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Analytical Methods

Detection of cow milk in cooked buffalo Mozzarella used as Pizza topping

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Abstract

One of the most popular worldwide meals is Pizza. Among the main ingredients, buffalo or cow Mozzarella are the cheeses most widely used. Different prices between buffalo and cow Mozzarella can stimulate frauds but it is unknown whether the European official method can differentiate Mozzarella from cow or buffalo milk after oven cooking when used as a Pizza topping. Preliminary experiments with Pizza dough and Mozzarella cheeses, followed by analysis of pure buffalo, pure cow and mixed Mozzarella separated from Pizza after cooking confirmed the reliability of the official method and the possibility to quantify low amounts of cow milk. The results of a survey on more than 50 Pizzas declaring only buffalo Mozzarella as cheese ingredient, showed the fraudulent use of mixed Mozzarella. This study showed that the official method is an effective tool to demonstrate this kind of fraud, protecting consumers against improper practices and guaranteeing fair trade.

Keywords: Buffalo Mozzarella; Pizza; Frauds; Gamma-casein; Plasmin

1. Introduction

One of the most popular worldwide meals is Pizza. Basically, Pizza is composed of a yeast–flour dough covered by tomato, cheese and oil, with the addition of other minor ingredients such as basil or oregano. Among the cheeses used as ingredients, pasta filata cheeses are the most widely added. Historically, Pizza has its origins in Southern Italy where buffalo or cow Mozzarella cheeses came from and are still widely diffused, so, in Italy, Mozzarella is an essential ingredient. In fact, the use of Mozzarella Bufala Campana Protected Designation of Origin (PDO) together with the use of San Marzano tomato variety and extra virgin olive oil, are necessary to be protected by European Regulation 2082/1992 (Council Regulation, 1992) with the recognition of "Pizza Napoletana Margherita extra", according to the proposal edited in the Official Journal of Italian Republic (Ministero Politiche Agricole Forestali, 2004). The cost of Buffalo Mozzarella is usually 20–50% higher than cow Mozzarella and this difference can stimulate the fraudulent use of cow or mixed Mozzarella. Many methods and reports involve the detection of cow casein in buffalo Mozzarella. The European official method (CE Regulation 213/2001, OJEC, 2001) detects the presence of cow milk in buffalo Mozzarella on the basis of the different electrophoretic mobility of cow and buffalo γ2 and γ3 caseins, originated by controlled plasmin hydrolysis of caseinate, extracted from the cheese (Addeo et al., 1990). Other methods for the detection of cow milk in buffalo Mozzarella are based on different milk components. The different composition of whey proteins (Cozzolino, Passalacqua, Salemi, & Garozzo, 2002; Pellegrino, De Noni, Tirelli, & Resmini, 1991) may be detectable by HPLC analysis; the different

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composition of triacylglycerols (Andreotti, Trivellone, Lamanna, Di Luccia, & Motta, 2000) may be easily detected by NMR analysis; the different patterns of cow and buffalo mytocondrial DNA from somatic cells is used to identify the cow milk addition by means of PCR analysis (Bottero, Civera, Anastasio, Turi, & Rosati, 2002; Lopparelli, Cardazzo, Balzan, Giaccone, & Novelli, 2007).

Despite of the abundance of reliable and sensitive methods to establish the purity of buffalo Mozzarella, yet no data are known about cooked Mozzarella used as a Pizza topping after oven cooking.

It is well-known that heat damages the protein structure by cross-linking, polymerisation or glycosylation reactions (Pellegrino, Resmini, De Noni, & Masotti, 1996; Pellegrino, Van Boekel, Gruppen, Resmini, & Pagani, 1999; Scaloni et al., 2002).

Until now, no information is available, if these modifications occur and how the varying heating conditions may impact the reliability of the methods for the detection of cow's milk.

According to the cited standard of production proposed for Pizza Napoletana, the temperature of Mozzarella should be 65–70 °C. This temperature is the result given by cooking Pizza using the following conditions: the thickness of the dough disk at its centre is not more than 0.3 cm, the cooking surface temperature of the oven is 485 °C, the oven dome temperature is 430 °C and the cooking time does not exceed 90 s. Such short cooking times prevent Mozzarella from completely dehydrating or forming a crust blocking vapour separation, conditions that allow Mozzarella to overcome the temperature of 100 °C. Changing one or more parameters, particularly the thickness of the dough and consequently the cooking time, the temperature of Mozzarella can rise as demonstrated by its browning due to Maillard reactions.

Scaloni et al. (2002) observed that lactosylation occurred specifically at Lys-107 in β casein even in moderately heat-treated milks. Because plasmin operated hydrolysis of peptide bond 107–108 of β casein generates γ3 casein, it is not known whether casein structure modifications due to Pizza oven cooking may affect the plasmin activity.

Finally, we do not know whether the other Pizza ingredients interfere with the plasmin activity and/or the electrophoretic detection of γ2 and γ3 caseins.

The aims of the study were to evaluate the ability of the European official method to detect the presence of cow milk in buffalo Mozzarella when oven cooked and to verify the extent of the fraud using cow Mozzarella instead of buffalo Mozzarella as a Pizza topping.

2. Material and methods

2.1. Modification of the official method for its application to the determination of cow casein in buffalo Mozzarella cheese from Pizza

The official method (OJEC, 2001), based on the different electrophoretic mobility of cow and buffalo γ2 and γ3 caseins, was used to determine the presence of cow casein in buffalo Mozzarella cheese. Some modifications were applied to the sample procedure and to the system of cow milk quantification, not established by the method.

2.1.1. Sample preparation

Pizza was cooled to 4 °C to allow easier separation of Mozzarella cheese from the other Pizza ingredients. All the cheese was separated and poured into a sieve where it was washed with tap water to eliminate the tomato sauce and the other minor ingredients used for the Pizza topping. An aliquot from the mass of the washed cheese was sampled for the analysis.

2.1.2. Estimation of the quantity of cow milk

The optical density of the protein bands separated by electrophoresis corresponding to cow and buffalo γ2 and γ3 casein were densitometrically measured using the GS-710 Calibrated Imaging Densitometer (Bio-Rad, Hercules, CA, USA) and the elaboration program Quantity One-41-1 (Bio-Rad).

The official method judges an unknown sample as added with cow milk on the basis of the comparison between the unknown sample and two standards, one of pure buffalo milk and the other obtained by adding 1% of cow milk to buffalo's milk. If both the areas of the γ2 and γ3 casein band or their ratio is equal to or higher than those of the 1% standard, the unknown sample is considered adulterated.

In Italy the official control laboratories also determine the amount of cow milk added to better evaluate the severity of the fraud. To perform this quantification, in a similar way to the one mentioned above, we also prepared other standards with 3%, 5%, 10%, 20%, 50%, 75% and 90% of cow milk. The values of the ratio among γ caseins of the different standards were used to make a calibration line, then used to calculate the amount of cow casein present in the unknown sample.

2.2. Cheese cooking trials for the evaluation of the method of detection of cow milk added to buffalo milk

To simulate Pizza production, wheat dough was purchased from a bakery and pressed by hand to create a pizza dough plate with a thickness of about 0.5 cm. This pizza dough plate was cut into disks with a radius of 5 cm. The disks were covered with a 0.5 cm slice of mixed Mozzarella (30% cow milk) and cooked in a ventilated electric muffle furnace at air temperature ranging from 375 to 450 °C for 1–2 min. To verify if heating can alter the performance of the method for the detection of *cow* milk, each sample of Mozzarella was analysed before and after the heat treatments. Each analysis was repeated five times.

To verify if the method can also detect low cow milk additions when Mozzarella is heated, tests were performed at air temperature of 425 °C for 2 min using mixed Mozzarella cheeses obtained by adding three different cow milk

quantities (about 5%, 10% and 30%). The measurements were repeated 11 times.

The mixed Mozzarella used for these trials, labelled as mixed cheese, were purchased from local dairies and furnished by Consorzio Tutela Formaggio Mozzarella Bufala Campana (San Nicola La Strada, Caserta, Italy).

2.3. Determination of the presence of cow casein in Mozzarella cheese from experimental Pizza

To verify the detection of cow milk in Mozzarella from oven cooked Pizza, three Pizza Napoletana were produced in a traditional Pizzeria at Caserta (Italy), under the supervision of the technicians of Consorzio. The ingredients of Pizza were: dough, tomatoes, extra virgin olive oil, basil, salt and cheese. Pure buffalo and cow Mozzarella, and Mozzarella deriving from the addition of about 40% of cow milk to buffalo milk were used. The cheeses were furnished to the Pizzeria directly by the technicians of Consorzio. The Pizza was cooked in a ventilated wood oven with air temperature of about 450 °C for 3 min. Both the fresh Mozzarella cheeses and the same cheese separated from the Pizza after cooking, were analysed. The analyses were made in duplicate and for the mixed Mozzarella were repeated 11 times.

2.4. Determination of the presence of cow casein in Mozzarella cheese from retail Pizza declared only with buffalo Mozzarella

To evaluate the conformity of Mozzarella cheese to what is declared on the label, a preliminary survey was made, sampling seven fresh Pizzas and seven frozen Pizzas produced by industrial bakeries. Seven samples of Pizza declaring only buffalo Mozzarella on the restaurant menu were purchased from seven Restaurants Pizzerias in Campania and Lodi. Frozen pre-cooked Pizzas, declaring the exclusive use of buffalo Mozzarella on the label, were purchased from food retails in Lodi and Campania. On the basis of the result of this first survey a second survey was made, sampling 50 Pizzas directly from 50 different Pizzeria Restaurants in Campania. All the samples were analysed in duplicate.

2.5. Statistical analysis

The significance of the difference between the results was statistically evaluated by analysis of variance (ANOVA) at one tailed significance level using Microsoft Office Excel 2003 (Microsoft Corporation, USA).

3. Results and discussion

3.1. Mozzarella cheese cooking trials at different air temperatures

The heat transmission during Pizza cooking is the result of different contributions, mainly radiating heat from the walls of the oven, conduction from the oven floor and convection. Cheese heating in particular is very complex and differentiated. The lower part of the cheese is heated by conduction from the tomato sauce and the baked dough, while the upper side is mostly heated by radiating energy and convection from the moist air in the oven and, when covered with oil, by conduction from the olive oil. To partially simulate this heat transmission Mozzarella cheese was put on a disk of dough before being heated in the ventilated muffle furnace.

The calculation of the ratio between cow and buffalo $\gamma 2$ and $\gamma 3$ casein from a sample of mixed Mozzarella cheese before and after oven heating, at air temperatures ranging from 375 to 450°C for 1–2 min, did not show significant differences (Table 1).

Three samples of Mozzarella produced with buffalo milk added with three decreasing amounts of cow milk were oven heated at an air temperature of 425 °C for 2 min and compared to raw samples (Table 2): heating did not affect the ability of the method to detect even the lowest amount of cow milk nor caused significant changes in the quantification of the cow milk added.

The presumable modifications of casein structure due to a further heat treatment, additional to curd stretching, did not seem to influence the plasmin ability to cleave the β casein peptidic bonds and release $\gamma 2$ and $\gamma 3$ casein. From these preliminary results we can conclude that even by heating Mozzarella in conditions similar to Pizza productions, the adulterated buffalo cheese can still be detected.

3.2. Determination of the presence of cow casein in Mozzarella cheese from experimental Pizza

To verify the results obtained with the model system, where only dough was used, three Pizzas were produced in a wood oven using pure buffalo, pure cow and mixed Mozzarella deriving from the addition of about 40% of cow milk to buffalo milk. The results of the analysis of fresh Mozzarella cheese and of the same Mozzarella after

Table 1
Evaluation of the ratio $\gamma 2 + \gamma 3$ cow casein to $\gamma 2 + \gamma 3$ buffalo casein in a sample of mixed Mozzarella cheese heated with different temperature and time conditions

Heating conditions		Heated Mozzarella	Fresh Mozzarella	*P* value
°C	min	Ratio $\gamma 2 + \gamma 3$ casein	Ratio $\gamma 2 + \gamma 3$ casein	
375	1.00	0.59	0.62	0.555
375	1.50	0.64	0.65	0.809
400	1.00	0.71	0.67	0.248
400	1.50	0.62	0.67	0.278
400	2.00	0.63	0.63	0.971
425	1.00	0.61	0.59	0.619
425	1.50	0.65	0.63	0.551
425	2.00	0.59	0.61	0.501
450	2.00	0.63	0.60	0.582

Each test was performed five times.

Table 2
Evaluation of % cow milk content of mixed Mozzarella cheese before and after heating at an air temperature of 425 °C for 2 min ($n = 11$)

% Cow milk added	% Cow milk found				*P* value
	Unheated		Heated		
	Mean	Standard deviation	Mean	Standard deviation	
5	2.9	0.5	3.1	0.9	0.538
10	7.8	1.9	8.1	1.7	0.711
30	34.7	2.9	34.4	4	0.830

separation from the other ingredients of the cooked Pizza did not show significant differences (Table 3). The cheeses obtained with pure buffalo or pure cow milk were recognized as 100% buffalo or cow, while the amount of cow milk in the mixed sample was measured at the same level before and after cooking. It can be concluded that the presence of other ingredients (tomatoes, basil, and olive oil) does not affect the performance of the method.

3.3. Determination of the presence of cow casein in Mozzarella cheese from retail Pizza declared with buffalo Mozzarella

Fourteen samples of Mozzarella from Pizzas were controlled. The results of this preliminary survey (Table 4) showed a different behaviour for the Pizzas made in a Pizzeria compared to those from industrial bakeries sold pre cooked and frozen at retail. The Mozzarella used for frozen Pizza complied to what was declared on the label in four out of seven samples, and in the other cases the presence of cow milk in buffalo Mozzarella was equal to or lower than 5%. The quality of Mozzarella used as Pizza topping in the Pizzeria was very variable and in many cases was really different from what was declared on the menu. In three out of the seven samples the amount of cow milk was higher than 75%.

Because of the mass of the cheese which was separated from Pizza in the sample preparation, it is difficult to understand where the fraudulent addition of cow's milk to cheese comes from, whether from mixed Mozzarella produced by the dairy or from a mixture of pure cow and buffalo Mozzarella made by the Pizza maker.

However the aim of this study was not to ascertain the responsibility, but to verify the reliability of the official method in detecting this fraud.

To better ascertain the relevance of the fraudulent use of pasta filata cheeses instead of buffalo Mozzarella a second survey was made sampling 50 Pizzas from different Pizzerias in Campania, the region of Italy where Pizza was born. The results (Table 5) showed a dramatic scenario, because 80% of the products did not comply to what was declared on the menu. Fourteen percent of the samples showed a presence of cow milk less than 10%, while one third of the samples were almost pure cow Mozzarella cheese instead of buffalo milk Mozzarella as written on the menu.

Table 3
Evaluation of % cow milk content of mixed Mozzarella cheese before and after oven cooking as Pizza topping ($n = 11$)

	Cheese		*P* value
	Fresh	Separated from Pizza	
Mean	38.6	39.2	0.830
Standard deviation	6.1	6.3	

Table 4
Evaluation of % cow milk in Mozzarella cheese separated from Pizza made in Pizzeria or in industrial bakery and sold frozen

Sample	From Pizzeria	Sample	Frozen
P1	3	F1	5
P2	79	F2	0
P3	88	F3	0
P4	91	F4	0
P5	0	F5	3
P6	0	F6	0
P7	25	F7	5

Table 5
Distribution on the basis of milk origin and relative composition of Mozzarella cheeses separated from 50 Pizza sampled from 50 different Pizzeria Restaurants in Campania (Italy), declaring on their menu the use of Buffalo Mozzarella as only one dairy product for the sampled Pizza variety

Milk origin and relative composition	Number of samples	% Distribution of the samples
100% Buffalo milk	10	20
<5% Cow milk	3	6
From 5% to 10% cow milk	4	8
From 35% to 50% cow milk	9	18
From 50% to 95% cow milk	8	16
From 95% to 99% cow milk	12	24
100% Cow milk	4	8

4. Conclusions

This study demonstrated that by applying the European official method it is possible to identify the presence of cow milk in buffalo Mozzarella even when Mozzarella is cooked as a Pizza topping. This method can be considered an effective tool to verify that buffalo Mozzarella cheese is really used as declared in the ingredient list, protecting consumers against improper practices and guaranteeing at the same time fair trade. Even if 57 Pizzas sampled from different sources are a relatively small sample when compared to the number of about 20,000 Pizzeria Restaurants working in Italy (FIPE, 2007), the fraud seems to be significant. Knowing that a method now exists that could identify this illegal practice could aid real pizza producers using high quality ingredients.

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