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Testing the authenticity of milk and milk products

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10.1 Introduction

The driving force behind any adulteration is to maximise revenues by either using a cheap ingredient to (partially) substitute a more expensive one, or to (partially) remove the valued component in the hope that the altered product passes undetected by the final user or consumer. Watering of milk or skimming off cream are good examples to illustrate the point, and these fraudulent operations have been practised for a long time. Dairy products account for a large share of the total value of agricultural production in the developed world. Major advances in agronomy, large-scale transport, processing, and the introduction of efficient distribution systems have resulted in increased technological complexity, a higher degree of globalisation and lower product prices. The economics of dairying are very complex and mostly governed by intricate guidelines and laws to balance production and demand, stabilise prices and protect local interests. A complicated market scheme for milk products is at the very heart of the European Union's Common Agricultural Policy (Rasmussen, 2003). Fraudulent malpractice creates unfair competition, leading to market distortions, which in turn may impact the local or even the international economy. Therefore, authentication of milk and milk products is of primary importance for both consumers and manufacturers, and at all levels along the process chain.

Extension of a product with a cheap ingredient, also known as 'economic adulteration', does usually not carry a health hazard for consumers. This statement is not generally valid regarding adulterated milk and milk products. Consumers allergic to cows' milk may suffer severely if they ingest, e.g., ovine or caprine milk fraudulently extended with bovine milk or whey. As a consequence most countries have set up a complicated legal framework to

ensure proper consumer protection and to foster fair trade practices. Compositional product standards, codified by national as well as supranational authorities, e.g. FAO/WHO Codex Alimentarius, International Dairy Federation (IDF) and the European Commission (EC), represent an integral part of food legislation. Product labelling is a vital instrument to inform consumers about the identity of a product, thereby obliging producers to conform to predefined product standards. Infringement of labelling regulations could lead to criminal charges in the courts. Therefore, analytical data used in court or in other disputes have to stand up to scrutiny. A large number of methods have been developed and standardised with a view to that particular purpose. They can be found in method manuals issued, e.g., by the IDF, the Association of Analytical Chemists International (AOAC Internat.), or the EC.

Most of the product standards refer *inter alia* to hygienic quality parameters, e.g. total plate count, number of various indicator micro-organisms, somatic cell count, etc., which are at the borderline of product authenticity and product safety. These issues are beyond the scope of this chapter and readers are referred to the respective literature (e.g. Robinson, 2002).

10.1.1 Milk and milk product authenticity issues

In general, authenticity issues fall into at least one of the following categories:

- Non-compliance with legal requirements (product standards) such as:
  - Maximum/minimum content of water, solids-non-fat, and fat in certain dairy products (butter, cheese, yoghurt, etc.)
  - Geographical origin of the product.
- Wrongful addition of certain ingredients of dairy or non-dairy origin such as:
  - Watering of milk
  - Milk of different species
  - Addition of non-dairy protein
  - Altering the casein/whey protein ratio
  - Addition of buttermilk or whey powder to milk powder
  - Addition of vegetable or animal fats to milk fat
  - Addition of reconstituted milk to fluid milk
  - Non-authorised preservatives.
- Non-compliance regarding use of certain technological processes:
  - Heat treatment
  - Cheese ripening
  - Membrane technology.

Whatever type of fraud is perpetrated, authenticity testing relies either on a fundamental difference between the original and the adulterant, or on an intimate knowledge of their composition and possible ranges of compositional variation. The former case is much more tractable than the latter. Detection of foreign proteins added to milk of a certain species by exploiting differences in their electrophoretic mobilities is an example where a fundamental dissimilarity

is used to check the purity of the product. If no tangible differences exist, compositional data of authentic samples have to be gathered, taking into account all possible natural variations, e.g. due to breed, stage of lactation, production systems, geographical origin, etc. The authenticity of a product is confirmed when its compositional data fit into the data space represented by authentic samples. This type of testing usually depends on some form of statistical decision-making procedure. As of lately, chemometrics, the discipline concerned with application of multivariate statistical methods, as well as those methods based on mathematical logic, to chemistry (Brown *et al.*, 1992), are increasingly applied to compositional data in order to solve authenticity and classification problems.

10.2 Detecting and quantifying foreign fats

Milk represents a very complex physico-chemical system, where virtually all components present contribute information that is valuable for authenticity testing (Table 10.1). Clear-cut distinctions of different principles are of course not always possible, e.g. heat treatment results in an increased formation of Maillard products which are derived from the reaction of proteins with reducing sugars (lactose).

Milk fat (MF) is perhaps the most valued milk component and therefore has been the target for dubious manipulations for a long time. Traditional physico-

Table 10.1 Analytes of indicative value for the detection of adulteration of milk and milk products

Milk component	Source of adulteration	Analyte(s)
Fat	Non-dairy fat or oil Buttermilk added to milk	Fatty acids Triglycerides Phospholipids Sterols Fat-soluble vitamins
Protein	Non-dairy proteins Milk of a different species Whey added to milk Heat load	Caseins Whey proteins Glucomacropeptide Casein bound-P Protein-N Denatured proteins
Lactose	Water Heat load	Freezing point Furosin Lysinoalanin HMF Glycosylated proteins
Minerals	Water	Freezing point

chemical methods to verify the authenticity of MF, e.g. by determining the iodine value (a measure of the total unsaturation of a fat), Reichert-Meissel value (titrimetric determination of steam-volatile, water-soluble fatty acids (FA)), or Polenske value (titrimetric determination of steam-volatile but water-insoluble fatty acids) are only successful to recognise massive adulteration of MF, or even its substitution by another fat (Collomb and Spahni, 1991). With the advent of gas-liquid chromatography (GLC) techniques, the classical fat values were substituted by the analysis of the complete FA spectrum.

Strategies to detect adulterated MF are based either on the concentration ranges of individual FA or on the concentration ratios of two or more FA. A large number of different FA ratios have been proposed (Fox *et al*., 1988; Hughebaert and Hendrickx, 1971; Muuse *et al.*, 1986; Toppino *et al.*, 1982; Ulberth and Rogenhofer, 1989; Younes and Soliman, 1986). The effectiveness of 19 such indices was compared using the FA composition of a large number of authentic MF samples (Ulberth, 1994). The addition of vegetable fats or oils was easier to detect than commingling of animal fats. Additions of coconut fat or linoleic acid-rich vegetable oils (sunflower seed oil, corn oil, safflower oil, etc.) were traced down to a level of 2% by the ratios C12:0/C10:0 and C14:0/C12:0, and C18:2/C8:0, respectively. At a level of 10% commingling, palm oil and olive oil were detected by using C14:0/C18:2 and C18:2/C8:0 ratios. At 5% commingling, 50% of the adulterated samples passed the test undetected. Tallow was particularly difficult to detect, the ratio C16:0/C14:0 being the most suitable. However, it was possible to detect only 15% of the cases where 5% tallow had been added to MF. If the information content of a FA chromatogram was exploited in a more efficient way, i.e. subjected to linear discriminant analysis instead of forming FA ratios, more than 95% of cases where either tallow, lard, olive oil or palm oil were added to MF at the 3% level were correctly classified (Ulberth, 1994).

FA ratios have been used with success to discriminate between MF of different species (bovine, ovine and caprine milk). Iverson and Sheppard (1989) used the C12:0/C10:0 ratio to detect addition of bovine to ovine or caprine cheese milk. For instance, the ratio for bovine MF averages around 1.16, while it is 0.46 for caprine MF and 0.58 for ovine MF. This ratio was employed to indicate the level of cows' milk in cheeses labelled as goats' or ewes' milk cheese. Other FA indicators, mostly based on the ratio of a medium-chain and a volatile, short-chain FA (e.g. C14:0/C8:0), have been proposed and were summarised by Ramos and Juarez (1984). These authors also reported that the limit of detection for cows' MF in mixture with goats' or ewes' MF is 5–10%. Applying a more sophisticated methodology (pattern recognition techniques) to evaluate certain FA ratios (C14:0/C8:0, C14:1/C8:0 and C14:1/C16:1) an even more sensitive limit for the detection of cows' milk in ewes' milk cheese was established (Schwaiger and Vojir, 1995).

The difficulty of detecting fat of animal origin added to MF has led to the development of so-called triglyceride (TG) formulae for MF purity control. Originally, Timms (1980) described an approach using the information content

inherent to the TG profile of MF and combined it with a multivariate evaluation of the results to allow the determination of non-MF, including animal depot fats, in MF down to a level of 5%. His basic idea was further refined by Precht and co-workers (Precht and Heine, 1986a, 1986b; Precht, 1992a, 1992b). Several collaborative studies organised by the EC demonstrated the general applicability of the approach (Precht, 1992c). It was adopted as a reference method for the detection of foreign fats in MF within the EC (Commission Regulation (EC) No 213/2001). Based on the TG profile of 755 different MF, so-called *S*-values were derived by regression analysis. The *S*-values for authentic MF fluctuate within a certain range. If these limits are transgressed, the presence of a foreign fat can be assumed with a given level of statistical confidence. All types of foreign fats can be detected using the formula:

$$S = -2.7575*C26 + 6.4077*C28 + 5.5437*C30 - 15.3247*C32 + 6.2600*C34 + 8.0108*C40 - 5.0336*C42 + 0.6356*C44 + 6.0171*C46$$

For authentic MF the *S*-value for the 'total formula' fluctuates within a range of 95.68 to 104.32 (99% confidence level). Typical values for the limit of detection are 4.5–5.0% for vegetable fats (soybean oil, olive oil, palm oil, etc.), 4.7% for lard and 5.4% for tallow. For a number of foreign fats (e.g. coconut fat, palm oil, lard, etc.) particular TG formulae have been developed which are more sensitive and allow detection at a level of 2–3% adulterant.

The validity and applicability of the TG formulae have been confirmed by others (Collomb *et al*., 1998a; Luf, 1988; Povolo *et al*., 1999; Ulberth *et al*., 1998; Van Renterghem, 1997). Although packed column GLC has been used to establish the TG formulae, certain types of capillary columns are fully equivalent and can be used without impacting the approach (Collomb *et al*., 1998b; Molkentin and Precht, 1994; Ulberth *et al*., 1998). Besides multiple linear regression analysis, other ways of multivariate treatment of TG data to detect foreign fats have been suggested (principal components analysis, partial least-squares regression, artificial neural networks), but have not found widespread acceptance (Collomb *et al*., 1998c; Lipp, 1996a, 1996b). Intensive lipolysis of MF, e.g. in (over)matured cheese, may lead to false-positive results when the TG formulae are applied, highlighting the need for the development of special formulae taking into account fat degradation (Battelli and Pellegrino, 1994). The TG formula approach to verify the authenticity of bovine MF was recently extended to caprine MF (Fontecha *et al*., 1998).

Sterol analysis is a straightforward way to detect vegetable fats added to MF, since phytosterols do not occur in MF in measurable amounts. Detection of β-sitosterol, stigmasterol, campesterol, etc. is taken as an unequivocal proof that a vegetable fat is present. However, care has to be exercised in drawing correct conclusions as minor components (e.g. lanosterol) in chromatograms of the unsaponifiable part of MF may elute closely to β-sitosterol (Homberg, 1991). Currently, two methods standardised by IDF exist for the determination of sterols in MF; one is based on the difference in melting points of phytosteryl

acetate and cholesteryl acetate (IDF Standard 32: 1965), the other on a GLC procedure (IDF Standard 54: 1970). A similar GLC procedure to detect β-sitosterol and stigmasterol was also described by the EC (Commission Regulation (EC) No 213/2001). As an alternative to the lengthy sample preparation described in those standards, a hyphenated LC-GC technique was introduced (Kamm *et al*., 2002). The limit of detection for the determination of β-sitosterol via LC-GC was found to be 2 mg/kg fat. This is considerably lower than detection limits of 40 mg/kg and 10 mg/kg, respectively, reported for the convential procedures (Homberg and Bielefeld, 1979). Considering the amount of β-sitosterol present in rapeseed oil (*ca.* 4000 mg/kg), this indicates that an addition of about 0.05% rapeseed oil to MF would be detectable. An adulteration at such a low level is of no practical concern. However, additions of only 1–2% vegetable oil will be detected with certainty. Even for palm oil, an example for a vegetable oil exhibiting only a relatively low content of β-sitosterol (200–400 mg/kg), the resulting limit of detection (0.5–1%) would be sufficient.

Misbranding of spreadable fats, which contain MF and suitable non-MF, is another problem area. Products introduced on the spreadable fats market within the EU must comply with Council Regulation (EC) No 2991/94. Up to now, no official method for the determination of the MF proportion in the fat blend has been specified. Usually, butyric acid (C4:0), which occurs exclusively in MF of ruminant animals, is used as a marker to estimate the amount of MF in the blend. A number of reliable methods have been suggested for the determination of C4:0 in spreadable fats by GLC (Molkentin and Precht, 1998a, 1998b; Pocklington and Hautfenne, 1986; Ulberth, 1998a, 1998b) or by HPLC (Christie *et al*., 1987). A major drawback of the approach is the natural variation of the C4:0 content in MF (Molkentin and Precht, 1997). For a representative number of samples taken all over Europe the figures varied between 3.07 g and 3.75 g per 100 g MF, with a mean value of 3.42 g per 100 g. The variations were due to differences in feeding regimen, lactation stage and breed. By using the average content of C4:0 in MF the proportion of MF in an unknown fat blend may thus deviate to up to $\pm$10% from the true value, without taking into account any additional analytical errors (Molkentin and Precht, 1998b). When a sample of the MF used for blend formulation is available for analytical testing, the performance of the method in terms of accuracy and precision can be improved. The EU Expert Group 'Milk and Milk Products' (Agriculture DG) has collaboratively tested the latter methodology in order to gain precision data. Reproducibility of the method was 1.7% MF for a mixture containing 25% MF, and 3.2% MF for a mixture containing 60% MF (Molkentin and Precht, 2000).

Alternatives to chromatographic techniques for MF authentication, such as differential scanning calorimetry (Bringer *et al*., 1991; Coni *et al*., 1994) and infra-red spectroscopic techniques (Sato *et al*., 1990; Laporte and Paquin, 1998), were proposed, but have not found wide application. In particular, spectroscopic techniques would be highly welcome, since they do not need lengthy sample preparation and therefore have a high throughput, and are non-destructive testing methods.

10.3 Detecting milk of different species

In most countries producers of dairy products are required to label the milk type (bovine, ovine, caprine) used for manufacture. Since the production volume of ovine and caprine milk is much smaller and their supply varies to a considerable extent, an incentive for economic adulteration exists. Moreover, certain traditional products that are highly valued by consumers on a worldwide scale, such as Mozzarella, Roquefort, Manchego, Pecorino or Feta cheese, are exclusively made from non-bovine milk. Hence, analytical methods are needed to check for the presence of cows' milk in products declared to be made solely from ewes', goats' or water-buffaloes' milk. In some production areas the addition of goats' milk to ewes' milk used for the production of traditional cheese varieties could also be an issue.

Differences in the molecular make-up of milk proteins are the primary route to discriminate milk of different species. Various forms of electrophoresis, chromatography and immunochemistry are used as analytical tools to track down those differences. The protein-based methods may be supplemented by the analysis of the fat phase (FA ratios, TG profile) as described in the preceding chapter.

Separation of milk proteins by various forms of electrophoresis is one of the most widely applied techniques in dairy products authentication. Early attempts focused on the higher electrophoretic mobility in polyacrylamide gels (PAGE) of the α_{s1}-casein of bovine milk as compared to ovine and caprine milk (Aschaffenburg and Dance, 1968; Foissy, 1976; Freimuth and Krause, 1968). A limit of detection of 1% of cows' milk in goats' milk was reported by those authors. As the caseins are partially degraded during cheese ripening, newly formed peptides obscure to a certain extent the region of the bovine α_{s1}-casein. As a result the sensitivity of the method drops, and data interpretation becomes much more difficult. Discontinuous electrophoresis of β-caseins was used as an alternative by Mayer and Hörtner (1992) for the determination of bovine caseins in milk and dairy products.

Due to its high separation efficiency, isoelectric focusing in thin polyacrylamide gels (PAGIF) has become a preferred technique for the separation of complex protein mixtures. Applying this technique, Krause *et al*. (1982) made use of the γ-caseins, proteolytic breakdown products of β-casein, as an indicator for the detection of an admixture of bovine to ovine and caprine milk and cheese. Ovine and caprine milk cannot be distinguished by this technique. The addition of plasmin to cheese caseins in order deliberately to create γ-caseins and their subsequent separation by PAGIF greatly enhances the sensitivity of the method (Addeo *et al*., 1990a). It has been officially adopted for the control of cheese within the EU (Commission Regulation (EC) No 213/2001). Evaluation is performed by comparing the protein patterns of the unknown sample with reference standards on the same gel. Detection of cows' milk in cheeses from ewes', goats' or water-buffaloes' milk and mixtures of ewes', goats' and buffaloes' milk is done via the γ_3- and γ_2-caseins, whose

isoelectric points range between pH 6.5 and pH 7.5. The limit of detection is less than 0.5% of cows' milk. The method is suitable for a sensitive and specific detection of native and heat-treated cows' milk and caseinate in fresh and ripened cheeses made from non-bovine milk, but it is not suitable for the detection of milk and cheese adulteration by heat-treated bovine whey protein concentrates.

PAGIF of para-κ-caseins allows not only the detection of an addition of bovine milk to cheese from other species but also the differentiation between cheese of ovine or caprine origin. However, in the case of ripened Roquefort cheese, a peptide migrating with cows' milk para-κ-casein was identified, leading to a false-positive response (Addeo *et al*., 1990b). Such difficulties were not noticed in another study with Camembert made from milk of different species (Mayer *et al*., 1997). The method can be extended to other dairy products. The addition of rennet to ewes' yoghurt to produce para-κ-caseins artificially and subsequent separation of the caseins by cationic PAGE allowed the detection of cows' milk down to 1% (Kaminarides and Koukiassa, 2002).

Whey proteins are not markedly altered by proteolysis during cheese ripening. In order to adequately exploit this feature, separation systems have been set up based on differences in electrophoretic mobilities of whey proteins, in particular of β-lactoglobulin (Addeo *et al*., 1989; Amigo *et al*., 1991; Rispoli *et al*., 1991). When silver nitrate was used for staining, cows' milk at a level of 1% was detectable in various types of cheese (Amigo *et al*., 1991). The drawback of the method is that heat treatment denatures whey proteins to a variable extent, thus affecting the test results. For example, heating to 90ºC for 30 min denatured the whey proteins and gave negative results when cows' milk was added to milk for cheese making (Amigo *et al*., 1991).

The increased availability of commercial capillary electrophoresis instrumentation has led to an increased transfer of traditional electrophoretic assay formats to this novel technique. The high resolution power and the speed of analysis are its most attractive features. Both the casein fraction and the whey proteins can be analysed and used for authentication purposes. As is the case with traditional PAGE, α_{s1}-casein of bovine milk had the highest mobility among the different caseins by applying capillary zone electrophoresis (CZE) in an uncoated tube, which proved to be useful for the detection of cows' milk in goats' milk (Lee *et al*., 2001). However, caprine para-κ-caseins and bovine β-casein were also found to be good markers for the presence of the milk of these species in Iberico-type cheese (Molina *et al*., 2000). The differences between the CZE patterns of the casein fraction of bovine, ovine and caprine milk allowed identification and even quantification of the milk of each species in binary and ternary mixtures by multivariate regression analysis (Molina *et al*., 1999). The mean errors in prediction were lower than 3% in all cases. A similar chemometric approach was reported by Vallejo Cordoba (1998).

Whey proteins of different species were successfully separated by CZE and can also serve as authenticity indicators (Cartoni *et al*., 1999; Cattaneo *et al*., 1996; Recio *et al*., 1995). The ratio of the corrected peak areas of bovine β-

lactoglobulin B to ovine α-lactalbumin was linearly related to the amount of cows' milk present within a range of 0–20% (Cartoni *et al*., 1999).

High performance liquid chromatography (HPLC) is another route to protein separation and was effectively used to determine individual milk proteins. Different chromatographic profiles are obtained for the proteins from different species. Ion-exchange as well as reversed-phase (RP) columns were applied to fractionate either the caseins or the whey proteins or total milk proteins. Bovine, ovine and caprine para-κ-caseins were baseline separated by cation-exchange HPLC and used for quantifying the mixture, i.e. the proportions of the milk types used for cheese making (Mayer *et al*., 1997). Others used RP columns to separate primarily α-, β- and κ-casein fractions of different species (Bordin *et al*., 2001; Urbanke *et al*., 1992; Veloso *et al*., 2002). By analogy to the official EC method, Volitaki and Kaminarides (2001) added plasmin to the isolated caseins to intensify the γ-casein fraction and separated the mixture by RP-HPLC. The caseinomacropeptides of different species are separable by HPLC and can serve as markers (Lopez Fandino *et al*., 1993). HPLC analysis of whey proteins is also of interest for species discrimination (Bobe *et al*., 1998a; de Frutos *et al*., 1991; de Noni *et al*., 1996; Romero *et al*., 1996).

When quantitative aspects regarding mixture proportions are considered, the different casein contents of bovine, caprine and in particular ovine milk used for cheese making have to be taken into account. Since ewes' milk has a much higher casein content than cows' milk, the resulting relationships are non-linear (Addeo *et al*., 1990a, 1990b; Mayer *et al*., 1997).

Recently, new strategies for the structural analysis of milk proteins based on mass spectrometric technologies, in particular matrix-assisted laser desorption–time of flight mass spectrometry (MALDI–TOF), have been developed. Owing to its speed and the minimum of sample preparation required, the MALDI–TOF technique is very attractive. Using particularly α-lactalbumin and β-lactoglobulin as markers, addition of cows' milk to the milk of other species and to water-buffalo Mozzarella were easily detected by MALDI–TOF (Angeletti *et al*., 1998; Cozzolino *et al*., 2001, 2002; Fanton *et al*., 1998).

Due to their excellent sensitivity and minimal sample preparation requirements, immunochemical methods have found wide acceptance for the discrimination of milk of different species. Various assay formats and antibodies directed against different antigens have been described (Table 10.2). Many of the assays target bovine β-casein, as this fraction has the highest allergenic potential of all caseins (Anguita *et al*., 1996a). The limit of detection for most of the assays is 0.1–1.0% depending on the assay format. If polyclonal antibodies were used, they were usually purified by affinity chromatography to eliminate cross-reactivity. Commercial test kits are now on the market and have found wide application.

Immunological methods can fail when the targeted antigen is substantially degraded by either heating or proteolysis. DNA from somatic milk cells (mostly leucocytes) is suggested to persist in ripened cheese and may be amplified by polymerase chain reaction (PCR) and analysed for species discrimination. Plath *et al*. (1997) used primers encoding a partial sequence of the β-casein gene to

Table 10.2 Immunochemical assay formats used for the discrimination of milk of different species

Assay	Antibody	Antigen	Reference
Competitive indirect ELISA	Polyclonal	Bovine γ-caseins	Richter *et al*. (1997)
Competitive indirect ELISA	Polyclonal	Native and heat denatured bovine β-lactoglobulin	Beer *et al*. (1996)
Indirect ELISA	Monoclonal	Bovine β-casein	Anguita *et al*. (1995)
Indirect ELISA	Monoclonal	Bovine α_{s1}-casein	Rolland *et al*. (1993)
Indirect ELISA	Monoclonal	Caprine α_{s2}-casein	Haza *et al*. (1996)
Indirect ELISA	Polyclonal	Bovine caseinomacropeptide	Bitri *et al*. (1993)
Indirect ELISA	Polyclonal	Caprine whey proteins	García *et al*. (1994)
Sandwich ELISA	Monoclonal	Bovine β-lactoglobulin	Levieux and Venien (1994)
Sandwich ELISA	Polyclonal	Bovine caseins	Rodriguez *et al*. (1993)
Immunostick ELISA	Monoclonal	Bovine β-casein	Anguita *et al*. (1996b)
Western blotting	Monoclonal	Bovine β-lactoglobulin	Molina *et al*. (1996)

detect the corresponding genomic DNA in milk and cheese. The PCR product from ovine or caprine β-casein DNA contained a specific restriction enzyme site that was not present in bovine β-casein DNA. After restriction enzyme analysis and subsequent separation of the fragments by PAGE, the undigested bovine β-casein fragment was detected as an additional band if cows' milk was present. A similar approach was described for the identification of water-buffalo, bovine, ovine and caprine milk in cheese, based on amplification of a 359 bp fragment of the cytochrome-b gene and restriction fragment chain length polymorphism analysis (Branciari *et al*., 2000). A single-step PCR method with bovine-specific primers for a fragment of the cytochrome-b gene to detect cows' in goats' milk was described by Bania *et al*. (2001).

Genomic DNA was extracted from cheese and PCR double-stranded amplifications were conducted using various suitable sets of primers for species-specific DNA amplification to detect the milk source (bovine, ovine and caprine) in cheese (Calvo *et al*., 2002). A duplex PCR was developed to identify cows' milk and buffaloes' milk in cheese products, particularly in buffalo Mozzarella cheese (Bottero *et al*., 2002).

Mitochondrial (mt) DNA shows also species-diagnostic sequence variations and, on top of that, the number of copies of mtDNA is much higher than that of genomic DNA. Cow-specific primers were designed to target the control region of mtDNA and the resulting PCR product of 413 bp separated by agarose gel electrophoresis. The limit of detection of cows' milk in goats' cheese was less than 0.1% (Maudet and Taberlet, 2001).

A DNA-based technique which combines PCR, ligase chain reaction (LCR) and an enzyme immunoassay (EIA) to detect the presence of cows' milk in

ewes', goats' and buffaloes' milk and corresponding cheeses was developed by Klotz and Einspanier (2001). It is based on subtle differences in the β-casein gene of cow, sheep, goat and buffalo species. DNA, extracted from milk or cheese samples, served as a template to amplify a universal β-casein PCR product. Subsequently, LCR with species-specific primers was performed using the PCR product as a template. LCR primers were labelled with biotin or digoxygenin for further sensitive detection by EIA. This screening technique allowed clear discrimination of cow species from sheep, goat and buffalo species in milk and cheese.

The sensitivity of the DNA-based methods is very high; limits of detection are reported to be better than 0.1% of the targeted species. However, quantification of mixture proportions seems to be difficult as the source of the DNA is somatic milk cells. It is well known that the somatic cell count is affected by a number of factors (number and stage of lactation, udder health) which are out of control.

10.4 Detection of non-milk proteins, watering of milk and alteration of the casein/whey protein ratio

Non-dairy proteins of vegetable or animal origin are generally cheaper than milk proteins and are sometimes added to extend the product (economic adulteration) or because of their functional properties. In particular, soy protein has good water holding and binding capacity and therefore can improve the texture of a product (e.g. soft cheese). A number of electrophoretic (Cattaneo *et al*., 1994; Kanning *et al*., 1993; Manso *et al*., 2002), chromatographic (Cattaneo *et al*., 1994; Espeja *et al*., 2001) and immunochemical (Turin and Bonomi, 1994; Sanchez *et al*., 2002) methods have been devised to detect the addition of non-milk proteins.

10.4.1 Watering of milk

Addition of water to a beverage is the epitome of food adulteration. Dilution with water alters the density of milk, the refractive index of the lactoserum and, most importantly, its freezing point. The thermistor cryoscopic determination of the freezing point of raw milk is probably the most widely and frequently applied technique for food authenticity testing. The freezing point of authentic raw milk varies only within narrow limits. Breed, stage and parity of lactation, feeding regimens, udder health, production region, season and milking time are seen as the most important factors influencing the freezing point (Buchberger, 1994, 2000; Rohm *et al*., 1991; Wiedemann *et al*., 1993). Mean values were reported for different countries in a range between -0.5310ºC and -0.5209ºC (Buchberger, 1990; Coveney, 1993; Rohm *et al*., 1992; Slaghuis, 2001). The procedure for the determination of the freezing point has been standardised (IDF Standard 108B:1991; Commission Decision 91/180/EEC). Although the method

is considered to be robust, a number of operating parameters may influence the test result and have to be controlled carefully (Rohm, 1993).

10.4.2 Alteration of the casein/whey protein ratio

The by-product of cheese making, rennet whey, is of low value but the volumes produced abound. Therefore, it is tempting to add whey fraudulently to other dairy products. Advances in membrane filtration technology opened up interesting possibilities to split skim milk into different protein fractions to give products with an added value on one hand and less valuable fractions on the other (Creamer *et al*., 2002). Furthermore, protein standardisation of milk and milk products is now permitted, provided that milk components only are added or removed and the ratio of casein to whey protein is not altered. Therefore, reliable methods are needed to check the protein composition of dairy products. The methods proposed are either indirect, for example by determining certain protein fractions not as such but in terms of an inherent characteristic feature, or direct, for example using electrophoretic or chromatographic methods to separate the protein mixture into individual components.

Whey proteins contain significantly more sulphur-containing amino acids (cysteine and cystine) and more aromatic amino acids (tryptophan, tyrosine and phenylalanine). Additions of whey proteins therefore increase those values as compared to the genuine product. The former can be determined by a modified ninhydrin reaction (De Koning and Van Rooijen, 1971) or by polarography (Mrowetz and Klostermeyer, 1976; Lechner and Klostermeyer, 1981). The limitation of the polarographic method, although very effective and reliable, is that it uses hazardous chemicals (methylmercury chloride). A very elegant way to determine the casein/whey protein ratio is the application of derivative spectroscopy. Second-order (Luf and Brandl, 1987) and even fourth-order derivatives (Lüthi-Peng and Puhan, 1999; Meisel, 1995; Miralles *et al*., 2000) have been proposed. The method quantifies aromatic amino acid residues of milk proteins and is unaffected by other absorbing non-protein material in the sample solution. The different content of protein-bound phosphorus of casein and whey protein is the basis of an effective though laborious testing principle that detects whey additions (Wolfschoon-Pombo and Furtado, 1989).

Direct measurement of all relevant protein fractions by electrophoretic (Basch *et al*., 1985; Meisel and Carstens, 1989; Miralles *et al*., 2000) or chromatographic (Bobe *et al*., 1998b; Bordin *et al*., 2001) methods is more laborious but gives a more detailed insight. The casein to whey protein ratio can be determined directly from the chromatographic trace obtained, after careful calibration using reference compounds.

The caseinomacropeptide (CMP), which results from the cleavage of κ-casein during renneting of milk, is a good indicator for the presence of rennet whey. Two methods based on gel-filtration HPLC have been adopted by the EC (Commission Regulation (EC) No 213/2001) to check skim milk powder for the

presence of rennet whey powder. Instead of UV detection, pulsed electrochemical detection of CMP, which was very sensitive and selective, was proposed (Van Riel and Olieman, 1995a). As an alternative to the HPLC procedures, CMP was determined by capillary electrophoresis (Recio *et al*., 2000; Van Riel and Olieman, 1995b). Proteolytic activity, particularly from psychrotrophic bacteria, during cold storage of milk produces peptides similar to CMP, which may interfere with CMP detection. This could give rise to false-positive results (Martinez Penagos *et al*., 1993; Recio *et al*., 1996). Other, less often applied techniques to estimate the casein/whey protein ratio are photoacoustic spectroscopy (Doka *et al*., 1999) and pyrolysis mass spectrometry (Schmidt *et al*., 1999).

10.5 Measuring heat load

The primary aim of heating milk is to ensure its microbiological safety and stability. However, heating of milk profoundly alters the physico-chemical state of its components, leading primarily to the denaturation of certain vulnerable protein fractions (immunoglobulins, enzymes, whey proteins) and the formation of so-called browning products (Maillard reaction).

According to Council Directive 92/46/EEC pasteurised milk is obtained by heat treatment, at least 71.7ºC for 15 seconds, or any other temperature–time combination producing an equivalent effect. Pasteurised milk has to show a negative reaction to the phosphatase test and a positive reaction to the peroxidase test. However, the production of pasteurised milk which shows a negative reaction to the peroxidase test is authorised, provided that the milk is labelled as 'high-temperature pasteurised'. The required tests (phosphatase and peroxidase) have been standardised (Commission Decision 91/180/EEC); alternative, more rapid testing methods (e.g. Fluorophos®, Reflectoquant®) have also been proposed (Berger *et al*., 2001; Lechner, 1996).

Higher heat loads result from a number of other processes applied during manufacture of dairy products, primarily UHT treatment, sterilisation, concentration by water evaporation and drying. Therefore, methods are needed that can (i) discriminate between the severity of heat treatment applied, and (ii) detect products with a high heat load that have been added to other milk products (e.g. addition of dried milk to fluid milk).

The American Dry Milk Institute (ADMI) has standardised a turbidimetric method developed by Harland and Ashworth to distinguish between different heat loads (low, medium, high) in skim milk powder (ADMI, 1971). In this test, casein and heat-denatured whey proteins are precipitated with NaCl at neutral pH; the supernatant is then acidified to coagulate the native whey proteins and the resulting turbidity is taken as a measure for the content of non-denatured whey proteins. More recently, the amount of non-denatured, soluble whey proteins has been quantified directly by HPLC procedures (IDF Standard 178: 1996; Kneifel and Ulberth, 1985; Resmini *et al*., 1989; Villamiel *et al*., 2000).

Immunochemical methods were also successfully employed to determine heat-denatured whey proteins (Jeanson *et al*., 1999; Rosenthal *et al.*, 1999).

Methods based on the determination of native whey proteins are particularly suited to discriminate products with rather low heat load (pasteurisation conditions). The presence of several Maillard reaction products such as lactose isomerisation and protein-glycation are indicative of severe heat treatment. The two main markers are furosine and lactulose. Furfurals are another group of heat treatment indicators, which have found wide application not only in the dairy industry (Albalá-Hurtado *et al*., 1997; Ferrer *et al*., 2000), but throughout the food industry.

Furosine is an amino acid obtained by acid hydrolysis of glycosylated proteins, in particular of the lysine–lactose adduct formed in the Maillard reaction. It can be determined by HPLC (Hartkopf and Erbersdobler, 1993; Henle *et al*., 1995; Nicoletti *et al*., 2000; Resmini *et al*., 1990) or by capillary electrophoresis (Corradini *et al*., 1996). Furosine has been used not only to distinguish between different types of heat treatment (Clawin-Raedecker *et al*., 2000; Pellegrino *et al*., 1995; Villamiel *et al*., 2000), but also to determine whether milk powder has been added to fluid milk or as an indicator for reconstituted milk (Ohta *et al*., 2002; Van Renterghem and De Block, 1996).

Lactulose is formed by isomerisation of lactose during the heating of milk, and has been proposed as an analytical index to distinguish UHT from sterilised milk (Clawin-Radecker *et al*., 1992); it is not found in pasteurised milk. A variety of methods were used for its determination: GLC (Martinez-Castro *et al.*, 1987), HPLC (Cataldi *et al.*, 1999; IDF Standard 147: 1991), capillary electrophoresis (Soga and Serwe, 2000), enzymology (Amine *et al*., 2000a; Kuhlmann *et al*., 1991), colorimetry (Amine *et al*., 2000b) and continuous-flow amperometry (Mayer *et al*., 1996; Moscone *et al*., 1999).

Another Maillard reaction product, lysinoalanine (LAL), was shown to be a sensitive indicator for heat treatment of milk and for addition of dairy-based substitutes rich in LAL (caseinates, etc.) to other milk products, in particular cheese (Faist *et al*., 2000; Moret *et al*., 1997; Pellegrino *et al*., 1996). Maillard products have fluorescent properties and this feature was used for a very sensitive and rapid determination of the heat load, which was in good agreement with more established procedures (Birlouez-Aaragon *et al*., 2002). Novel strategies for the estimation of heat load are the direct determination of glycosylated proteins, either by immunology (Pallini *et al*., 2001), by HPLC (Pellegrino and Cattaneo, 2001) or by mass spectrometry (Cozzolino *et al*., 2001).

10.6 Identifying geographical origin

Products manufactured in a particular way in a specific geographical region have always found a following, although they usually command a higher price. Marketing of agricultural products has recently focused on promotion of premium goods in affluent countries. Traditional cheese varieties such as

Camembert, Parmesan or Stilton are in high demand, and to protect their market legal instruments have been introduced in the EU (Council Regulation (EEC) 2081/92). To be eligible to use a protected designation of origin (PDO) or a protected geographical indication (PGI), an agricultural product or foodstuff must comply with strict specifications. On-site inspections by a control authority are currently the only accepted way to safeguard the PDO/PGI label, as reliable and validated analytical testing methods do not exist yet.

The most promising approach seems to be to characterise the products by determining stable isotope ratios ($^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{16}O/^{18}O$) and subsequent application of mathematical pattern recognition techniques. This has been applied to the characterisation of the geographical origin of milk (Kornexl *et al*., 1997), butter (Rossman *et al*., 2000) and Pecorino Sardo cheese (Manca *et al*., 2001). It was found that the feeding regimen, in particular maize silage, can influence the $^{13}C/^{12}C$ ratio, use of industrial fertilisers the $^{15}N/^{14}N$ ratio, and the water supply the $^{16}O/^{18}O$ ratio of milk and milk products.

A number of chemical (fat content and pH value), biochemical (L- and D-lactate, and pyruvate), microbiological (lactobacilli and enterococci), colour and sensory parameters were investigated to discriminate between Emmental cheeses of different origin (Pillonel *et al*., 2002). Although some promising results have been obtained, the analytical approach is in its infancy and much needs to be done to give a reliable indication that verifies the origin of a product.

10.7 Conclusions

Detection of fraud is complicated by the fact that the quantities of certain indicators vary due to biological, climatic, agronomic and temporal factors. Moreover, processing can dramatically change the composition of minor constituents. Therefore, too stringent specifications cannot be set by food inspection, as this will eventually increase the number of false-positive results. Since unscrupulous manufacturers or vendors have developed an excellent understanding of the underlying principles to detect fraud, they have managed in many cases to tailor blends in such a way that they comply with product specifications.

In many cases no fundamental differences, ideally the lack or presence of a product-specific component, between the genuine and the adulterated product exist. Consequently, purity criteria have to be empirically determined by analysing a wide array of genuine products and creating and regularly updating a database holding information about the concentration ranges of certain indicative components of the commodity concerned. In order to solve difficult cases more than one analyte has to be considered for detecting fraud. Likewise, a combination of different analytical techniques to determine dissimilar characteristics of a commodity (e.g. a combination of spectroscopic and chromatographic methods) could be more useful than relying on one single methodology. Given the complexity of some problems, univariate statistics

(measures of location and dispersion) have to be substituted by intricate statistical algorithms to aid in pattern recognition and classification of genuine and adulterated products. The merits of such procedures, though scientifically sound, are difficult to comprehend for those not familiar with advanced statistical data interpretation techniques, and might, therefore, find little acceptance in a court of law.

The challenge for food law enforcement agencies is to be a step ahead and to develop constantly new methods to get a better insight into the complex chemical mixture representing food, in order to identify a set of possible marker components for authentication purposes.

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