14

Rapid on-line analysis to ensure the safety of milk

A. Amine, Université Hassan II-Mahammedia, Morocco and L. Micheli, D. Moscone and G. Palleschi, Università di Roma 'Tor Vergata', Italy

14.1 Introduction

With the increasing requirement for pre-prepared and processed food, as consumers become less willing to spend significant periods of time preparing fresh food, problems with the quality and safety of prepared or processed foods will increase. A major issue is food safety, highlighted by recent outbreaks such as those concerning BSE, foot and mouth, and *E coli* 0157. There is a general and urgent need for rapid procedures, applicable to process control, to monitor food safety and quality. This chapter addresses this issue in relation to milk quality. It first discusses the safety of the product at the initial point of product collection: the farm. It then looks at ensuring safety and quality during processing by monitoring a number of key parameters, and investigates the possibilities of new markers of product quality.

14.1.1 Contamination monitoring in the milking parlour

Concerns over milk contamination are growing as the labour available for milking cows diminishes and milking systems are increasingly automated. Companies and research institutions within the EU lead the world in the development and introduction of robotic milking. Although high standards have been set for animal welfare and milk hygiene by legislation, the technical means of achieving these standards are still lacking. Close control, in real time, of milk contamination markers will improve the milking system by allowing operators to make quick decisions about milk quality, ensuring that freshly-collected milk is hygienically acceptable for human use.

The two most important contamination factors in fresh collected milk are faecal contamination and mycotoxin contamination. As a result of the cow's anatomy and the farm environment, faecal contamination during the production of milk is an enduring problem. There is a need to avoid introducing undesirable colour and flavouring taints into the milk, combined with the ever-present risks associated with the introduction of faecal pathogens. Milk is also susceptible to contamination from external sources. One of the most important hazards is aflatoxin M_1, a hepatocarcinogenic mycotoxin that could occur in the milk of cows fed with aflatoxin B_1-contaminated feedstuffs (Miraglia, 1998).

14.1.2 Monitoring processed milk

There is a need to define and develop fast and reliable procedures to identify markers of milk quality in order to distinguish effectively-processed milks (heated/lactose hydrolysed) from those for which treatments have been omitted or carried out unsuccessfully. Developments in dairy technology have introduced new heating processes and new time/temperature combinations for the thermal treatment of milk (for example thermized, pasteurised, UHT in bottle sterilised). This trend is leading to the availability of milks with different characteristics, which may be marketed under the same designation (EU Commission Doc VI/5726/92, 1992; EU Directive 92/46, 1992; Pellegrino *et al*., 1995).

Some thermal markers, such as alkaline phosphatase (ALP) and lactoperoxidase (LPO), are already in use as indices of adequate milk heat treatment (EU Commission Doc VI/844/93, 1993; Griffiths, 1986; IDF Doc 442, 1990; IDF, 1991). Other indices, such as lactulose and lactosilated proteins, have been proposed as indices of heat damage (Andrews, 1984; Burton, 1984; Erbersdoler and Dehn-Müller, 1989; Geier and Klostermeyer, 1983; Henle *et al*., 1991; O'Brien, 1995; Staal, 1986) and are still being studied by working groups of both the International Dairy Federation (IDF) and the European Union (EU) (EU Commission Doc VI/5726/92, 1992; EU Commission Doc VI/844/93, 1993; EU Directive 92/46, 1992; EU Commission Doc VI/CG/1018/94, 1994; IDF Doc 557, 1993a). There is therefore a continuing need for the development of fast and reliable thermal markers to monitor process effectiveness and product safety.

In this chapter we will report on a number of new procedures based on monitoring key markers for processed milk. These include rapid procedures for lactulose based on an electrochemical sensor and spectrophotometric analysis and an immunosensor procedure for the detection of lactosilated milk proteins. The detection of these marker compounds in fresh and processed milk has been carried out using biosensor and immunosensor technologies. Currently, not many bio- and immunosensor devices are used in the food industry. However, the increasing concern for more efficient detection of chemical contaminants and pathogens and their metabolites in milk has stimulated interest in the use of rapid methods of analysis based of biosensors and immunosensors (Turner, 2000).

14.2 Monitoring contamination during milking: faecal contamination and mycotoxins

The main point of contamination of milk by faecal material during milking is the teats of cows. Teats could be washed automatically within the teat cup and electronic cameras could be used to examine the cleanliness of the teats of cows standing in a stall (Bull *et al*., 1996; Mottram, 1997). Close inspection would require sensors mounted above a teat cup and an automated monitoring system. However if the quality/cleanliness of the wash water can be verified, the absence of milk contamination downstream can be assured without direct sensing. Chlorophyll derived from bovine faeces is a recognised marker of faecal contamination of milk. The development of a sensor for chlorophyll determination in washing water before milking is a part of an EU funded project: ROSEPROMILK (EU Project, 2002).

Spectroscopic techniques have been used successfully to detect contamination in marine and freshwater (Arar, 1997). However, they are not practical when dealing with the pre-milking washing water, which may contain absorbing components. More recently, the ROSEPROMILK team have developed an electrochemical sensor based on screen-printing technology for the rapid electrochemical determination of chlorophyll. Cyclic voltammetric studies using screen-printed carbon electrodes showed that chlorophyll could not be determined directly in a quiescent buffer solution. However, using a medium exchange protocol which involved substrate accumulation through stirring in phosphate buffer solution containing acetone, followed by brief rinsing in water and final cyclic or linear sweep voltammetry in a phosphate buffer, a clear peak response was obtained for chlorophyll at an applied potential of +400 mV versus Ag/AgCl. To obtain an optimum detection of chlorophyll required an accumulation for 60 s at open circuit in 0.1 M phosphate buffer pH 7.0 containing 1% acetone, followed by rinsing in water and cyclic voltammetry at 50 mV/s in 0.1 M phosphate buffer at pH 7.0. Calibration plots of chlorophyll concentration were linear over the range 0.25–2.25 μM ($r^2 = 0.998$). The oxidation peak measured at around +500mV is directly proportional to a concentration of chlorophyll in the submicromolar range. The pH has no effect on response of chlorophyll oxidation in the range of pH 5.0–8.0. The development of an operational on-line sensor needs further research

14.2.1 Mycotoxin contamination

Mycotoxins are a group of chemically diverse secondary fungal metabolites that induce a variety of toxic responses in humans and animals when foods or feeds containing these compounds are ingested. Aflatoxins are a class of mycotoxins produced by the fungal strains *Aspergillus flavus* and *Aspergillus parasiticus* during growth, harvest or storage. In particular aflatoxin B_1 has been implicated in lethal episodic outbreaks of mold poisoning in exposed human and animal

populations (Miraglia, 1998). Aflatoxin M_1 a hepatocarcinogenic mycotoxin, could occur in the milk of cows fed with aflatoxin B_1-contaminated feedstuffs.

The maximum content of aflatoxin M_1 allowed in milk under EU directives is 0.05 ppb (Rosner, 1998). The level of aflatoxin M_1 can be kept so low only if the analytical controls are accurate and sensitive. At present, aflatoxin analysis is carried out using ELISA kits for screening M_1 and B_1 (Biancardi, 1997), with confirmation by HPLC (which is the official method) using post-columns derivatisation after sample clean-up (Markaki and Melisseri, 1997). Dragacci *et al*. (2001) have reported on 'proficiency testing for the evaluation of the ability of EU-national reference laboratories to determine aflatoxin M_1 in milk at levels corresponding to the new EU legislation'. They tested samples of milk powder and liquid milk at various levels of aflatoxin M_1 contamination. Two trials were conducted in 1996 and 1998 according to ISO guide 43, in particular for the homogeneity testing of sample batches and for the calculation of laboratory z-scores. The samples used were naturally-contaminated milk obtained by feeding cows with aflatoxin B_1-contaminated feed. The levels of aflatoxin M_1 in the samples ranged from 0.2 to 0.7 μg/kg in milk powder and from 0.05 to 0.07 μg/kg in liquid milk. These levels were chosen as being close to the EU-regulated limit of 0.05 μg/kg of aflatoxin M_1 per litre. Results produced by the participating laboratories were compiled and statistically analysed to detect variations and to calculate the individual z-scores. Except for one laboratory in each exercise, all laboratories exhibited acceptable z-scores. The interlaboratory relative standard deviation for reproducibility (RSD_R) obtained were in the range 15.7–30.3%. Compared with other published studies, this indicated a very good precision for the performance of this laboratory network in the analysis of traces of aflatoxin M_1 in milk. Tables 14.1 and 14.2 report the raw data and their statistical summary in the 1998 exercise.

These aflatoxin M_1 tests were carried out using a HPLC procedure including an immunoaffinity clean-up step. However this procedure, standardised by the International Dairy Federation (IDF) in 1995 (IDF, 1995) and then by the ISO and CEN (ISO, 1998) is slow and requires highly-qualified personnel, expensive instrumentation and reagents. Recent research has concentrated on developing more rapid methods with a comparable level of sensitivity. Andreou and Nikolelis (1998) have reported the application of lipid-based biosensors for monitoring aflatoxin M_1 in milk and milk preparation. This is based on electrochemical flow injection monitoring of aflatoxin M_1 using stabilized systems of filter-supported bilayer lipid membranes. Injections of aflatoxin M_1 were made into flowing streams of a carrier electrolyte solution, and a transient current signal appeared less than 10 s after exposure of the lipid membranes to the toxin. The magnitude of this signal was linearly related to the concentration of aflatoxin M_1, with detection limits at the subnanomolar level. The mechanism of signal generation was investigated by differential scanning calorimetric experiments. Using this technique aflatoxin M_1 could be determined in continuous flowing systems with a rate of at least 4 samples min. Figure 14.1 reports some experimental results involving injection of aflatoxin M_1 into milk

Table 14.1 Raw data (blind duplicated 1 and 2) and laboratory means in the 1998 exercise. The results are expressed in μg of aflatoxin M_1L

	Batch A			Batch B		
Laboratory codes	Blind duplicate 1	Blind duplicate 2	Mean	Blind duplicate 1	Blind duplicate 2	Mean
1	0.038	0.031	0.035	0.067	0.073	0.070
2	0.037	0.036	0.037	0.056	0.058	0.057
3	0.163	0.180	0.172	0.210	0.284	0.247
4	0.053	0.053	0.053	0.081	0.079	0.080
5	0.046	0.044	0.045	0.075	0.071	0.073
6	0.042	0.059	0.051	0.104	0.081	0.093
7	0.030	0.025	0.028	0.072	0.055	0.064
8	0.033	0.042	0.038	0.076	0.056	0.066
9	0.042	0.043	0.043	0.063	0.063	0.063
10	0.058	0.054	0.056	0.082	0.085	0.084
11	0.053	0.049	0.051	0.074	0.075	0.075
12	0.046	0.045	0.046	0.069	0.071	0.070
13	0.048	0.061	0.055	0.082	0.078	0.080
14	0.041	0.050	0.046	0.080	0.050	0.065
15	0.044	0.040	0.042	0.066	0.064	0.065
16	0.052	0.054	0.053	0.080	0.083	0.082

and Table 14.3 reports some results of aflatoxin M_1 added in commercial milk preparations.

Sibanda *et al*. (1999) reported a membrane-based flow-through enzyme immunoassay for the detection of aflatoxin M_1 in milk. The assay comprised nylon Immunodyne ABC membrane spotted with anti-mouse antibodies, a plastic snap-fit device, absorbent cotton wool, mouse anti-aflatoxin M_1, monoclonal antibodies and aflatoxin B_1-horseradish peroxidase conjugate. This assay was coupled to an immunoaffinity column. The visual detection limit was 0.05 ng/g AFM_1 in milk. Assay time for the immunoaffinity column clean-up was 12 min, and 18 min for the flow-through assay, making the total assay time 30 min. This method allowed for a rapid screening of milk consignments not conforming to the maximum permissible limit of 50 ppt, hence enabling their

Table 14.2 Statistical summary for precision parameters in the 1998 exercise. Assigned values for batches A and B were respectively 0.050 and 0.071 μg of aflatoxin M_1/L

Batch	*N*	*m* μg/l	SD_r (μg/l)	*r* (μg/l)	RSD_r (%)	SD_R (μg/l)	*R* (μg/l)	RSD_R (%)
A	16	0.045	0.0050	0.0142	11.1	0.0090	20.1	0.0256
B	16	0.072	0.0086	0.0244	11.9	0.0114	15.7	0.0322

m: overall mean; SD_r: standard deviation for repeatability; *r*: repeatability value; RSD_r: relative standard deviation for repeatability; SD_R: standard deviation for reproducibility; *R*: reproducibility value; RSD_R: relative standard deviation for reproducibility.

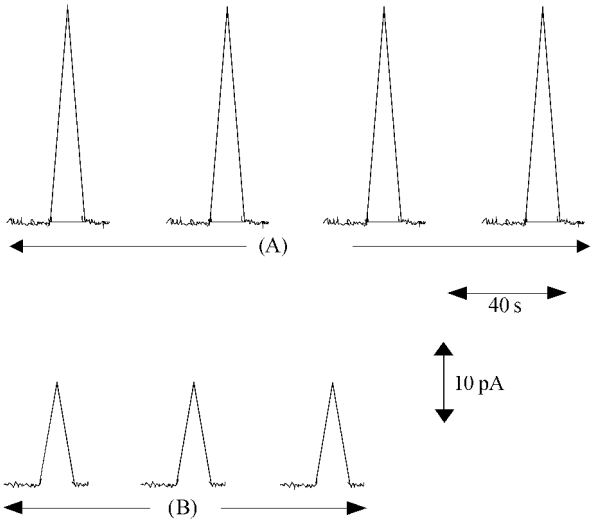

Fig. 14.1 Recordings showing the variability of response of the BLMs to repetitive AFM,-spiked milk samples injections at a flow rate value of 4.0 ml min^{-1}. AFM_1 concentrations were (A) 6.09 and (B) 3.05 nM. The injection of each sample was made at the beginning of each recording.

immediate rejection at the farm. Laboratory validation was done using certified reference materials (CRM) with aflatoxin M_1 concentrations of <0.05, 0.09 and 0.76 ppb. The high precision of the assay was shown by the high repeatability of the assay results. There were no significant differences in recovery between standard in buffer and CRM ($P > 0.05$), and assay responses for these two were highly correlated (99.63%). Tables 14.3 and 14.4 report respectively the quantification of aflatoxin M_1 from commercial spiked milk, and the comparison of the recovery of aflatoxin M_1 from spiked milk compared to toxin in buffer at concentrations of 0.00, 0.05, 0.1 and 1 ppb. Table 14.5 shows the summary statistics.

Table 14.3 Results of quantification of AFM_1 added in commercial milk preparations (numbers in parentheses are the spike amounts of AFM_1)[a]

Sample ID	AFM_1 content (nM)
FAGE Dairy Products S.A., skimmed milk	(7.15) 6.61 ± 0.30
Delta Dairy Products S.A., half cream milk	(3.81) 4.14 ± 0.21
Delta Dairy Products S.A., full cream milk	(14.6) 14.7 ± 0.97
Carnation instant non-fat dry milk, Société des Produits Nestlé S.A. (reconstituted)	(11.1) 10.2 ± 0.58
Noulat, Fiesland Dairy Foods	(11.1) 11.6 ± 0.70

[a] Results presented are the average of five determinations ±1SD.

Table 14.4 Recovery of aflatoxin M_1 from spiked milk compared to toxin in buffer at concentrations of 0.00, 0.05, 0.1 and 1.0 ng/ml[a]

	AFM_1 in buffer	AFM_1 spiked milk	AFM_1 in buffer	AFM_1 spiked milk	AFM_1 in buffer	AFM_1 spiked milk	AFM_1 in buffer	AFM_1 spiked milk
AFM_1 concentration (ng/ml)	0.0	0.0	0.05	0.05	0.1	0.1	1.0	1.0
$(\bar{\chi})\Delta E^{*b}_{ab}$	9.13	9.48	4.73	4.87	4.58	4.84	4.56	5.04
SD (±)	0.82	0.33	0.16	0.59	0.20	0.14	0.20	0.86
Sample variance	0.67	0.10	0.03	0.35	0.04	0.02	0.04	0.75

[a] n = 10 (number of samples/assays ran for each concentration).
[b] Colour development.

A new immunoaffinity fluorimetric biosensor has been developed by Carlson *et al*. (2000) to detect and to quantify all aflatoxins including aflatoxin M_1. The handheld, self-contained biosensor is fully automatic, highly sensitive, rapid and requires no special storage. Approximately 100 measurements can be made before refurbishment is required, and concentrations from 0.1 ppb to 50 ppb can be determined in less than 2 minutes with a 1 ml sample volume. The device operates on the principles of immunoaffinity for specificity and fluorescence for a quantitative assay. The analytical procedure is flexible so that other chemical and biological analytes could be detected with minor modifications to the current device. Figure 14.2 illustrates the immunochemical-based capture, purification and detection process.

Recently, Micheli *et al*. (2002) reported on the development of a disposable immunosensor for aflatoxin M_1 detection in spiked milk, which can combine the high selectivity of immunoanalysis with the convenience of electrochemical probes. Immunoassay parameters, such as amounts of antibody and labelled antigen, buffer and pH, length of time and temperature of each precoating,

Table 14.5 Summary statistics of the flow-through assay for the detection of AFM_1 in buffer and (CRM)[a]

	Standard in buffer	Zero level CRM No. 282	Standard in buffer	Zero level CRM No. 283	Standard in buffer	Zero level CRM No. 285
AFM_1 concentration (ng g^{-1})	0.00	<0.05	0.09	0.09 (±0.04, 0.02)	0.76	0.76 (±0.05)
	10.44	10.40	5.66	6.01	5.69	5.58
SD (±)	1.08	2.07	0.78	0.70	0.50	0.46
Sample variance	1.16	4.29	0.60	0.48	0.25	0.20

[a] n = 10 (number of samples/assays ran for each concentration).
[b] Colour development.

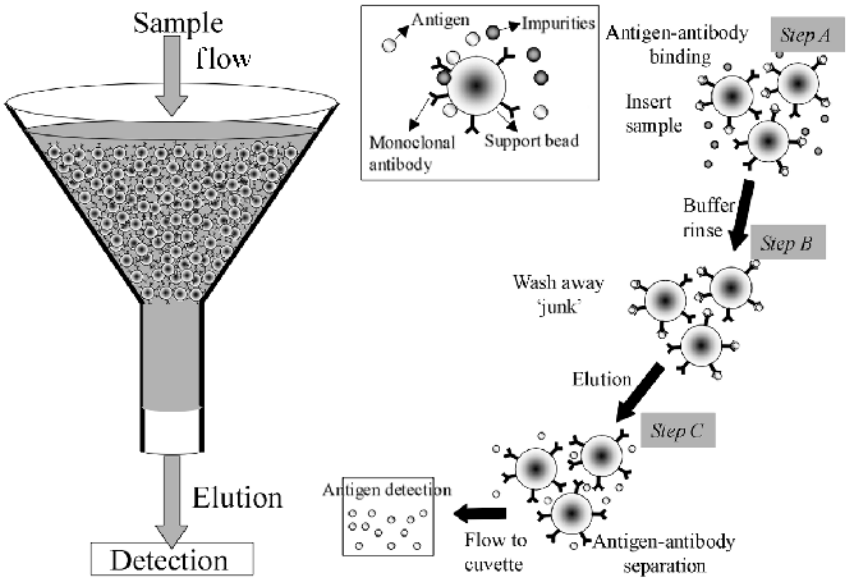

Fig. 14.2 Pictorial representation of the immunochemical-based capture, purification and detection process. The antibody coated beads capture the antigen (step A) as the liquid is passed over the beads. In step B, the beads/antibodies/antigen are rinsed clean of any impurities. Once clean, the antigens are then released back into solution (step C), where their concentration is measured.

coating, binding and competition steps were evaluated and optimised in setting up a spectrophotometric Enzyme-Linked ImmunoSorbent Assay (ELISA) procedure, a powerful tool in biochemical trace analysis. A working range between 0.03 and 0.12 ppb was obtained in a direct competitive format. Electrochemical immunosensors have been fabricated immobilising the antibodies directly on the surface of screen-printed electrodes (SPEs), and allowing competition between aflatoxin M_1 free and that conjugated with HRP. Electrochemical techniques, such as Chronoamperometry and Differential Pulse Voltammetry (DPV) have been evaluated and the most sensitive selected for the final detection step. The sensor has been evaluated for the analysis of aflatoxin M_1 directly in milk. Figure 14.3 illustrates a screen-printing electrode detection system (SPE, used as support and transducer, and direct competitive ELISA format) and Fig. 14.4 a calibration curve for aflatoxin detection in spiked milk.

14.3 Measuring the effectiveness of heat treatment

Milk is heat treated to ensure a longer shelf-life and to guarantee its microbiological safety. However, heat treatment can damage the milk's nutritional properties. It should therefore meet the minimum time/temperature combinations required to make the milk safe without significant heat damage.

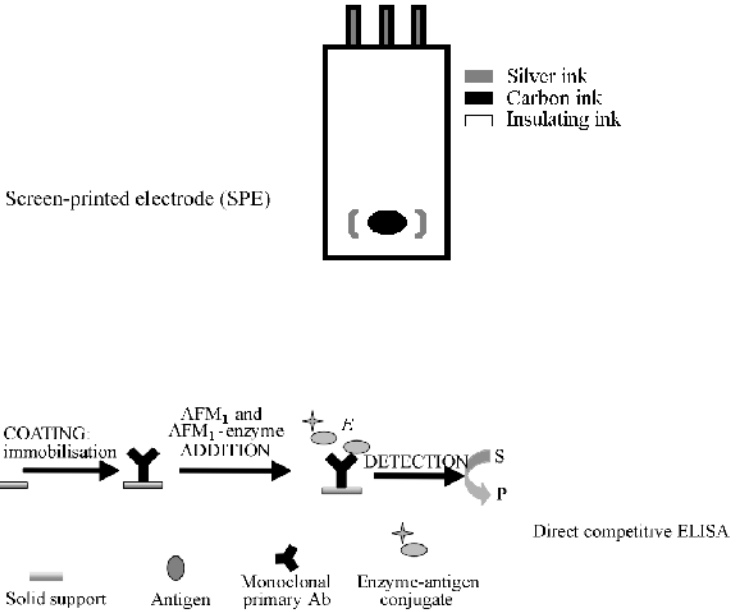

Fig. 14.3 Scheme of a screen-printing electrode detection system: SPE, used as support and transducer, and direct competitive ELISA format.

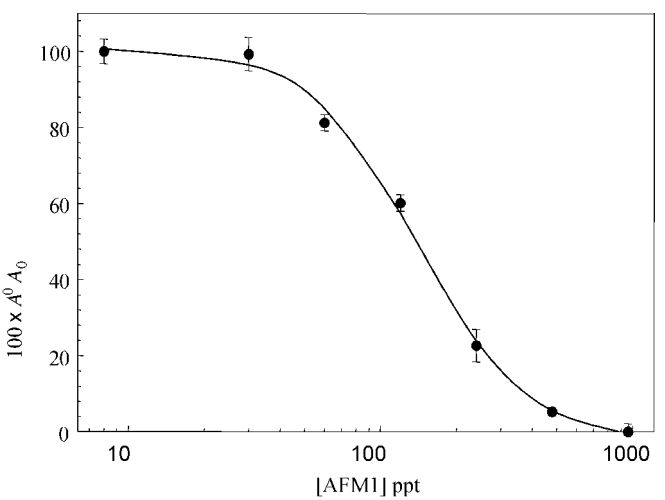

Fig. 14.4 Typical calibration curve for direct competitive ELISA for aflatoxin M_1 detection, calculate with the '4 -parameter logistic equation' $[f(x) = \{[a-d]/[1+(X/c)^b]\} + d]$.

Table 14.6 Example of heat treatments commonly used by the dairy industry

Heat treatment	Conditions
Thermisation	65ºC/30 s
Pasteurisation	72ºC/15 s
High temperature	90ºC/5 min
Ultrapasteurisation	120ºC/2–4 s
UHT	140ºC/3–8 s
Sterilisation	110ºC/5–10 min

So far there is no one universal marker, which can distinguish differing degrees of milk heat treatment from thermisation to in-bottle sterilisation. The dairy industry urgently needs the development of rapid, sensitive and low-cost analytical methods to discriminate milk heat treatment effects. Examples of industrial heating processes commonly used by the dairy industry are presented in Table 14.6.

The evaluation of heat treatment is possible if irreversible changes are induced in the product. These can then be identified by suitable chemical markers. Chemical markers should in principle be easy to determine by rapid instrumental techniques preferably at an early stage than when food deterioration becomes detectable by sensory methods. Two types of chemical markers may be used to assess heat treatments (Mortier *et al*., 2000):

- the degradation, denaturation and inactivation of heat labile components, e.g. whey proteins or enzymes
- the formation of 'new' substances, such as lactulose or products of the Maillard reaction (MR), which are not present, or present only at trace levels, in the raw milk.

It is useful to consider three stages in the MR (Van Boekel, 1998): the initial, advanced and final stages. The initial stage of the MR, in which Amadori compounds are formed, does not give rise to colour. Upon prolonged heating, the Amadori rearrangement products undergo dehydration and fission and yield colourless reductones as well as fluorescent substances, some of which may be pigmented. The final stage of MR is where most of the colour is produced. This stage is characterised by the formation of unsaturated brown polymers (IDF, 1971).

14.3.1 Measurement of alkaline phosphatase and lactoperoxidase activity in milk

Alkaline phosphatase (EN, 1984a) is a thermolabile enzyme which is indigenous to all dairy products, including raw milk. It has an inactivation temperature slightly above that which is required to destroy the most resistant disease organisms likely to be found in milk. This method can be used to determine

whether or not the pasteurisation process was adequate or to detect post-process contamination of pasteurised products with raw milk. However false-positive results might occur in three different situations:

- Microorganisms present in milk after pasteurisation may produce phosphatases.
- Reactivation of phosphatase enzyme may be observed in milk processed by high-temperature/short-time (HTST) pasteurisation or may be caused by exposure of the product to the room temperature in the presence of Mg^{++}.
- Interfering substances reacting directly with reagent assays and producing a background coloration.

Confirmation tests are required when positive phosphatase results are observed. The standard method uses sodium phenylphosphate as substrate and quantitative determination of phenol by the 'indophenol reaction'. The qualitative statement 'phosphatase negative' means an alkaline phosphatase activity (ALP) lower than 4 mg phenol/mL of milk (IDF, 1971). The new IDF standard (IDF Method 155A, 1999) specifies the use of the fluorimetric method for determination of ALP in milk and milk-based drinks. The cost of this method is higher than colorimetric tests. However, the fluorimetric method is more sensitive and can be used to measure ALP not only in milk but also in many milk and dairy products. It can be used to measure ALP in coloured/flavoured pasteurised products whereas colorimetric tests cannot.

Lactoperoxidase (LPO) is grouped under the general class of peroxidases (EN, 1984b) which catalyse the oxidation of suitable electron donors by hydrogen peroxide. Since lactoperoxidase is a heat stable enzyme and is very sensitive to temperature changes around 80ºC, its activity has been used for the determination of the upper limit of pasteurisation. Pasteurised milk must show a positive LPO reaction and must be labelled as 'high-temperature pasteurised' when a negative result is obtained. Before measuring LPO activity spectrophotometrically at 412 nm with ABTS (2,2′-azinobis (3-ethyl-benzothiazoline-6-sulphonic acid)) and H_2O_2 as substrate, milk proteins require precipitation and filtration steps for turbidity elimination (Hernandez *et al*., 1990). Blel *et al*. (2001) have described a very easy and rapid colorimetric method which avoids preliminary casein precipitation and filtration steps using a clarifying Reagent^R.

14.3.2 Determination of lactulose in processed milk

Lactulose, which is formed during milk heat treatment, is the most widely studied index for differentiating heated milks (Pellegrino *et al*., 1995; Corzo *et al*., 1996). It has been proposed by the IDF (IDF, 1993b) and by the European Commission (EC) (EU Commision Doc VI/844/93, 1993) as an analytical index to distinguish UHT milk from in-bottle sterilised milk. There are several analytical methods for detection of lactulose:

- gas chromatography (Martinez-Augustin *et al*., 1995)
- the IDF official method based on liquid chromatography (IDF, 1991),
- enzymatic methods based on spectrophotometric detection (De Block *et al*., 1996) or amperometric detection (Mayer *et al*., 1996; Sekine and Hall, 1998)

As with other established methods, these techniques are time-consuming. An example is provided by a kit commercially available from Roche Diagnostic (formerly Boehringer Mannheim, Germany) based on an enzymatic procedure (Boehringer Mannheim, 1995). One drawback of this method is the potential interference from glucose, which is quite high in milk where the molar ratio lactose/lactulose could be equal to 1000/1. While it is possible to minimise the effects of glucose interferences by sample pre-treatment with glucose oxidase and catalase, this approach makes the assay more difficult to automate, because it requires six different enzymes, expensive reagents and about 15 hours to perform the analysis.

Moscone *et al*. (1999) have developed a simple and rapid flow method based on the use of an electrochemical biosensor and microdialysis. It is based on the hydrolysis of lactulose to galactose and fructose by the enzyme β-galactosidase immobilised in a reactor. The amount of fructose produced was measured with an electrochemical biosensor based on the fructose dehydrogenase enzyme, $K_3[Fe(CN)_6]$ as mediator and a platinum-based electrochemical transducer (Fig. 14.5). The use of a microdialysis probe as the sampling system permitted the direct measurement of lactulose in milk samples without pre-treatment in the range 4–1700 mgL. The sensitivity of the procedure allows pasteurised, UHT and in-container sterilised milks to be distinguished.

Amine *et al*. (2000a) have developed a new enzymatic spectrophotometric method for the determination of lactulose according to the following reactions:

$$\text{Lactose} \xrightarrow{\beta\text{-galactosidase}} \text{D-fructose} + \text{galactose}$$

$$\text{D-fructose} + \text{MTT} \xrightarrow[\text{PMS}]{\text{FDH}} \text{5-Keto-D-fructose} + \text{MTT Formazan}$$

This method entailed the use of β-galactosidase, which hydrolyses lactulose to fructose and galactose, and fructose dehydrogenase (FDH), which reacts with fructose in the presence of a tetrazolium salt (MTT), giving a coloured compound which can be detected spectrophotometrically at 570 nm. The assay showed a lactulose detection limit in milk of about 10 mgL^{-1}, a linear range of 20–800 mgL^{-1} and a relative deviation of 5%. The correlation with the determination of lactulose in milk using reference procedures was good (Tables 14.7 and 14.8). Moreover this procedure was found suitable for the quantification of lactulose in milk after the heat treatment process, and more convenient for the rapid and sensitive estimation of lactulose if compared with previous published enzymatic methods.

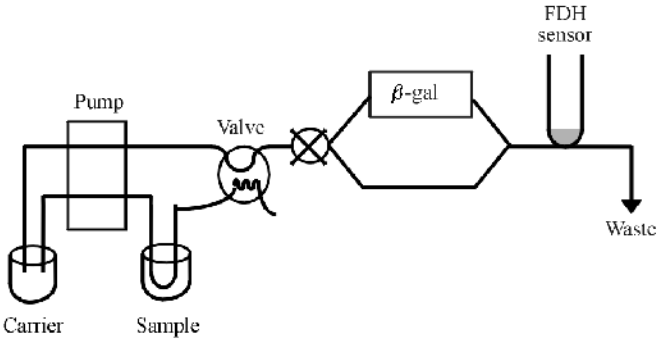

Fig. 14.5 Flow manifold for determination of lactulose in milk samples. β-galactosidase immobilised in a reactor and fructose dehydrogenase immobilised on platinum electrode. Carrier is citrate buffer and mediator, ferricyanide. The sampling system is microdialyse probe. B = buffer + mediator; P = peristaltic pump; S = sample ; M = microdialysis probe; T = three-way stopcock; β-gal = β-galactosidase reactor; FDH = fructose dehydrogenase biosensor; C = cell; D = detector; R = recorder; W = waste.

Table 14.7 Total lactulose content of different UHT milk samples determined by the Roche spectrophotometric kit and the proposed method

Milk samples	Spectrophotometric kit **A** (mgL^{-1})	Proposed method **B** (mgL^{-1})	(A − B)/A %
UHT A	131	120	8
UHT B	140	131	6
UHT C	116	128	−10
UHT D	75	82	−9
UHT E	124	134	−8
UHT F	166	173	−4

Table 14.8 Recovery of lactulose added to commercial low pasteurised milk determined by the official HPLC method and by the proposed method[a]

Added lactulose (mgL^{-1})	HPLC official method **A** Recovery (mgL^{-1})	(%)	Proposed method **B** Recovery (mgL^{-1})	(%)
225	206	92	217	96
280	246	88	267	95
300	287	96	325	108
450	472	105	417	93
600	581	97	577	96

[a] Correlation coefficients A – B, $r = 0.980$.

Table 14.9 Absorbance measurement using Seliwanoff's reaction of raw milk and of different types of milk commercially available. Number of samples (n) and minimum, maximum, mean, median and relative standard deviation (RSD) of the absorbance values are indicated

	Raw ($n = 7$)	Pasteurised ($n = 15$)	UHT ($n = 12$)	Sterilised ($n = 4$)
Mean	0.152	0.174	0.240	0.367
RSD %	7	16	18	10
Min	0.136	0.143	0.185	0.340
Max	0.165	0.223	0.326	0.422
Median	0.154	0.158	0.239	0.350

A more rapid and simple method to distinguish between UHT and sterilised milk has also been developed (Amine *et al*., 2000b). This method uses a Seliwanoff's reagent and is based on the assumption that lactulose is the only source of ketose (fructose) in milk. This method determines lactulose directly in milk with no treatment. This method showed a linear range between 17 and 170 mg dL^{-1} and a detection limit lower than that of the official HPLC method. This novel procedure was compared with a commercially available enzymatic method and the results correlated well (Table 14.9).

14.3.3 Glycated proteins in milk

Lactose reacts non-enzymatically with lysine residues of milk proteins to form a Schiff base, which is stabilized through an Amadori rearrangement in the first stage of the MR. So far, the most widely used method to evaluate the Amadori compound (lactuloselysine), the major form of modified lysine during the initial stage of glycosylation in thermally treated milk, is the furosine method (Resmini *et al*., 1990). The main drawbacks of this procedure are the time of analysis and the fact that only part of lactuloselysine is converted into furosine. The formation of hydroxymethylfurfural (HMF) from the Amadori compound has also been studied (Furth, 1988; Morales *et al*., 1997). However the yield of HMF is only about 10% of the content of the Amadori component. A direct method for measuring lactuloselysine after complete enzymatic hydrolysis by an amino acid analyser has also been proposed (Henle *et al*., 1991).

Pizzano *et al*. (1998) have developed an immunological approach to direct detect protein bound Amadori compounds. The polyclonal antibodies raised against a lactosylated synthetic peptide were used to specifically detect the Amadori compounds in milk. More recently, monoclonal antibodies for lactosylated proteins were produced, characterised and used in a competitive assay format (Pallini *et al*., 2001). The data obtained indicate that the ELISA is applicable to diluted milk samples and is able to distinguish between milk samples that have undergone different heat treatments (UHT and pasteurised

milk). The main drawback of the quantification of lactuloselysine is the lack of a pure standard for comparison.

A new fluorimetric FAST (Fluorescence of Advanced Maillard products and Soluble Tryptophan) method to estimate the intensity of heat treatment applied to milk from thermization to ''in-bottle'' sterilisation has also been developed (Leclere and Birlouez-Aragon, 2001; Birlouez-Aragon *et al*., 2002). The FAST method is a global approach for quantifying the MR by measuring the formation of fluorescent advanced products (AMP) in the pH 4.6 milk supernatant. The FAST method gives an estimate of nutritional damage and it is a rapid alternative for measuring the furosine concentration of heat treated milk.

14.4 Future trends

Public concern for milk safety and quality, and increased general demands for information about food, are likely to provide more impetus for innovative approaches to food analysis in the future. In this context, rapid methods of analysis are increasingly important, particularly where they involve portable instrumentation, have a high sensitivity, need no reagents, and are cost effective, reproducible and accurate. One technology, which matches most of the features reported, is that of bio- and immunosensors not only for fresh milk contamination control but also to improve milk process control. This is particularly important in improving UHT milk so that it matches the nutritional and sensory quality of fresh milk while remaining safe for the consumer.

14.5 References

AMINE A, MOSCONE D, BERNANDO R, MARCONI E and PALLESCHI G (2000a), 'A new enzymatic spectrophotometric assay for the determination of lactulose in milk', *Anal Chim Acta*, **406** 217–224.

AMINE A, MOSCONE D and PALLESCHI G (2000b), 'Rapid determination of lactulose in milk using Seliwanoff's reaction', *Anal Lett*, **33**(1) 125–135.

ANDREOU VG and NIKOLELIS DP (1998), 'Flow injection monitoring of aflatoxin M1 in milk and milk preparations using filter-supported BLMs', *Anal Chem*, **68** 1735.

ANDREWS GR (1984), 'Distinguishing pasteurized, UHT and sterilized milks by their lactulose content', *J Soc Dairy Technol*, **37** 92–96.

ARAR EJ (1997), 'In vitro determination of chlorophylls a,b,c_1 + c_2 and pheopigments in marine and freshwater algae by visible spectrophotometry', *US Environmental Protection Agency*, Method 446.0.

BIANCARDI A (1997), 'Determinazione di aflatossina M1 nel latte', *Industrie Alimentari XXXVI*, 870–876.

BIRLOUEZ-ARAGON I, SABAT P and GOUTI N, (2002), 'A new method for discriminating milk heat treatment', *Int Dairy J*, **12**(1) 59–67

BLEL M, GUINGAMP -F, GAILLARD JL and HUMBERT G (2001), 'Improvement of a method for the measuring of lactoperoxidase activity in milk', *Int Dairy J*, **11** 795–799.

BOEHRINGER MANNHEIM (1995), 'Lactulose in milk', in: Keesey J (ed.), *Methods of Enzymatic Bioanalysis and Food Analysis*, Indianapolis (USA), Boehringer Mannheim Biochemicals, Cat. N 139106

BULL CR, MCFARLANE NJB, ZWIGGELAAR R, ALLEN CJ and MOTTRAM TT (1996), 'Inspection of teats by colour image analysis for automatic milking systems', *Computers and Electronics in Agriculture*, **15** 15–26.

BURTON H (1984), 'Reviews of the progress of dairy science: the bacteriological, chemical, biochemical and physical changes that occur in milk at temperatures of 100-150ºC', *J Dairy Res*, **51** 341–363.

CARLSON MA, BARGERON CB, BENSON RC, FRASER AB, PHILIPS TE, VELKY JT, GROOOPMAN JD, STRICKLAND PT and KO HW (2000), 'An automated, handheld biosensor for aflatoxin', *Bios Bioel*, **14** 841–848.

CORZO N, VILLAMIEL M and MARTINEZ-CASTRO I (1996), 'Lactulose, monosaccharides and undenatured serum protein contents in commercial UHT creams and their usefulness for thermal treatment assessment', *Food Chem*, **56**(4) 429–432.

DE BLOCK J, MERCHIERS M, VAN RENTERGHEM R and MOERMANS R (1996), 'Evaluation of two methods for the determination of lactulose in milk', *Int Dairy J*, **6**(2) 217–222.

DRAGACCI S, GROSSO F, PFAUWATHEL-MARCHOND M, FREMY JM, VENANT A and LOMBARD B (2001), 'Proficiency testing for the evaluation of the ability of European Union-National Reference laboratories to determine aflatoxin M1 in milk at levels corresponding to the new European Union legislation', *Food Add Contam*, **18(**5) 405–415.

EU COMMISSION (1992), Dairy Chemistry Group. Doc VI/5726/92: Projet de decision de la commission fixant les limites et les méthodes permettant de distinguer le differents types de lait de consom traites thermiquement.

EU COMMISSION (1993), Dairy Chemistry Group. Doc. VI/844/93: Peroxidase inactivation and β-lactoglobulin in commercial samples of pasteurised milk.

EU COMMISSION (1994), Dairy Expert Group. Doc VI/CG/1018/94. Determination of furosine (ϵ-furoylmethyl-lysine) content.

EC DIRECTIVE 92/46, 16-6-1992. EC O.J. NO L268, 14-9-1992 (1992), Council Directive laying down the health rules for production and placing on the market of raw milk, heat-treated milk and milk-based products.

EN (ENZYME NOMENCLATURE) (1984a), Alkaline Phosphatase – EC 3.1.3.1, Webb EC (ed.), Academic Press, London.

EN (ENZYME NOMENCLATURE) (1984b), Lactoperoxidase – EC 1.11.1.7, Webb EC (ed.), Academic Press, London.

ERBERSDOBLER HF and DEHN-MÜLLER B (1989), 'Formation of early Maillard products during UHT treatment of milk', in: *Heat-induced Changes in Milk*, Fox PF (ed.), *Int Dairy Fed Bul*, **238** 62–70.

EU PROJECT (2002), 'Quality of life and management of living resources – ROSEPROMILK Contract n. QLK1-CT-2001-01617 ROBUST CHEMICAL SENSORS AND BIOSENSORS FOR RAPID ON-LINE IDENTIFICATION OF FRESHLY COLLECTED MILK', First Annual Report: 1 December 2001–30 November 2002.

FURTH AJ (1988), 'Methods for assaying non enzymatic glycosylation', *Anal Biochem*, **175**, 347–360.

GEIER H and KLOSTERMEYER H (1983), 'Formation of lactulose during heat treatment of milk', *Milchwissenschaft*, **38** 475–477.

GRIFFITHS MW (1986), 'Use of milk enzymes as indices of heat treatment', *J Food Prot* **49**

696–705.

HENLE R, WALTER H and KLOSTERMEYER H (1991), 'Evaluation of the extent of the early Maillard reaction in milk products by direct measurement of the Amadori product lactuloselysine', *Z. Lebensm Unters. Forsch*, **193** 119–122.

HERNANDEZ M, VAN MARKWIJK B and VREEMAN H (1990), 'Isolation and properties of lactoperoxidase from bovine milk', *Met Milk Dairy J*, **44** 213–231

INTERNATIONAL DAIRY FEDERATION (IDF) (1971), Standard 63. 'Milk and milk powder, buttermilk and buttermilk powder, whey and whey powder: determination of phosphatase activity (reference method)'.

INTERNATIONAL DAIRY FEDERATION (IDF) (1990), E-Doc 442: Phosphatase activity.

INTERNATIONAL DAIRY FEDERATION (IDF) (1991), 'Alkaline phosphatase test as a measure of correct pasteurisation', *Int Dairy Fed Bul*, **262** 32–35.

INTERNATIONAL DAIRY FEDERATION (IDF) (1993a), E-Doc 557: 'Lactulose determination and other methods for distinguishing heat treatment of milk'.

INTERNATIONAL DAIRY FEDERATION (IDF) (1993b), B-Doc 235. 'Influence of technology on the quality of heated milk and fluid milk products'.

INTERNATIONAL DAIRY FEDERATION (IDF) (1995), E-Doc 171. 'Immunoaffinity column/ HPLC determination of AFMI'.

INTERNATIONAL DAIRY FEDERATION (IDF) (1999), Milk and milk-based drinks. 'Determination of alkaline phosphatase activity using a fluorimetric method. Method 155A'.

ISO (INTERNATIONAL STANDARD ORGANIZATION) (1998), EN ISO 14501, 'Milk and milk powder – determination of aflatoxin M1 content: Clean up by immunoaffinity chromatography and determination by high-performance liquid chromatography'.

LECLÈRE J and BIRLOUEZ-ARAGON I (2001), 'The fluorescence of advanced Maillard products is good indicator of lysine damage during the Maillard reaction', *J Agric Food Chem*, **49** 4682–4687.

MARKAKI P and MELISSARI E (1997), 'Occurrence of aflatoxin M1 in commercial pasteurized milk determined with ELISA and HPLC', *Food Add Contam*, **14**(5) 451–456.

MARTINEZ-AUGUSTIN O, BOZA JJ, ROMER JM and GIL A (1995), 'A rapid gas-liquid chromatography method for the determination of lactulose and mannitol in urine: clinical application in studies of intestinal permeability', *Clin Biochem*, **28**(4) 401–405.

MAYER M, GENRICH M, KÜNNECKE W and BILITEWSKI U (1996), 'Automated determination of lactulose in milk using an enzyme reactor and flow analysis with integrated dialysis', *Anal Chim Acta*, **324**(1) 37–45

MICHELI L, GRECCO R, PALLESCHI G, and MOSCONE D (2002), 'Development of a disposable immunosensor for aflatoxin M1 detection in milk', in: *Euroanalysis 12*, Dortmund, Germany 8–13 September 2002, 326.

MIRAGLIA M (1998), 'Mycotoxins and phycotoxins – developments in chemistry, toxicology and food safety', in: Miraglia M, van Egmond HP, Bresa C and Gilbert J (eds), *Proceeding of the International IUPAC Symposium on Mycotoxin and Phycotoxin*, Colorado (USA), Alaken, Inc. Fort Collins, 67, 151, 181.

MORALES FJ, ROMEO C and JIMENEZ-PEREZ S (1997), 'Chromatographic determination of bound hydroxymethylfurfural as an index of milk protein glycosylation', *J Agric Food Chem*, **45** 1570–1573.

MORTIER L, BRAEKMAN A, CARTUYVELS D, RENTERGHEM RV and BLOCK JD (2000), 'Intrinsic indicators for monitoring heat damage of consumption milk', *Biotechnol Agron*

Soc Environ, **4**(4) 221–225.

MOSCONE D, BERNARDO RA, MARCONI E, AMINE A and PALLESCHI G (1999), 'Rapid determination of lactulose in milk by microdialysis and biosensors', *Analyst*, **124** 325–329.

MOTTRAM TT (1997), 'Requirements for teat inspection and cleaning in automatic milking systems', *Computers and Electronics in Agriculture*, **17** 63–77.

O'BRIEN J (1995), 'Heat-induced changes in lactose: isomerization, degradation, Maillard browning', in *Heat-induced Changes in Milk* (2nd edition), Int Dairy Fed., Brussels (Belgium), 134–170.

PALLINI M, COMPAGNONE D, DI STEFANO S, MARINI S, COLETTA M and PALLESCHI G (2001), 'Immunodetection of lactosylated proteins as a useful tool to determine heat treatment in milk samples', *Analyst*, **126** 66–70.

PELLEGRINO L, RESMINI P and LUF W (1995), 'Assessment (indices) of heat treatment of milk', in: *Heat-induced Changes in Milk* (2nd edition), Fox PF (ed.), Int Dairy Fed. Special Issue n 9501, 409–453.

PELLEGRINO L, DE NONI I and RESMINI P (1995),'Coupling of lactulose and furosine indices for quality evaluation of sterilized milk', *Int Dairy J*, **5**(7) 647–659.

PIZZANO R, NICOLAI MA, SICILIANO R and ADDEO F (1998), 'Specific detection of the Amadori compounds in milk by using polyclonal antibodies raised against a lactosylated peptide', *J Agric Food Chem*, **46** 5373–5379.

RESMINI, P, PELLEGRINO L and BATELLI G (1990), 'Accurate quantification of furosine in milk and dairy products by a direct HPLC method', *Ital J Food Science*, **3**, 173–183.

ROSNER H (1998), 'Mycotoxin: limits in European union and effects on trade', in: *Mycotoxin and Phycotoxins – Developments in Chemistry, Toxicology and Food Safety*, Miraglia M, van Egmond HP, Brera C and Gilbert J (eds), Alaken, Inc. Fort Collins, Colorado USA, 203–212.

SEKINE Y and HALL EAH (1998), 'A lactulose sensor based on coupled enzyme reactions with a ring electrode fabricated from tetrathiafulvalen-tetracyanoquinodimetane', *Bios Bioel*, **13**(9) 995–1005.

SIBANDA L, DE SAEGER S and VAN PETEGHEM C (1999), 'Development of a portable field immunoassay for the detection of aflatoxin M1 in milk', *Int J Food Microbiol*, **48**(3) 203–209.

STAAL PFJ (1986), 'Legislation/statutory regulations applicable to pasteurized fluid milk in a selected number of countries', *Int Dairy Fed Bull*, **200** 71–89

TURNER APF (2000), 'Biosensors – sense and sensitivity', *Science*, **290**, 1315–1317.

VAN BOEKEL MAJS (1998), 'Effect of heating on Maillard reactions in milk', *Food Chem*, **62**(4) 403–414.