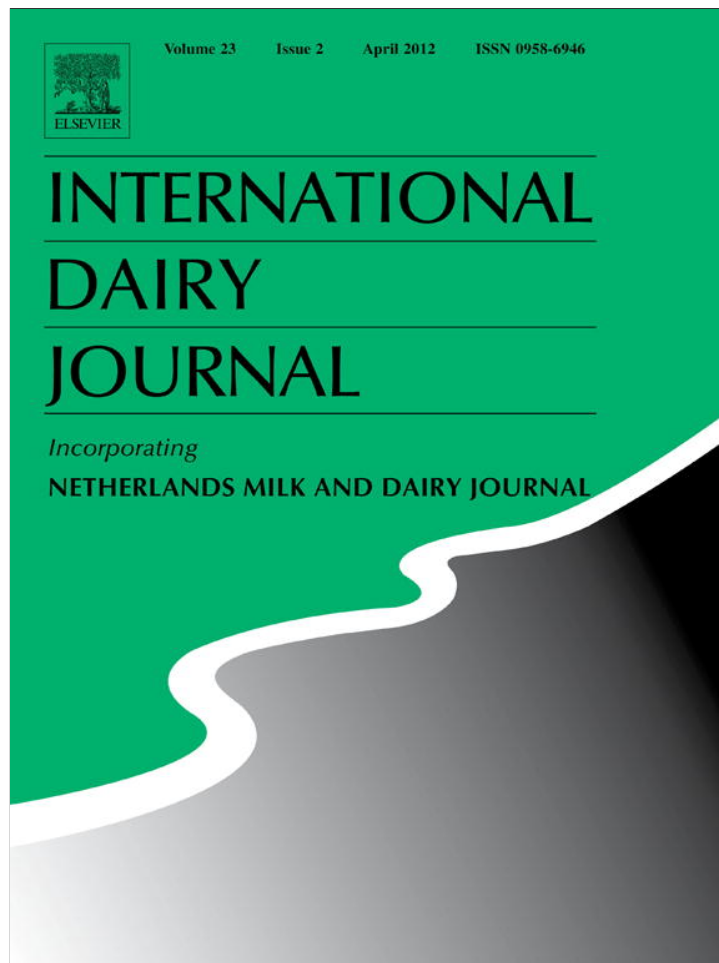


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## Discrimination of grated cheeses by Fourier transform infrared spectroscopy coupled with chemometric techniques

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### ABSTRACT

Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR), combined with chemometric analysis, was used to classify grated Parmigiano-Reggiano cheese from other grana-type cheeses (so called for their granular texture) from Italy, central and northern Europe. A total of 36 grated cheese samples (21 Parmigiano-Reggiano and 15 Italian and non-Italian non-Protected Designation of Origin) were analysed. Samples were scanned in the range of 4000–700 cm<sup>-1</sup>. Two attenuated total reflectance accessories were utilised. Linear discriminant analysis (LDA) and principal component analysis were used to analyse spectral data after applying a moving windows algorithm for wavelength selection. Both methods successfully classified the four classes of grated cheese samples, and LDA was found to be the best chemometric approach. ATR-FTIR spectroscopy coupled with LDA is a promising technique, and merits further investigation as a reliable and rapid classification tool that does not require chemical analyses for discrimination of cheese.

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### 1. Introduction

In recent years, many traditional and artisanal food products have been increasingly rediscovered by consumers as they are considered beneficial for daily nutrition due to their genuineness and authenticity. In the hard cheese, or “grana-type” category (so called for their granular texture), there are a large number of cheeses that have remained unchanged over time, maintaining the link with tradition and local methods of production. This linkage is the result of the interaction between natural and human factors, namely animal fodder and the technology used for production (Karoui & De Baerdemaeker, 2007).

One such case is the Protected Designation of Origin (PDO) Parmigiano-Reggiano cheese, one of the oldest traditional cheeses produced in Italy (Zannoni, 2010). The factors that make this product unique include the local fodder and pasture consumed by the dairy cows where any type of silage is forbidden, the quality of milk (Summer et al., 2003), the use of raw and partly skimmed cows' milk, supplementation with a natural whey starter before curd formation (Bottari, Santarelli, Neviani, & Gatti, 2010; Gala

et al., 2008), the exclusive use of calf rennet and the prohibition of preservatives such as lysozyme, the use of copper vats, cheese master know-how (Bellesia et al., 2003), and aging for 12–36 months.

Parmigiano-Reggiano, as well as a wide number of grana-type cheeses, can be sold as packaged pieces or grated; the former are easily recognizable because the emblem embossed on the rind certifies its origin (Regattieri, Gamberi, & Manzini, 2007). However, when grated, there may be some doubt about composition and authenticity. Therefore, it is necessary to have the analytical means of verifying that the origin of a grated cheese corresponds with its labelling.

The development of novel and sophisticated analytical techniques have been used for determination of authenticity and geographical origin of various foodstuffs, including dairy products (Luykx & van Ruth, 2008; Reid, O'Donnell, & Downey, 2006). In particular, Fourier transform infrared spectroscopy (FTIR) combined with multivariate data analysis such as principal component analysis (PCA), Fisher discriminate analysis (FDA), linear discriminate analysis (LDA), support vector machine, k-nearest neighbour and partial least squares have all been successfully used to monitor the quality parameters or authenticity of olive oil (Maggio, Cerretani, Chiavaro, Kaufman, & Bendini, 2010; Maggio et al., 2009), honey (Hennessy, Downey, & O'Donnell, 2008), fruit

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(Vardin, Tay, Ozen, & Mauer, 2008), wine (Tarantilis, Troianou, Pappas, Kotseridis, & Polissiou, 2008), meat (Argyri, Panagou, Tarantilis, Polysiouand, & Nychas, 2010) and dairy products (Nicolaou, Xu, & Goodacre, 2010). With a special concern towards cheeses, FTIR coupled with chemometrics has been used to investigate the composition of aged cheese (Subramanian, Alvarez, Harper, & Rodriguez-Saona, 2011), sensory and texture parameters (Blazquez et al., 2006), geographic origin (Karoui et al., 2004; 2005) and aging time (Lerma-García, Gori, Cerretani, Simó-Alfonso, & Caboni, 2010; Martin-del-Campo, Picque, Cosío-Ramirez, & Corrieu, 2007).

Moreover, FTIR is a non-invasive and non-destructive technique that offers a fast and cost effective method of food analysis (Karoui et al., 2004), and can be easily applied in research, quality control laboratories and industrial settings. One of the main advantages of the FTIR spectrometer is the ability to scan spectra ( $4000\text{--}700\text{ cm}^{-1}$ ) directly on the cheese sample by collecting an interferogram of a sample signal with an interferometer, which measures all infrared frequencies simultaneously. The spectrum recorded thus provides a “fingerprint” of each cheese sample since it contains information about its physicochemical characteristics (Coates, 2000).

In this study, attenuated total reflectance (ATR) FTIR spectroscopy combined with PCA and LDA was used to discriminate grated Parmigiano-Reggiano cheese from other grana-type cheeses from Italy, central and northern Europe, with the purpose of establishing a fast, highly reproducible and non-invasive method for quality control of grated cheese. Since the non-homogeneity of components in various cheeses is well-known (McQueen, Wilson, Kinnunen, & Jensen, 1995), a further aim of this investigation was to test the reproducibility of the analytical method with two type of ATR accessories: an ATR accessory used for liquids that permits analysis of 1.5–2 g of sample, and an ATR accessory used for the analysis of 0.5–1 g of solid sample.

## 2. Material and methods

### 2.1. Grated cheeses

A total of 36 plastic-sealed grated cows' milk cheese samples were used for FTIR analysis. The cheese samples were manufactured in Italy, the Czech Republic and Lithuania, and classified as follows: 21 Parmigiano-Reggiano at 12 and 24 months of age, and 5 Italian non-PDO, 5 Czech non-PDO and 5 Lithuanian non-PDO that were aged for unknown time. Parmigiano-Reggiano cheese samples were kindly donated by the Cheese Consortium (CFPR), whereas others were purchased in blocks from local markets. Each sample were grated with an industrial toothed grater roller prior to the direct determination of the content of fat, protein, moisture and salt with a Foss FoodScan analyzer (FoodScan Lab, Type 78800, FOSS, Hillerød, Denmark) by the CFPR personnel. The grated samples were then kept in a sealed box until analysis to prevent changes in relative composition due to moisture loss. The origin and status of conservation of all samples were guaranteed by the suppliers.

### 2.2. FoodScan calibration

The instrument calibration was carried out by introducing into the ISScan management software of FoodScan (Routine Analysis Software, FOSS), a data set consisting of spectral data and analytical data obtained by wet chemistry analysis, such as moisture (drying the sample at  $105\text{ }^{\circ}\text{C}$  for 6–8 h), fat, protein and salt (Careri, Spagnoli, Panari, Zannoni, & Barbieri, 1996). A brief description of the data set is shown in Table 1. After calibration, a periodic external validation was carried out which evaluates parameters obtained by the wet chemistry analysis and FoodScan for 20 samples (data not

**Table 1**

FoodScan parameters for calibration and validation procedures of moisture, fat, protein and salt content.

Parameter	Moisture	Fat	Protein	Salt
<i>Calibration</i>				
Number of samples	197	165	237	812
Mean	34.1	29.2	31.8	1.31
SD <sup>a</sup>	4.2	2.4	2	0.44
Calibration range	21.3–46.8	21.9–36.5	25.53–38.0	0.00–2.64
<i>Internal validation</i>				
SEC <sup>b</sup>	0.3323	0.3686	0.4640	0.0665
R <sup>2</sup>	0.9939	0.977	0.9501	0.9776
SECV <sup>c</sup>	0.3531	0.3958	0.4613	0.0697

<sup>a</sup> Standard deviation.

<sup>b</sup> Standard error of calibration.

<sup>c</sup> Standard error during cross validation.

shown). In all cases no significant statistical differences between both methods were observed.

### 2.3. Instrumentation and spectral acquisition

Spectra were acquired using a Tensor 27 FTIR spectrometer system (Bruker Optics, Milan, Italy), fitted with a Rocksolid interferometer and a DigiTect detector system, and coupled to an ATR accessory. Two ATR accessories were tested for the purpose of verifying the intensity of the absorption and the reproducibility of the measurements: an ATR for analysis of solid food matrices (MIRacle accessory, Pike Technologies, Madison, WI, USA) coupled to a MIRacle high-pressure clamp, and an ATR accessory for the analysis of liquid matrices (Specac Inc., Woodstock, GA, USA) with a larger surface of contact and path length. The latter was adapted for the analysis of solid samples by using an ad-hoc clamp and a Teflon form to allow good contact of the sample against the crystal. Both ATR accessories were equipped with a ZnSe reflection crystal. Analyses were carried out at room temperature, and spectra were acquired (32 scans per sample or background) in the range of  $4000\text{--}700\text{ cm}^{-1}$  at a resolution of  $4\text{ cm}^{-1}$ , using OPUS r. 6.0 software (Bruker Optics).

### 2.4. Sample treatment and data acquisition

Prior to analysis, each sealed box containing grated cheese was shaken vigorously. Samples were treated differently depending on the ATR accessory used. For ATR accessories destined for liquid sample analysis, about 1.5–2 g of grated cheese was spread with a spatula across the cell. The cheese was worked gently with the spatula to ensure complete coverage. The cell was then closed with a Teflon form while exerting pressure by the ad-hoc clamp to allow complete contact of the grated cheese against the crystal. Three spectra were recorded for each portion.

For the ATR accessories destined for solid sample analysis, three aliquots of grated cheese (10 g) were taken from the bottom, the centre and the surface of the box, and 0.5–1 g from each aliquot was deposited on the ATR surface and a pressure clamp was applied to obtain a thin cheese layer. Each aliquot was measured in triplicate. In both the cases, the absorbance spectrum was collected against a background obtained with a dry and empty ATR cell. Before acquiring each spectrum, the ATR crystal was cleaned with a cellulose tissue soaked in n-hexane and then rinsed with acetone.

### 2.5. Chemometric techniques

LDA is a probabilistic classification technique that searches for directions (canonical variables) with maximum separation among

categories (Massart et al., 1998); the first canonical variable is the direction of maximum ratio between inter-class and intra-class variances. Classification of spectral data was carried out after applying a moving windows algorithm strategy for wavelength selection (Xiaobo, Jiewen, Povey, Holmes, & Hanpin, 2010) as a feature selection technique. This evaluates the performance of LDA classification on the basis of predictive ability in the cross-validation groups (internal prediction rate), and searches for the range of variables with the largest classification success. Performance of LDA classification was evaluated on internal prediction rate in the cross-validation procedure for each class.

A PCA is concerned with explaining the variance–covariance structure of a set of variables through linear combinations of these variables. The general objectives are data reduction and thus simplification of interpretation. PCA was carried out after applying the moving windows algorithm strategy for wavelength selection (Jolliffe, 2002) as a feature selection technique. Performance of PCA classification was evaluated on the basis of predictive ability in the cross-validation groups (internal prediction rate). All chemometric techniques were carried out using MATLAB 7.0 (The Mathworks, Natick, MA, USA, 2007).

### 2.6. Data processing

LDA and PCA were carried out on the spectral data. ATR-FTIR spectra were standardized by using mean centre pre-treatment to remove or minimise any unwanted baseline contribution. Data matrices were reduced by taking a minimal number of spectral point that allowed effective discrimination; only one of ten spectral points was used for the calculations (reducing the calculation time). To apply classification methods, grated cheese samples were divided into four classes: class 1 (Parmigiano-Reggiano), class 2

(Italian non-PDO), class 3 (Czech non-PDO) and class 4 (Lithuanian non-PDO).

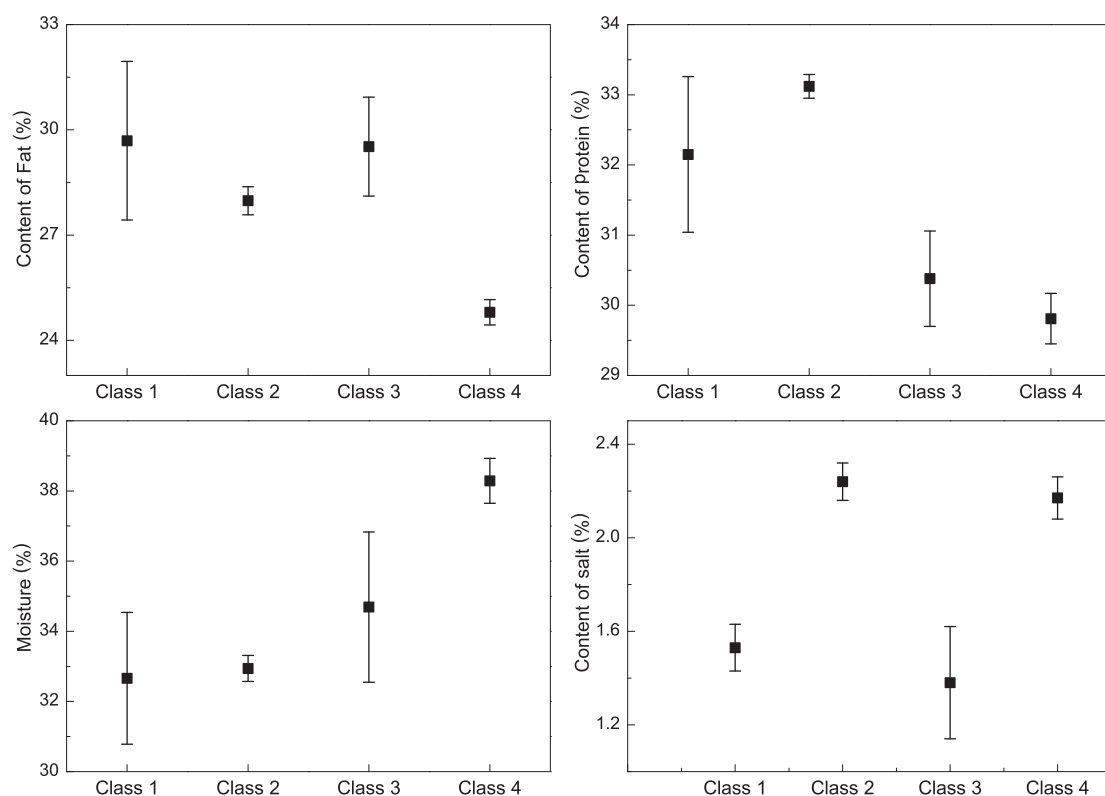
## 3. Results and discussion

### 3.1. Grated cheeses composition

The content of fat, protein, moisture and salt was determined by near infrared transmission in the range of 1050–850  $\text{cm}^{-1}$ , and the composition is reported in Fig. 1. The Parmigiano-Reggiano grated cheeses were produced by the approved method of the CFPR that is restricted to grated cheese having the technical and technological parameters as reported in the regulation (Council Regulation, 1992). Nevertheless, the information about the content of fat, protein, moisture and salt was insufficient to verify the affiliation of each grated cheese sample to their group, and to discriminate the Parmigiano-Reggiano from the others grated cheese groups.

### 3.2. Choice of the ATR accessory

To assess analytical performance, two ATR accessories were tested. This phase was necessary to evaluate which of the two better met the requirements of repeatability, and was therefore able to best represent the sample. The Pike MIRacle accessory coupled to the clamp permits analysis of a small and constant amount of solid food matrix on which a uniform force is exerted, so that the sample adheres to the crystal as a thin film. On the other hand, the Specac accessory was conceived for analysis of liquid food matrices, and therefore has no clamp to distribute the sample as the liquid spreads itself on the crystal, although it is possible to measure higher quantities of sample due to the larger surface of the crystal.



**Fig. 1.** Mean values (square symbols) and standard deviation (bars) of fat, protein, moisture and salt content of the grated cheeses: class 1 (Parmigiano-Reggiano), class 2 (Italian non-PDO), class 3 (Czech non-PDO) and class 4 (Lithuanian non-PDO).

Fig. 2 shows a typical ATR-FTIR spectrum and the estimated background noise of a grated cheese sample, acquired with the two ATR accessories. For this procedure a random sample of Parmigiano-Reggiano was measured ten times using each ATR. The mean spectrum and the standard deviation of absorbance were calculated at each wavelength.

The mean spectra acquired with the ATR accessory for solid food matrices generally showed a lower absorbance than the ATR accessory for liquid food matrices, which was higher due to the larger surface of the crystal, however, the estimated background noise was always lower for the ATR accessory for solid food matrices, probably due to homogeneous pressure applied by the clamp on a small surface. Therefore, considering the decreased inter-sample variation as the most robust classification model, for analysis of grated cheese samples, the Pike MIRacle accessory coupled to the high-pressure clamp was adopted.

### 3.3. FTIR data and chemometric analysis

FTIR spectra were dominated by several typical peaks. Absorbance in different wavenumber ranges could be assigned to the following contributions: –O–H stretching in hydroxyl groups (3700–3000  $\text{cm}^{-1}$ ), –C–H stretching in fatty acids (3000–2800  $\text{cm}^{-1}$ ), –C=O of acids and esters (1750–1650  $\text{cm}^{-1}$ ), amide I and amide II of proteins (1650–1450  $\text{cm}^{-1}$ ), esters and aliphatic chains of fatty acids (1460–1150  $\text{cm}^{-1}$ ) and C=O and C–C stretching of acids (1200–800  $\text{cm}^{-1}$ ) (Karoui et al., 2005; Lerma-García et al., 2010). Additionally, the bands between 1450 and 1410  $\text{cm}^{-1}$  include absorbance from acidic amino acids, such as glutamic acid, and the aliphatic chains of fatty acids (Subramanian et al., 2011).

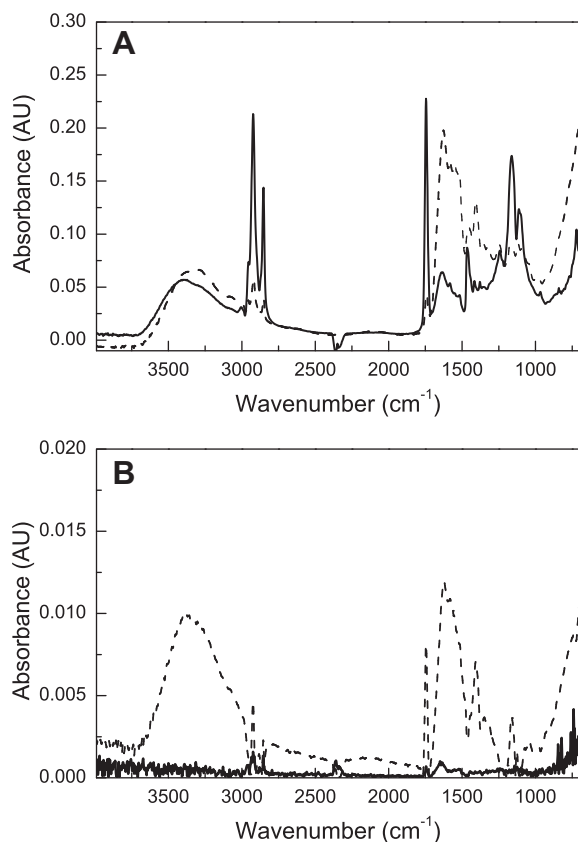


Fig. 2. Mean IR spectra overlay ( $N = 10$ ) of Parmigiano-Reggiano grated cheese (A) and instrumental background noise (B) using an ATR accessory for solid (—) and liquid samples (---).

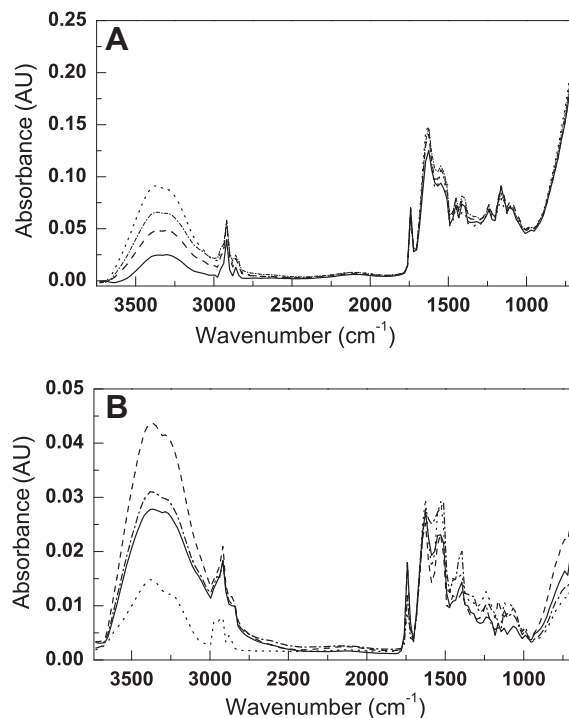


Fig. 3. Mean FTIR spectra using the ATR accessory for solids (A), and intra-class absorbance variation (B) of four grated cheese samples: Parmigiano-Reggiano,  $N = 21$  (—); Italian non-PDO,  $N = 5$  (---); Czech non-PDO,  $N = 5$  (···) and Lithuanian non-PDO,  $N = 5$  (-·-·).

Monitoring multiple functional groups simultaneously offers advantages over other spectral methods, thus favouring its use for rapid analysis by spectroscopy. However, as shown in Fig. 3A, the spectral data of the four classes of cheese, Parmigiano-Reggiano, Italian non-PDO, Czech non-PDO and Lithuanian non-PDO, did not reveal obvious differences from visual inspection according to the origin of cheese. Additionally, the regions with higher differences among classes also had higher variations within the same class, as can be seen in Fig. 3B.

A chemometric method, namely the PCA model, was applied to detect differences between samples (Table 2), although classification procedures were not acceptable for analytical purposes. The best results were obtained in the full spectral range for class 1 (Parmigiano-Reggiano) with a correct classification of 68%. Class 2 (Italian non-PDO) was the worst class, achieving a prediction rate of 48%, thus demonstrating that PCA was not able to group these grated cheeses according to origin.

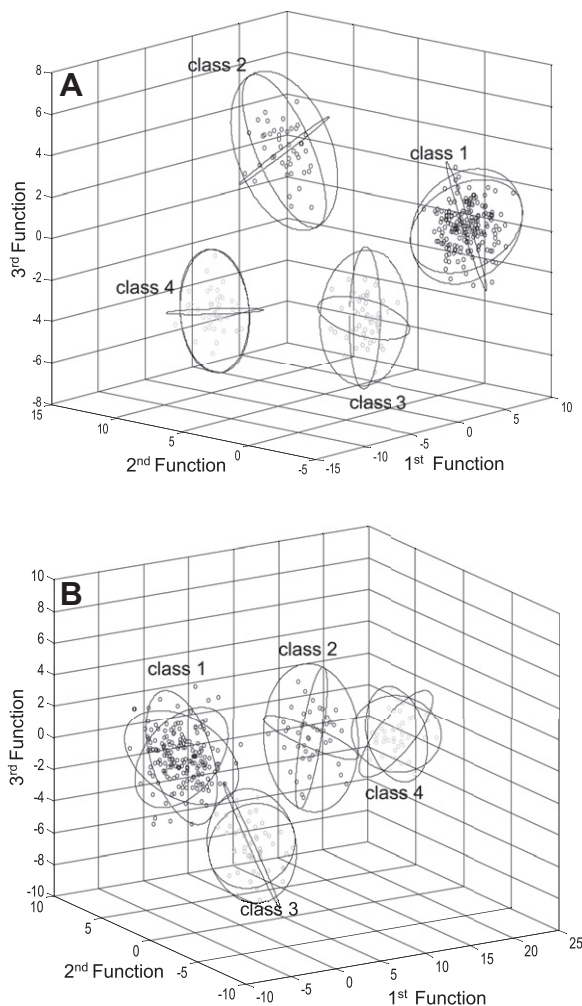
Table 2 and Fig. 4 show the results obtained by LDA classification in the calibration and cross-validation procedures. The moving windows algorithm strategy for wavelength selection was applied in order to obtain the best spectral range for the classification. This

Table 2

Results of classification for principal component analysis (PCA) and linear discriminant analysis (LDA) and predictive ability in cross-validation procedures.

Class	Classification ability (%)		Prediction ability (%)	
	LDA	PCA	LDA	PCA
Class 1	100	68	99	68
Class 2	100	48	100	48
Class 3	100	59	100	61
Class 4	100	60	100	60
Means	100.0	59.1	99.9	59.6





**Fig. 4.** Three-dimensional score plots of discriminant analysis for (A) calibration and (B) cross-validation determined by discriminant factors 1, 2 and 3 for mid-infrared spectra (3361.7–1317.3  $\text{cm}^{-1}$ ) for grated cheeses with four different origins: class 1 (Parmigiano-Reggiano), class 2 (Italian non-PDO), class 3 (Czech non-PDO) and class 4 (Lithuanian non-PDO).

strategy evaluated the performance of LDA classification by a cross-validation procedure along all spectral range, varying systematically the initial wavelength and width of the window. The best results were obtained in the 3361.7–1317.3  $\text{cm}^{-1}$  spectral range. The percentages of correct classification and prediction were 100% and 99%, respectively. In this case, the worst in terms of classification was class 1 (Parmigiano-Reggiano) with a correct prediction rate of 99%, also demonstrating that the LDA results are in agreement with the origin of grated cheese samples.

#### 4. Conclusions

In this investigation, a total of 36 grated cheese samples were discriminated according to origin by ATR-FTIR. Two ATR accessories were used to evaluate reproducibility of FTIR spectra obtained from non-homogenous samples. The ATR accessory for solid food matrices was chosen to assess minor variations in inter-sample classes. Spectral data was analysed using two different chemometric approaches: PCA and LDA. The LDA method was successfully applied to spectral data after used the moving windows algorithm strategy for wavelength selection. LDA-FTIR analysis had a predictive classification ability approaching 100% during the cross-validation procedure whereas PCA could not successfully classify

the different types of cheese. The results showed that LDA-FTIR is a promising technique that could potentially be a reliable and fast classification tool for PDO grated cheeses. Lastly, such a rapid analysis of grated cheese may provide information on overall differences between hard cheeses, which could complement results from other analytical identification and quantification methods.

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