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DETERMINING THE AUTHENTICITY OF PDO BUFFALO MOZZARELLA: AN APPROACH BASED ON FOURIER TRANSFORM INFRARED (MIR-FTIR) SPECTROSCOPY AND ON CHEMOMETRIC TOOLS

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***Abstract** – The potential of Mid-infrared spectroscopy coupled with chemometric tools was evaluated for the authentication and discrimination of PDO (Protected Denomination of Origin) buffalo mozzarella produced by traditional and industrial cheese-making processes.*

Samples of mozzarella provided by local producers and supermarkets were analysed through both official destructive methods and Attenuated Total Reflectance-Fourier transform infrared spectroscopy (FTIR/ATR). In particular, destructive methods allowed to determine the content of fatty substances, proteins, moisture and total nitrogen.

The results show that only the conjunction of MID-infrared spectroscopy with chemometric analysis can provide a satisfying solution to discriminate between the different types of mozzarella.

***Keywords**:* Attenuated total reflectance infrared spectroscopy, PDO Buffalo mozzarella, Italian cheese, food authenticity, cheese-making processes.

I. INTRODUCTION

Food authenticity assessment is an arduous challenge for food research. In this context, European Union's schemes for geographical indications and traditional specialties are becoming increasingly important. Protected Designation of Origin (PDO) is a quality control system created to protect the integrity of food and drinks produced in a specific area using a unique method. This label refers to the region and production area of a specific cheese, whose characteristics are strictly related to the geographical area in which the product is produced. All PDO products are subject to a set of traditional codified rules within the framework of a detailed Production Specification provided by the European Commission[1,2,3].

This procedure establishes the product's characteristics, ranging from production techniques to labeling and marketing rules. The EU countries that follow such a system are mostly from Southern Europe. In Italy there are 47 PDO and 1 PGI (Protected Geographical Indication) cheeses and dairy products, which makes it the second world producer of PDO cheese.

The PDO cheeses have specific characteristics and enhanced quality and are defined according to their geographical area of production, as well as in terms of the material and technology used in their manufacturing. Certainly, the determination of origin is a key component for PDO.

New tools and strategies need to be developed to assess the authenticity of these high-quality products, protecting consumers against cheaper industrial imitations. Moreover, regulatory agencies are concerned with the prevention of economic fraud, while food processor needs confirmation of added-value claims showed on the product label (i.e. food ingredient authenticity, geographic origin, processing history etc.) in order to protect a brand [4,5].

One of the world's most popular Italian products with Protected Designation of Origin is the buffalo mozzarella, a stretched curd cheese exclusively made with buffalo milk from Campania and produced using a process that meets the PDO specification. Buffalo milk's composition is different from cow's or sheep's milk, in that it is rich in protein, fat and calcium. The peculiarity of this cheese is entirely due to the technology used in its traditional preparation. Furthermore, since it does not contain carotenoids, the mozzarella is very bright, almost a porcelain white colour.

Many factors such as composition of the milk, moisture, starter cultures, manufacturing process, and biochemical events occurring during maturation affect the quality of mozzarella. There are sensible differences between traditional and industrial buffalo cheese-making processes, and the

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market prices of the two final products reflect such differences.

Thus, a considerable interest is increasing in the development of instrumental techniques to enable faster, more objective and less expensive assessment to define and control the qualitative characteristic of typical cheeses. That is done in order to secure the consumer's choice, protect traditional products against cheaper industrial imitations, and limit the phenomenon of *Italian sounding*. The methods used for the verification of food authenticity and traceability have to be suitable for the prevention of deliberate or accidental mislabeling.

They are important for commercial reasons and play a considerable role in the assurance of public health.

Rapid, inexpensive and reliable analytical methods are required to measure food characteristics for authenticity and traceability purposes, and to link a finished product to its ingredients. These analytical techniques have to be applied to the food manufacturing industry for process performance evaluation, detection of faults, and achievement of persistently high-quality food product [4].

Infrared spectroscopy is an attractive technology for rapid, inexpensive, sensitive, and high-throughput analysis of food components which does not require special skills by the users.

In these last years, Infrared spectroscopy has been applied for the authentication of products by commodity, variety and geographical origin. The potential of IR spectroscopy has been shown in monitoring the geographic origin of cheeses [6,7] and for discriminating them according to the nature of the milk used in the cheese-making process [8,9]. Specifically, mid-infrared spectroscopy (4000- 700 cm^{-1}) has been used not only to monitor the geographic origin, but also to determine the major components in cheese, such as protein, fat, moisture, lactic acid, sugars, casein and cholesterol[10,11], each with a distinct and reproducible biochemical fingerprint.

The objectives of the present research are the following:

- developing a simple and rapid screening tool for the definition of the qualitative characteristics of Italian buffalo-cheese by means of Fourier transform infrared spectroscopy (FTIR);
- discriminating between traditional and large scale industrial cheese-making processes by means of a statistical model working on measurements of the absorbance at different wavelengths.

II. MATERIALS AND METHODS

Samples

A total of twenty samples of PDO Italian buffalo mozzarella were purchased from authorized manufacturers and supermarkets, and categorized into two groups according to their cheese-making process.

In particular, ten samples of traditional cheese-making mozzarella and ten samples of industrial cheese-making mozzarella were collected in different production dates and analysed on the same day of purchase.

Two sizes of Italian buffalo cheese were analysed: 125 gr and 250 gr. A part of each sample was used for pH determination and FTIR/ATR Spectroscopy measurements, whereas the remainder was homogenized to define the content of fatty substances, the level of moisture, lactic acid, total nitrogen and proteins.

Physico-chemical analysis

The percentage compositions of moisture, fat, and protein were determined using the reference methods. The fat content was determined via Gerber reference method using Soxtec system, the moisture content via vacuum-oven method, and the protein content via Kjeldahl method [12,13]. All samples were tested in duplicate.

FTIR/ATR ANALYSIS

The PDO buffalo mozzarella samples were analysed using Fourier Transform Infrared spectrometer (Spectrum1000 PerkinElmer), mounted with an ATR accessory equipped with a grip.

Thin sections of samples were taken from the centre of the mozzarella and pressed through the high-pressure clamp to ensure good contact between the sample and the crystal. Infrared spectra were recorded in the 3000-900 cm^{-1} region, and 64 scans were conducted for each spectrum at 4cm^{-1} resolution in order to improve the signal to noise ratio. The regions of the mid-infrared spectra located between 3000-2800 cm^{-1} (fat region), 1700-1500 cm^{-1} (protein region) and 1500-900 cm^{-1} (fingerprint region) have been considered in this study [14].

STATISTICAL AND CHEMOMETRIC ANALYSIS

The data analysed concern the values of absorbance registered at different wavelengths for the two categories of PDO buffalo mozzarella. The data are intended to reflect the spectral range of 3000–1000, which is the typical range of measurements suggested by literature [10,15,16,17]. In particular, such a spectral range was divided into three regions : a) 3000–2800; b) 2800–1700; d) 1700–1000.

To discriminate between these two types of mozzarella given the values of absorbance and the wavelengths at which such values were estimated, a logistic regression model[18] was exploited.

III. RESULTS AND DISCUSSION

FTIR technique was applied to identify the main types of structure present in the analyzed samples, through the correct association between absorption bands and fundamental vibrations of functional groups of molecule. The intensities of the different bands are correlated with the concentration of the chemical functional groups. Figure 1 shows the mid infrared spectra of traditional and industrial PDO buffalo mozzarella between 3000 cm^{-1} and 900 cm^{-1}.

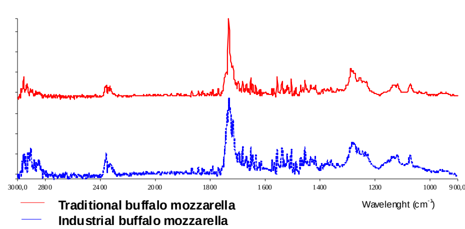

Fig. 1: Mid Infrared spectra of traditional (red solid line), and industrial (blue dotted line) PDP buffalo mozzarella between 3000 cm^{-1} and 900 cm^{-1}.

Three spectral regions were identified to be the most important to assess the quality attributes of cheese:

- 3000-2800 cm^{-1} region corresponding to lipids,
- 1700-1500 cm^{-1} corresponding to amide I and II bands,
- 1500-900 cm^{-1} corresponding to carbohydrates region.

It is worth noting that the spectral values of 1700-700 cm^{-1} are considered to be "fingerprint" values, specific fingerprint of each compound [17,19,20].

From literature, it is known that the area has a coverage between 800-1200 and 1000-1500 cm^{-1} [15,21] but, this study has extended it to 1700 cm^{-1}. The region between 3000-2800 cm^{-1} consists of absorbance from C-H stretching vibrations of $–CH_3$ and $>CH_2$ functional groups of fatty acids [16]. Specifically, the intervals 2900-2827 cm^{-1} and 1782 to 1705 cm^{-1} correspond to fat content in cheese. Figure 2 shows two major bands corresponding to methylene/methyl groups, that were observed at λ 2963 and at λ 2955 cm^{-1}. The observed changes in methyl and methylene bands were attributed to difference in nature concentration and physical state of fatty acids [14].

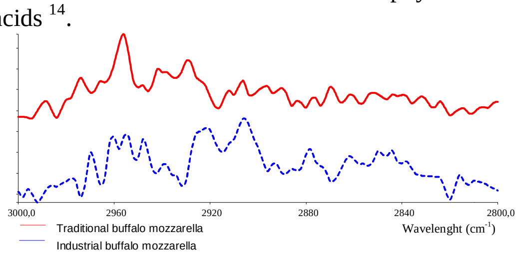

Fig. 2: Spectra of traditional buffalo mozzarella (red solid line), and industrial buffalo mozzarella (blue dotted line) in the region between 3000 and 2800 cm^{-1}

The spectral region between 1701-1507 cm^{-1} corresponds to protein content. Figure 3 shows spectra of traditional buffalo cheese (solid line), and industrial buffalo cheese (dotted line) in the region between 1850 and 1500 cm^{-1}. Two well-defined peaks were observed at 1715 and 1730 cm^{-1} associated with esters and organic acids. Moreover, the solid line in figure 3 shows many other picks, two of which are associated with the Amide I at ~ 1651 cm^{-1} , and Amide II at ~ 1556 cm^{-1} [10,22]. According to Bertrand and Dufour[16], these two peaks are probably associated with hydrolysed proteins. Changes in intensity and position of these bands in the 1700-1500 cm^{-1} range were associated with changes in casein secondary structure, protein aggregation and protein-water interaction [16,17,23,25].

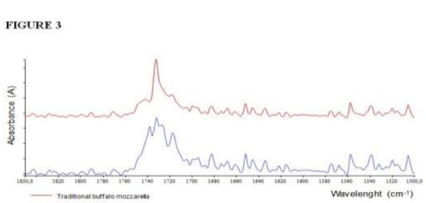

Fig. 3: Spectra of traditional buffalo mozzarella (red solid line), and industrial buffalo mozzarella (blue dotted line) in the region between 1850 and 1500 cm-1.

In the third region of FTIR spectra (1500-900 cm^{-1}), wavelengths for specific chemical groups were recognised: 1477-1400 and 1195-1129 cm^{-1} for ester carbonyl C-H and C-O group [26]. The solid line in figure 4 depicts more peaks in the range between 1477 and 1400 cm^{-1}, but we can find in both spectra (solid line and dotted line) peaks at λ 1420, 1435, 1470 cm^{-1}, in spite of a different absorbance. The highest peak was observed at ~ 1289 cm^{-1}.

In both spectra were observed two peaks: at ~ 1073 cm^{-1} and at ~ 1120 cm^{-1}, probably related to sum of lactose, monosaccharide [24,27] and the ester linkage of lipids [28,29]. No well-defined peaks were attributed to glucose and galactose - which may be located at ~ 1377 cm^{-1}, according to Othman *et al.* [23].

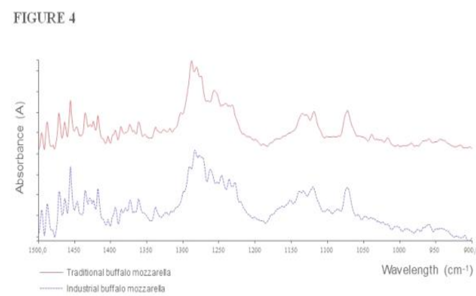

Fig. 4: Spectra of traditional buffalo cheese (red solid line), and industrial buffalo cheese (blue dotted line) in the region between 1500 and 900 cm^{-1}.

Throughout the MIR region (3000-900 cm^{-1}), and specifically in the intervals 2900-2827 and 1782-1705 cm^{-1} (corresponding to fat content in cheese), in the range between 1701 and 1507 cm^{-1} (corresponding to protein content), and finally in that from 1200 to 967 cm^{-1} (carbohydrates), the "traditional" buffalo cheese samples showed higher absorbance, corroborating the chemical and physical results according to which these samples possess a higher content of protein and fat respect those obtained through industrial buffalo cheese-making processes. Considering that the absorbance intensity of fat-and-protein-related bands increased as a consequence of an increase in fat and protein contents, these findings highlight the importance of discriminating among different regions across the entire spectral range to predict the quality attributes of buffalo mozzarella.

The absorbance areas of fat related and protein-related peaks for different cheese samples were compared with the fat and protein contents by physico-chemical analysis. Absorbance of fat-related bands decreased with a reduction of fat level in cheese as expected. The change due to protein related bands was opposite to that due to fat-related bands. Results were in agreement with proximate analysis, since a reduction in fat constituted a slight increase in protein content [22,30].

Although the results obtained by physico-chemical analysis were in accordance with the FTIR spectroscopic findings, they did not show substantial differences between the two cheese-making processes (Table 1) and did not allow to discriminate between the two types of mozzarella.

TABLE 1. MEAN (±SD) OF THE PHYSICOCHEMICAL CHARACTERISTICS OF TRADITIONAL AND INDUSTRIAL BUFFALO-CHEESE MAKING PROCESSES.

Parameters	***Traditional buffalo mozzarella***	***Industrial buffalo mozzarella***
pH	5,30 (±0,09)	5,22 (±0,08)
Lactic Acid %	0,47 (±0,02)	0,48 (±0,03)
Fat%	23,2 (±0,82)	22,6 (±0,57)
Protein%	16,2 (±0,38)	12,5 (±0,32)

However, it seems possible to check any variations in buffalo cheese samples analysing and comparing the absorbance values registered at the different wavelength by FTIR spectroscopy. This tool could be also used to implement the traceability of products (especially when they have to be marketed abroad) and identify substances (such as soda and bleaching) commonly used in phenomena like "Italian sounding", therefore protecting Italian products and consumer choices.

The main issue of FTIR spectroscopy application in buffalo mozzarella analysis is related to the homogeneity of samples. Although every effort was made to obtain uniform samples, the mozzarella samples used in FTIR experiments were not homogeneous: a probable reason could be the non-homogeneity of fat and bound moisture in the protein matrix, and the presence of voids in the cheese matrix. Even though several pieces from each cheese slab were analysed and results averaged in order to overcome this problem, only the conjunction of MID-infrared spectroscopy with chemometric tools can provide satisfying results for the intended purpose.
Figure 5 depicts a bivariate density of wavelength (λ) and absorbance (A) estimated from the sample for four ranges: a) 3000–2800 cm^{-1}; b) 2800–1700 cm^{-1}; c) 1700–1000 cm^{-1}.

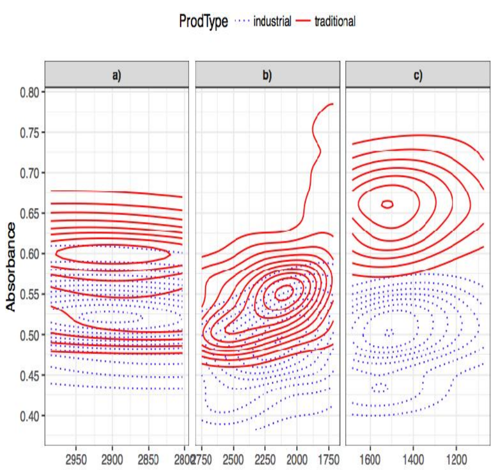

Fig.5: Bivariate density of wavelength and absorbance for the ranges of measurement considered.

The figure shows that the absorbance in traditional products tends to be higher than in industrial products for a given wavelength. The figure also confirms that in the fingerprint region there is a clear discrimination between types of products [17,19,20]. For greater values of λ, the overlap of densities becomes more and more problematic. In particular, region a) is the one with the highest overlap.
In order to discriminate between the two types of buffalo mozzarella, a logistic regression[18] was exploited. Through such a model, it is possible to predict the probability that a unit belongs to a given category as a function of some explanatory variables. For the current study, the chosen category was the traditional DOP type of mozzarella, whereas the explanatory variables were absorbance and wavelength. Introducing also a random component accounting for the specific mozzarella on which each measurement was performed did not seem to alter the model substantially, and therefore all the measurements were finally treated as independent. In particular, the probability $\Pr(.)$ of a traditional DOP product was modelled as a function of the absorbance and the wavelength using the following specification:

$$\Pr(t \mid \mathrm{A}, \lambda) \frac{1}{1+\exp\left[-(\beta_0+\beta_1 \mathrm{A}^4+\beta_2\lambda^5+\beta_3\lambda^6)\right]}$$

where t indicates the traditional category, **A** the absorbance, λ the wavelength, and β_i $(i = 1,2,3,4)$ are the model coefficients to be estimated.

This model seems to be able to fit the data properly in the spectral ranges considered, and account for the nonlinear patterns. This comes with almost no loss in interpretability. Table 2 shows the model summary, where all the coefficients are highly significant.

TABLE 2: SUMMARY OF THE LOGISTIC REGRESSION

Coefficient	Estimate	Std. Error	z value	Pr (>|z|)
β_0	-1.05×10^{1}	8.13×10^{-1}	−12.86	<0.001
β_1	8.40×10^{1}	6.30×10^{0}	13.32	<0.001
β_2	3.22×10^{-16}	3.34×10^{-17}	9.65	<0.001
β_3	-1.06×10^{-19}	1.27×10^{-20}	−9.66	<0.001

The absorbance has a remarkable positive impact on t: the higher the absorbance, the higher the probability that the cheese is traditional.

The effect of λ is estimated through $\hat{\beta}_2$ and $\hat{\beta}_3$. When the absorbance is kept at the mean value in the dataset (i.e. ~0.55), the behaviour of the wavelength against the odds of traditional product is the one depicted in Figure 6: the marginal effect of λ increases from 3000 cm^{-1} to 2528 cm^{-1} and decreases from 2528 cm^{-1} to 1000 cm^{-1}, being negative before 2890 cm^{-1} and after 1912 cm^{-1}.

This would be noticeable in Figure 5 if one drew a horizontal line at the average value of absorbance: such a line would clearly cross the density of the traditional category on the left side of panel b), and cross the density of the industrial category in panels c), i.e. in the fingerprint area.

Figure 7 shows the scatterplot of absorbance against λ, with the predicted boundary separating the two categories of product. Such a boundary corresponds to a fitted probability of 50%. The figure shows that the misclassification error decreases to 0 when moving to the fingerprint area.

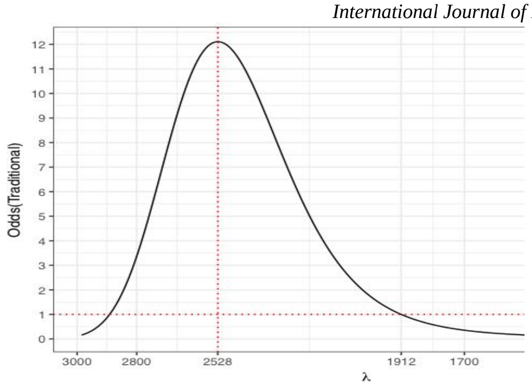

Fig 6 : Odds of a traditional product as a function of the wavelength (absorbance is kept at its mean value)

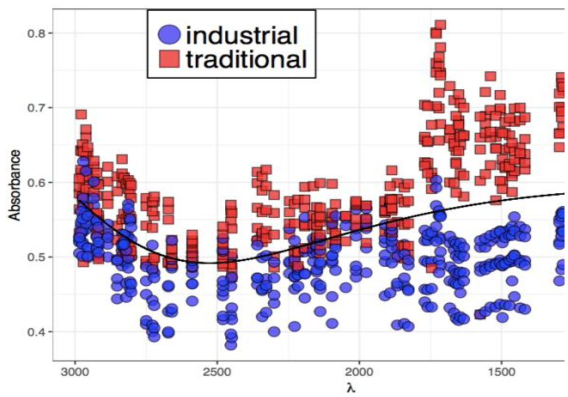

Fig.7: Scatterplot of absorbance vs wavelength with fitted decision boundary

IV. CONCLUSION

Results from this study showed that industrial technology has a large impact on Italian buffalo mozzarella quality, significantly affecting the texture attributes of cheeses, despite similar characteristics in composition. FT-IR spectroscopy could contribute to the development of simple and rapid protocols for monitoring complex biochemical changes, and predicting the final quality of cheese. Variation in absorbance intensity of fat and protein-related bands changed greatly due to variation in cheese-making procedure. However, the main difficulty in the application of FTIR spectroscopy to buffalo mozzarella analysis was related to the homogeneity of samples. Although several pieces form each cheese slab were analysed and results averaged in order to overcome this problem, only the conjunction of MID-infrared spectroscopy with chemometric analysis can provide an exceptional tool to confirm cheese quality and classify products according to their manufacturing process. This could also represent an opportunity to implement the dietary product quality traceability system, and contrast the adulteration, the sophistication, and other phenomena of "Italian sounding". The worthy intent is to protect both the local economy and the consumer choices.

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