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Article

Application of a novel S3 nanowire gas sensor device in parallel with GC-MS for the identification of rind percentage of grated Parmigiano Reggiano

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Abstract: Parmigiano Reggiano cheese is one of the most appreciated and consumed food worldwide, especially in Italy, for its high content of nutrients and for its taste. However, these characteristics make this product subject to counterfeiting in different forms. In this study, a novel method based on an electronic nose has been developed in order to investigate the potentiality of this tool to distinguish rind percentage in grated Parmigiano Reggiano packages that should be lower than 18%. Different samples in terms of percentage, seasoning and rind working process were considered to tackle the problem at 360°. In parallel, GC-MS technique was used to give a name to the compounds that characterize Parmigiano and to relate them with sensors responses. Data analysis consisted of two stages: multivariate analysis (PLS) and classification made in a hierarchical way with PLS-DA ad ANNs. Results are promising in terms of correct classification of the samples. The classification rate is higher for ANNs than PLS-DA, reaching also 100% values.

Keywords: electronic nose, nanowire gas sensors, food quality control, Parmigiano Reggiano, multivariate data analysis, artificial neural network

1. Introduction

Parmigiano Reggiano (PR) cheese is one of the most typical Italian food and one of the oldest traditional cheeses produced in Europe. It is also the most important Protected Designation of Origin (PDO) Italian cheese in terms of commercial importance [1]. Its production is regulated by the Parmigiano Reggiano Cheese Consortium (CFPR). According to European Regulation 510/2006, this designation can be exclusively assigned to the cheese made following a traditional established production technology in a restricted area of Italy (provinces of Parma, Reggio Emilia, Modena, Mantova and Bologna) from milk produced in the same area [2].

PR can be found on the market in different forms. It can be portioned or grated and cannot be subjected to any treatment like lyophilization, drying and freezing [3]. All the procedures, that must be followed to obtain the original PR, make this cheese a high-value product. That leads to a final product that has various nutritional properties: its dry weight is mostly composed of proteins and lipids, is lactose- and galactose-free and rich in organic acids, such as lactic acid, acetic acid, propionic and butyric acids [4]; the semi-fat composition due to natural creaming of skimmed unpasteurized milk [5] is produced by cattle that consumes only locally grown forage because supply of silage and fermented feeds is not permitted [6].

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For these reasons PR has a high cost, if compared to similar hard cheeses. This encourages the appearance on the market of counterfeited products that bear PR brand at a lower price. The rate of fraud is estimated between 20% and 40%, the latter predominantly in the grated form. [7].

As established in the procedural guideline, grated PR cheese must follow some technical and technological parameters: moisture no less than 25% and no more than 35%, at least 12 months of ripening, rind not over 18%, typical amino-acid composition of the cheese, absence of additives, not crumbly in aspect and with homogeneous particles with a diameter inferior to 0.5 mm and not exceeding 25% [8].

In order to distinguish if a PR cheese package conforms to the rules, the aromatic profile of grated PR can be analyzed thanks to the volatile organic compounds (VOCs). VOCs of various dairy products have received a great deal of attention in the last years. Till now, about 600 volatile compounds have been identified for cheese [9]. However, only a small part of these compounds is responsible for cheese flavor [10]. Cheese aroma is considered the result of the equilibrium between various VOCs that separately do not reflect the overall odor [11]. Hydrocarbons, alcohols, aldehydes, ketones, esters and lactones were the major classes of compounds found in the neutral fraction of cheese [12].

In this work, an electronic nose has been used in order to analyze rind percentage in grated PR cheese through emitted VOCs. In the last years, this kind of devices has received numerous attentions for its potentialities; it has been applied in various fields, as environment [13-14], health [15-16] and food with excellent results. Regarding food applications, some examples of electronic nose applications are the detection of microorganisms in tomato sauce [17] and of different molds in coffee [18], the determination of shelf life of milk [19], the finding of additives in fruit juices [20]. These few examples show how e-noses have the potentiality to be used in different ways to assess food quality and identity.

Placed side by side with e-nose analysis, Gas Chromatography coupled with Solid Phase Micro Extraction (SPME) was used. SPME has received much attention in the literature to find VOCs that characterize food matrices. Many foods have been studied, including dairy products, such as milk [21], butter [22] and cheese [23-24].

The aim of the work is to recognize rind percentages of the sample under analysis with an innovative and rapid methodology in order to identify eventual frauds and therefore have an affordable and reliable instrument to reduce them, making the most of the possible differences between the products such as VOCs presence and amount.

2. Materials and Methods

2.1. Samples preparation and experimental design

Analyzed samples were packaged under vacuum at the headquarters of CFPR. They came from two different ripening stages: 12 and 24 months. For each of these, five different combinations of pulp-rind were prepared (expressed in rind percentage): 0%, 18%, 26%, 45% and 100%. In addition, two kind of rind working processes were considered: washed-rind (WR) and scraped-rind (SR). The only exceptions are represented by 0% samples, for which only the 24 month ripening was taking into account, and 100% samples, for which there is one for WR and one for SR that correspond to 24 month and 12 month seasoning respectively. For each sample, 14 several replicas were arranged for a total of 210 (14 replicas x 15 samples).

Samples were stored at 4°C until the moment they were prepared for the analysis. The amount of 2 g of grated cheese were positioned in 20 mL glass headspace vials and sealed with a metal cap with a PTFE-silicon membrane, crimped with an aluminum crimp.

2.2 GC-MS Analysis

The Gas Chromatograph (GC) used during the analyses was a Shimadzu GC2010 PLUS (Kyoto, KYT, Japan), equipped with a Shimadzu single quadrupole Mass Spectometer (MS) MS-QP2010 Ultra

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(Kyoto, KYT, Japan) and with an autosampler HT280T (HTA s.r.l., Brescia, Italy). The GC-MS analysis was coupled with the Solid-Phase Micro Extraction (SPME) method in order to find the most significant VOCs that allow to recognize the different kinds of cheeses.

The fiber used for the adsorption of volatiles was a DVB/CAR/PDMS – 50/30 µm (Supelco Co. Bellefonte, PA, USA). The fiber was exposed to the headspace of the vials after heating the samples in the HT280T oven thermostatically regulated at 50°C for 15 min with the aim to create the headspace equilibrium. The length of the fiber in the headspace was kept constant. Desorption of volatiles took place in the injector of the GC-MS for 6 min at 250°C.

The gas chromatograph was operated in the direct mode throughout the run with the mass spectrometer in electron ionization (EI) mode (70 eV). GC separation was performed on a MEGA-WAX capillary column (30m x 0.25mm x 0.25µm, Agilent Technologies, Santa Clara, CA, USA). Ultrapure helium (99.99%) was used as the carrier gas at the constant flow rate of 1.3 mL/min. The following GC oven temperature programming was applied: at the beginning, the column was held at 40 °C for 8 min, and then it was programmed from 40 to 190 °C at 4 °C/min; then, the temperature was maintained at 190°C for 5 min. Next, the temperature was raised from 190°C to 210°C with a rate of 5°C/min; finally, 210°C were maintained for 5 min.

The GC-MS interface was kept at 200 °C. The mass spectra were collected over the range of 45 to 500 m/z in the Total Ion Current (TIC) mode, with scan intervals being 0.3 s. The identification of the volatile compounds was carried out using the NIST11 and the FFNSC2 libraries of mass spectra.

Each sample was analyzied one time.

2.3 S3 Analysis

The innovative Small Sensors System S3 device used in the present work has been completely designed and constructed at SENSOR Laboratory (University of Brescia, Italy) in collaboration with NASYS S.r.l., spin-off of the University of Brescia. The tool comprises a metal oxide (MOX) gas sensors array, flow sensors, temperature and humidity sensors, fluidodinamic system, electronic control system. In particular, the sensor used in this study are 8 MOX gas sensors. Three of them are nanowires of MOX as presented in [25-26]. Two of them are tin oxides nanowires sensors, both grown by means of Vapor Liquid Solid technique [27] using a gold catalyst on the alumina substrate, but one has also been functionalised with gold clusters; the third sensor has an active layer of copper oxide nanowires. The working temperature is 350°C, 350°C and 400°C respectively. Other three sensors are prepared with Rheotaxial Growth and Thermal Oxidation (RGTO) thin film technology; one is tin oxide functionalised with gold clusters (working at 400°C), while the other two are pure tin oxide (working at 300°C and at 400°C respectively).

Finally, the last two are commercial MOX sensors produced by Figaro Engineering Inc. (Osaka, Japan). In particular, they are the TGS2611 and TGS2602, that are sensitive to natural gases and to odorous gases like ammonia, respectively, according to the datasheet of the company. Commercial sensors have been mounted on our e-nose in order to evaluate the performances of nanowire sensors. Details of S3 sensors made at SENSOR Laboratory are summarised in Table 1.

The MOX nanowires are gas sensors with a high sensitivity to a broad range of chemicals; they exhibit physical properties that are significantly different from their polycrystalline counterpart. The nanowires have a high degree of crystallinity, atomically sharp terminations and an extraordinary length-to-width ratio, resulting in enhanced sensing capability as well as long-term material stability for prolonged operation. In addition, the three-dimensional network formed by the nanowires increases the adsorption surface and the catalytic activity, improving the response and decreasing the instrument threshold [28].

S3 anlayses the head space (HS), i.e. the volatile fraction of the samples formed when the equilibrium of the solid-liquid phase and the vapor phase of all volatile compounds is reached. The creation of the HS depends on the test substance (vapor pressure) and the conditioning temperature of the sample. The compounds are extracted at the equilibrium point between the solid phase and the vapor in a dynamic head space. This characteristic allows a non destructive samples analyses. In this case, the sensor base line is obtained with the air of the surrounding environment, no gas cylinder of chromatographic air is required (an essential feature that makes it a portable instrument). The

environmental air was filterd using a small metal cylinder (21.5 cm in length, 5 cm of diameter) filled with activated carbons.

Table 1. Type, composition, morphology and operating temperature of S3 sensors made at SENSOR Laboratory.

Type	Composition	Morphology	Operating temperature (°C)
SnO2Au	SnO2 functionalised with Au clusters	RGTO	400°C
SnO2	SnO2	RGTO	300°C
SnO2	SnO2	RGTO	400°C
SnO2Au+Au	SnO2 grown with Au and functionalised with gold clusters	Nanowire	350°C
SnO2Au	SnO2 grown with Au	Nanowire	350°C
CuO	CuO	Nanowire	400°C

The volatile fraction is then aspirated and transported to the sensor chamber to be analyzed. In order to avoid any influence of the surrounding environment to the sensor response, the chamber has been thermostated and isolated. To prevent the absorption of volatile substances that could be released during subsequent analysis, the chamber and the connection between the elements tires are made using steel. The air is flown into the sensor chamber using a pump through a needle valve. That is used to adjust the total airflow, which is measured by a flowmeter downstream of the pump.

The instrument was also provided with the auto-sampler head space system HT2010H, supporting a 42 loading sites carousel and a shaking oven to equilibrate the sample head space. The vials were placed in a randomized mode into the carousel. Each vial was incubated at 50°C for 5 minutes into the auto-sampler oven, by shaking it for 1 minute during the incubation. The sample head space was then extracted from the vial in dynamic head space path and released into the carried flow (80 sccm).

The analysis timeline can be divided in three different steps, for a duration of 420 seconds (7 minutes) per sample, that are preceded by a step of warm-up that allows the achievement of the base line for the entire system:

- *injection*: the sample HS is flowed in the sensor chamber for 60 seconds (it is the actual analysis time); then, for 30 seconds environmental air flows through the same tube to clean it from any residual VOCs;
- *restore*: it starts when *injection* period is finished and, in this step, filtered air is flowed into the sensors camber. In this time (330 seconds) the sensors restore the original condition of the base line.

Thanks to the processor integrated in the S3 instrument, the frequency at which the equipment works is equal to 1 Hz.

2.4 Data Analysis

Data analysis was performed using MATLAB® R2015a software (MathWorks, USA). First of all,

sensors responses in terms of resistance (Ω) were normalized compared to the first value of the acquisition (R_0). As a feature for all the sensors, the difference between the first value and the minimum value during the analysis time was calculated. Hence, the dataset was composed by $\Delta R/R_0$ parameters.

In the second step, the normal distribution of the variables was checked using the Jarque-Bera (JB) test with a significance level chosen equal to 0.05. This test is a goodness-of-fit test of whether sample data have the skewness and kurtosis matching a normal distribution. The null hypothesis is a joint hypothesis of both the skewness and the excess kurtosis being zero.

Based on the test result, Partial Least Squares (PLS) method was used both to view how the groups of samples were represented thanks to the considered volatile compounds and to sensors responses, respectively, and to build the model that was used to classify the samples themselves. PLS is a statistical method that combines features from Principal Component Analysis (PCA) and multiple regression (MR). PCA is a statistical method that uses an orthogonal transformation in order to pass from the variable space X to a space of uncorrelated variables called principal components (PC). The aim is to reduce the number of variables to obtain a better representation of data. In addition to this, in PLS also the response matrix Y is decomposed and the principal component of X are rotated in the direction of maximum correlation with the principal components of Y. The new calculated variables are called latent variables (LV). Another advantage of using PLS instead of PCA is that it is not required that variables have a normal distribution.

Finally, classification was performed comparing two different classifiers: Partial Least Squares Discriminant Analysis (PLS-DA) and Artificial Neural Networks (ANNs). PLS-DA was successfully applied in different fields where products had to be recognized according to their place of origin or the presence of contamination, as in milk [29], honey [30], wine [31] and cheese [32]. ANNs are complex structures that try to mimic what human brain does. They are formed by elemental units called neurons that works like real neurons: once information arrives, they elaborate it and give an output. Each neuron id characterized by an activation function and coefficients of connectivity called weights. Overall structure is mainly composed by an input layer, hidden layers and an output layer [33]. ANNs can be used to resolve regression and classification problems or function approximation. They found a lot of space in food applications in order to analyze data collected with electronic noses [34-37]. In this work, a feed-forward ANN trained with Levenberg-Marquardt algorithm is used.

For PLS, dataset was splitted in two parts, training set and test set, using Venetian Blinds (VB) as cross-validation procedure. This method divides the whole dataset in j cross-validation groups; in each one, one sample is put in the test set and the others in the training set on the first step. Subsequently, in every group the sample after the previous one is taken into the test set and the others in the training set, and so on. In this work, the number of cross-validation groups was chosen equal to 10.

For the classification with PLS-DA, a toolbox made for MATLAB® and released by Milano Chemometrics was used [38]. Instead, ANNs were created using the function *nntool* of the same software. This tool allows to do a random split of dataset in test and training set by default.

3. Results and Discussion

3.1 GC-MS Analysis results

From the comparison between samples chromatograms, substantial differences were found. The main difference between 12 months and 24 months ripened grated PR lies in the amount of fatty acids that characterize this product. They are acetic acid, butanoic acid, hexanoic acid, octanoic acid, n-decanoic acid and their presence is much greater in 24 months PR. This result is widely confirmed from literature. Indeed, it is well known that these fatty acids are the results of fermentation processes especially in butter and seasoned cheese. Some studies revealed that the amount of acetic acid and butanoic acid doubles up in the period from 12 and 24 months [39-40].

Differences were also found comparing samples with different percentage of rind and same rind working process; the same trend is valid both for 12 months and 24 months ripened PR. It turned out

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that increasing the quantity of rind, the presence of three compounds increases, too. Besides butanoic and hexanoic acid, 2-nonanone has the same behavior. It is a member of the class of methyl ketones and it can be found in several foods, among which milk and cheese [41]. It is produced by oxidative degradation of fatty acids [42]. These results suggest that both the fermentation and the degradation happen more near the rind that in the central part of the cheese.

3.2 S3 Analysis results

Once data were acquired, at first sensors responses were checked. Since the first measures of each session were very different from the others, they were discarded. Consequently, there is a different number of replicas for each sample. Most likely, experimental conditions of first acquisitions were not the same of the following measures in terms of temperature of the autosampler oven where vials were put in, as explained in section *2.3 S3 Analyis*. In Table 2, a detailed description of number of samples that were considered for the following analysis is shown.

Table 2. Considered samples divided for ripening stage, rind percentage and rind working processes.

	0%	**18%**		**26%**		**45%**		**100%**	
12 month	-	11	12	13	11	12	14	-	14
24 month	12	14	14	13	13	11	13	13	-
		WR	**SR**	**WR**	**SR**	**WR**	**SR**	**WR**	**SR**

In Figure 1, boxplots of TGS2602 response that include $\Delta R/R_0$ for each sample are shown. This sensor represents the general trend that can be observed for all the sensors. Obviously, since different sensing materials are used, there are differences in terms of the highlighted groups overlapping. On the left part of the figure, there are 24-months seasoned samples: in the upper part grated cheese with SR while in the lower PR with WR. On the right part, there are 12-months ripened samples and they follow the same trend. The first boxplot is relative to samples of 0% rind; its $\Delta R/R_0$ is different respect to all the other group, but it is more similar to WR grated PR both seasoning stage. This result reflects the fact that they are characterized by a bigger amount of humidity.

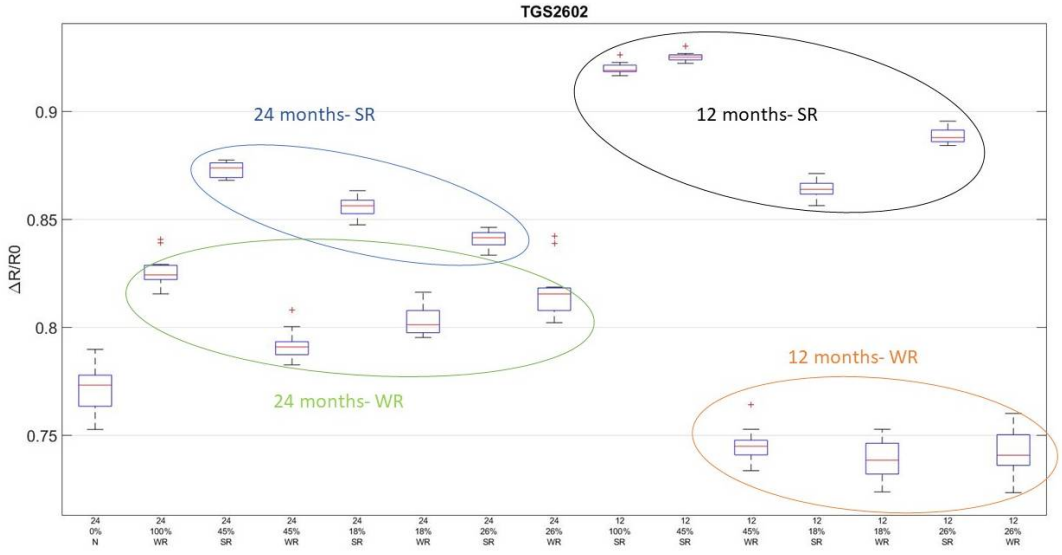

Figure 1. Boxplots of TGS2602 feature $\Delta R/R_0$. Four groups are highlighted: in blue 24 months-SR, in green 24 months-WR, in black 12 months-SR and in orange 12 months-WR.

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After checked general sensors performances, JB-test was applied to the dataset. It results that only 4 of the eight parameters followed a normal distribution ($p<0.05$); they correpond to the features extracted by the two tin oxide nanowires and RGTO sensors. That is the main reason for which PLS has been chosen. In Figure 2, PLS score plot was made considering the first two LV, for a total explained variance equal to 99.95% (99.87% for LV1 and 0.08% for LV2). In the plot measures are divided by seasoning degree. It can be observed that 24 months class is in the central part of the graph, while the other one is divided in the left and right part.

For this reason, classification techniques are used in a hiercachical way. In addition, another motive for this choice is to simplify classification models since this is a 15-class problem. Hence in the first step, classifiers were used to distinguish the seasoning degree; in a second step, for each ripening state the different working processes were discriminated; finally, ring percentage has been taken into account. In Figure 3, a scheme of the steps is shown.

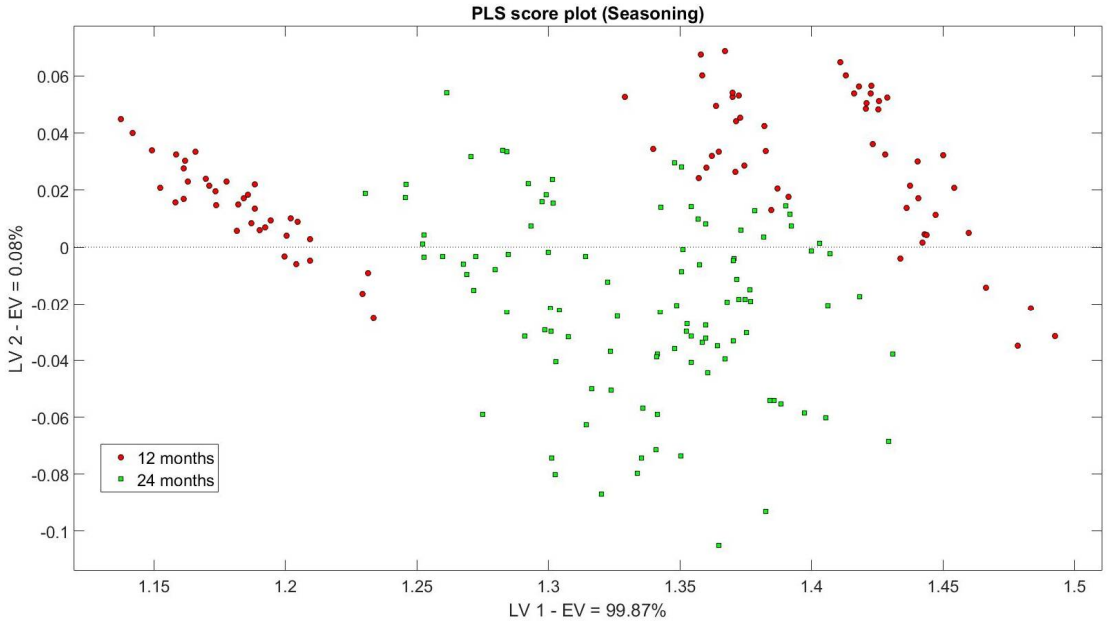

Figure 2. PLS score plot for all the measures divided for seasoning degree: in red circle 12 months, in green square 24 months. Total explained variance equal to 99.95% in first two LV.

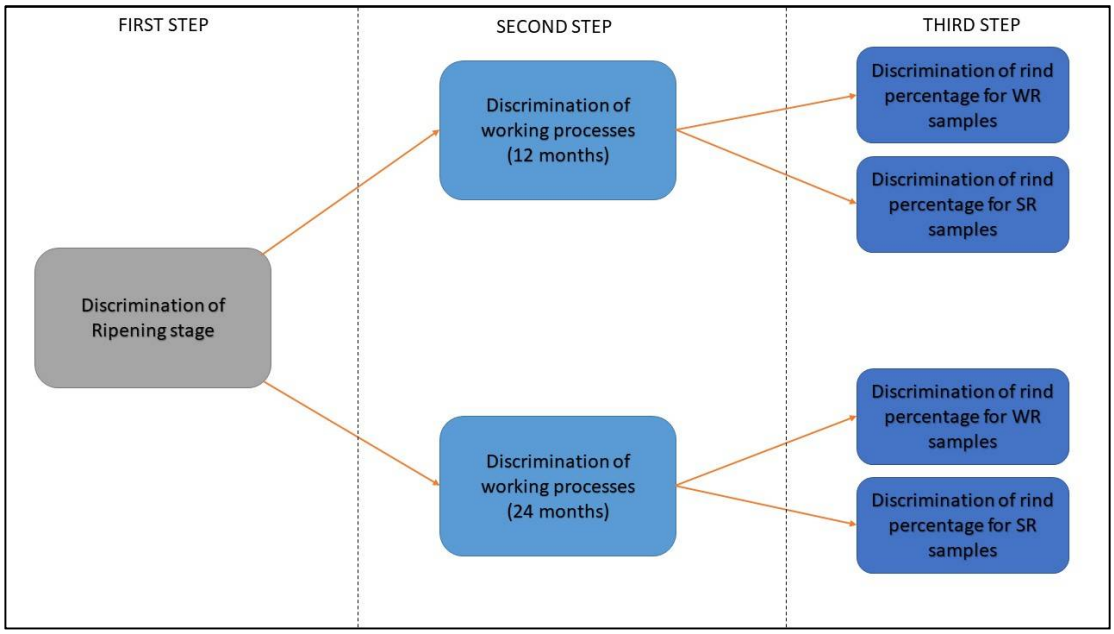

Figure 3. Step by step scheme for classification analysis.

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Regarding ANNs structures, three different ones were considered, one for each step. In the first case, a two layers architecture with 3 neurons in the input layer and 1 in the output layer was considered. For the second stage, the same number of layers was used, but in the first one two neurons were put. Finally, the third ANN had the same structure of the previous ones, but with 6 neurons in the input layer. For all the neurons, hyperbolic tangent sigmoid transfer function was chosen.

In Table 3, overall classification rate of the two classifiers is put side by side. In general, ANN classification rates are better than those of PLS-DA. Indeed, ANN is able to recognize correctly all the samples based on seasoning and rind working processes. Although PLS-DA performances are lower, it can reach good classification rates. The distinction between rind percentage shows that both classifier can classify samples with SR better than those with WR. A possible interpretation of this results could be the different amount of humidity: WR samples have a higher content of humidity because of water treatment and the adsorption sites this could cause the occupation of by water molecules instead of the ones that characterize the volatile fingerprint of the samples.

Table 3. Classification ratess of PLS-DA and ANNs divided per steps.

	First step Ripening stage	**Second step** Working processes	**Third step** Rind percentage
PLD-DA	94.7%	*12 months*:100%	*WR*: 61.1%
			SR: 90.2%
		24 months:79%	*WR*:90.2%
			SR: 95%
ANN	100%	*12 months*:100%	*WR*: 63.8%
			SR: 96.1%
		24 months:100%	*WR*: 58.8%
			SR: 100%

4. Conclusions

This study was aimed to verify the possible discrimination between grated PR with different rind percentage with an electronic nose, taking into account other two variables: seasoning degree and working processes of rind. In parallel, a consolidated technique, *i.e.* SPME GC-MS, has been used to understand which VOCs characterized analyzed samples. This combined analysis has produced promising results that pave the way to assess cheeses quality and avoid frauds.

First of all, with GC-MS, the VOCs that characterize grated cheeses have been individuated. The results regarding PR are compliant with those found in literature. Indeed, fatty acids that describe aroma and taste profile of PR have been found in greater quantity for 24 months seasoned samples as compared to 12 months ones. In addition, VOCs, whose amount is bigger in rind compared to pulp, was found and they are acquiescent with chemical reactions that take places in this product.

The multivariate statistical analysis made with PLS indicated how to proceed during the classification stage. A hierarchical approach was used, both for PLS-DA and ANNs. ANNs classification rates are the highest, suggesting that in future they could be improved to increase their performances. These first results are encouraging and further research is in progress in order to add more samples and to have more statistical significance of the achieved results.

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