Authentication of dairy products by immunochemical methods: a review

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REVIEW PAPER

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Abstract Antibody-based techniques for the assessment of authenticity of dairy products are reviewed in this paper. Because of the inherent complexity of the protein and peptide fractions from milk and even more so from cheese, the use of immunoreagents which are more selective than polyclonal antibodies is usually required for the assaying dairy authenticity by immunochemical methods. Significant advances in this area have been achieved over the last decade thanks to advances in the antipeptide antibody technology, based on the use of properly designed peptides which mimick specified protein substructures as model antigens. Tailor-made antibodies have been developped either for the detection of single protein components or for recognizing protein adducts created by the technological processes employed. Different reagent configurations and immunoassay formats have been devised for a number of analytical applications relevant to the quality control of dairy products, ranging from the monitoring of molecular markers to the tracing of technological processes applied to milk for cheese-making.

免疫化学方法鉴定乳制品: 综述

摘要 本文综述了基于抗体技术鉴定乳制品的真实性。由于乳和干酪中蛋白质和肽组成和结构的复杂性，采用免疫化学方法鉴定乳制品时多采用免疫试剂而不采用多克隆抗体。这项技术的成功应用得益于在过去十几年中抗多肽抗体技术的发展，该技术基于模拟特定蛋白亚结构的完全设计的肽作为模式抗原。由模式抗原得到的抗体可以用来检测单一蛋白组分或者识别在加工过程中引入的外源性蛋白质。采用不同的试剂和不同的免疫测定方式可以进行各种蛋白质的分析，这种分析技术可以应用于相关乳制品的质量控制，进而可以作为分子标记应用在干酪生产用乳的可追溯体系中。

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关键词 干酪鉴定 · 抗多肽抗体 · ELISA · 免疫印迹 · 在线控制

1 Introduction

Immunoassay technology, already extensively used in clinical diagnostics, has been exploited in food science since the early 1980s, both for research purposes and, more significantly, for routine analysis. In comparison to conventional methods such as chromatographic or electrophoretic techniques, immunochemistry can provide valuable solutions to specific problems in food analysis in terms of performance (sensitivity, reproducibility, and reliability) and convenience (rapidity, cheapness, minimal sample clean-up, throughput, and reduced environmental impact) (Allen 1990). The fundamental difference between immunoassays and other analytical techniques lies in the fact that in immunoassays the high level technology is built into the design of the reagents and not in the apparatus, thus allowing detection of analytes, present even in trace amounts, without recourse to expensive equipment or highly skilled operators. In addition, the physical and chemical features of the samples have little impact on the results thanks to the selectivity and sensitivity of the immunoreagents. This may be particularly advantageous in food analysis. Indeed, food matrices range from liquids and pastes to solids and powders, and food ingredients, used either singly or in mixtures, may be processed by a variety of heat treatments or extraction procedures. Furthermore, progress in food chemistry and technology has led to an ongoing manufacture of new specialized food products. Despite the chemical complexity and variety of food products, highly selective and sensitive reagents such as antibodies can generally recognize a specific food analyte in either simple dilutions or rough extracts of food samples, thus allowing interferences from the food matrixes to be minimized. After all, every immunochemical method is designed for the detection of the primary antigen–antibody reaction. Therefore, its value is heavily dependent on the binding specificity of the antibody used as the reagent. The selection of an appropriate immunogen is a crucial factor in determining the reactivity spectrum of the antibody. Analytes of low molecular weight (<2,000 Da) are usually poorly immunogenic and can elicit an antibody response only if they are conjugated to carrier molecules, whereas larger molecules, such as polysaccharides and proteins, are immunogenic on their own. As a rule, a protein used as an immunogen in ordinary immunogenic procedures gives rise to a mixed population of antibodies recognizing the different epitopes of the protein. The resulting antiserum can display cross-reaction in immunoassays whenever the sample includes a molecular species having even only one epitope similar to one of the epitopes of the species used as the immunogen. Nevertheless, such antisera can be effective reagents for the immunodetection of contaminants of exogenous origin in food (i.e., mycotoxins, pesticides, antibiotics, anabolics, foodborne pathogens, and their toxins) (Rittenburg and Grothaus 1992). In contrast, immunoreagents which are far more selective than simple polyclonal antibody antisera are required for the detection of molecular indicators of food authenticity.

The need is for reagents which are capable of (a) identifying ingredients, (b) evaluating their quality, (c) defining their genetic origin, and (d) recognizing markers of production technology and storage conditions of food products.

Analytical dairy research is strongly engaged in developing methods for the assessment of authenticity of milk-derived products. The challenge is to ascertain the full compliance of high-quality dairy products, in products such as cheeses registered as protected designation of origin (PDO), which conform to the regulations and labeling claims. The contribution of immunochemistry to analytical research which addresses issues related to the authenticity of milk-derived products has not been fully acknowledged in the existing literature. Only the antibody-based methods concerning the discrimination of milk species have been reviewed among the analytical procedures developed for authenticity assessment of dairy products (De la Fuente and Juarez 2005) and the enzyme-linked immunosorbent assay (ELISA) techniques applied to the determination of food authenticity (Asensio et al. 2008). The present review is focused on recent advances in assaying for the authenticity of milk-derived products by immunochemical analysis of their protein content. About 80% of the total protein content in bovine milk is composed of four proteins, i.e., α_{s1}-, α_{s2}-, β-, and κ-casein. The remaining 20% is formed by whey proteins, so called because they are released in serum by milk clotting. As shown by high-resolution gel electrophoresis of milk proteins, casein fractions are markedly microheterogeneous, due to the occurrence of genetic variability, discrete phosphorylation, glycation, and limited hydrolysis by native milk proteinases (Swaisgood 1992). Apart from the inherent complexity of the protein composition of milk used for cheese-making, the peptide fraction in cheese is actually a mixture of a multitude of closely related products arising from the proteolysis of each casein fraction. In addition, single proteins and/or peptide components of cheese may be further modified by dairy processes. Consequently, specific detection of a molecular species recognized as a marker of authenticity in the protein fraction from milk and/or dairy products is generally a difficult task.

The first immunoassays designed for the control of authenticity of milk and dairy products employed polyclonal antibodies purified by immunoaffinity chromatography of antisera recovered from immunized laboratory animals (Harlow and Lane 1988b). In order to overcome issues related to the use of affinity-purified polyclonal antibodies, which mainly concerned the limited availability of selected reagents of constant quality, the second generation of antibodies targeting milk proteins was produced using hybridoma technology (Köhler and Milstein 1975). Finally, the third generation of immunoreagents for analysis of milk-derived products was obtained by exploiting the antipeptide antibody technology, which allowed the pre-determination of the antibody binding specificity towards specified substructures within protein chains using synthetic peptides as immunogens (Pizzano et al. 1999). Antipeptide antibodies proved to be especially valuable tools in the different analytical applications for the quality control of milk and dairy products, acting as real “smart reagents”.

Analytical advancements in the authentication of dairy products using properly developed immunochemical reagents have almost exclusively focused on the following issues:

1. Milk species identification in milk mixtures and cheese

2. Evaluation of the intensity of heat treatments applied to milk
3. Monitoring of proteolysis in cheese
4. Immunodetection of formaldehyde–casein adducts in cheese

2 Milk species identification in milk mixtures and cheese

A number of methods have been proposed to detect the fraudulent practice of blending milks in the production of ovine, caprine, and water buffalo cheeses. They are essentially based on chromatographic or electrophoretic or, more recently, PCR analysis of the protein fractions of the cheese (Mayer 2005; Mininni et al. 2009). Bovine milk is most frequently used as an adulterant owing to its low price and its consistent and continuous market availability. The reference method for the evaluation of cows' milk casein in ovine, caprine, and water buffalo cheeses was established by the Commission of the European Communities (Commission Regulation (EC) 2008). This method is based on the densitometric determination of bovine γ_2-caseins in the gel isoelectric focusing profiles of the in vitro plasmin hydrolysates of the cheese samples. The first attempts to develop immunochemical methods for milk species identification were based on the use of polyclonal antibodies capable of immunoprecipitating whey proteins, mainly immunoglobulins (Aranda et al. 1993; García et al. 1990). Quantitative determination of bovine (García et al. 1993) or caprine (García et al. 1989) milk in ovine milk as well as of bovine milk in caprine milk (Castro et al. 1992) was achieved using affinity-purified polyclonal antibodies and ELISA formats. Next, monoclonal antibodies against bovine β-lactoglobulin (Levieux and Venien 1994) and bovine immunoglobulins (Hurley et al. 2003, 2006) were applied in ELISA to quantify the amount of bovine milk added to caprine and ovine milks. Progress in the immunodetection of species-specific whey proteins has allowed more reliable speciation analysis of milk mixtures but only from raw milks, as whey proteins are susceptible to heat treatments applied to milk. Moreover, the significance of immunodetection of species-specific whey proteins in cheese samples is poor as whey proteins are only present in small amounts, depending on the cheese-making procedure. In contrast, all caseins are highly thermostable and are all retained in the curd during cheese-making. Consequently, antibodies specifically recognizing one of the four caseins are choice reagents for milk speciation in milk mixtures as well as in adulterated cheeses, regardless of the thermal treatments applied. Unfortunately, owing to the strict homology which exists between the caseins from the milk of the main animal species of dairy interest, i.e., cow, sheep, goat, and water buffalo, polyclonal antibodies against each of the caseins from any of these milks also recognize the respective counterparts in milk from all of the other species.

Nevertheless, immunoadsorption of an antiserum developed against whole bovine casein on insolubilized whole ovine casein allowed polyclonal antibodies which specifically recognized bovine casein in mixtures of bovine and ovine milk by an immunodotting method to be obtained (Aranda et al. 1988). Two similar antisera, one raised against whole bovine casein and the other against whole caprine casein, after immunoaffinity purification, were successfully employed in ELISA determi-

nation of bovine and caprine casein in ovine milk and cheeses (Rodríguez et al. 1993, 1994). As expected, the results were not affected by the heat treatments applied to milk (Aranda et al. 1988), but immunochemical analysis of the cheese samples based on such antibody preparations was not fully reliable. Indeed, the molecular species actually targeted by these antibodies have not been identified and consequently no information regarding the preservation of the antibody-recognized epitopes, depending on protein hydrolysis during cheese ripening, was available. Afterwards, a well-designed ELISA for specific detection of bovine casein in ovine and caprine cheese samples was then proposed (Richter et al. 1997). It was based on the use of affinity-purified polyclonal antibodies raised against the bovine γ-caseins generated by plasmin-mediated β-casein hydrolysis, previously recognized as the most suitable indicators of bovine adulteration in ovine, caprine, and water buffalo cheeses (Commission Regulation (EC) 2008). A number of monoclonal antibodies raised against caseins have been produced for different purposes relevant to dairy research (i.e., casein quantitation, hormonal regulation of casein production during lactogenesis, structural and topological studies, and recognition of genetic variants). Some of these monoclonal antibodies targeting caseins, displaying no cross-reactivity towards the homologous caseins from milk of the other animal species, were selected for developing analytical methods for assurance of cheese authenticity. The first, named AH4, directed against bovine β-casein, was advantageously used to detect bovine casein in ovine and caprine milk and cheese in different ELISA formats (Anguita et al. 1995, 1996, 1997a, b). More recently, it has been shown that the results of AH4-based ELISA matched unequivocally with those obtained by PCR analysis (López-Calleja et al. 2007), but immunoassays are less expensive and more practical for routine use in comparison to PCR, as they allow faster processing of a high number of samples without the need for digestion and/or DNA purification steps. The second monoclonal antibody, named B2B, directed against caprine α_{s2}-casein, was used for the detection of caprine milk in ovine milk (Haza et al. 1996, 1997) and cheese (Haza et al. 1999). Finally, two monoclonal antibodies (MAb) which were raised against bovine γ-casein have been applied to the immunochemical evaluation of bovine milk in ovine and caprine milk using an automated optical Biacore biosensor (Haasnoot et al. 2004). More recently, a low-cost alternative optical biosensor was used instead of the Biacore instrument to determine the amount of cow's milk in ewe's and goat's milk and bovine rennet whey powder in milk powder, and the results were found to be comparable. The low-cost analytical system also proved to be useful for the detection of fraudulent water additions to milk (Haasnoot et al. 2006). However, monoclonal antibodies are not necessarily the best choice for any analytical application. The production of monoclonal antibodies is quite expensive, clone screening is time-consuming, and additional work is required for the selection and characterization of antibodies displaying the highest affinity for the antigen (Harlow and Lane 1988a). Alternatively, novel immunoreagents suited to milk speciation have been developed according to the antipeptide antibody technology. By comparing the primary milk casein structures of the main animal species of dairy interest, despite having an overall high degree of homology, short stretches of amino acid sequences with species-specific features can be found. Synthetic peptides reproducing some of the cow-specific sequences were chemically synthesized and used as immunogens. Using this approach, many different antibody

preparations capable of selectively detecting bovine casein in a mixture with ovine, caprine, and water buffalo casein have been obtained. However, even if an antipeptide antibody apparently shows good performances in immunoassays, its binding specificity has to be carefully evaluated by a controlled technique, such as immunoblotting of protein profiles obtained by electrophoresis (Pizzano et al. 1997). The reactivity of the antipeptide antibodies raised against the three sequence stretches reported in Fig. 1 has been studied in detail. The antibodies directed against the two fragments of bovine β-casein (i.e., the plasmin-released 1–28 phosphopeptide and the synthetic peptide reproducing the β-casein 176–185 amino acid sequence) were found to exclusively recognize the parent β-casein from both bovine and water buffalo milk (Pizzano et al. 2000, 2001). Consequently, they were suitable reagents for the development of direct immunoassays for the detection of bovine milk fraudulently used as an ingredient in ewes' and goats' cheese-making. An unexpected complexity of the α_{s1}-casein fraction has been shown by polyclonal antibodies raised against the bovine α_{s1}-casein 139–149 fragment. A similar antibody preparation, directed against the bovine α_{s1}-casein 140–149 fragment,

β-casein (1-28)

	cow	water buffalo	ewe	goat
1	R	R	R	R
2	E	E	E	E
3	L	L	Q	Q
4	E	E	E	E
5	E	E	E	E
6	L	L	L	L
7	N	N	N	N
8	V	V	V	V
9	P	P	V	V
10	G	G	G	G
11	E	E	E	E
12	I	I	T	T
13	V	V	V	V
14	E	E	E	E
15	S	S	S	S
16	L	L	L	L
17	S	S	S	S
18	S	S	S	S
19	S	S	S	S
20	E	E	E	E
21	E	E	E	E
22	S	S	S	S
23	I	I	I	I
24	T	T	T	T
25	R	H	H	H
26	I	I	I	I
27	N	N	N	N
28	K	K	K	K

β-casein (176-185)

	cow	water buffalo	ewe	goat
176	K	K	K	K
177	A	A	A	A
178	V	V	V	V
179	P	P	---	---
180	Y	Y	---	---
181	P	P	P	P
182	Q	Q	Q	Q
183	R	R	R	R
184	D	D	D	D
185	M	M	M	M

α_{s1}-casein (139-149)

	cow	water buffalo	ewe	goat
139	N	N	N	N
140	Q	Q	Q	Q
141	E	E	E	E
142	L	L	L	L
143	A	A	A	A
144	Y	Y	Y	Y
145	F	F	F	F
146	Y	Y	Y	Y
147	P	P	P	P
148	E	Q	Q	Q
149	L	L	L	L

Fig. 1 Three of the peptides reproducing sequence stretches of bovine caseins successfully used as immunogens in rabbits to induce production of antibodies specifically detecting bovine casein in milk mixtures from different animal species. The amino acid differences within the sequences from the different species are *boxed*

had previously been proposed for specific detection of bovine casein in ovine and caprine cheeses by ELISA and the single amino acid residue at site 148 of α_{s1}-casein (E in bovine α_{s1}-casein and Q in ovine and caprine α_{s1}-casein) was supposed to be responsible for antibody recognition of bovine casein (Rolland et al. 1993, 1995). Based on electrophoresis and immunoblotting results obtained in further studies regarding the antigenicity of the 139–149 α_{s1}-casein region in different animal species, it has been established that antipeptide antibodies directed against the bovine α_{s1}-casein 139–149 fragment recognized a minor α_{s1}-casein component, occurring only in bovine milk (Pizzano et al. 1997). This component, subsequently identified as a C-terminally truncated α_{s1}-casein form, was found in fresh bovine milk and young bovine cheese, but it was mostly degraded after 3 months of cheese ripening and disappeared in the 15-month-old cheese sample giving rise to species undetectable by immunoblotting. An overview of the results obtained by these antipeptide antibodies is given in Fig. 2. In contrast, a constant level of antibody-reactive species has been detected in bovine cheese samples using ELISA, irrespective of the ripening age. Analysis by ELISA of the chromatographic fractions obtained by reversed-phase high-performance liquid chromatography (RP-HPLC) of the pH 4.6 soluble fraction of chymosin hydrolysate of whole bovine casein demonstrated that the chymosin-mediated degradation of the short bovine α_{s1}-casein gave rise to a pH 4.6 soluble peptide. This antibody-reactive peptide was found in a single chromatographic peak, also recognized by antipeptide antibodies directed against the bovine 23–34 α_{s1}-casein fragment, but unresponsive to antipeptide antibodies directed against the bovine 1–22 α_{s1}-casein fragment (Pizzano et al. 1998a). According to these findings, the adulteration of ovine and caprine cheeses by bovine casein could be properly quantified by ELISA using the sole pH 4.6 soluble fraction extracted from cheese as sample (Pizzano et al. 1999). ELISA analysis of four caprine cheese samples experimentally adulterated by bovine casein is shown in Fig. 3. The

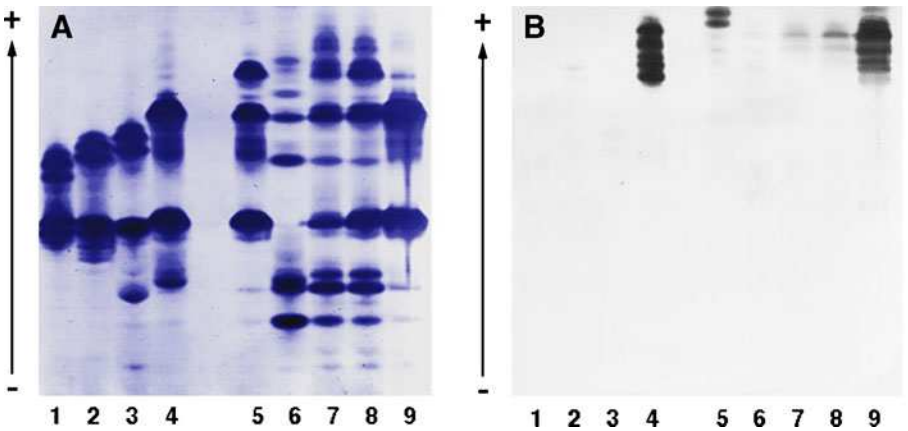

Fig. 2 Polyacrylamide gel electrophoresis at pH 8.6 in denaturing conditions and immunoblotting analysis of proteins from caprine (*lane 1*), ovine (*lane 2*), water buffalo (*lane 3*), and bovine (*lane 4*) whole caseins; 1-h chymosin (*lane 5*) and 1-h plasmin (*lane 6*) hydrolysates of whole bovine casein; 15-month-old (*lane 7*), 105-day-old (*lane 8*), and 1-day-old (*lane 9*) Parmigiano Reggiano cheese samples. **a** Coomassie brilliant blue R-250 stained gel; **b** immunodetection using anti-bovine α_{s1}-casein (139–149) antibodies. (This figure was previously published (Pizzano et al. 1999) and reprinting was authorized by A. Gayathri, Publication Manager of Research Signpost, Trivandrum, India.)

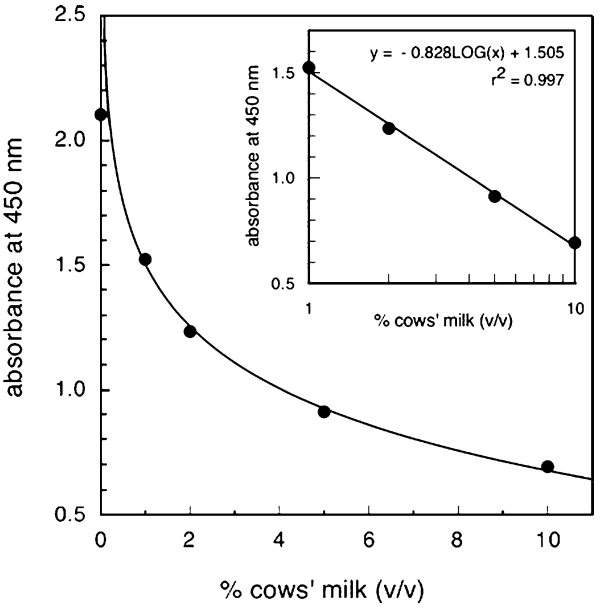

Fig. 3 Competitive ELISA of the pH 4.6 soluble fraction of experimental cheese samples from caprine milk adulterated with bovine milk using polyclonal antibodies raised against the 139–149 sequence of bovine α_{s1}-casein. In the *insert*, the linear relationship found by reporting ELISA results vs logarithm of percent cow's milk was shown. (Unpublished results)

main features of the above-mentioned ELISAs designed to determine the animal species in milk mixtures and cheese are summarized in Table 1.

Even though the C-truncated minor α_{s1}-casein component is a suitable marker of bovine milk and in mixtures with water buffalo milk, the authenticity of water buffalo Mozzarella cheese could not be evaluated by immunodetecting the peptide raised by hydrolysis of this bovine-specific component. According to the standard production regulation of Mozzarella cheese, a PDO product (Commission Regulation (EC) 1996), the curd from rennet-coagulated milk is kneaded and stretched in a large quantity of hot water at 70 °C. Most likely the antibody-reactive pH 4.6 soluble peptide generated by the bovine-specific α_{s1}-casein component is released in the liquid phase during this step of cheese-making. At present, the official European reference method for the evaluation of cows' milk casein in ovine, caprine, and water buffalo cheeses is the most frequently used method for the assessment of water buffalo Mozzarella cheese authenticity (Commission Regulation (EC) 2008). As previously mentioned, this method is based on the detection of bovine γ_2-casein in plasmin-digested cheese proteins separated by isoelectric focusing. Reliable results, irrespective of the degree of proteolysis, can be obtained in the analysis of ovine and caprine cheeses, whereas the evaluation of cows' milk casein in water buffalo cheese is sometimes uncertain. Indeed, a peptide co-migrating with bovine γ_2-casein is frequently generated by plasmin-mediated hydrolysis of pure water buffalo casein samples. The occurrence of this peptide may lead to false-positive detection of cows' milk in a genuine water buffalo cheese, when it is analyzed by the European official method. Recently, this problem has been solved by an immunochemical approach (Addeo et al. 2009). Immunoblot analysis with antipeptide antibodies raised against three sequence stretches of bovine β-casein has been performed to select a reagent capable of distinguishing between γ_2-casein and the interfering bovine γ_2-casein-like peptide along the electrophoretic profiles of pure water buffalo casein. One of these antibody

Table 1 Milk species identification in milk mixtures and cheese by ELISA

Type of immunoreagent	Target	Analytical performance	Reference
Polyclonal antibodies	Whole bovine casein	0.1% of bovine milk in ovine milk	Aranda et al. (1988)
Polyclonal antibodies	Caprine IgG	0.5% of caprine or bovine milk in ovine milk	Aranda et al. (1993)
Polyclonal antibodies	Bovine whey proteins	1% of bovine milk in caprine milk	Castro et al. (1992)
Polyclonal antibodies	Caprine whey proteins	0.5% of caprine milk in ovine milk	García et al. (1993)
Polyclonal antibodies	Bovine whey proteins	3% of bovine milk in ovine milk	García et al. (1989)
Polyclonal antibodies	Bovine whey proteins	1% of bovine milk in ovine milk	García et al. (1990)
Polyclonal antibodies	Bovine γ-casein	0.1% of bovine milk in ovine and caprine milk and cheese	Richter et al. (1997)
Polyclonal antibodies	Whole bovine casein	1% of bovine milk in ovine milk and cheese	Rodríguez et al. (1993)
Polyclonal antibodies	Whole caprine casein	1% of caprine milk in ovine milk and cheese	Rodríguez et al. (1994)
Monoclonal antibody	Bovine β-casein	0.5% of bovine milk in ovine and caprine milk and cheese	Anguita et al. (1997a)
Monoclonal antibody	Caprine α_{s2}-casein	0.25% of caprine milk in ovine milk	Haza et al. (1997)
Monoclonal antibody	Caprine α_{s2}-casein	0.5% of caprine milk in ovine cheese	Haza et al. (1999)
Monoclonal antibody	Bovine IgG	0.1% of bovine milk in ovine, caprine, and buffalo milk	Hurley et al. (2003)
Monoclonal antibody	Bovine IgG	0.001% of bovine milk in sheep or buffalo milk and 0.01% bovine milk in goat milk	Hurley et al. (2006)
		0.001% of bovine milk in goat soft cheese and 0.01% in sheep and buffalo soft cheese	
Monoclonal antibody	Bovine β-lactoglobulin	0.001% of bovine milk in ovine and caprine milk	Levieux and Venien (1994)
Polyclonal antipeptide antibodies	Bovine α_{s1}-casein 139–149 peptide	0.5% of bovine milk in ovine and caprine cheese	Pizzano et al. (1999)
Polyclonal antipeptide antibodies	Bovine α_{s1}-casein 140–149 peptide	0.125% of bovine milk in ovine milk and 0.5% of bovine milk in ovine cheese	Rolland et al. (1993)
Polyclonal antipeptide antibodies	Bovine α_{s1}-casein 140–149 peptide	0.5% of bovine milk in caprine cheese	Rolland et al. (1995)

preparations, the anti-β-casein (106–110) antiserum, cross-reacted with the native and plasmin-generated γ_2-casein from bovine and water buffalo casein but did not recognize the majority of peptides resulting from the water buffalo β-casein plasminolysis and, in particular, the bovine γ_2-casein-like band in the electrophoretic profile of pure water buffalo casein. Based on the use of the anti-β-casein (106–110) antiserum, immunoblotting analysis of experimental Mozzarella cheese samples from

water buffalo milk mixed with bovine milk was sensitive to 0.25% (*v*/*v*) of bovine milk added to the water buffalo milk, whereas a detection limit of 0.5% (*v*/*v*) could be achieved by the European official method. At present, this immunoblotting procedure can be considered as the "gold standard" method for detecting bovine casein in water buffalo milk and cheese and might be included in the current official method of analysis as a second assurance of water buffalo milk purity and cheese authenticity.

3 Evaluation of the intensity of heat treatments applied to milk

During thermal processing and storage of milk, free amino groups of proteins can be non-enzymatically glycated by lactose according to the Maillard reaction (Finot et al. 1981). The early stable products resulting from the rearrangement of the primary adducts are named "Amadori compounds" (the Amadori compounds are actually N-substituted deoxylactulosyl-derivatives of the amino acid residues attacked by lactose). The extent of the reaction depends on the intensity of the heat treatment applied to milk. Higher concentrations of Maillard compounds than those lawfully expected in milk and dairy products can either be due to excessive or repeated heat treatments, thus indicating milk of inferior microbiological quality, or fraudulent use of milk powders. Current procedures for the evaluation of the extent of the Maillard reaction in heat-treated milk are based on the detection of the products obtained by chemical conversion of lactosylated lysine residues. These procedures are generally cumbersome and time-consuming and are not appropriate for online routine controls. For example, the determination of furosine, widely considered to be the most suitable indicator of the presence of Amadori compounds in milk and dairy products, requires a RP-HPLC analysis of the 24-h acid-hydrolyzed samples (Henle et al. 1995). In order to develop immunochemical methods for the detection of lactose–protein adducts in milk, polyclonal antibodies have been produced against ovoalbumin (Matsuda et al. 1992) or poly-L-lysine (Fogliano et al. 1997) heated in the presence of lactose. Unfortunately, the binding specificity of these antibody preparations has not been defined since the antigen preparations used as immunogens were actually mixtures of both early and advanced Maillard products in unknown proportions. Antipeptide antibody technology has been exploited to obtain immunoreagents capable of selectively recognizing early Maillard products, that is the Amadori compounds, in heat-treated milk (Pizzano et al. 1998b). The peptide RPKPQQFFGLM has been used as a lactosylation model substrate to mimic Amadori lactose–protein adducts in milk. Indeed, the free amino group of its N terminus and the ε-amino group of the lysine residue at position 3 of its amino acid sequence could simulate the main glycation sites of proteins. The kinetics of peptide lactosylation was monitored by RP-HPLC and the formation of the Amadori lactose–peptide adducts was ascertained by mass spectrometric analysis of the reaction mixture. The lactose-treated peptide, after conjugation to a protein carrier, was used as an immunogen in rabbits. The binding specificity of the polyclonal antibodies raised against the lactosylated peptide was analyzed by competitive ELISA. The antibodies were found to be selectively directed against the specific structural features of the glycosidic moiety of the immunogen (Pizzano et al. 2006, 1998b). The antibody reactivity towards lactosylated caseins included in different heat-treated milk samples has been evaluated by immunoblotting experiments. Strong and widespread

immunostaining along the electrophoretic profiles of both UHT-treated and bottle-sterilized milk samples indicated that consistent amounts of variously lactosylated heat-induced proteins were included in these milk samples. In contrast, very poor antibody reactivity was found in the profiles of both raw and pasteurized milk samples. An ELISA procedure based on the use of this antipeptide antibody preparation has been developed. Analysis by this competitive ELISA method of commercial milk samples is reported in Fig. 4. The index of lactosylation of proteins in milk samples treated from 60 to 100 °C, as determined using ELISA results, was linearly correlated to the furosine content. Accordingly, this ELISA-based procedure has been suggested as a suitable alternative to the current method based on furosine detection for the evaluation of Maillard compounds in milk and cheese samples (Pizzano et al. 1998b). Thanks to its rapidity and reliability, this procedure can be especially appropriate for online control of milk processing and extension before cheese-making.

According to the results described above, the intensity of heat treatments involved in the UHT treatment of milk has been shown to trigger the Maillard reaction, whereas pasteurization is not a harsh enough treatment to promote the formation of stable Amadori adducts in milk. Consequently, the ELISA procedure using antipeptide antibodies raised against the lactosylated peptide was unsuited to distinguishing between raw and pasteurized milk samples. Mild time/temperature treatments of milk, including pasteurization, can be evaluated by a specific immunoassay using a monoclonal antibody capable of monitoring heat-induced conformational changes in bovine milk alkaline phosphatase (Levieux et al. 2007). Similar results have also been obtained by quantifying separately the native and "heat-denatured" forms of α-lactalbumin using monoclonal antibodies in different assay formats (Dupont et al. 2004; Jeanson et al. 1999). In contrast, thermal treatments which are more severe than pasteurization can be classified on the basis of lactoferrin denaturation (Indyk et al. 2007).

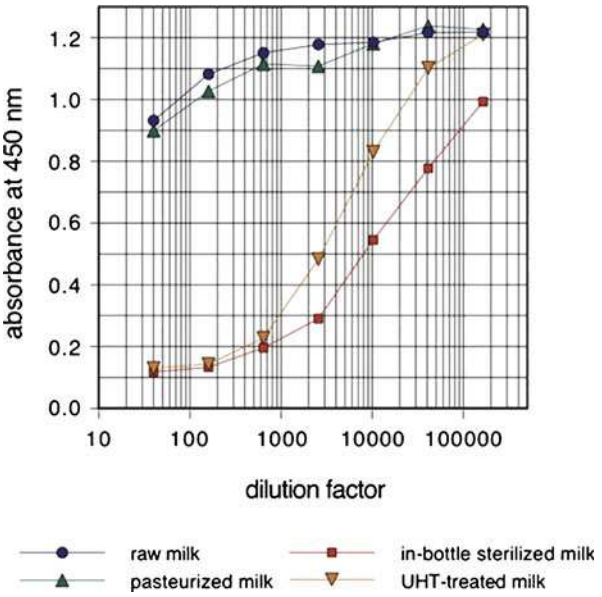

Fig. 4 Competitive ELISA of defatted raw, pasteurized, UHT-treated, and in-bottle-sterilized milk samples. Three aliquots of 50 μL of the serially diluted milk samples ($1:4^n$, n from 1 to 7) were tested, starting from a 1:40 (*v*/*v*) dilution in 50 mmol · L^{-1} NH_4HCO_3, pH 8.5 and the mean values of ELISA absorbance were reported. Microtiter plates were coated with 2 mg · mL^{-1} whole casein from UHT-treated bovine milk and 1:500 (*v*/*v*) diluted anti-lactosylated peptide antiserum was used. (This figure was previously published (Pizzano et al. 1999) and reprinting was authorized by A. Gayathri, Publication Manager of Research Signpost, Trivandrum, India.)

Unfortunately, improper use of heat-treated milk for raw cheese production cannot be detected by these assays, as the antibody-targeted proteins are not included in the final cheese. In contrast, in milk as well as in cheese samples, the pH 4.6 insoluble β-lactoglobulin (βlg) has proven to be an especially valuable marker for the detection of modifications to milk proteins induced by mild heat treatments such as pasteurization (Manzo et al. 2008). Thermal denaturation of the major whey proteins is currently regarded as the initial step leading to the heat-induced aggregation of milk proteins (Law et al. 1994). In particular, denatured βlg was found to be linked to casein through intermolecular disulfide bonds in heat-treated milk (Manzo et al. 2008). Immunoblotting experiments using polyclonal antibodies directed against βlg have been performed in order to check the possibility of relating the amount of βlg–casein aggregates insoluble at pH 4.6 to the severity of heat treatments applied to the milk. More than 90% of the pH 4.6 soluble βlg included in raw milk has been recovered in the pH 4.6 insoluble protein fraction of UHT-treated milk samples. Moreover, a significant fraction of the βlg content of raw milk was found to be linked to caseins even in pasteurized milk samples. Accurate evaluation of heat-induced βlg–casein aggregates in commercial milk samples has been achieved by determining the βlg content in the pH 4.6 insoluble fraction from differently heat-treated milk samples using a competitive ELISA. Subsequently, the same ELISA procedure has been applied to cheese analysis using Mozzarella cheese as a model system. The pH 4.6 insoluble βlg content in cheese from pasteurized milk was about 90 times higher than that found in cheese made from raw milk (9.1 vs. 0.1 mg of βlg in 1 g of Mozzarella cheese). According to the ELISA results, about 36% of the total βlg milk content was transferred from the pasteurized milk to the Mozzarella cheese, whereas less than 0.5% was transferred from the raw milk. Determination of the pH 4.6 insoluble βlg by ELISA has allowed the distinguishing of raw and pasteurized milks and to detect the use of pasteurized milk instead of raw milk in cheese-making. It is worth noting that milk pasteurization can alter the indigenous milk microflora and consequently can affect the specific sensory characteristics of cheeses traditionally made from raw milk (Grappin and Beuvier 1997). The ELISA-based procedure described in this paragraph has proven to be appropriate for the quality control of milk used in the manufacture of traditional raw milk cheeses. Thanks to the strictly heat-induced βlg partition between the two fractions recovered from milk after precipitation at pH 4.6, a simple commercially available antibody preparation raised against bovine βlg, is an immunoreagent which is more than adequate for labeling the intensity of heat processing of milk by ELISA.

4 Monitoring of proteolysis in cheese

A descriptive overview of the milk proteins included in cheese is currently obtained by gel electrophoresis which can be improved by immunostaining the electrophoretic profiles (Addeo et al. 1995). As a rule, polyclonal antibodies raised against each single casein simultaneously recognize the native casein and all of the high molecular mass peptides generated by the proteolysis of that casein in immunoblotting patterns. Consequently, such antibodies cannot be used to develop immuno-

assays for cheese analysis. Several antibody preparations suited to monitor cheese proteolysis have been obtained using appropriately designed peptides as immunogens (Pizzano et al. 1999). Most antibodies raised against peptides reproducing either a C- or N-terminal casein region are capable of recognizing the respective parent casein, thus indicating that casein chain extremities usually carry epitope structures which are easily accessible to antibodies. In agreement with this general finding, antibodies raised against the 1–28 phosphopeptide of bovine β-casein are capable of detecting native β-casein. These antibodies have allowed plasmin-mediated bovine β-casein breakdown to be monitored in Parmigiano Reggiano cheese ripened from 1 day to 24 months. The amount of residual β-casein, as determined by competitive ELISA, has been proposed as a suitable indicator of the ripening age of cheese. In contrast, ELISA analysis of the pH 4.6 soluble fraction of Parmigiano Reggiano cheese has shown that the 1–28 phosphopeptide released from β-casein, which is the actual peptide targeted by antibodies, can be further broken down by proteolysis and hence cannot be used as an indicator of the stage of ripening of hard cheeses (Pizzano et al. 2000, 2001). By a similar approach, based on the use of antipeptide antibodies directed against the plasmin-sensitive cleavage sites included in β-casein, ELISA determination of residual native β-casein allowed the plasmin-mediated hydrolysis of β-casein during the ripening of Comté cheese to be monitored (Dupont et al. 2003). More recently, proteolysis of α_{s1}-casein in a cheese matrix has been visualized by confocal laser scanning microscopy using fluorescently labeled antibody-like fusion proteins generated by antibody phage display and directed towards three small synthetic peptides of α_{s1}-casein (Duan et al. 2009).

It is worth noting that some of the antipeptide antibodies raised against inner sequence stretches of caseins were unable to cross-react with the parent caseins, likely due to conformational constraints acting on the native structures. Frequently, the antipeptide antibodies which are insensitive to the parent caseins are capable of recognizing their targeted sequences after proteolysis of the parent caseins, thus giving rise to an ideal reagent for a one-step evaluation of the extent and progression of proteolysis in a cheese sample by an immunological approach, irrespective of the content of the residual native parent casein (Pizzano et al. 1999).

5 Immunodetection of formaldehyde–casein adducts in cheese

New analytical opportunities can be developed using antipeptide antibody technology whenever a milk protein is chemically attacked by xenobiotics and the reaction product has been identified. A modified peptide, which mimicks the novel haptenic structure formed by exposing milk proteins to the xenobiotic agent, can be used as a model immunogen to obtain antipeptide antibodies which are capable of tracing the addition of a xenobiotic to milk. This procedure has been applied to detect formylated protein adducts which occur in formaldehyde-treated milk (Pizzano et al. 2004).

Formaldehyde and its precursor, hexamethylene-tetramine, food additives known respectively as E240 and E239, are used as bacteriostatic agents in the dairy industry. The addition of formaldehyde to milk in cheese-making prevents blowing

and cracking (i.e., "late blowing") of hard cheese, which arises from the germination of *Clostridium* spores contained in milk. The toxicity of formaldehyde by ingestion has been well documented but the consumption of foods treated with formaldehyde in amounts that are required to prevent "late blowing" (25–30 ppm of formaldehyde in milk) is generally not regarded as a significant dietary risk factor. However, the use of formaldehyde in cheese-making is an indication of the inferior microbiological quality of the raw milk and so the treatment of milk with formaldehyde is normally not included in standard production regulations of PDO cheeses. Over 80% of the formaldehyde added to milk has been found to be linked to γ_2-casein in cheese (Restani et al. 1989). In particular, formaldehyde can react with the alpha-amino group of the histidine residue at the N terminus and the principal reaction product is a spinacine residue. An HPLC-based method for determination of spinacine residues in cheese has been developed to detect the improper use of formaldehyde-treated milk in cheese-making (Pellegrino and Resmini 1996). A novel immunochemical reagent for selectively detecting formylated adducts of γ_2-casein in cheese from formaldehyde-treated milk has been developed by exploiting antipeptide antibody technology (Pizzano et al. 2004). The peptide HKEMPGGC was chemically synthetized. The first five amino acid residues (i.e., the HKEMP sequence) constituted the N-terminal sequence of γ_2-casein and contained the histidine residue which was found to be the site which was attacked by formaldehyde. In addition, the C-terminal cysteine residue provided the sulfhydryl group required for correctly orienting the linkage of the peptide to a protein carrier in order to improve the immunogenicity. The two glycine residues were included to form a spacer arm in the peptide–carrier conjugate. As schematized in Fig. 5, an aliquot of this peptide was formylated and the peptide and its formylated adduct were separately used as immunogens to obtain polyclonal antibodies at respectively predetermined specificities towards the native γ_2-casein site mainly involved in formylation and the same site which had been modified by formaldehyde addition. The performances of the antipeptide antibodies towards their putative protein targets are shown in Fig. 6. The protein fraction extracted from a 12-month-old Grana Padano cheese sample (panel a) and that from the same sample treated with 26 ppm

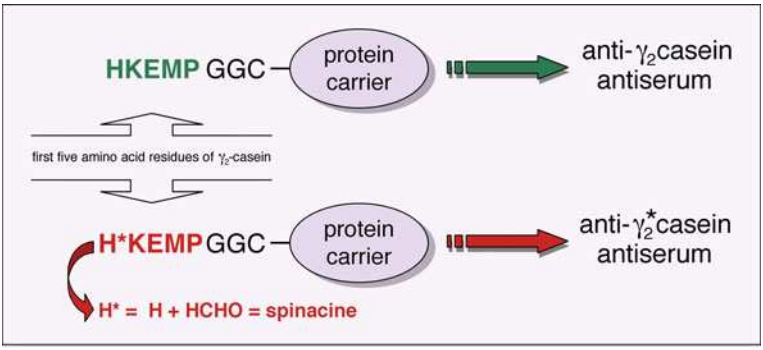

Fig. 5 Production of antisera recognizing specifically either native or formaldehyde-modified γ_2-casein. The peptide HKEMPGGC and its formylated adduct, after conjugation to a protein carrier, were separately used as immunogen in rabbits. Analysis of the binding specificity of the resulting antipeptide antibodies towards their putative protein targets was reported in Fig. 6

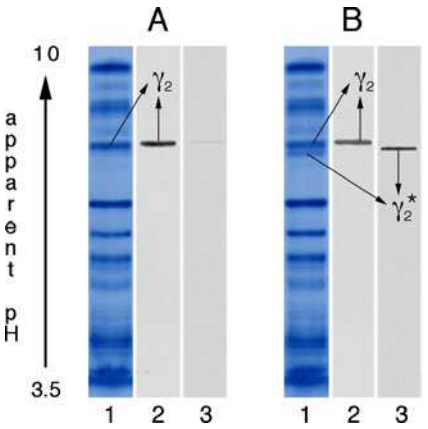

Fig. 6 Isoelectric focusing of the protein fraction extracted from an 18-month-old Grana Padano cheese sample (**a**) and from the same protein fraction incubated at 37 °C with 26 ppm of formaldehyde (**b**). The profiles were stained by Coomassie brilliant blue G-250 (*lanes 1*) or immunostained by anti-γ_2-casein antiserum (*lanes 2*) or by anti-γ_2*-casein antiserum (*lanes 3*). (Unpublished results)

of formaldehyde (panel b) were separated by electrophoretic focusing and stained by Coomassie brilliant blue (lane 1 of panels a and b) or immunostained after blotting the electrophoretic profiles onto nitrocellulose. The antibodies raised against the native peptide selectively recognized the protein band currently considered as γ_2-casein (Commission Regulation (EC) 2008) on the complex protein profiles of both cheese samples (lane 2 of panels a and b). The antibodies raised against the formylated adduct of the peptide specifically detected the protein band previously recognized as the formylated adduct of γ_2-casein (Restani et al. 1989) on the profile of the formaldehyde-treated cheese (lane 3 of panel b). In contrast, these antibodies did not react with any of the proteins in the control cheese sample (lane 3 of panel a), thus showing to be able to distinguish between the formaldehyde-modified γ_2-casein and the native γ_2-casein. Based on the use of this antibody preparation, immunoblot analysis of a number of commercial Grana Padano cheese samples has been performed to evaluate the extent of improper use of formaldehyde-treated milk in the Grana Padano cheese industry (Pizzano et al. 2004). Unfortunately, γ_2-casein can be modified by reactive aldehydes resulting from microbial metabolism in very mature cheese (Restani et al. 1996). These aldehyde γ_2-casein adducts are electrophoretically and immunologically indistinguishable from the formylated γ_2-casein. Consequently, the immunoblot analysis described above can only be appropriately applied for the detection of formaldehyde-treated milk in hard cheeses aged up to 15 months.

6 Conclusion

Immunochemistry has evolved in clinical environments and clinicians were the first to appreciate the performance and use of antibody-based techniques in medical research and diagnostics. The development of immunochemical methods for food analysis has been delayed, most likely due to the fact that food researchers are normally trained as chemists and are not as aware of the potentialities of immunoassays. Indeed, at present, immunochemistry still has little impact on analysis concerning cheese authentication. Results presented in this review have

shown that immunochemical methodology can provide suitable solutions to the analytical challenges presented by milk and cheese samples and fulfill the major requirements for the quality control analysis of dairy products. In the near future, new powerful immunoreagents will more than likely be provided by gene technology. Repertoires of antibody-like molecules directed to almost any antigen have been generated in phage display libraries. Such libraries are collections of recombinant phages, each containing a gene segment encoding the antigen-binding variable domain of an antibody fused to the gene encoding the protein coat. Consequently, each recombinant bacteriophage displays a different antigen-binding domain on its surface and can be isolated according to its antigen-binding specificity (McCafferty et al. 1990). Antibody-like reagents obtained by screening a phage display library have been used for monitoring proteolysis in cheese, as previously mentioned (Duan et al. 2009).

Advancements in dairy analysis by immunochemical methods are also expected with regard to the development of new assay formats. Nowadays, an attractive alternative to ELISA for food analysis by immunochemical means is based on the use of immunosensors, allowing fast, specific, and accurate determinations of food components. Immunosensors are analytical devices composed of antibodies immobilized on a solid support and connected to transducers converting the binding of the antigen into a physical signal. Applications of immunosensors to the control of milk and dairy products have been reviewed (Dupont et al. 2006), as regards the detection of antibiotics, drug residues, chemical contaminants, hormones, pathogens and toxins, single caseins and also monitoring of α-lactoglobulin denaturation to evaluate severity of heat treatment applied to milk (Dupont et al. 2004). Immunosensors might be especially valuable tools for direct online analysis as they give a response in real time, but the development of portable low-cost devices is required for field measurements by immunosensor-based techniques. The ideal solution for online controls is actually provided by one-step strip tests. Such tests are based on the migration of the sample on a membrane by capillarity and the immunodetection of the analyte of interest using an antibody labeled by latex or gold particles. The procedure is very fast and user-friendly, even if only a qualitative or a semi-quantitative response can be obtained. Simple and portable strip tests have been developed for the immunodetection of antibiotic residues, pathogens, and toxins in milk and dairy products, as recently reviewed (Jin et al. 2006).

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