



FT-NIR and FT-MIR spectroscopy to discriminate competitors, non compliance and compliance grated Parmigiano Reggiano cheese

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ABSTRACT

In this investigation the potential of infrared spectroscopy, coupled to different statistical methods, were used to estimate the authenticity of grated Protected Denomination of Origin (PDO) Parmigiano Reggiano cheese (P-R). The feasibility of the analytical approach in the prediction of cheese authenticity without the use of wet chemistry was evaluated. A total of 400 plastic-sealed grated cheese samples classified as: compliance P-R, competitors, non-compliance P-R (defected P-R), and P-R with rind content of > 18%. PCA was conducted for an explorative spectra analysis. Soft independent modelling of class analogy (SIMCA) analysis and artificial neural networks (ANNs) were used to classify samples, according to different cheese categories. For both the spectroscopic techniques, PCA correctly discriminated compliance P-R from competitors, but not the P-R as a function of the rind percentage and months of ripening. SIMCA analysis accurately classified the compliance and competitors' P-R samples, while samples belonging to the classes of defected P-R and P-R with rind content > 18% were not accurately classified. ANN was more efficient than SIMCA in the classification of all the cheese classes. The results showed that NIR and MIR combined with different statistical approaches can be suitable for a sensitive, non-destructive, rapid and inexpensive screening of grated P-R cheese authenticity.

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1. Introduction

Parmigiano Reggiano (P-R) cheese is a reduced-fat, additive free hard cheese made from partly skimmed, unpasteurised milk in north Italy (Malacarne et al., 2008). It carries the prestigious appellation of Protected Denomination of Origin (PDO), and the production is regulated by the Parmigiano-Reggiano Cheese Consortium (CFPR). The denomination of origin is extended to the grated cheese when manufactured exclusively from whole Parmigiano-Reggiano cheese wheels that respond to the production standard. The grated cheese must be characterized by the absence of additives, moisture no less than 25% and no more than 35%, matured for a period of at least 12 months, rind not over 18%, typical amino-acid composition of the cheese, not crumbly in aspect and with homogeneous particles with a diameter inferior to 0.5 mm and not exceeding 25% (D.P.C.M. 4/11/1991), in addition to the specific rules and compliance to the standard that local processors must attain to during production (Cevoli, Ragni, Gori, Berardinelli, & Caboni, 2012).

The determination of authenticity of high-value foods that carry the PDO, plays a crucial role in quality control and safety (Luykx & van Ruth, 2008; Reid, O'Donnell, & Downey, 2006). Food frauds and

deceptive practices, carried out by unscrupulous producers solely oriented to the misrepresentation, have always been a serious problem because of the economic advantages deriving from partial replacement of high-priced ingredients with low cost ones. In particular, for foodstuffs produced, processed and prepared in a specific geographical area using recognized methods, objective and exhaustive food authenticity information is the major concern for food producers, retailers and consumers, against fraudulent practices. The main concern during the cheese grating operations, is the loss of the stamp embossed on the rind as a symbol of authenticity and guarantee to costumers, that will be printed afterwards on the package. In this way, the grated P-R can be easily mixed with low quality cheeses, extra rind and non-compliant P-R samples. The traditional analytical analysis applied by the CFPR to differentiate P-R cheese from the major commercial hard type cheese brands, rely on the determination by wet chemistry of some technological parameters that distinguish the P-R cheese manufacturing features (Panari, Filippi, Caroli, Pecorari, & Nocetti, 2009). The long ripening time, the lack of additives and the use of raw milk are evaluated by the determination of soluble nitrogen, the presence of the added lysozyme and the measure of the alkaline phosphatase activity respectively. However, this approach has the disadvantage of the limited knowledge of the range of each parameter in the different brands of competitor cheeses. More efficient but expensive

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spectroscopic techniques to determine the quality and/or geographical origins of P-R cheese are: nuclear magnetic resonance and stable isotope analysis (Camin et al., 2012; Consonni & Cagliani, 2008).

The demand for non destructive, fast, sensitive, high-throughput and cheaper methods for direct quality analysis of food and food ingredients has recently led to the increased popularity of infrared spectroscopy (IR) in food science (Luykx & van Ruth, 2008; Reid et al., 2006). In particular, Middle- and Near-IR (MIR and NIR respectively) coupled to chemometric techniques, have been widely used for dairy product analysis, because it is possible to extract chemically based information related to composition and conformation of cheese components from the spectra, generating a reliable fingerprint (Woodcock, Fagan, O'Donnell, & Downey, 2008). MIR analysis, which reveals information about the molecular fundamental vibrations and associated rotational-vibrational structure in the spectral range from 4000 to 700 cm^{-1} , have been efficiently applied in combination with pattern recognition techniques for the determination of coagulation and chemical parameters of milk (De Marchi et al., 2009), composition of aged cheese (Subramanian, Alvarez, Harper, & Rodriguez-Saona, 2011), microbial proteolytic activity (Vannini, Baldi, & Lanciotti, 2001), ripening time and manufacturing technique (Lerma-García, Gori, Cerretani, Simó-Alfonso, & Caboni, 2010), texture parameters (Boubellouta & Dufour, 2012), and geographic origin (Gori, Maggio, Cerretani, Nocetti, & Caboni, 2012; Karoui et al., 2005). With regards to NIR, which provides more complex structural information because it can excite overtone or harmonic vibration as the vibrational behaviour of bond combinations in the wavelength range from 10,000 to 4000 cm^{-1} , investigations combined with chemometric methods were carried out for cheese authentication (Ottaviana et al., 2012; Pillonel et al., 2003), prediction of sensory attributes (González-Martín et al., 2011), and determination of physicochemical properties of cheese (Sánchez-Macías et al., 2010).

Since confirmation of authenticity of high value cheeses represents an emerging topic within the food sector, and the demand for fast and low-cost analytical techniques for quality control in industrial setting is receiving great attention, the purpose of this study is to investigate the potential of the use of MIR and NIR coupled to different chemometric techniques to discriminate grated PDO P-R cheese from non-compliance P-R (defected P-R; P-R with rind > 18%) and competitors.

2. Materials and methods

2.1. Samples

A total of 400 plastic-sealed grated cheese samples were analysed. As reported in Table 1, the samples were classified as follow: compliance P-R, competitors, non-compliance P-R (defected P-R) and P-R with rind content > 18%.

Compliance P-R samples were derived from certified whole cheese wheels, grated and packaged in the area of origin as quoted in the product specification (D.P.C.M. 4/11/1991).

Non-compliance P-R were grated samples derived from certified whole cheese wheels, but characterized by the presence of extra rind

content (from 25% to 50%). A further class of non-compliance P-R was represented by the grated cheese samples derived from defected uncertified whole P-R cheese wheels. The compliance and non-compliance grated P-R cheese samples were of known month of ripening (from 12 to 32 circa), and provided by the CFPR which guaranteed the origin.

The competitor group embraced 25 major commercial brands of Italian, European and extra-European grated cheeses of unknown age, collected at local markets.

All samples were packaged in about 200 g plastic sealed boxes and the particle size ranged between 0.5 and 1.5 mm.

2.2. FT-NIR spectroscopy

NIR spectra were obtained using a spectrophotometer Vector 22/N (Bruker Optics, Ettlingen, Germany) in diffuse reflectance mode. The instrument was equipped with a rotating integrating sphere that allows a wide illumination of the sample and a better reduction of scattering due to the irregularity of the surfaces. Before every analysis, each box containing grated cheese was stirred. NIR spectrum was obtained by average of 32 scans of each sample and three replicates were collected for each individual sample (50 g of grated cheese) by using standard cups with quartz base. Background was defined by acquiring the spectrum of the quartz support without the sample, in the same instrumental conditions (25 °C). The speed of acquisitions was 10 kHz in a range from 12,500 cm^{-1} –4000 cm^{-1} to 8 cm^{-1} of spectral resolution.

2.3. FT-MIR spectroscopy

FT-MIR spectra of each sample of cheese were acquired using a Tensor 27 TM FTIR spectrometer system (Bruker Optics, Ettlingen, Germany) equipped with an interferometer Rocksolid and a DigiTect detector system, coupled to an attenuated total reflectance accessory (ATR), that was used to analyse directly the grated cheese. The ATR is equipped with reflective crystal zinc-selenide (PIKE Technologies, Madison, WI, USA).

Before every analysis, each box containing grated cheese was stirred, and approximately 1 g of grated cheese was deposited on the ATR surface, and a pressure clamp was applied to obtain a thin cheese layer. The spectrum of each sample was obtained through the average of 64 scans with a speed of 10 kHz in a range from 4000 to 650 cm^{-1} and with 4 cm^{-1} of spectral resolution. For each sample 3 replicates were performed using the air as background, measured in the same instrumental conditions (25 °C). Before acquiring each spectrum, the ATR crystal was cleaned with a cellulose tissue soaked in *n*-hexane and then rinsed with acetone.

2.4. Data processing

FT-MIR data were treated with the first derivative using the Savitzky-Golay algorithm (Savitzky & Golay, 1964). Subsequently the regions between 4000 and 3050 cm^{-1} , and between 2410 and 2260 cm^{-1} , were deleted because they contained no useful chemical information (Maggio et al., 2009; Pillonel et al., 2003). Mean-centred spectral data were subjected to PCA, SIMCA and ANN analysis.

To remove the effects of light scatter, NIR spectra were pre-treated with multiplicative scattering correction (MSC). The first part of the spectra until 9000 cm^{-1} , was deleted before processing because it contains no useful chemical information, but only instrumental noise. Data were also treated by applying the first derivative (Savitsky-Golay) to the absorbance data. Also in this case, mean-centred spectral data were subjected to PCA, SIMCA, and ANN analysis.

Principal component analysis (PCA) was applied as an exploratory analysis in order to define a possible discrimination among samples of

Table 1
Classification of the 400 grated cheese sample analysed by infrared spectroscopy.

Months of ripening	Compliance P-R	Non-compliance P-R				Competitors
		Rind percentage			Defected	
		25%	30–33%	50%		
<12	0	0	1	1	19	
From 12 to 18	51	7	2	5	18	
From 19 to 25	43	15	15	15		
From 26 to 32	44	13	14	12		
>32	2	2	2	2		
Total	140	37	34	35	37	116

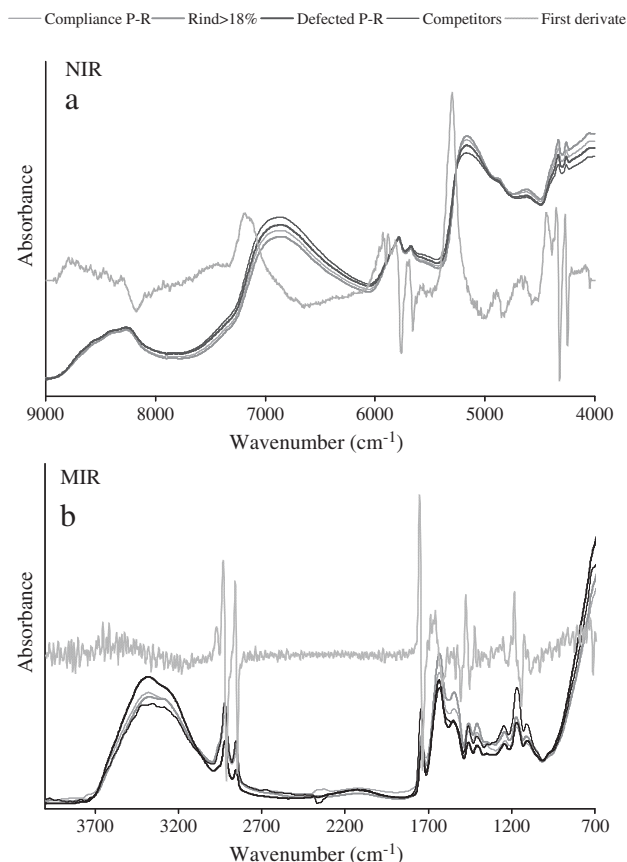


Fig. 1. Averaged NIR and MIR spectra of four classes of cheese and relative fist derivate.

compliance P-R and non-compliance P-R or competitors (The Unscrambler ver. 9.7, CAMO, Oslo, Norway).

To discriminate among specific classes through the use of a linear method, SIMCA classification (Soft independent modelling of class analogy) was performed (The Unscrambler ver. 9.7, CAMO, Oslo, Norway). Disjoint class-modelling is the first step in the classification SIMCA procedure, called training stage. Here individual models of the data classes were made. The subsequent classification stage then uses these established class models to asses to which classes new objects belong. Results from classification stage allow us to study the modelling and discrimination power of the individual variables. In

this work four different classes were individually modelled by PCA, according to four sample categories (compliance P-R, competitors, defected P-R and P-R with rind > 18%). The number of significant PCs were determined for each class by *full cross validation* by using 80% of the data set, selected as representative of the population variance. The remaining 20% of the data set was used to classify spectra from the previous classes' modelling (referring to a significance level of 10%).

Subsequently, artificial neural network models (ANNs) were performed by using STATISTICA Neural Networks 4.0 (StatSoft Inc., Tulsa, OK, USA). A Multi-Layer Perceptron (MLP) neural network was built to predict the specific classes to which the samples belong by a non-linear method. To perform classification tasks, four nominal output variables (V1, V2, V3 and V4) were used: V1 for compliance P-R samples, V2 for competitors' samples, V3 for defected P-R samples (not-compliance) and V4 for P-R samples with a rind percentage higher than 18% (non-compliance). For input and hidden layers, linear and logistic activation functions were used, respectively, while for output layer the *softmax* function was used. From a statistical point of view, with the *softmax* activation function and the cross-entropy error, neural network model can be seen as a multilogistic regression model, and the outputs are then interpretable as posterior probabilities for categorical target variables (Bishop, 1995).

Looking for the best classification ability, different node numbers in the hidden layer and combinations of momentum and learning rate were tested. The convergence of ANNs was ruled by a back propagation algorithm.

The original data sets were randomly divided into training set (70%), verification set (15%) and test set (15%). It was checked that samples from all classes were contained in the test set.

3. Results and discussion

3.1. Spectra characterization

The FT-NIR and FT-MIR spectral band characterization was accomplished by comparing wavelengths with published infrared region data.

The NIR light molecular absorption of the 400 grated cheeses provided characteristic broad and overlapping peaks between the 9000 and 4167 cm^{-1} spectral region. Fig. 1a presents averaged spectra of the four classes. All the samples showed the same spectral behaviour, and are characterized by the main absorption bands of water and fat respectively. The same spectral areas could be visually identified according to earlier investigations (Karoui et al., 2005, 2006a). The Savitzky–Golay first derivative (see Fig. 1) allowed the extraction of useful band information at

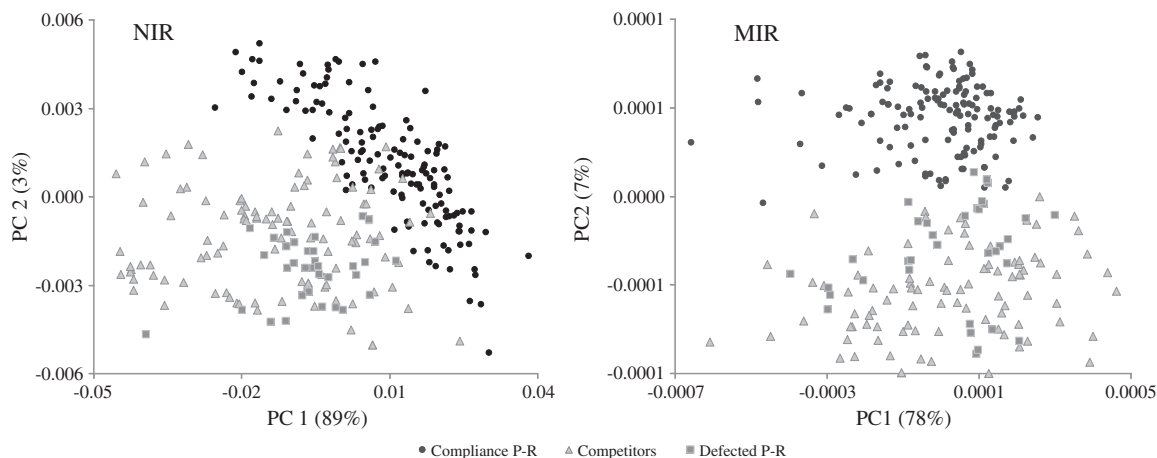


Fig. 2. Score plot obtained by PCA analysis of the NIR and MIR spectra to discriminate the compliance P-R from non compliance (competitors and defected P-R).

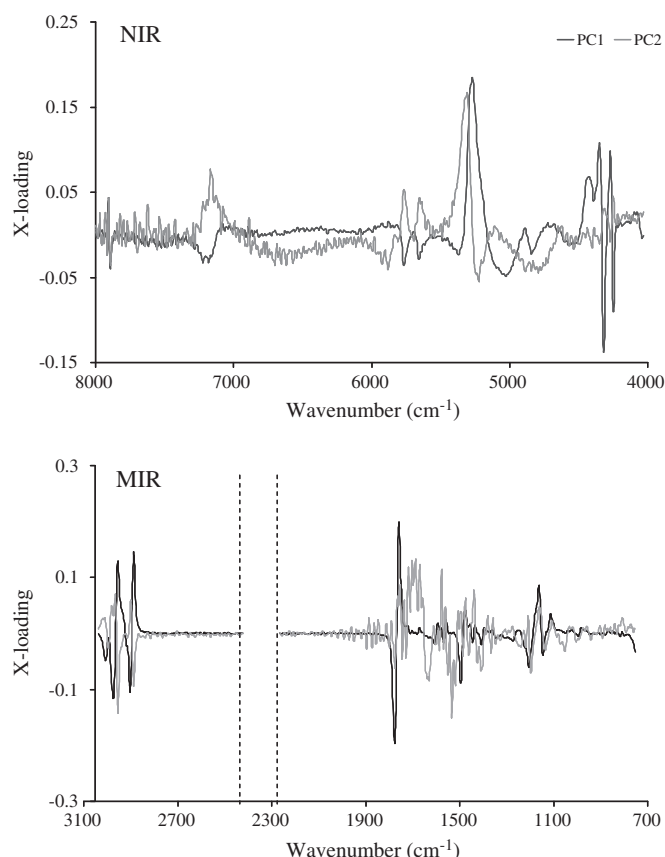


Fig. 3. X-loading spectra of the first and second principal components obtained by PCA analysis of all NIR and MIR spectra.

5882–5656, 5305, 4322–4243, and 4566 cm^{-1} related to the first and second C–H and O–H overtones.

The absorption peaks being reported to correspond to the lipid contribution at 5903, 5858, 5770 and 5656 cm^{-1} , were attributed to the first overtone of a C–H stretch, while bands at 4394, 4320, 4274 and 4246 cm^{-1} were addressed to the C–H₂ group. Moreover the absorption peaks originating at 5305 and 4566 cm^{-1} were assigned to the combination band of the asymmetric and scissor stretch O–H vibrations of water and the combination of N–H and O–H bonds respectively.

In Fig. 1b averaged spectra of the four classes acquired in the 4000–700 cm^{-1} spectral region are reported. In this case, the samples showed the same spectral behaviour. According to the Savitzky–Golay

first derivative the major spectral information computed in the statistical analysis were encompassed between 3000–2800, 1700–1500 and 1500–900 cm^{-1} .

A strong absorption of bands were observed between 3000 and 2800 cm^{-1} , in particular at 3000 cm^{-1} arose the weakest band which was associated in earlier investigations on butter and vegetable oils with –C=C–H stretching groups of *cis*-unsaturation (Koca, Kocaoglu-Vurma, Harper, & Rodriguez-Saona, 2010), while the predominant bands located at 2919 and 2851 cm^{-1} were ascribed to the symmetric and anti-symmetric stretching vibration of CH₂ and CH₃ groups of fatty acids respectively (Karoui, Mazerolles, Bosset, de Baerdemaeker, & Dufour, 2007). The contribution to the lipids can also be noticed at 1765–1730 cm^{-1} , which was associated with C=O stretching of carbonyl groups of acids and esters (Subramanian et al., 2011).

The 1650–1540 cm^{-1} spectral region was attributed to amide I and II vibrations of proteins, despite the H–O–H bending vibration of water absorbed in this region that may affect the interpretation of the spectra (Karoui, Mazerolles, & Dufour, 2003).

Difference between the grated cheese samples were also noticed in the so called fingerprint spectral region (between 1500 and 900 cm^{-1}), that represent the result of the absorption of several chemical bonds (Lerma-García et al., 2010). In particular, the spectral band located between 1477–1400 and 1170 cm^{-1} were associated in Emmental cheese with O–C–H, C–C–H and C–O–H bending modes, and C–O and C–C stretching modes respectively (Karoui et al., 2006b).

3.2. PCA

The score plots of the PCA performed to discriminate between compliance P-R, competitors and defected P-R samples are reported in Fig. 2. For both the spectroscopic techniques, a clear separation between compliance P-R samples and the rest of the sample was observed. The competitors' samples and the defected P-R samples were grouped together.

For the FT-MIR, the best discrimination was obtained using the following spectra regions: 3000–2800 cm^{-1} , 1760–1730 cm^{-1} and 1650–1540 cm^{-1} . The samples of grated compliance P-R cheese were grouped along the positive values of PC2 (y-axis) and the other samples were grouped on the negative side of the axis.

For the FT-NIR, both the PC1 (89%) and the PC2 (3%) contributed to the discrimination between compliance P-R samples and the other samples. The loading plots (Fig. 3) suggested that the discrimination might be attributed to spectral regions that previous investigations associated with moisture and lipid contribution (Karoui et al., 2005, 2006a). A possible explanation may be related to the moisture content of defected P-R samples, that were ripened for a maximum of 13 months (Malacarne et al., 2009). Furthermore, during the ripening, complex chemical-physical modification might occur: particularly proteolysis and lipolysis

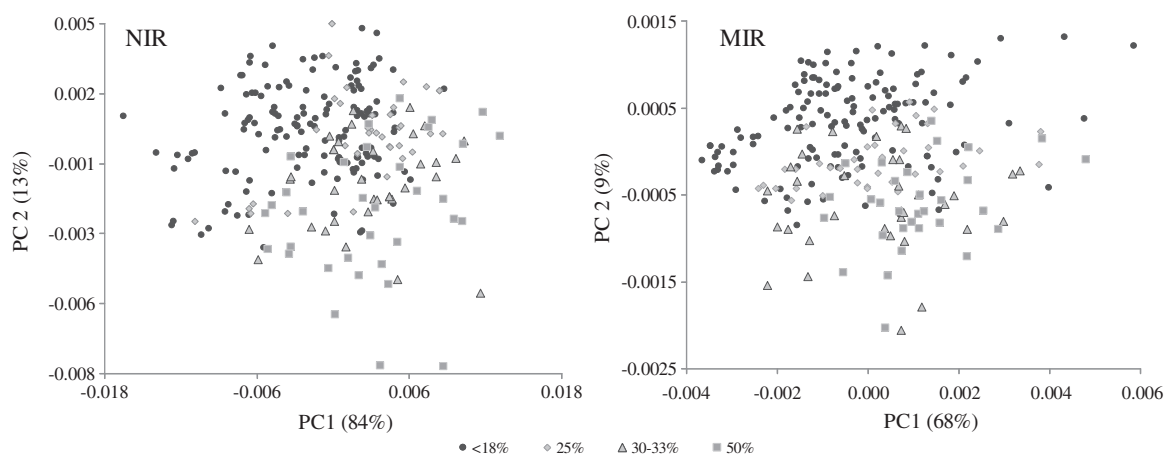


Fig. 4. Score plot obtained by PCA analysis of the NIR and MIR spectra (compliance P-R and P-R with rind > 18%).

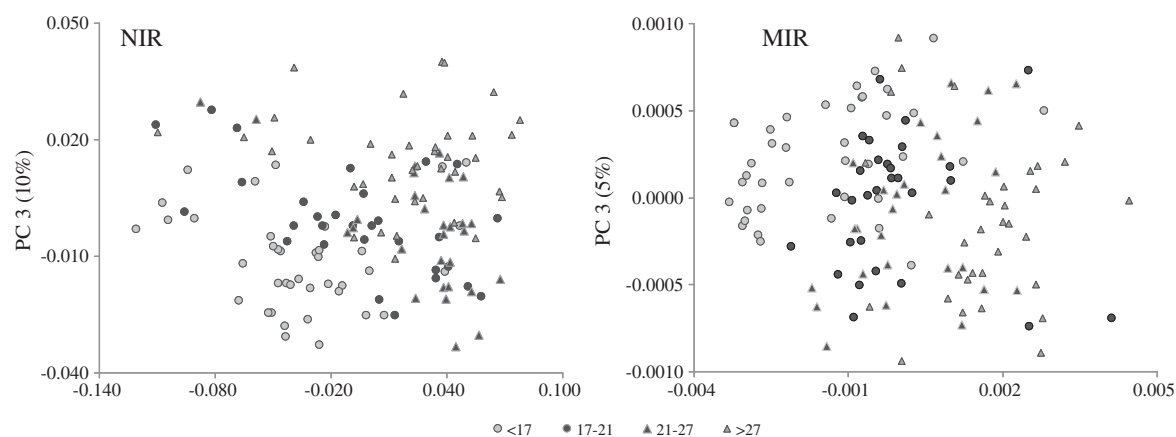


Fig. 5. Score plot obtained by PCA analysis of the NIR and MIR compliance P-R spectra to discriminate the months of ripening.

are the most important biochemical processes, which cause a variation in the state of the nitrogen fraction as well as a modification of the lipid composition (McSweeney, 2004; Pellegrino et al., 1997; Tosi, Sandri, Tedeschi, Malacarne, & Fossa, 2008).

The score plots concerning the discrimination between samples characterized by different rind percentage, are reported in Fig. 4. Both spectroscopic techniques were unable to achieve a clear separation between samples with different rind percentage. Only the samples characterized by a rind content of 18% were grouped. An insufficient separation may be due to the fact that in addition to the rind percentage, also the months of ripening varied (from 12 to 32 months).

Considering only the compliance samples (rind content <18%), it is possible to observe for both spectroscopic techniques (Fig. 5) that the sample distribution takes place according to the months of ripening. Particularly, the samples with less than 17 months of ripening and the sample with more than 27 months of ripening are grouped separately.

3.3. SIMCA

The results of SIMCA analysis conducted to classify the cheese samples are summarized in Table 2. The percentage of samples correctly classified and the model to model distance are reported.

The object distance to the model and the distance from the model centre were used to evaluate the class membership of new objects. The first distance is a measure of how far the sample lies from the modelled class and it is computed as the square root of the sample residual variance, while the second distance is a measure of how far the projection of a sample into the model is from the class centre, i.e. it expresses how different the sample is from the other class members, regardless of how well it can be described by the class model.

Further model to model distance is an important factor for the evaluation of SIMCA results because it gives a measure of the separation between classes. The value of this measure should be compared to this 1

(distance of a model to itself). A model distance larger than 3 shows that the two models are quite different, which in turn implies that the two classes are likely to be well distinguished from each other (Esbensen, 2006). In this case, the model to model distances range from 1.566 to 12.220, and from 3.193 to 9.593 for FT-NIR and FT-MIR data respectively.

For both the spectroscopic techniques, the compliance P-R and competitors' samples were correctly classified with a minimum percentage of 80. However the percentage of correctly classified defected P-R and with a rind content > 18% samples was low (from 55 to 78%). The incorrectly classified defected P-R samples were assigned to competitor class, while the incorrectly classified samples with rind > 18% were assigned to compliance P-R. This result may depend on the lower model to model distance between the model of compliance P-R and P-R with rind content > 18%, and between the model of defected P-R and competitors.

3.4. ANN

An early stopping technique was used to select the number of training cycles to avoid overfitting, using the verification set to monitor the prediction error. Error graphs of training and verification set was reported in Fig. 6. The minimum values at about 1600 iterations indicated that the learning had reached its maximum level. Over these points, the error increased again. This is a clear evidence that the ANN tends to overfit. Consequently the results of ANN are related to these iterations.

Training was repeated 5 times and the network's predictions were averaged, since with ANNs convergence is influenced by the initial weight value. The learning rate and momentum parameters were used to control the size of weight adjustment along the descending direction and for dampening oscillations of the iterations.

The success of the classification model was evaluated in different ways: (a) recognition ability as percentage of the samples in the training and verification set correctly classified; (b) predictive ability

Table 2
SIMCA results for the classification of compliance and not compliance P-R (competitors, defected and rind > 18%) in terms of correct classified case percentage and model to model distance.

Technique	Classes	PCs	Corrected classified cases (%)	Uncorrected classified cases (%)	Model to model distances			
FT-NIR	Compliance P-R	3	80	20 (rind <18%)	1			
	Competitors	3	100		4.848	1		
	Defected P-R	4	55	45 (competitors)	3.778	1.799	1	
	Rind > 18%	2	62	38 (compliance P-R)	1.566	12.220	9.426	1
FT-MIR	Compliance P-R	5	90	10 (rind > 18%)	1			
	Competitors	5	90	10 (defected)	7.119	1		
	Defected P-R	4	55	45 (competitors)	9.593	3.519	1	
	Rind > 18%	5	78	22 (compliance P-R)	3.193	4.115	5.968	1

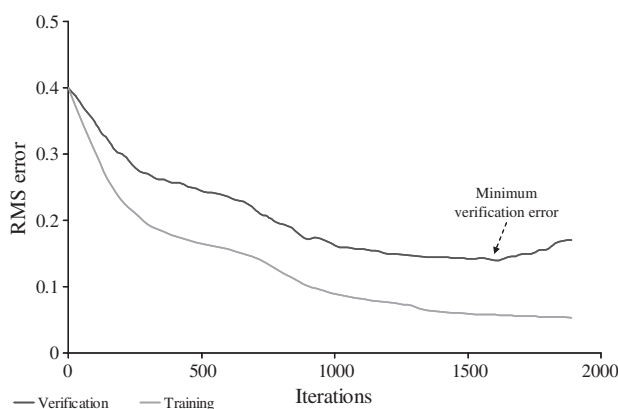


Fig. 6. Root mean square error (RMSE) graphs of training and verification set.

as percentage of the samples in the test set correctly classified by using the developed model during the training step; and (c) classification ability as percentage of samples in both training, verification and test sets correctly classified by the model (Cajka, Hajslova, Pudil, & Riddellova, 2009).

The results of the neural networks tested to discriminate the compliance P-R, the competitors, the defected P-R and P-R with rind content > 18% are reported in Table 3.

For the FT-NIR data, the best prediction results were obtained with a three layer network, having 5 and 7 nodes, respectively for the FT-NIR and FT-MIR data. A large number of nodes did not increase the network performance. All the ANNs were obtained with a learning rate of 0.01 and a momentum of 0.8.

For the FT-NIR data, the predictive ability for compliance P-R, competitors, defected P-R and P-R with rind content > 18% was 95.5, 95.5, 100, and 91% respectively, while the classification ability was 98.5, 97, 100 and 97% respectively. The average probability of correct identification (test set) was 0.974.

For the FT-MIR data, the predictive ability for compliance P-R, competitors, defected P-R and P-R with rind content > 18% was 96.7, 100, 100, and 93.2% respectively, while the classification ability was 98.9, 100, 96.7 and 95.5% respectively. The average probability of correct identification (test set) was 0.969.

4. Conclusions

In this study, middle and near infrared spectroscopy coupled with advanced statistical techniques (PCA, SIMCA, and ANN), were able to discriminate in a fast and reliable way the grated samples of the PDO P-R cheese from non-compliance P-R (samples with defect, and with extra rind content) and major commercial brands of European and

extra-European grated cheeses. However the differentiation of grated cheese groups, while considering contemporaneously their extra rind content and the months of ripening, resulted difficult to estimate.

For the determination of P-R conformity, ANN resulted to be the best performing statistical technique able to classify correctly in the test set the 95.5% and 96.7% of compliance P-R for FT-NIR and FT-MIR respectively, while SIMCA the 80% and 90% respectively. Furthermore, ANN was more efficient than SIMCA in the classification of non-compliance P-R.

Finally, considering the classification capability of the grated cheese groups analysed in the current investigation, FT-MIR coupled with ANN was demonstrated to be the best performing analytical approach. The results obtained by both NIR and MIR techniques agreed with each other confirming the use of spectroscopic methods as reliable, fast and non-destructive techniques, which could assist cheese manufacturers during quality control.

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Table 3

Artificial neural networks results for the classification of compliance and not compliance P-R (competitors, defected and rind > 18%) in terms of correct classified case percentage and probability.

Technique	Classes	Corrected classified cases (%)			Probability (test set)	Classification ability (%)
		Training set	Validation set	Test set		
FT-NIR	Compliance P-R	100	100	95.5	0.974	98.5
	Competitors	100	95.5	95.5	0.987	97
	Defected P-R	100	100	100	0.941	100
	Rind > 18%	100	100	91	0.993	97
FT-MIR	Compliance P-R	100	100	96.7	0.972	98.9
	Competitors	100	100	100	0.972	100
	Defected P-R	100	100	90	0.953	96.7
	Rind > 18%	100	93.2	93.2	0.978	95.5

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