Research of volatile compounds in cheese affected by different technological parameters

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Summary

Nanos cheese is a hard type of cheese produced from milk obtained from cows grazing in a limited specific geographic area (Vipava valley, Slovenia). This study is the first report about the influence of certain technological parameters and their impact on the Nanos cheese flavour, which can be meant as a model of hard-type cheeses. Apart from collecting the milk from different geographic areas, ripening location, ripening method (classical vs in grape marc), ripening temperature (12 °C, 18 °C) and the amount of starter culture were changed and evaluated. For the determination of the profile of volatile organic compounds (VOCs) in the cheese, solid phase micro extraction (SPME) coupled to gas chromatography – mass spectrometry (GC-MS) was used. Doubling the addition of the starter culture contributed to higher levels of certain VOCs (e.g. benzaldehyde responsible for nutty flavour). Higher temperature during ripening increased the content of some ethyl esters and alcohols from 2 to 10-fold and it had an effect on appearance of the cheese. It was also observed that cheeses ripened in grape marc had a higher content of most esters. The differences in VOCs profile were not significant in cheeses from different geographic areas and ripening locations.

Keywords

cheese; volatile compound; starter culture; technological parameter

Cheese quality, chemical, technological and microbiological, depends mainly on the characteristics of milk and the process of cheese production [1, 2]. The characteristic flavour of individual cheeses is commonly defined by a complex balance of volatile organic compounds (VOCs) as well as non-volatile organic and inorganic compounds [3]. The analysis of VOCs in foods, including dairy products, is nowadays one of the most common methods for the determination of their quality. VOCs in cheeses could be differentiated by chemical classes, such as fatty acids, alcohols, ketones, esters and aldehydes [4].

VOCs are affected by many factors among which the animals' (e. g. cows') feed, season and cheese ripening method are the most important [2, 5, 6]. During cheese ripening, VOCs are formed through various biochemical processes such as lipolysis, proteolysis, metabolism of lactate and citrate, metabolism of fatty acids and amino acids [7]. In the study of Stefanon and Procida [2], the presence and the contents of individual odour-active compounds were found to be due to the diet. The indigenous microflora is a key factor in the development of the volatile profile of cheese [2]. On the other hand, heat treatment may partially destroy technologically beneficial microorganisms of raw milk coming from different areas [8]. Centeno et al. [5] found that the higher storage temperature of milk probably favoured the growth of bacterial strains with a strong proteolytic activity, which enhanced the cheese proteolysis during ripening. Some compounds could derive also from non-enzymatic reactions, they come rather from methodology applied or they depend on production area [6].

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A suitable analytical method for determining VOC profiles in cheese is gas chromatography (GC) with mass spectrometry (MS) [9]. During the last twenty years, the improvement of GC-MS analysis has been achieved by introducing micro-extraction on solid phase (solid phase micro extraction, SPME) [4, 10]. The method was efficiently implemented in cheese analysis [3, 4, 11].

Instrumental determination of cheese flavour employing GC-MS was the main tool used in this study to assess the impact of VOCs, since it is known that VOCs' profile is one of the most powerful tools in preventing adulteration of cheeses with protected designation of origin (PDO) [3]. The model cheese for the study was Nanos cheese from the Vipava valley in Slovenia, which has a PDO status granted by EU Regulation No. 987/2011 [12]. This hard-type cheese was chosen due to its unique flavour and national importance for the region and the country. The aim of the experiments was evaluation of the effects of five main technological parameters on the flavour and the quality of Nanos cheese, namely, origin of milk, ripening location, ripening temperature, the amount of added starter culture and finally ripening under various conditions (ripening in grape marc compared to classic ripening). The parameters were changed individually in order to facilitate statistical analysis.

MATERIALS AND METHODS

Experimental design

The Slovenian traditional Nanos cheese is a cheese of hard type. Its production follows a specific procedure described in detail in the specification [13], but it is briefly described in the following text.

The milk for Nanos cheese can only be obtained from a specific breed of cows, also all the animals must graze on the pastures on Nanos plateau located in the Vipava valley, the Karst area (Slovenia) and the surrounding highlands. The Nanos cheese for this study was produced in the Vipava dairy (Vipava, Slovenia) from the milk standardized at 3.1–3.2 % milk fat content, thermized at 63–69 °C. The milk was inoculated with a thermophilic starter culture of *Streptococcus thermophilus* and *Lactobacillus helveticus* at 32–34 °C and then rennet (Chr. Hansen Holding, Hoersholm, Denmark) was added. The mass was left to coagulate for 30–40 min. After that, the coagulum was cut and the curds were scalded at 45–50 °C. When cheese grains were properly dry, whey was removed. Cheese grains were then pressed at up to 0.6 MPa for 20–40 min. Following this, the curd was cut into blocks, placed into round hoops (diameter 35 cm, height 15 cm) and placed under pressure again, this time under three consequential graduated compressions (first: 30 min at 0.25 MPa; second: 30 min at 0.4 MPa; third: 30 min at 0.5 MPa). During the compressions, the curd was checked for the pH value. When the cheese wheels reached the appropriate pH value (pH 5.4–5.0), they were put into 18–24 % brine (NaCl) for three days. Cheese ripening lasted for at least 60 days at 9–15 °C and 80–90% humidity. The cheese wheels weighed 8–10 kg.

The experiment was divided to four parts explained in detail in Tab. 1. Five parameters were changed individually. Parameters which were not changed during the experiment were left according to the specification. In this study, the milk for cheesemaking (CP1), the ripening locations (CP2), the amount of starter culture (CP3), the ripening temperature (CP4) and the mode of ripening (CP5) were changed in separate parts of experiment.

Milk for cheesemaking coming from different areas

In part A, the cheeses were made according to the specification [13] and the origin of milk was the only changeable parameter (Tab. 1). Nanos cheese made from milk coming from different areas was compared: milk from the original area of Nanos cheese (geographical area limited in south-west by Karst plateau and Vipava Valley, in the north by Trnovo Plateau and in the east by the Karst area of Postojna Basin) and from the outside areas (milk coming from the areas outside the original area specified above). Since our primary tool was GC, we could not examine other components such as solids, proteins or amino acids, which could serve as substrates for bacteria during fermentation. Nevertheless, as it was shown in the specification [13], individual VOCs that were predominant in summer or winter season feed or milk (e.g. 3-methyl-1-butanol, 3-methyl butanal, hexanal), were not decisive for differentiation of the cheeses according to the season.

That is why this study is focused only on VOCs of the cheese. For this purpose, two batches of Nanos cheese made from "selected" milk from the area of Nanos cheese, and two batches of cheese made from milk from the outside range, the so called "unselected" milk, were compared. Within each batch, three cheese wheels were selected as a sample on 63rd day of ripening. The results were analysed via principal component analysis (PCA) in order to compare the cheeses made from "se-

Tab. 1. Process flow chart of experimental setup.

Changeable parameters			Part A	Part B	Part C	Part D
CP 1	Milk for cheesemaking	"Selected" milk "Unselected" milk	CP 1	AS	AS	AS
CP 2	Ripening location of cheese	Presence of grape wine Absence of grape wine	AS	CP 2	AS	AS
CP 3	Starter culture	Regular amount Double amount	AS	AS	CP 3	AS
CP 4	Ripening temperature	12 °C 18 °C	AS	AS	CP 4	AS
CP 5	Mode of ripening	Classical In grape marc	AS	AS	AS	CP 5

CP – changeable parameters; AS – according to specification.

lected" and "unselected" milk. VOCs profile of each cheese sample was analysed in three replicates.

Different ripening locations

In part B, the cheeses were produced according to the specification for Nanos cheese production [13] and then ripened at two different locations (wine region Vipava, not a wine region Gorenja Vas, Slovenia). The ripening location was the only parameter that was changed according to specification for Nanos cheese production, which describes in detail the location of ripening. The first ripening location was the maturation room in the Vipava dairy (in the presence of grape wine, as it is required in the specifications for Nanos cheese; GPS coordinates 45°50'45.0" N, 13°57'44.4" E), whereas the second ripening location was chosen elsewhere and in the absence of grape wine (Gorenja Vas; GPS coordinates 46°06'20.6" N, 14°08'23.6" E). Four wheels of Nanos cheese from the same batch were chosen and two were ripened in the Vipava dairy for 63 days, whereas the other two were transported to Gorenja Vas and ripened there also for 63 days. VOCs profiles of each cheese wheel were analysed in five replicates.

Different amount of starter culture and ripening temperature

In part C, two batches of Nanos cheese were produced following the specification [13]. The first batch was added a regular amount of starter culture (*Str. thermophilus* 5.3 mg·l^{-1}; *Lb. helveticus* 1 mg·l^{-1}), whereas the second batch contained a double amount of starter culture (*Str. thermophilus* 11 mg·l^{-1}; *Lb. helveticus* 2 mg·l^{-1}). Two cheese wheels from each batch were selected and ripened during 63 days at two different temperature ranges. One cheese wheel with the regular amount of starter culture and one with a double amount of starter culture were ripened at the specified temperature of 12 °C, while other two cheese wheels were ripened at a higher temperature of 18 °C. At the end of the ripening process, the effect of different amount of starter culture and the effect of ripening temperature could be compared at the same time. VOCs profiles of each cheese wheel were analysed in five replicates.

Ripening in grape marc

In part D of the experiment, classical ripening (introduced in the technological process of Nanos cheese) [13] and ripening in the grape marc ("Drunken" cheese) were compared. The cheese wheels (diameter 32–34 cm, height 7–12 cm) were put in a wooden container and were completely submerged in a layer of grape marc (approximately 10 kg) for 17 days. Then, cheese wheels were put in a wooden barrel (50 l) and immersed in red wine of Barbera Merlot genre (Vinska klet Vipava, Vipava, Slovenia) for 50 h. Ripening in grape marc was done in two ways:

– Cheese placed in grape marc immediately after salting (D1) – Immediately after salting, cheese wheels were put in Cabernet Sauvignon grape marc for 17 days and then immersed in red wine of Barbera Merlot genre for 50 h. After that, the last 58 days of ripening took place in a classical way in the Vipava dairy maturation room. Thus, the production of cheese following this method took in total 77 days, i.e. 17 days in grape marc + 2 days (50 h) in wine + 58 days classical ripening in the Vipava dairy maturation room.
– Cheese placed in grape marc after 6 weeks of the classical ripening process (D2) – After 6 weeks of classical ripening process, cheese

wheels were placed in Cabernet Sauvignon grape marc for 17 days and then immersed in red wine of Barbera Merlot genre for 50 h. Following this method, the total time of ripening was 61 days, i.e. 42 days classical ripening in Vipava dairy maturation room + 17 days in grape marc + 2 days (50 h) in wine.

For each method of ripening, VOCs profile of each cheese wheel was analysed in five replicates.

Determination of volatile compounds

Analytical standards

Analytical standards of VOCs were purchased from several suppliers: hexanal, octanal, 1-hexanol, 2-octanone from Alfa Aeser (Karlsruhe, Germany); 2-ethyl 1-hexanol, 3-methyl 1-butanol, acetic acid, α-pinene, 2-pentanone, 2-butanol, ethyl ethanoate, ethyl hexanoate, 2-pentanone from Fluka (Buchs, Switzerland); δ-decalactone, 1-octanol (internal standard), 2,3-butanediol, 1-butanol, 3-methylbutanoic acid, butanoic acid, β-carryophyllene, sabinene, *p*-cymene, heptanoic acid, 2-butanone, benzaldehyde, octanoic acid, 3-methyl butanal from Sigma-Aldrich (St Louis, Missouri, USA); 2-methylbutanoic acid, decanoic acid, 2-pentanol, nonanal, 2-heptanone, 2-nonanone, limonene and 3-hydroxy-2-butanone from Merck (Darmstadt, Germany). The chromatographic purity was in all cases above 95 %.

Sample preparation

Cheeses were sampled by cutting the cheese wheel with a conical cheese borer from the lateral surface of the cheese wheel towards the centre. The outer 10 mm layer of the cheese sample was discarded while the rest was grinded in a blender and the amount of 4 g was immediately put into a 20 ml headspace vial according to our previously developed procedure [14].

Extraction of volatile compounds

VOCs present in cheese samples were analysed using solid phase micro-extraction (SPME) kit (Supelco, Bellefonte, Pennsylvania, USA) following the supplier‘s instructions. The chosen SPME fibre was 20 mm long, coated with divinylbenzene, carboxen and polydimethylsiloxane (50/30 μm). Every fibre was prior to exposure to the sample conditioned and activated by inserting it into the GC injector at 270 °C for 30 min, according to producer‘s instructions. The fibre was then exposed for 24 h (above the cheese sample to volatile compounds in vapour phase of gas-tighed head-space vial) at (25 ± 1) °C. Then the fibre was transferred to the gas chromatograph for analysis.

The relatively long extraction time was used to improve the yield of semi-VOCs. Further, alteration of the extracted compounds was reduced at the low temperature. In preliminary testing, the fibre exposure was carried out, in triplicate, for different duration of 45 min, 16 h [15] and 24 h, where the duration of 24 h proved to be the best.

Identification of volatile compounds with GC-MS

The SPME device (manual holder with the extracted VOCs on the fibre) was placed into the splitless injector at 270 °C for 10 min with purge flow closed. This procedure was previously confirmed to provide complete desorption. The gas chromatograph with a mass-selective detector Agilent 6890 Series GC System with Agilent 5973 Mass Selective Detector (Agilent Technologies, Santa Clara, California, USA) was used. Volatiles were separated on Rtx-20 column (60 m length, 0.25 mm inner diameter, 1 μm stationary film thickness; Restek, Bellefonte, Pennsylvania, USA). The temperature programme used was: initial temperature 50 °C (2 min), gradient 10 °C min^{-1} to 150 °C (3 min), gradient 10 °C min^{-1} to 250 °C (5 min). The total run time was 30 min. The mass spectrometer was operated in the electron ionization mode at 70 eV, the temperature of the MS Quad was set at 150 °C, the interface 270 °C and the ion source at 230 °C (50–400 amu; scan mode 10 000 amu·s^{-1}).

Compounds were identified in two ways. Some of them were identified by comparison with standards, the others were identified via comparison of experimental mass spectra with mass spectral library (NIST02, Gaithersburg, Maryland, USA). The peak area for quantification was measured in total ion current (TIC) chromatogram. In order to monitor the instrument and analytical performance, 1-octanol (5 μl of 1.37 mg ml^{-1} ethanol solution) was added to 4 g of grinded cheese immediately before SPME. Statistical analysis was done using data obtained from peak areas in chromatograms.

Statistical analyses

Statistical analyses were performed using Statistica software (Dell Software, Round Rock, Texas, USA).

RESULTS AND DISCUSSION

Milk from different areas

PCA was applied in order to define which VOCs contributed the most to distinguishing

between the cheese wheels made from milk from different areas. Cheese wheels W1–W6 were the Nanos cheese made from "selected" milk, normally used for the Nanos cheese production, and cheese wheels W7–W12 were cheese made from "unselected" milk (Fig. 1). The results presented in Fig. 1 show that the wheels, depending on the origin of the milk they are made from ("selected" or "unselected"), are distinguished by component 2 (Factor 2, which explained 11.0 % of the variability). If both Factor 1 and Factor 2 were included, 22.3 % of the variability could be explained.

VOCs with the highest potential of differentiation between cheese wheels made from "selected" milk and "unselected" milk were butanoic acid, 2-pentanol, 2-ethyl-1-hexanol, 2-nonanol, octanal and limonene, all presented in Tab. 2.

According to PCA analysis, the most important compound defining the geographical footprint was the terpene β-caryophyllene (data not shown), which was only found in the Nanos cheese made from "selected" milk. The compound is of plant origin [16, 17]. However, a more detailed study including two powerful analytical techniques GC-MS coupled with GC-olfactometry could give us a more detailed insight in odour or aroma activity of individual VOCs.

Our results unambiguously showed β-caryophyllene as a powerful geographic marker for Nanos cheese. However, analysis of other types of cheeses should be carried out to confirm this hypothesis.

Despite the explanation, 22.3 % of the variability on the basis of PCA analysis of VOCs, which was defined by some VOCs' content in the text above, cheese wheels made from "selected" and "unselected" milk were not completely separated according to Factor 1 and Factor 2. This could be explained by the fact that Nanos cheese is made from thermized milk. Milk heating (including thermization) is, in fact, the most common way to standardize milk quality in the cheesemaking process. The heat treatment reduces natural microorganisms of raw milk, which could form aromatic VOCs during maturation and thus could be involved in the formation of the cheese flavour. The first part of the results shows that the quality of raw material from different regions did not contribute significantly to the differences in the aromatic profile of cheeses.

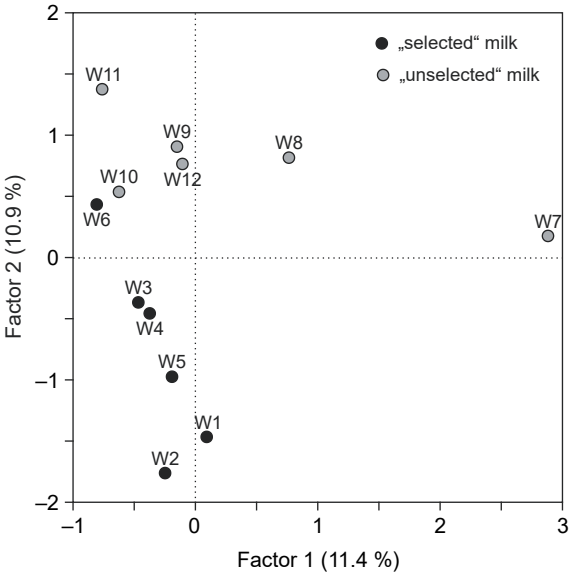

Fig. 1. Principal component analysis of Nanos cheese made from "selected" and "unselected" milk.

Effects of different ripening location

VOCs profiles of cheeses ripened in two different locations did not differ significantly for most substances. A reason might be the the parameters of maturation (moisture and temperature in the maturation room) were controlled and set equal in order to facilitate the comparison. However, the non-parametric Mann-Whitney U-test showed significantly different values for some compounds found in cheese ripened in the wine region Vipava ($p < 0.05$) (Tab. 2). For example, 3-methylbutanoic acid, butanoic acid and 2-methylbutanoic acid in the group of acids; 2-nonanol as a representative of alcohols; octanal and nonanal in the group of aldehydes; ethyl butanoate, ethyl hexanoate and ethyl octanoate in the group of esters; 2-pentanone as a representative of ketones and δ-decalactone as a representative of lactones. During ripening, cultures of moulds, yeasts and various bacteria grow on the surface of the cheese wheels. The composition of the surface microbiota depends on many factors such as the technology of cheese production (e.g. type of starter culture or dryness of grains), environmental conditions (temperature, humidity, salt concentration) and microbiota of the brine and of the ripening room. Microorganisms that grow on the surface can secrete a variety of substances. For example, yeasts could secrete alkaline metabolites, such as ammonia. This can lead to a rise of pH value on the surface of the cheese wheel. However, the composition of the microbial population on the surface of Nanos cheese wheels and its role are still not adequately addressed.

Tab. 2. Content of volatile organic compounds in Nanos cheese ripened at various conditions.

Volatile organic compounds	Grape wine				*p*	Regular amount of starter culture				Double amount of starter culture				*p* (two-factor ANOVA)		
	Presence		Absence			12 °C		18 °C		12 °C		18 °C				
	Mean	*RSD*	Mean	*RSD*		Mean	*RSD*	Mean	*RSD*	Mean	*RSD*	Mean	*RSD*	*T*	SC	*T* × SC
Fatty acids																
Acetic acid	3.34×10^7	a	3.22×10^7	c	ns	1.45×10^8	a	2.67×10^8	a	1.14×10^8	a	1.49×10^8	a	***	***	***
Butanoic acid	1.95×10^8	a	3.27×10^8	d	ns	5.11×10^8	a	4.35×10^8	a	4.30×10^8	a	6.26×10^8	a	ns	ns	**
3-Methylbutanoic acid	1.90×10^6	a	1.10×10^7	d	*	7.13×10^7	a	6.46×10^7	a	1.11×10^8	a	7.22×10^7	a	***	***	**
2-Methylbutanoic acid	nd		3.34×10^6	d	*	1.70×10^7	a	2.22×10^7	a	2.24×10^7	a	1.81×10^7	a	ns	ns	***
Hexanoic acid	1.77×10^8	a	1.94×10^8	d	ns	3.51×10^8	a	4.26×10^8	a	4.38×10^8	a	1.65×10^6	a	***	***	***
Heptanoic acid	1.87×10^6	a	2.56×10^6	a	ns	3.69×10^6	a	4.27×10^6	a	4.12×10^6	a	4.72×10^6	a	**	*	ns
Octanoic acid	7.70×10^7	a	3.72×10^7	d	*	1.64×10^8	a	1.61×10^8	a	2.54×10^8	a	2.56×10^8	a	ns	***	ns
Decanoic acid	1.76×10^7	a	1.02×10^7	d	ns	5.55×10^7	a	6.23×10^7	a	1.13×10^8	a	9.45×10^7	b	ns	***	ns
2-Methyl-propanoic acid	nd		nd			9.24×10^6	b	5.71×10^6	d	nd		nd		*	***	*
Alcoholes																
Butanol	1.78×10^6	b	1.58×10^6	d	ns	1.05×10^6	a	1.49×10^7	a	6.45×10^5	a	1.20×10^6	a	***	***	***
2-Pentanol	1.38×10^6	d	2.79×10^6	d	ns	2.39×10^6	d	7.19×10^7	a	8.84×10^5	c	5.81×10^6	a	***	***	***
3-Methylbutanol	nd		1.13×10^6	d	ns	8.50×10^6	a	1.49×10^7	a	1.24×10^7	b	1.81×10^7	a	***	**	ns
2,3-Butanediol	nd		nd			2.46×10^7	a	4.39×10^7	a	7.34×10^6	b	1.39×10^7	a	***	***	***
Hexanol	3.88×10^6	a	5.51×10^6	a	ns	nd		nd		2.11×10^7	c	1.45×10^7	b	ns	***	ns
2-Heptanol	2.39×10^6	a	1.11×10^7	c	ns	4.38×10^6	a	5.93×10^7	a	1.10×10^6	a	5.97×10^6	a	***	***	***
2-Ethylhexanol	nd		1.18×10^6	d	ns	1.33×10^7	a	1.47×10^7	a	9.91×10^6	b	1.03×10^7	d	ns	*	ns
2-Nonanol	4.22×10^6	a	1.69×10^6	d	*	1.71×10^6	d	1.09×10^7	a	2.78×10^6	b	3.53×10^6	c	***	***	***
2-Furanmethanol	nd		nd			nd		nd		nd		5.55×10^8	a	***	***	***
Aldehydes																
3-Methylbutanal	nd		nd			1.83×10^6	a	nd		3.76×10^6	b	3.73×10^6	c	*	***	*
Heptanal	1.31×10^6	b	6.29×10^5	a	ns	3.61×10^6	a	nd		4.19×10^6	b	nd		***	ns	ns
Octanal	3.40×10^7	a	1.61×10^7	c	*	1.31×10^7	d	1.93×10^7	a	1.11×10^7	a	1.66×10^7	c	ns	ns	ns
Benzaldehyde	1.56×10^7	a	1.38×10^7	c	ns	2.93×10^7	a	3.28×10^7	a	1.64×10^8	a	5.36×10^7	d	***	***	***
Nonanal	1.56×10^6	b	7.48×10^5	d	*	4.67×10^6	a	4.13×10^6	c	7.74×10^6	b	4.84×10^6	a	*	**	ns
Benzene acetaldehyde	nd		nd			1.92×10^6	a	1.51×10^6	b	4.30×10^6	a	5.59×10^6	c	ns	***	ns
Decanal	2.15×10^6	a	nd		ns	4.35×10^6	a	4.81×10^6	b	7.16×10^6	b	5.85×10^6	a	ns	**	ns
Esters																
Ethyl ethanoate	4.10×10^7	d	6.49×10^6	d	ns	4.71×10^6	a	2.00×10^7	b	7.47×10^6	c	8.83×10^6	a	***	**	***
Ethyl butanoate	1.02×10^7	a	3.21×10^7	d	*	3.88×10^7	a	1.21×10^8	a	2.17×10^7	a	2.97×10^7	c	***	***	***
3-Methylbutyl ethanoate	nd		nd			nd		5.58×10^6	a	nd		nd		***	***	***

Tab. 2. *continued*

Volatile organic compounds	Grape wine				*p*	Regular amount of starter culture				Double amount of starter culture				*p* (two-factor ANOVA)		
	Presence		Absence			12 °C		18 °C		12 °C		18 °C				
	Mean	*RSD*	Mean	*RSD*		Mean	*RSD*	Mean	*RSD*	Mean	*RSD*	Mean	*RSD*	*T*	SC	*T* × SC
Ethyl hexanoate	9.90×10^6	b	1.78×10^7	d	*	2.11×10^7	a	3.54×10^7	a	1.48×10^7	a	1.95×10^7	a	***	***	**
Ethyl octanoate	7.66×10^6	c	4.04×10^6	b	*	1.16×10^7	a	2.25×10^7	a	1.62×10^7	a	2.95×10^7	a	***	**	ns
Ethyl 3-methyl-butanoate	nd		nd			nd		nd		nd		1.22×10^6	b	***	***	***
Ethyl 2-methyl-butanoate	nd		nd			nd		nd		nd		1.99×10^6	b	***	***	***
Pentyl ethanoate	nd		nd			nd		nd		nd		3.93×10^8	b	***	***	***
Ethyl-2-methyl propanoate	nd		nd			nd		9.77×10^6	a	nd		3.85×10^6	a	***	***	***
Ketones																
2,3-Butanedione (diacetyl)	2.22×10^6	a	4.06×10^6	d	ns	2.23×10^7	a	nd		2.05×10^7	a	1.16×10^7	a	***	***	***
2-Butanone	3.32×10^6	c	5.32×10^6	d	ns	4.56×10^6	a	1.09×10^7	a	3.01×10^6	d	5.95×10^6	a	***	***	**
2-Pentanone	1.20×10^7	a	6.14×10^6	d	*	2.31×10^7	a	3.76×10^7	a	1.72×10^7	a	1.36×10^8	d	***	**	**
3-Hydroxy 2-butanone (acetoin)	1.15×10^6	a	3.97×10^6	d	ns	7.34×10^7	a	9.35×10^6	b	1.16×10^8	a	7.19×10^7	a	***	***	**
2-Hydroxy-3-pentanone	nd		nd			8.27×10^6	a	nd		6.21×10^6	a	4.61×10^6	a	***	**	***
2-Heptanone	8.07×10^7	a	8.62×10^7	b	ns	2.90×10^8	a	2.47×10^8	a	2.03×10^8	a	8.13×10^6	c	***	***	***
2-Octanone	1.77×10^6	d	1.18×10^6	b	ns	nd		nd		nd		2.71×10^6	c	***	***	***
2-Nonanone	3.36×10^7	b	1.11×10^7	d	ns	2.71×10^7	a	2.37×10^7	a	2.76×10^7	a	5.81×10^7	b	**	***	***
2-Undecanone	1.98×10^6	a	8.13×10^5	c	ns	1.39×10^7	a	1.75×10^7	a	6.11×10^6	d	1.07×10^7	c	*	***	ns
Terpenes																
Pinane	nd		nd			2.87×10^7	a	2.23×10^7	a	9.34×10^6	b	2.93×10^6	b	***	***	ns
α-Pinene	3.10×10^5	a	1.00×10^6	d	ns	9.03×10^7	a	7.32×10^7	a	3.17×10^7	a	8.49×10^6	b	***	***	ns
Limonene	5.47×10^6	c	6.50×10^6	a	ns	1.51×10^7	b	1.19×10^7	c	7.16×10^6	b	6.76×10^6	a	ns	***	ns
p-Cymene	9.41×10^5	c	2.01×10^6	d	ns	2.80×10^7	a	2.71×10^7	a	1.90×10^7	a	1.61×10^7	b	ns	***	ns
β-Caryophyllene	7.77×10^5	a	4.68×10^5	d	ns	8.41×10^6	a	7.89×10^6	a	8.15×10^6	a	7.72×10^6	a	ns	ns	ns
Camphene	nd		nd			4.51×10^6	a	3.81×10^6	b	2.45×10^6	b	nd		***	***	**
Carene	nd		nd			3.42×10^6	a	2.30×10^6	a	nd		nd		***	***	ns
2-Pinene-10-ol	nd		nd			3.74×10^6	a	3.55×10^6	a	2.23×10^6	a	2.45×10^6	a	ns	***	ns
Miscellaneous																
Ethylbenzene	6.57×10^5	b	1.65×10^6	d	ns	1.01×10^6	a	3.92×10^6	a	nd		nd		***	***	***
δ-Octalactone	3.21×10^6	a	1.80×10^6	d	ns	6.95×10^6	a	7.45×10^6	a	7.24×10^6	a	2.20×10^7	d	*	*	*
δ-Decalactone	3.78×10^6	a	1.65×10^6	d	*	8.70×10^6	a	8.24×10^6	a	1.81×10^7	a	1.74×10^7	c	ns	***	ns

Content is expressed in abundance units.
RSD – relative standard deviation represented by the letters (a – *RSD* < 10 %, b – *RSD* 10–20 %, c – *RSD* 20–30 %, d – *RSD* > 30 %); *T* – temperature; SC – starter culture; nd – not detected.
p – significance *p*-values (ns – not significant at $p > 0.05$, * – significant at $p < 0.05$; ** – significant at $p < 0.01$; *** – significant at $p < 0.001$).

Effects of the amount of starter culture and of ripening temperature

Nanos cheese was made with the addition of a regular amount of starter culture and ripened at two temperatures, 18 °C or 12 °C. More and bigger cheese eyes were observed from the cross-sections of cheeses ripened at the higher temperature (18 °C) (Fig. 2). Increasing the temperature during ripening can accelerate cheese ripening [18], which can influence the formation of cheese eyes. The formation of eyes in hard and semi-hard type cheeses is also an important quality parameter [19]. The formation of eyes is caused by the release of gaseous CO_2. This is generated by different ways, namely, by the metabolism of lactose (by heterofermentative bacteria belonging to genera *Leuconostoc* or *Lactobacillus* [20, 21]), by the metabolism of lactate (by propionic bacteria) or of citrate (in Dutch-type cheeses), by decarboxylation of glutamic acid and by metabolizing urea [7]. In Nanos cheese, there are smaller amounts of CO_2 release. The formed Nanos cheese eyes, however, come mainly from the increased activity of lactic acid bacteria.

GC-MS analysis revealed 55 VOCs in cheeses made with different amounts of starter culture (regular, double) and ripened at different temperatures (12 °C, 18 °C) (Tab. 2). The cheese ripened at the higher temperature contained higher contents of esters and alcohols. The results also indicated that ripening at the higher temperature led to a reduction in the contents of 2,3-butanedione (diacetyl) and 3-hydroxy-2-butanone (acetoin), which are important aroma-active compounds as well as precursors of other VOCs.

The maximal content of all compounds (Tab. 2) was determined in cheese manufactured with an increased amount of starter culture and ripened at a higher temperature. The increased amount of starter culture also increased the levels of benzaldehyde, which has a characteristic nutty flavour [22].

Both factors, temperature (12 °C, 18 °C) and the amount of starter culture, significantly influenced the content of most identified VOCs in cheese. Significant interactions were found between temperature and the amount of starter culture in most VOCs in cheese (Tab. 2) with the exception of aldehydes and terpenes. Significant interactions indicated that the amount of starter culture in various temperature environments acts differently in the ripening process. However, they do not show a uniform pattern. The decrease in individual VOCs is expected if this VOC is a precursor for another VOC. However, in a real situation all interactions are not easily explained.

Ripening in grape marc

Nanos cheese, ripened in grape marc had a unique aroma profile mainly due to the content of esters. Esters contribute to fruity notes [15], which was also confirmed in the sensory analysis. For this reason, this experiment was focused on esters, despite the fact that the complete analysis of VOCs was done.

The preliminary sensory analysis (three-member committee) noticed the pleasant fruity (probably coming from the nature of esters) in case of "Drunken" cheese ripened in the mode D1 (cheese placed in grape marc immediately after salting), whereas cheese ripened in the mode D2 (cheese placed in grape marc after 6 weeks of the classical ripening process) had a little more stuffy fruity flavour and aroma of fermented fruit. Moreover, the consistency of cheese ripened in the mode D2 was harder and more firm in comparison with cheese ripened in the mode D1.

In addition, seven new esters were identified only in cheeses ripened in the grape marc (Tab. 3). From these esters, butyl ethanoate was identified

Fig. 2. Cheese wheels matured at different temperature.

Left cheese wheel – maturation at 18 °C, right cheese wheel – maturation at 12 °C.

only in "Drunken" cheese ripened in the mode D2. Similarly, Di Cagno et al. [23] found the content of butyl ethanoate higher in cheese ripened in the grape marc, compared with cheeses that were ripened classically [23]. Esters present in cheese ripened in grape marc are probably the result of esterification of free fatty acids and alcohols present in grape marc or wine. The formation of esters is also influenced by the presence of alcohols. In "Drunken" cheese, higher levels of ethyl ethanoate and ethyl hexanoate were found. They are characterized by a fruity orange note [17]. In addition, in "Drunken" cheese there was a higher content of ethyl butanoate and lower content of butanoic acid (data not shown). This can be explained by esterification of free fatty acids with ethanol present in wine or grape marc. In particular, the alcohol ethanol is a limiting reactant for the esterification [24].

Tab. 3. Average peak areas of esters found in Nanos cheese and in cheese ripened in grape marc.

Esters	Nanos cheese	Cheese ripened in grape marc	
		D1	D2
Ethyl ethanoate	5.15×10^{6a}	2.94×10^{7b}	8.19×10^{6a}
Ethyl butanoate	1.55×10^{7a}	6.57×10^{7c}	2.80×10^{7b}
3-Methylbutyl ethanoate	nd[a]	1.00×10^{6c}	5.67×10^{5b}
Ethyl hexanoate	1.17×10^{7a}	1.63×10^{8c}	7.62×10^{7b}
Methyl octanoate	2.39×10^{6a}	5.32×10^{6c}	4.06×10^{6b}
Ethyl octanoate	3.30×10^{6a}	1.47×10^{8c}	1.12×10^{8b}
Ethyl decanoate	nd[a]	3.48×10^{7c}	5.45×10^{7b}
Ethyl 2-nitropropanoate	nd[a]	3.48×10^{6b}	4.38×10^{7a}
Ethyl propanoate	nd[a]	1.51×10^{6c}	8.28×10^{6b}
Methyl butanoate	nd[a]	4.47×10^{5b}	nd[a]
Butyl ethanoate	nd[a]	nd[a]	8.99×10^{5b}
Ethyl-2-methyl propanoate	nd[a]	2.49×10^{6c}	1.41×10^{6b}
Ethyl nonanoate	nd[a]	1.83×10^{6c}	1.02×10^{6b}
Ethyl dodecanoate	nd[a]	nd[a]	9.91×10^{6b}

Means followed by the same letter in superscript are significantly different at $p > 0.05$ according to Tukey's test.
D1 – cheese placed in grape marc immediately after salting,
D2 – cheese placed in grape marc after 6 weeks of the classical ripening process.
nd – not detected.

CONCLUSION

The results of this study confirmed some important changes of VOCs profile of Nanos cheese that were induced by changing technological cheesemaking parameters. A double amount of added starter culture contributed to higher levels of certain VOCs (benzaldehyde) and the presence of some new VOCs (2-furanmethanol). A higher temperature of ripening contributed to larger size and higher number of eyes formed in cheese, as well as to an increase in the content of some esters and alcohols. Ripening in grape marc led to an increase in the content of esters, which could be associated with a fruity cheese flavour. While factors such as the amount of starter culture, ripening temperature and ripening in grape marc do alter the aroma profile of Nanos cheese, the geographical origin of raw milk for production as well as the location of ripening do not affect the Nanos cheese aroma profile. This implies that the origin of milk could be extended to a wider geographical area. This is the first study which deals with the changes in cheese due to the above mentioned technological cheesemaking parameters in the case of the traditional Nanos cheese. The study provided novel information, in particular on VOCs profile of cheese ripened on grape skins. The analytical approach applied to Nanos cheese in this study can be used also for other hard type cheeses.

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