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# Volatile compounds of traditional Bagnolese Pecorino cheese

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## 1. Introduction

The term "pecorino" typically refers to a family of cheeses made from ewe's milk. The most famous is Pecorino Romano DOP, produced in the regions of Lazio and Sardinia. The strong and robust flavour, combined with the long aromatic persistence, are some of the most appreciated sensory characteristics of pecorino cheese. The sensory properties of pecorino cheese differ based on the region and specific cheese-making methodologies employed (Braghieri et al., 2014). The positive reputation of pecorino cheese is mainly based on tradition and origin from specific territories, where consumers express a liking especially for small-scale local productions. The willingness of consumers to request local dairy products made with milk from pasture-raised animals lies in the greater perception of animal welfare and environmental sustainability, as well as a better nutritional quality of the resulting cheese (Balivo et al., 2023). In fact, Serrapica et al. (2020) found that Pecorino Bagnolese obtained from sheep raised on mountain pastures had a higher amount of unsaturated fatty acids, in particular of the omega-3 series and trans 11-based fatty acids (i.e., rumenic acid and vaccenic acid).

Pecorino Bagnolese is manufactured with raw milk from the local Bagnolese sheep breed raised in the Picentini Mountains, in the Alburni Mountains, in the Piana del Sele and in the Vallo di Diano of Irpinia in Campania region (Comendador et al., 2012). The flock is reared extensively or semi-extensively, taking advantage of the mountain pasture during the summer season. In addition to fresh forage, the sheep's diet may include hay and concentrate. The milk must be processed within 24 hours of milking and is curdled with paste lamb chymosin. The annual production is small and varies between 150-250 quintals of cheese (Comendador et al., 2012). Pecorino Bagnolese cheese is recognised as a traditional agri-food product (PAT) by the Italian Ministry of Agriculture and as a slow food presidium (ONAF, 2020). Maturation lasts from 20 days to over 6 months, generating a soft-to-hard cheese.

In a previous study, Pecorino Bagnolese was distinguished from Pecorino Sardo and Toscano applying multivariate statistical techniques to sensory data assessed by a trained panel (Comendador et al., 2012). So far, there have been no studies examining the volatile compounds (VOCs) in Pecorino Bagnolese. We chose the analysis of volatile compounds since the VOCs in cheese are affected by factors such as animal breed, diet, production methods, maturation, and microbial interactions, and therefore can represent a chemical fingerprint for distinguishing the various types of Pecorino cheeses.

The aim of this research is to differentiate Pecorino Bagnolese cheese from other kinds such as Pecorino Romano, Toscano, Sardo, del Pastore, Canestrato Pugliese and Fiore Sardo based on volatile organic compounds (intrinsic characteristics) and other productive socio-economic variables as breed, rennet, paste, ripening, feeding system and certification (extrinsic characteristics) using multivariate statistics approaches.

The following part of the paper's structure is as follows: Section 2 introduces data sources, outlines SPME-GC/MS[1] for VOCs data collection, and the statistical analysis model. In Section 3, main statistical findings are presented. Section 4 discusses the results with the

[1] Solid-Phase Micro Extraction (SPME) Gas Chromatography-Mass Spectrometry (GC/MS).

existing literature.

## 2. Data and methods

Thirty-seven samples of Pecorino cheese were purchased from local supermarkets in the Campania region, Italy. Seven types of pecorino cheese, listed below, were included in our study: Pecorino Bagnolese, Pecorino Romano, Pecorino Toscano, Pecorino Sardo, Canestrato Pugliese, Fiore Sardo and Pecorino del Pastore. The samples were randomly selected to ensure a representative assortment of Pecorino cheeses available in the local market. Upon collection, the Pecorino cheese samples were immediately transported to the laboratory under refrigerated conditions and frozen at -20°C.

### 2.1. SPME-GC/MS

The VOCs were extracted from cheese samples using the Headspace-SPME technique following the procedure described by Sacchi et al. (2020). Frozen samples (25 g) were finely grated, suspended with distilled water (25 mL), and mixed in a 100 mL glass bottle with 50 µL of 2-methyl-3-heptanone (99% purity, Sigma-Aldrich, St. Louis, MO, USA) as internal standard (510 mg $L^{-1}$, in water solution) and 6.25 g of sodium phosphate (NaH2PO4) (Sigma-Aldrich). The mixture was heated (50 °C for 10 min, then 40 °C for 20 min) and magnetically stirred (150 rpm). VOCs were adsorbed onto a 50/30 µm thick divinyl-benzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) 2 cm length fibre, which was exposed to sample headspace for 30 min at 40 °C while stirring.

The VOCs were desorbed directly in the injector port of GC kept at a temperature of 250 °C in split mode with a 4:1 split ratio, for 10 minutes. Volatile compound analysis was performed on an Agilent 7890A GC System gas chromatograph coupled to an Agilent 5975C VL MSD with Triple-Axis-Detector mass spectrometer (Agilent Technologies, Inc., Palo Alto, CA, USA). GC was equipped with a Zebron ZB-WAX capillary column (60 m × 0.25 mm i.d. × 0.25 µm film thickness 100% polyethylene glycol; Phenomenex, USA). The carrier gas was helium with a flow of 1 mL $min^{-1}$. The temperature program was 40 °C for 10 min, then raised at 5 °C min-1 to 240 °C and held for 11 min (Balivo et al., 2023). Mass spectra were recorded at 70 eV. The source temperature was 230 °C, the quadrupole temperature was 150 °C and the interface temperature was 250 °C. Identification of VOCs was based on retention times, mass spectra, and reference compounds. Quantitative data were normalised to the internal standard and analysed using MSD ChemStation 5975 TAD data analysis software (Agilent Technologies, Palo Alto, CA, USA). The SPME-GC/MS procedure allowed us to extract a total of 64 different VOCs molecules from 37 distinct samples of Pecorino cheese. The VOCs of cheese samples were collected in triplicate for each sample type.

### 2.2 The statistical model

The analysis of the data collected on Pecorino cheese includes the classification variable as a criterion variable and dichotomised as follow: $Y = 1$ if the pecorino cheese is Bagnolese, and $Y = 0$ otherwise. We performed a linear discriminant analysis in order to find a linear combination of variables that characterises or separates two classes of units. Discriminant analysis is also useful in determining whether a set of variables is effective in predicting the class.

If the observed variables are denoted by $x_1, x_2, \ldots, x_p$, then in discriminant analysis the row vector of coefficients $\boldsymbol{a'}$ is sought, which maximizes $\boldsymbol{a' Ba} \,/\boldsymbol{a' Wa}$, where $\boldsymbol{B}$ and $\boldsymbol{W}$ are the between and the within sum of squares and cross-products matrices. The linear combination of the observed variables involving the elements of $\boldsymbol{a'}$ as coefficients is the best discriminant function, in that it provides for maximum separation on the groups.

In order to obtaining the discriminant function, the coefficients (the $a_j$) are scaled so that $\boldsymbol{a'a} = 1$ for each composite (the so-called unit norm condition). The discriminant function ($\boldsymbol{d}$)

then is given by:

$$\boldsymbol{d} = \boldsymbol{a}'\boldsymbol{x} = \sum (a_j\, x_j).$$

The similarities and differences among the groups is achieved through the relative contribution of variables towards the separation of groups. When the classes are well-separated, linear discriminant analysis does not suffer of unstable parameter estimates.

The stepwise discriminant analysis with prior probability for the groups calculated from the size of the groups was performed on the VOCs data to evaluate the differences between Pecorino Bagnolese and other Pecorino cheeses. The first variable to enter is the one that maximizes separation among the groups. The next variable to enter is the one that adds the most to further separating the groups, and so on.

Statistical analysis was performed on the 64 VOCs and 8 socio-productive dummy variables (dichotomised in terms of presence = 1 or absence = 0) using the SPSS package (IBM, 2020).

## 3. Results

The SPME-GC/MS technique involves extracting and analysing the volatile compounds in cheese, which constitute the mixture of odorous molecules perceived by the sense of smell when eating cheese. In particular, SPME is used to sample the volatile compounds released by cheeses in the headspace of a bottle, while GC/MS is used to separate, identify and quantify the individual volatile compounds in the odorous mixture, providing detailed insight of the aroma composition of cheese samples.

Table 1 shows the results of the stepwise selection of discriminant variables. The discriminant function is statistically significant (canonical correlation = 0.941, Wilks' lambda = 0.115, chi-square = 68.152, $p < 0.001$). The square of the canonical correlation for this composite, when converted to a percent, indicates that about 89% of the score variation for the first function is between groups. The fitted discriminant function with seven variables do provide support for the presence of large differences between the Bagnolese and the other Pecorino cheeses.

**Table 1.** Forward stepwise discriminant analysis, selection of observed variables with the classification variable in two groups (Bagnolese Pecorino cheese; Other pecorino cheese), n =37.

| Step | | Variables | Wilks'Lambda | F | Sig. | Canonical discriminant function coefficients |
|---|---|---|---|---|---|---|
| **1** | Included | 2-pentanol | 0.546 | 29.142 | < 0.001 | 1.331 |
| **2** | Included | 2-undecanone | 0.390 | 26.567 | < 0.001 | 1.814 |
| **3** | Included | 2-pentanone | 0.321 | 23.307 | < 0.001 | ------- |
| **4** | Included | 2-heptenal | 0.257 | 23.101 | < 0.001 | -1.353 |
| **5** | Included | ethyl-hexanoate | 0.212 | 23.039 | < 0.001 | 1.203 |
| **6** | Included | ethanol | 0.173 | 23.820 | < 0.001 | - 1.191 |
| **7** | Included | 2-octanone | 0.147 | 24.094 | < 0.001 | - 1.315 |
| **8** | Removed | 2-pentanone | 0.155 | 27.286 | < 0.001 | ------- |
| **9** | Included | 1-hexanol | 0.115 | 31.908 | < 0.001 | 0.765 |

*At each step, the variable that minimizes the global Wilks' Lambda is included – $P(F_{to\_Enter})=0.05$; $P(F_{to\_Remove})=0.10$.*

Pecorino Bagnolese exhibited higher levels of 2-undecanone, 2-pentanol, and ethyl hexanoate. Conversely, it showed lower levels of 2-heptenal, 2-octanone, and ethanol.

## 4. Discussion and conclusion

The aim of our work was to differentiate Pecorino Bagnolese from other pecorino cheeses. The first result highlighted by the statistical analysis is that the extrinsic characteristics of the cheeses are secondary compared to the intrinsic ones.

The development of volatile compounds in cheese is the result of complex biochemical reactions during fermentation and maturation/ripening process (Andiç et al., 2015). 2-undecanone is a methyl ketone formed by the reaction of β-oxidation of free fatty acids followed

by the subsequent decarboxylation of the resulting β-ketoacid (Bertuzzi et al., 2018). Labows et al. (1980) reported that *Pseudomonas aeruginosa* strains are mainly responsible of the production of a series of odd carbon methyl ketones. Methyl ketones in cheese also derive from the metabolism of mesophilic non-starter lactic acid bacteria, i.e., mesophilic *Lactobacillus*, *Pediococcus*, *Enterococcus* and *Leuconostoc* spp. (De Pasquale et al., 2019).

*Pseudomonas* spp can reduce methyl ketones to secondary alcohols, like 2-pentanol (Cormier et al., 1991). In Pecorino Bagnolese cheese, 2-pentanol resulted from the reduction of 2-pentanone, which derives from the β-oxidation and decarboxylation of hexanoic acid. Hexanoic acid is the main free volatile fatty acid in cheese (Randazzo et al., 2010). High amounts of secondary alcohols derived from methyl ketones have been detected in cultures of *Leuconostoc* spp. and *L. fermentum* (Pogačić et al, 2016).

Ethyl hexanoate is an ester derived from the esterification of hexanoic acid and ethanol. *Pseudomonas* spp are also known for their significant lipolytic activity, producing large amounts of ethyl esters. In addition, *Lactococcus lactis* subsp. *cremoris* thanks to the presence of alcohol acyltransferase, enzyme that catalyse the esterification reaction between free fatty acids and alcohols, favours the production of ethyl hexanoate in cheese (Liu et al., 2004).

Pecorino Bagnolese had a lower amount of ethanol, consumed mainly to produce ethyl esters. A higher quantity of ethanol was found with the addition of starter cultures of *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* (Kondyli et al., 2002). The other Pecorino cheeses were characterised by a higher abundance of 2-octanone and 2-heptenal derived from oxidation reactions of fatty acids.

These findings suggest that the abundance of these VOCs in Pecorino Bagnolese may be directly linked to its unique microbial composition and enzymatic activity during cheese ripening. These compounds result from lipid catabolism, influenced by the indigenous microflora specific to the production area, particularly shaped by the terroir.

In conclusion, our study identifies seven variables that effectively discriminate Pecorino Bagnolese from other Pecorino cheeses. Plausibly, these intrinsic variables could be attributed to the characteristic microbiota of Pecorino cheese, such as the presence of *Pseudomonas* and other indigenous microorganisms, whose enzymatic activity might explain the production of these VOCs.

Further analyses are required to elucidate the specific enzymatic processes responsible for generating these VOCs in Pecorino Bagnolese cheese. These intrinsic characteristics could serve as crucial factors for authenticating Pecorino Bagnolese cheese and hold promising applications within the dairy industry.

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