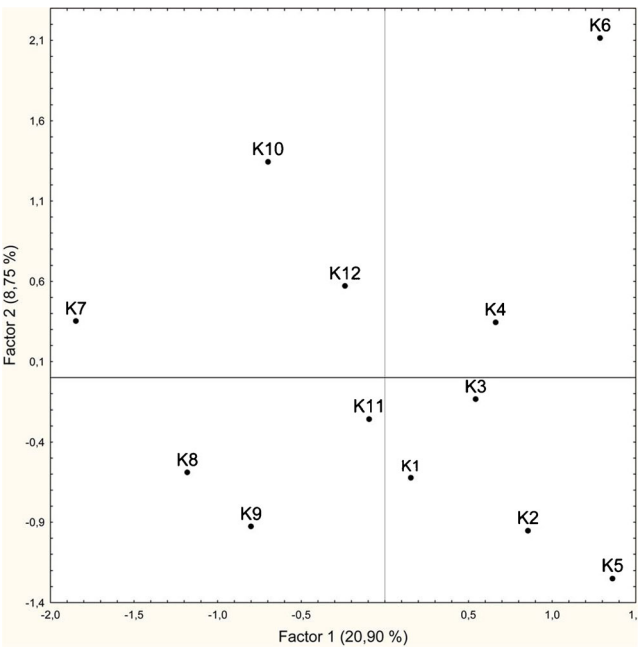

Fig. 2 Principal component analysis for some VCs' components identified on the 63th day of ripening, separated by wheels: summer wheels (K1-6), winter wheels (K7-12)

Table 3 The calculated weight of the components of certain variables-volatile components (V) for the first two components (Componet 1, Componet 2) on the 63th day of ripening

	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10	V11	V12	V13	
Component 1	−0,91	−0,50	−0,97	0,35	0,81	−0,92	−0,90	−0,92	0,27	0,11	0,75	0,55	−0,77	
Component 2	0,17	0,79	0,02	−0,32	−0,05	0,02	−0,07	0,05	−0,33	0,81	0,27	0,19	0,15	
	V14	V16	V17	V18	V19	V22	V23	V24	V25	V26	V28	V29	V30	
Component 1	0,23	−0,92	0,12	−0,48	−0,64	0,38	−0,88	−0,59	−0,52	0,31	0,66	0,00	−0,08	
Component 2	0,89	0,16	−0,26	−0,14	−0,26	−0,24	−0,08	0,20	0,17	0,27	0,03	0,95	0,86	
	V32	V33	V34	V35	V36	V37	V38	V40	V41	V43	V44	V46	V47	
Component 1	−0,91	−0,82	−0,92	−0,83	−0,21	−0,90	0,54	−0,22	0,14	0,55	0,73	0,47	0,55	
Component 2	0,07	−0,03	0,05	0,05	0,92	0,23	0,35	0,92	0,94	−0,27	−0,19	−0,45	−0,28	
	V48	V49	V50	V51	V52	V53	V54	V56	V57	V58	V59	V60	V61	V62
Component 1	0,35	0,72	0,61	0,82	−0,64	−0,33	0,36	0,58	0,03	0,74	0,75	0,30	−0,91	−0,35
Component 2	−0,47	0,03	0,62	0,25	−0,08	−0,28	−0,21	−0,13	0,06	0,32	−0,20	−0,33	0,15	0,18

V, identical number of volatile compounds are the same as in Table 2

also originates from amino acids, while some of acetic acid is probably transferred from feed to milk. In our case in feed from winter season (grass silage) it reached higher value in comparison to the summer feed. However, acetic acid was not identified in milk in our case. For this reason it is difficult to confirm its transfer from feed to milk and from there to cheese. Of course, it could not be ignored, that the major source of acetic acid in cheese is its production due to metabolism of certain microorganisms, whose activity might be higher in the winter season.

Butanoic acid (V2), which was in our case higher in winter season, occurs in cheese due to the activity of lipases or microbiota (McSweeney and Sousa 2000). It is also the main product of clostridia (Stefanon and Procida 2004) particularly *Clostridium tyrobutyricum* (Martin et al. 2005) in fermentation of lactose (Stefanon and Procida 2004). Clostridia spores can be transferred from feed via milk into cheese. The result is a higher concentration of butanoic acid in cheese (Danner et al. 2003). But it depends also on the quality of silage (Martin et al. 2005) which could also be one of the reasons why it was higher in the winter season (silage feed) compared to the summer season. In fact, the butanoic acid was identified only in grass silage, whereas it was present in milk of both seasons with similar quantities. 3-methyl butanoic acid (V3) with a rotten cheesy note (Frank et al. 2004) also had higher amount in grass silage, but in the milk sample it was not identified as acetic acid.

Hexanoic acid is responsible for sharp-goaty odour (Frank et al. 2004), while ethyl esters possess pleasant sweet and fruity notes (Van Leuven 2008). Hexanoic acid (V6) was identified in grass silage and milk from both seasons. It is interesting that hexanoic acid ethyl ester (V32) was only present in grass silage and milk from winter season which indicates possible transfer of these compounds from feed to cheese. It is interesting that Carpino et al. (2004) in their study found that hexanoic acid was not present in cheeses made from the milk of cows that were grazing, whereas hexanoic acid ethyl ester was present in significant amount. Since, it is the ethyl ester of low threshold (Curioni and Bosset 2002), it could be one of the most important component defining the aroma of Nanos cheese. Also Curioni and Bosset (2002) argue that hexanoic acid ethyl ester is important for formation the final cheese flavor of various hard cheeses: Cheese Cedar, Grana Padano, Regusano. It is known, that the amount of ethyl hexanoate can be increased by the addition of lactic acid bacteria, including *Str. thermophilus* (Liu et al. 2004). *Str. thermophilus* affect the formation of ethanol (Liu et al. 2004), which is involved in the esterification with the fatty acids, and it is an important factor in the formation of esters (McSweeney 2004). Hexanoic acid ethyl ester is also produced by psychrophilic bacterium *Pseudomonas fragi* (Liu et al. 2004). Octanoic acid ethyl ester (V30) was not detected in any samples of feed but its content in milk samples was higher from the winter season. So for these compounds the most possible passage is thus from milk. Decanoic acid ethyl ester (V35), with a fruity odour (Liu et al. 2004; Mallia et al. 2005), was only identified in the grass silage. The octanoic acid ethyl ester is referred to be as comprehensive ester in Grana Padano cheese, and it was identified also in other hard cheeses: Parmigiano-Reggiano cheese, Cheddar cheese (Wolf et al. 2010) and Emmental cheese (Curioni and Bosset 2002).

In the case of 2-butanone (V37) there were higher concentrations also in feed from winter season (grass silage). 2-butanone which has sap-acetone odour (Table 2, column Flavour notes) (Van Leuven 2008) is the VC of grass silage and occurs in milk through air, inhaled by cows (Urbach

1990). In literature we did not specifically observed the impact of grass silage on the content of 2-butanone in cheeses. In fact, 2-butanone is formed from 2,3-butanediol, which derives from the citrate, due to the activity of certain bacteria (Keen et al. 1974). It was already found that the formation of 2-butanone is influenced by different bacteria added such as starter culture (e.g. *Lactococcus lactis* ssp. *cremoris*, *Leuc. mesenteroides*) and also wild strains of lactic acid bacteria (e.g. *Lb. plantarum*, *Lb. mesenteroides*, *Lb. casei*, *Lb. brevis*, *Lb. rhamnosus*) (Randazzo et al. 2007). From the results of preliminary studies, it is evident that the higher impact on the content of 2-butanone have microorganisms.

Terpenes have impact on fruity hazelnut aroma (Bugaud et al. 2001) and intense flavour (Urbach 1997). Some also have herbaceous and citrus-like note (Mariaca et al. 1997). Terpenes such as pinane (V49) and sabinene (V51) marked the summer season with a higher content. They were present in feed and milk in both seasons, but their amount in the summer season was higher.

Conclusion

In this study we evaluated changes during ripening time and seasons on the VCs profiles in Nanos cheese. This study will enable profound characterization of cheeses with PDO status based on nutritional profile and seasonal feed variance given to animal.

The results of our study confirmed changes of VC profile during ripening with the highest amounts of 2-heptanone, 2-nonanone, butanoic acid and butanoic acid ethyl ester at the end of ripening. During cheese ripening (including the 99th day) 62 VCs were detected which were not present at all times in both seasons (summer and winter). The amount of some VCs was changing probably due to their precursor role for other compounds (e.g. 2-heptanone is a precursor of 2-heptanol). Most of VCs content increased during ripening (especially fatty acids and esters). But in contrast it was found that some VCs (e.g. 2,3 butanedione/diacetyl, 3-hydroxy 2-butanone /acetoine) had higher amounts at the beginning of ripening and then a downward trend. Probably because these VCs (diacetyl, acetoin) are precursors for other components (2,3 butanediol, 2-butanone) as in cheese during ripening many biochemical changes occurred.

We also found differences between seasons (summer, winter) in the quantity rather than quality of individual VCs of Nanos cheese, despite the fact that the feed in both season is from the same region, milk is heat-treated (thermised) and the technological process of cheese making is always the same. For example in winter there were higher concentrations of some fatty acids (hexanoic acid, octanoic acid, decanoic acid) and esters (hexanoic acid ethyl ester, octanoic acid ethyl ester) compared to the summer season. For some VCs of Nanos cheese we also noticed possible links with VCs of feed and/or milk what confirms impact of feed and milk on studied cheese.

All the possible transfers of VCs from feed to milk did not always reflect in the cheese (e.g. ethyl acetate) but we noticed some VCs possible transfer from feed and milk to cheese (e.g. terpenes, toluene). Some of the VCs were present only in a particular season (e.g. in summer pentanoic acid, 2,3 heptadione, 6-methyl 5-hepten-2-one; in winter octanoic acid, methyl ester and decanoic acid, ethyl ester) but for these VCs we did not find any link with the feed or milk.

With the PCA method, we found some VCs that discriminate samples of Nanos cheese from two different seasons (summer, winter). For some of these VCs a possible connection with feed and milk was also found (acetic acid, butanoic acis, 3-methyl butanoic acid, hexanoic acid, hexanoic acid ethyl ester, octanoic acid ethyl ester, decanoic acid ethyl ester, 2-butanone, pinane, sabinene).

The final profile of VCs in cheese at the end of the cheese ripening is due to the interaction of numerous factors and reactions (by the microorganisms due to different feed). Because of this it is difficult to examine all VCs in Nanos cheese and predict origins and impacts for individual VCs. Our study presented aromatic profile not only of cheese but also of milk and feed which could be key factors of cheese aroma formation especially in cheese with PDO status. Results of our study strongly support the use of instrumental analysis in VCs profiling of cheeses, alone or possible in combination with sensorial analysis. Namely, the main advantage of instrumental analysis is avoidance of possible discrepancies resulting from sensory evaluations. Our study is an important contribution to the developing field of combined approach using instrumental and sensory methods to assure more accurate, precise and reliable information on VCs in cheeses, with special focus to the traditional cheeses. This is the first report which deals with the changes of VCs in cheese during ripening influenced by season and feeding system, and with possible transfers of VCs from feed and milk to cheese in the case of traditional Nanos cheese which could also serve as a model system in other hard type cheese studies especially those with PDO status.

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