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Occurrence of Aflatoxin M1 in Dairy Products

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1. Introduction

Aflatoxin M1 (AFM1) is a major metabolite of aflatoxin B1 (AFB1), which is formed when animals ingest feed contaminated with aflatoxin B1. The AFB1, once ingested by the animal, is rapidly absorbed by the gastrointestinal tract and is transformed into the metabolite AFM1, which appears in the blood after 15 minutes and is then secreted in the milk by the mammary gland (Van Egmond, 1989; Battacone, et al. 2003). The amount of AFM1 which is found in milk depends on several factors, such as animal breed, lactation period, mammary infections etc… It has, anyway, been demonstrated that up to 6% of the ingested AFB1 is secreted into the milk as aflatoxin M1 (Van Egmond & Dragacci, 2001) and, because AFM1 is relatively resistant to heat treatments (Yousef & Marth, 1989; Galvano et al., 1996), it is almost entirely retained in pasteurized milk, powdered milk, and infant formula. Moreover, only a limited decrease of AFM1 content has been verified in UHT milk after long storage (Galvano et al., 1996; Martins & Martins, 2000; Tekinsen & Eken, 2008). The hepatotoxicity and carcinogenic effects of AFB1 have been clearly demonstrated, thus it has long been classified as a group 1 human carcinogen by the International Agency on Research on Cancer (IARC, 2002). Initially, the IARC classified AFM1 as a possible carcinogen for humans (group 2b) since toxicological data was limited (IARC, 1993). However, genotoxicity and cancerogenity of AFM1 have been observed in vivo, although lower than those of AFB1, and its cytotoxicity has been definitively demonstrated (Caloni et al., 2006). As a result of these and other further investigations, the IARC moved aflatoxin M1 from group 2B to group 1 human carcinogen (IARC, 2002).

Considering that milk and milk derivatives are consumed daily and, moreover, that they are of primary importance in the diet of children, most countries have set up maximum admissible levels of AFB1 in feed (European Commission, EC, 2003a) and of AFM1 in milk, which vary from the 50 ng/kg established by the EU, to the 500 ng/kg established by US FDA (EC, 2003b; U.S. Food and Drug Administration, FDA, 2011). More restrictive MRLs have been implemented by the EU for the presence of AFM1 in baby food (EC, 2004) Regulations for aflatoxin M1 existed in 60 countries by the end of 2003, most of them being EU, and candidate EU countries, but some other countries in Africa, Asia and Latin America also apply the limit of 50 ng/kg. The higher regulatory level (500 ng/kg) is applied in the United States and in several countries in Asia and in Latin America, where it is also established as a harmonized MERCOSUR limit (FAO, 2011).

Based on admissible levels, on measured values in milk obtained in various monitoring programs and on typical diets, the intake of aflatoxin M1 from milk has been calculated to

vary between 0.1 ng/person per day in Africa to 12 ng/person per day in the Far East (Europe: 6.8 ng/person per day, Latin America: 3.5 ng/person per day, the Middle East: 0.7 ng/person per day) (Creppy, 2002). The level of attention in the control of AFM1 contamination in milk is high all over the world, as attested by the number of scientific papers dealing with development and validation of analytical methods for measuring such a contaminant, the published survey studies on this argument, and by the attention paid by various international organisms.

However, the stability of AFM1 determines the persistence of such toxic compound in a number of other foodstuffs of wide human consumption, which are subject to less scrutiny, except in some geographical regions such as in the Middle East. In particular, the resistance to heat treatment and mild acidic conditions used in the production of cheese or other dairy products (such as, for example, yogurt, butter, cream and ice cream) has been accounted for the contamination of such products (Oruc et al., 2006; Colak, 2007). In addition, several authors have demonstrated that AFM1 is bound to milk proteins (Kamkar et al., 2008; Mendonca & Venancio, 2005; Prandini et al., 2009), mainly casein, and that therefore the toxin is more concentrated in cheese than in the milk used to produce it. As a result of the affinity of AFM1 for milk proteins, the toxin is distributed unevenly between whey and curd. In 2001, Govaris et al. (Govaris et al., 2001) first discussed the contrasting results reported until then, which regarded the distribution of AFM1 between whey and curd during cheese manufacturing. Differences in published results were attributed both to the variability of cheese-making processes investigated by the various authors and to the method of analysis employed to measure AFM1. More recent papers report results in greater agreement among themselves and demonstrates that the highest concentration of the toxin is found in the curd, regardless of the procedures applied in cheese-making and the method of analysis employed (Colak, 2007; Kamkar et al., 2008; Motawee et McMahon, 2009; Deveci, 2007; Manetta et al., 2009). According to Motawee et al and Deveci et al. approximately 60% of the AFM1 is found in the curd. Kamkar et al. found an even greater amount of AFM1 in the curd (3-times the content of whey). Accordingly, about half of the AFM1 from contaminated milk is found in cheese (Oruc et al., 2006; Colak, 2007), which means that levels of contamination could be very high, given that a kilogram of cheese is produced from several litres of milk, depending on type and maturity level of cheese (for example, 4.5 l of milk give 1 kg of mozzarella cheese, while as much as 16 l of milk are needed to obtain 1 kg of parmesan). As a matter of fact, AFM1 has been found in dairy products at levels which are 2-5 times higher than in the milk (Kamkar et al., 2008; Govaris et al., 2001, Motawee & McMahon, 2009; Deveci, 2007; Manetta et al., 2009). Moreover, substantially all authors who investigated the fate of AFM1 during cheese-making and cheese maturation agree to conclude that AFM1 content does not change significantly during these steps. These findings have also been confirmed by recent survey studies (Table 2) regarding the incidence of AFM1 contamination in cheese, which demonstrate the presence of AFM1 at various levels with a relevant incidence of positive samples (> 50 ng/kg), and in some case of highly contaminated samples (> 250 ng/kg). Occurrence of AFM1 in dairy products other than cheese has also been assessed (Kim et al., 2000; Maqbool et al., 2009; Lin et al., 2004; Martins & Martins, 2004) and demonstrates the potential risk for consumer health due to the widespread contamination of milk-derived products.

Despite this evidence, an adequate regulation about admissible limits of AFM1 in dairy products is still lacking in most countries. The strategy applied by several countries (i.e: EU and USA) is based on the assumption that a strict control of milk would prevent

contamination of derived products. Therefore, the establishment of admissible limits for aflatoxin B1 in feed and of very severe MRLs in milk are judged to be sufficient to protect consumers from risk due to aflatoxin intake. On the other hand, specific maximum admissible levels for AFM1 in cheese have been set up in some countries and are summarised in Table 1 (Creppy, 2002; Italian Health Department, 2004; Dashti et al., 2009; Amer & Ibrahim, 2010; Sarımehmetoglu et al., 2004). The majority of countries which established a limit fixed it at 250 ng/kg, which corresponds to the assumption that cheese is made with milk which complies to regulations (i.e: contaminated at a level below 50 ng/kg) and that AFM1 concentration could rise up to 5-fold due to dehydration. However, some countries have decided on a zero tolerance strategy (Rumania and Egypt), to give the maximum consumer health protection at the expense of milk and cheese producers. Contrarily, in 2004, Italy raised the limit applicable to hard cheese to 450 ng/kg to protect parmesan production, which was generally highly contaminated in that year as the result of a foregoing peak of AFB1 contamination in feed. Interestingly, the vast majority of surveys on the occurrence of AFM1 in cheese have been carried out in those countries that in fact set up an admissible level in cheese and not just in milk (Table 2). Particularly noteworthy is that most studies have been carried out on Turkish cheese or on cheese consumed in Turkey.

Country	MRL (ng/kg)	Ref
Argentina	500	Dashti et al., 2009
Austria	250	Dashti et al., 2009
Switzerland	250	Creppy, 2002, Dashti et al., 2009
Egypt	0	Amer & Ibrahim, 2010
Honduras	250	Dashti et al., 2009
Italy	250 (450[a])	Italian Health Department, 2004
Rumania	0	Dashti et al., 2009
The Netherlands	200	Creppy, 2002
Turkey	250	Sarımehmetoglu et al., 2004

[a] limited to hard cheese

Table 1. International admissible levels for aflatoxin M1 in cheese

2. Methods of analysis of AFM1 in dairy products

Several methods for aflatoxin M1 determination have been developed, including high-performance liquid chromatography associated with fluorescence or mass spectrometric detection. Immunochemical methods have also been described and are employed as screening methods in routine analysis, mainly because of their simplicity and rapidity. However, the rate-determining step and the major source of errors in the analysis of cheese is the extraction of AFM1, which, in fact, strongly limits the number of samples to be analysed, with its being the most time-consuming, tedious and costly step of the entire analytical protocol.

2.1 Confirmatory and validated methods of analysis
Analytical methods for measuring aflatoxin M1 in milk have been widely described and a lot of HPLC-based methods are available. Some of them have been validated both in inter-

laboratory trials (Dragacci, & Grosso, 2001; Gallo et al., 2006; Gilbert & Anklam, 2002) and according to latest EU rules (Muscarella et al., 2007). In past years, TLC methods have also been widely used and, even more recently, a TLC protocol to determine AFM1 in milk has been reported and validated (Grosso et al., 2004). As regards cheese and other dairy products, some instrumental analysis methods have been described (Oruc et al., 2006; Kamkar et al., 2008; Mendonca & Venancio, 2005; Govaris et al., 2001; Deveci, 2007; Hisada et al., 1984; Pietria et al., 1997; Manetta et al., 2005). Validation according to EU regulation has been reported for an LC-FLD method applied to yogurt (Tabari et al., 2011). Schematically, confirmatory analytical protocols consist of: (i) extraction of the toxin with some organic solvent (dichloromethane, chloroform, methanol, acetonitrile); (ii) clean-up, which usually exploits the affinity and selectivity of antibodies immobilized in a solid-phase extraction (SPE) column (Immuno Affinity Chromatography) to reduce matrix interfering components and to strongly concentrate the target compound; alternatively C18-SPE is used for the purpose; (iii) chromatographic separation by reverse-phase HPLC; (iv) detection of the native fluorescence of AFM1. In 2005, Manetta and co-workers described a particularly sensitive method of analysis (LOD as low as 1 ng/kg in cheese) which used post-column derivatisation to enhance AFM1 fluorescence (Manetta et al., 2005). Mass spectrometric detection has also been successfully applied for the determination of AFM1 in different types of cheese samples (Cavaliere et al., 2006) and for the simultaneous detection of the toxin with other eight mycotoxins (Kokkonen et al., 2005). The exploitation of a very selective detection, such as tandem mass spectrometry, moreover permitted the application of simplified extraction procedures (Cavaliere et al., 2006).

2.2 Rapid techniques for measuring AFM1 in cheese

Historically, the first visual and rapid methods for the detection of AFM1 in milk were TLC methods. TLC-based analytical methods were developed for the measurement of the toxin present in cheese and dairy products too and were recognized as reference methods (see for example: AOAC 980.21 and 947.17 visual methods and Bijil et al., 1987). Nevertheless, immunoassays nowadays play a major role in the monitoring of AFM1 as a first level screening analysis. A number of commercial immunoassay kits (mainly ELISA methods) (International Standards Organisation, ISO, 2002) are available, which state their applicability not only in milk, but also in yogurt, cheese and any other sort of dairy products. However, since the lack of specific regulations in most countries, ELISA kits are principally intended for milk analysis. Therefore, their performances are valued for this purpose, as for example in the work of Rubio et al. who compared five commercial immunoassay kits aimed at the measurement of the target toxin in milk (Rubio et al., 2009). Each of the five kits was singularly evaluated and compared with the other, emphasising strong limitations in some of them. Immunoassay techniques which regard AFM1 determination in milk have been also reported in literature (Pestka et al., 1981; Tihrumala-Devi et al., 2002; Magiulo et al., 2005), while few papers report results aimed at demonstrating that immunoassays are reliably applicable for measuring AFM1 in dairy products: examples are represented by the work of Kim et al. who demonstrated the applicability of the developed ELISA in yogurt samples (Kim et al., 2000) and of a previously published work of our group where the modification of a commercial ELISA intended for milk analysis for measuring AFM1 in cheese was described (Anfossi et al., 2008). On the other hand, commercial ELISA kits have been widely used to study the fate of AFM1 during cheese-making or the occurrence of the toxin in various cheeses by

several authors. Lopez et al. evaluate the performance of one of these commercial kits (Ridascreen Aflatoxin M1, R-Biopharm, Darmstadt, Germany) in the determination of the target compound in cheese and validate it by comparison with a thin layer chromatographic reference method, according to AOAC (Lopez et al., 2001). A nice approach for the rapid detection of AFM1 in milk, which exploits components of an immunoassay, carried out "on-column" instead of in a microtitre plate, was proposed by Sibanda et al. (1999). This visual assay has been extended and applied to yogurt and kefir by Goryacheva et al. (2009). Briefly, a specific antibody is immobilized on a gel-support, which is packed into a cartridge to form an immune-layer. A solution containing the toxin is mixed with an antigen-peroxidase conjugate and passed through the immune-layer, thus, a competition between the toxin and the antigen-peroxidase is established for binding to the immobilized antibody to take place. After washing, a chromogenic substrate solution of the peroxidase is added to the column, and the developed colour is observed. In the absence of the toxin, the antigen-peroxidase conjugate present is bound by antibodies and remains in the immune-layer; therefore intense colour development is observed. In the presence of the toxin, the binding of the antigen-peroxidase conjugate is inhibited, and, consequently, colour intensity would be lower or completely absent. The on-column assay coupled with the pre-concentration obtained by the same immune-layer allowed AFM1 detection at a level low enough to raise regulatory concern. The latest goal of researchers in the development of new rapid techniques in mycotoxin analysis is the exploitation of the immunochromatographic assay, also called lateral flow immunoassay (LFIA) or gold-colloid-based immunoassay, to produce fully-portable devices, which require no laboratory equipment, minimum skilled personnel, minimum sample preparation, and no hazardous chemicals (Krska & Molinelli, 2009). The assay can be typically concluded in few minutes and results can be both visually estimated or read by an appropriate reader. A commercial LFIA for the quantitative detection of AFM1 in milk is available (Rosa Aflatoxin M1 SL, Charm) and has been validated in an interlaboratory trial, confirming its reliability in the 300-550 ng/kg range. A more sensitive one-step device is also available from the same supplier (Rosa aflatoxin M1 MRL, Charm). Very recently, Wang et al. published the first LFIA for the assessment of AFM1 in milk. Nevertheless, AFM1 could only be detected at levels higher than 1 µg/kg. The requirement of extracting the toxin in a liquid medium from cheese samples, which would involve the use of organic solvent and laboratory equipment, together with the lack of specific regulations, has, until now, discouraged researchers from developing LFIA for measuring AFM1 which could be applicable to cheese.

2.2.1 Extraction of AFM1 from cheese samples to be analysed by rapid techniques

As discussed above, often a rapid and simple analytical method of measurement loses part or all of its advantages because for the need of time-consuming and laborious sample treatments. In addition, sample manipulation often involves the use of hazardous chemicals and laboratory equipment (centrifuge, evaporation systems, etc). As a typical example, the extraction protocol required before measuring the target toxin by means of the Ridascreen ELISA kit (R-Biopharm, Germany), which is the most widely used in AFM1 monitoring in cheese (Tekinsen & Eken, 2008; Colak, 2007; Dashti et al., 2009; Amer & Ibrahim, 2010; Sarimehmetoglu et al., 2004; Lopez et al., 2001; Virdis et al., 2008; Yapar et al., 2008; Ardic et al., 2009; Gurbay et al., 2006; Fallah et al., 2009), consists of the following procedures:

(i) a 2g-portion of cheese is homogenised and extracted with 40 ml of dichloromethane for 15 min; (ii) 10 ml of the extract is filtered and the solvent is evaporated under a nitrogen flux at 60°C; (iii) the residue is re-dissolved in a methanol-phosphate buffer mixture (50/50); (iv) the fat components are removed by adding an equal volume of hexane to the methanol-phosphate solution, shaking for 1 min, and (v) separating from the organic layer by centrifugation (15 min); (vi) finally, after discarding the upper organic layer, the lower aqueous-methanolic layer is diluted with a buffer and used in the assay.

Some other authors used a different commercial ELISA kit (Tecna srl, Trieste, Italy), whose extraction procedure is almost identical to that described above, except for the volume of dichloromethane used in the first step. These procedures evidence three major drawbacks: (i) large volumes of organic solvent are used particularly chlorinated ones, which means too that samples should be small (to limit the volumes of hazardous solvents being used) thus limiting representativeness; (ii) analysis should be conducted in an equipped laboratory; (iii) the procedure is long and laborious, with several steps, therefore increasing the sources of possible errors. Recently, we described a very simple and fast procedure for the extraction of AFM1 from dairy products, which uses an aqueous extracting medium and which allows the processing of several samples at the same time (Anfossi et al., 2008). The proposed method is based on the observation that AFM1 is bound to milk proteins, thus a protocol aimed at re-dissolving proteins from cheese (routinely employed in cheese analysis with the purpose of measuring total protein content) has been applied. The procedure involves: sample homogenisation and addition of a citrate solution; 15-min heating (50°C) under stirring; followed by centrifugation (15 min). The upper fat layer is discarded and the underlying layer is directly used in the ELISA. The validity of the approach has been verified on yogurt samples and different types of cheese: fresh, cream, soft, semi-hard, hard, blue, and elastic cheese. Validation of the described extraction has been made by comparing results on naturally contaminated cheeses with those obtained through a HPLC-FLD reference method. The extraction method is simple, relatively rapid and does not involve the use of any hazardous chemicals. Noteworthy is, the extraction medium, being completely aqueous and buffered at pH 8, makes it easy to combine with immunoassays.

3. Incidence of contamination of AFM1 in cheese

Since the late nineties of the last century, when the toxicity of aflatoxin M1 was brought to light and global regulations regarding aflatoxins started to be defined, monitoring of aflatoxin M1 in milk has been carried out. Some authors also investigated the occurrence of AFM1 in dairy products, although to a much lesser extent. These works have been already reviewed elsewhere (Govaris et al., 2002), therefore the latest five-years results have been summarised here.

3.1 Survey studies from 2006 to date

Several surveys have been conducted over the last five years on the occurrence of AFM1 in dairy products and, in particular, in cheese. The latter have been summarised in Table 2.

The first self-evident observation is that the problem of AFM1 contamination in cheese is mostly perceived in a specific geographical area, as almost all investigations have been carried out in the Middle East, except from the study conducted by Oliveira et al. (2011) in

Brasil and by Virdis et al. (2008) and Montagna et al. (2008) in Italy. In part, this may be explained by the fact that admissible levels in cheese have been set up by countries of the same region. Another factor which could be accounted for is the typical diet and the commercial relevance of cheese within various countries. Habits and the typical diet are difficult to quantify. However, there is a mismatch between the attention paid to the risk of aflatoxin contamination in cheese and geographical distribution of cheese production and consumption. More than 99% of the global production of cheese in 2010 was attributable to only 11 countries; in details, 47% in European countries (EU together with Switzerland) and 32% in the USA. Within the European Union, France (13.3%), Germany (8.3%), and Italy (7.8%), play the major role as cheese producers. In parallel, data on cheese consumption confirms the prominence of European countries (45% of global consumption of cheese in 2010) and the USA (32%); and specifically of France (10.7%), Germany (8.5%) and Italy (9.6%) within the European Union. Interestingly, Brasil and New Zealand are strong cheese consumers (5% of the cheese globally consumed is attributable to each of these countries) and Brasil is also a producer of a certain relevance (4% of total cheese produced annually in the world) (United States Department of Agriculture Foreign Agricultural Service, 2011a; 2011b). In this context, from one point of view, the interest in monitoring cheese safety in Italy and Brasil is not surprising, on the other hand a lack of data regarding other countries (for example United States, France and Germany) is evident.

Considering which analytical methods have been employed to conduct survey studies which have been published in the last five years, almost all use ELISA immunoassays to measure AFM1, thus confirming that the availability of simple and cost-effective techniques allows large monitoring programs to be carried out. The exception established by the work of Oliveira et al. who carried out a survey program by exploiting an HPLC method for measuring AFM1 in 48 samples, further confirms that the use of instrumental techniques limits the number of samples to be considered. In conclusion, there is a strong consistency in the analysis methods and a certain territorial homogeneity in considered samples, although this does not mean that samples are similar to each other concerning cheese-making, maturation and composition. In contrast, results on the level and incidence of AFM1 contamination are highly variable. Some authors found very low contamination levels and a great incidence of negative samples (Amer & Ibrahim, 2010; Dashti et al., 2009; Montagna et al., 2008; Er et al., 2010). On the contrary, other authors, who use the same analytical method and even analysed samples coming from the same country found a much larger incidence of positive samples and generally a much higher level of contamination (Tekinsen & Eken, 2008; Yapar et al., 2008; Ardic et al., 2009). A partial explanation of the discrepancy of results on Turkish cheese is the number of samples analysed which is, in some cases, too limited to be really representative. According to Govaris et al. (2001), the type of cheese-making could also influence toxin amount and, in fact, works have been done of different types of cheese.

However, the most populated level is the one which corresponds to AFM1 < 50 ng/kg in most works; some noticeable exceptions are represented by the level of AFM1 occurrence in Iran in 2008-2009 (Fallah et al., 2009; Rahimi et al., 2009) and in Turkey in 2008, according to Teckinsen & Eken (2008) and Ardic et al. (2009). Finally, we can observe that samples with AFM1 contamination beyond the admissible limits (where they exist) have been found in not insignificant percentages and that very high AFM1 concentrations (> 450 ng/kg) have been measured in 58 samples (4.6% of the total), both of which highlight the need for further and continuous control to preserve consumer health.

	Analysed samples (TOT)	Types of cheese	N of samples with AFM1 at level/TOT (%)				Ref
			<50 ng/kg	51-250 ng/kg	251-450 ng/kg	>450 ng/kg	
Turkey 2006	39	1	71.8	28.1	0	0	Gurbay et al. 2006
Turkey 2008	105	5	28.6	33.3	35.2 [a]	2.8 [a]	Yapar et al., 2008
Italy 2008	265	15	83.3	16.6	0	0	Montagna et al., 2008
Iran 2008	210	2	23.3	52.2	14.7	9.4	Fallah et al., 2009
Turkey 2008	132	1	17.4	55.3	19.7 [b]	7.6 [b]	Tekinsen & Eken, 2008
Italy 2008	41	1	9.8% positives, range 79.5-389 ng/kg				Virdis et al., 2008
Kuwait 2009	40	28	70.0	27.5	0	2.5	Dashti et al., 2009
Turkey 2009	193	1	17.6	56.0	14.0 [b]	12.5 [b]	Ardic et al., 2009
Iran 2009	88	1	53.4% positives, range 82-1254 ng/kg				Rahimi et al., 2009
Turkey 2010	70	1	92.9	7.1	0	0	Er et al., 2010
Egypt 2010	150	3	66.7	33.3	0	0	Amer & Ibrahim, 2010
Brasil 2011	48	2	77	18.8 [c]	4.2 [c]	0	Oliveira et al., 2011
Iran 2010	80	2	Average contamination: 22.3 (creamy cheese) and 43.3 ng/kg (feta cheese)				Mohamadi & Alizedeh, 2010

[a] reported contamination levels : 250-400 and >400 ng/kg

[b] reported contamination levels : 250-500 and >500 ng/kg

[c] reported contamination levels : 51-200 and 200-400 ng/kg

Table 2. Survey of AFM1 contamination in cheese from 2006 to date.

3.2 Occurrence of AFM1 in Italian cheese: results of a survey study conducted in 2010

The occurrence of AFM1 in Italian cheese was investigated during a one-year monitoring program in 2010. More than a hundred samples, belonging to different milking animal (cow, sheep, goat), manufacturing (industrial or traditional), feeding of dairy cattle (grazing or composite feed), and cheese maturation (long maturation, medium maturation, fresh) have been collected and analysed. Samples were extracted by using the above described aqueous approach and were analysed by a commercial ELISA kit. The complete method of analysis – extraction and quantification - had been validated in a previous work through comparison with a HPLC-FLD reference method on various classes of Italian cheese (Anfossi et al., 2008). In these conditions, the ELISA was demonstrated to have a limit of detection of 25 ng/kg, a dynamic range of 30-500 ng/kg

and relative standard deviations lower than 20%. It should be noted that the described method contemplates a corrective factor in the AFM1 quantification which makes results independent from the water content of samples. Indeed, different cheese could have very variable water content (usually indicated by the humidity percentage), depending on the preparation process and ripening, however this parameter has been included in the calculation of the amount of the target toxin, as discussed. The first aim of the work was the assessment of the occurrence of the aflatoxin M1 in Italian cheese. Italy is a producer of cheese of global importance and, in the meantime, Italians are strong consumers of both national and imported cheeses. In addition, there is a countless variety of the types of cheese that can be found on the Italian market; several of them originate from small producers who follow ancient recipes and traditional cheese-making methods. The complexity of this situation makes it difficult to generalize and classify samples so as to find exhaustive information regarding samples. Besides this first purpose of snapshotting the amplitude of the risk associated to AFM1 contamination of Italian cheese, the main objective of the work has been the identification of correlations between levels of contamination and some external factors which were identified as potentially influencing the presence and the concentration of the toxin. For this purpose, samples were divided into four categories according to: the animal which supplied the milk used to produce the cheese, the type of manufacturing, the season of production, and the maturation of the cheese. Within each category, samples were further sub-divided into groups (Table 3), which were compared with each other by statistic tests to evidence significant differences between groups.

3.2.1 Materials and methods

Samples classified as industrial were obtained from local supermarkets, while samples classified as small-scale were kindly provided by the Slow Food association (Cuneo, Italy) and by Eataly Distribuzione srl (Cuneo, Italy). Hard and medium maturing cheese samples were stored at -18°C until analysed. Soft cheese samples were immediately analysed without freezing. All samples were analysed before their expiry dates. A portion of sample (100 g *ca*) was roughly cut and then thoroughly minced and homogenized in a kitchen mixer. Aflatoxin M1 extraction was performed as previously described. In details, 5 g of homogenised cheese sample was weighed in a 50-mL conic tube, 20 mL of the extraction solution was added and the combination was maintained at 50°C for 15 min under vigorous stirring. The slurry was then centrifuged in a refrigerated centrifuge (25°C) for 15 min at 3200 x g. The fatty semi-solid upper layer was discarded and the liquid serum was withdrawn and directly analysed. Samples were extracted in single and analysed in triplicate. ELISA analyses were carried out as previously described (Anfossi et al., 2008). Briefly, 60 μL of AFM1 standard solutions or sample extracts was added to the same amount of the diluted antiserum and incubated in non-coated wells for 50 min. One hundred microliters of the mixture were transferred into coated wells and incubated for 15 min. After washes, 100 μL of the diluted anti-rabbit antibody labelled with the peroxidase was incubated in wells for 15 min. Colour development was obtained by a 20 min incubation with the TMB solution, followed by the addition of the stop solution. Finally, absorbance was recorded at 450 nm. Aflatoxin M1 concentrations were determined by interpolation on a linear calibration curve. Linearization of the calibration curve was performed by the logit-log transformation, by plotting the logit of the ratio (in percent) between the absorbance at each concentration of analyte (B) and the absorbance in the absence of analyte (B_0) against

the log of analyte concentration. The best data fit was obtained by linear regression of the standard points. Statistical analysis of data was carried out by the SigmaPlot 11.0 software (Systat Software Inc., CA, USA). First, the Shapiro-Wilk test on distribution of data was carried out. To be able to include undetectable samples (AFM1 concentration below 25 ng/kg, which is the detection limit of the method) in the statistical analysis, they were randomly ordered and a concentration value comprises between 0 and 25 ng/kg was attributed to each of them, by random number generation. Statistical differences between groups were evaluated by means of the Mann- Withney test on ranks (for the comparison between two groups) and of the extended Kruskall-Wallis ANOVA test on ranks (for the comparison between more than two groups) (Massart et al., 1988).

3.2.2 Correlation of aflatoxin M1 contamination with type of manufacturing, season of production, species of the animal that produced the milk, and cheese maturation

More than 83% of analysed samples showed detectable levels of toxin (> 25 ng/kg); most of the positive samples were measured to contain AFM1 between 50 and 150 ng/kg, with the exception of fresh cheese and of cheese made with goats' milk alone or mixed with other types of milk (Table 3). These groups generally showed a lower AFM1 content. Cheeses made with sheeps' milk have an equal distribution between the contamination levels below 50 ng/kg and between 50 and 150 ng/kg.

Statistical data analysis brought in light that the only factor which determined significant differences among groups was the origin of the milk. More specifically, cheese made with cows' milk showed itself to be more contaminated than cheese made with goat or sheep (or mixed goat/sheep, mixed goat/cow and sheep/cow) milk. This result agrees with the previous observation that milk from goats and sheep is less contaminated than cows' milk, both because of the different digestive apparatuses and mechanism of AFB1 assimilation of animals, and for the different feeding used in cow's breeding compared to ovine and caprine (Barbiroli et al., 2007; Hussain et al., 2010; Fallah et al., 2011). In fact, cattle fodders are more likely to be contaminated with AFB1 than those used to feed sheep and goats. This finding also confirms previous observations of other authors (Montagna et al., 2008), who also reported that cow's cheeses are more contaminated than others.

As a consequence of this first observation, samples made with cow's milk (82 samples) were isolated from the rest and the statistical analysis was repeated on them for the other three identified categories: manufacturing, cheese ripening, and production season. In this way, a further significant difference could be emphasized; industrial products were discovered to be less contaminated than small-scale products, probably because checks conducted on milk to be used in cheese production are more stringent in industrial scale production than in artisanal contexts. In addition, artisans often makes use of only one milk source, which can occasionally be contaminated with high AFM1 levels (although within the legal limit) thus determining a peak of contamination which would be found also in the derived cheese, while industrial production uses dilution of milk from various sources. This finding is in contrast to that recently obtained by Fallah et al. (2011). On the other hand, contrary to what appears at first sight from the data shown in Table 3, maturation does not influence AFM1 content in cheese. Several other authors observed that maturation does not significantly alter the AFM1 concentration, as would be reasonable to expect, given an appropriate correction of concentration values for the water content of the cheese analysed. A decrease of aflatoxin M1 concentration rather than an increase during maturation could be assumed, because of degradation of the toxin with time. Nevertheless, this degradation has not been pointed out

in any previous works aimed at assessing the fate of the toxin (Oruc et al., 2006; Colak, 2007; Kamkar et al., 2008; Mendonca & Venencio, 2005; Prandini et al., 2009; Govaris et al., 2001; Motawee & McMahon, 2009; Deveci, 2007).

Category	Group	Analysed samples (TOT)	N of samples contaminated at a level/TOT (%)			
			<50 ng/kg	50-150 ng/kg	150-250 ng/kg	>250 ng/kg
Maturation	Long (>3 months)	29	31.0	62.1	3.4	3.4
	Medium (>45 days; <3 months)	46	43.5	54.3	2.2	0.0
	Fresh (<45 days)	27	55.6	40.8	3.7	0.0
Manufacturing	Big brands	38	47.4	51.4	0.0	0.0
	Small-scale	64	52.6	53.1	4.7	1.6
Production season	Winter-spring	65	38.4	56.9	3.1	1.5
	Summer-autumn	37	51.4	45.9	2.7	0.0
Milk from	Cow	82	39.0	56.1	3.7	1.2
	Sheep	6	50.0	50.0	0.0	0.0
	Goat	6	83.3	16.7	0.0	0.0
	Buffalo	3	33.3	66.7	0.0	0.0
	Mix [a]	5	60.0	40.0	0.0	0.0
TOTAL		102	44	54	3	1

Table 3. Number of cheese samples analysed for the various groups identified as potentially influencing the level of AFM1 contamination and distribution of samples between these groups as a function of the level of AFM1 contamination.

The production season is also irrelevant according to statistical analysis. The factor "season of production" was defined to evaluate the influence of animals feeding (grazing or composite feed) on the assumption that animals fed on pasture would be less exposed to AFB1 ingestion and, consequently, would produce less AFM1 contaminated milk. Accordingly, cheeses made during summer and autumn, which belong to milk from grazing animals, would be less contaminated than cheese made during winter and spring, which belong to milk from animals fed with composite and stored fodder. Actually, according to information (when available) provided by producers of samples analysed in our work, animals were fed in pastures during summer and autumn, whereas they consumed stored feed during most of the spring. Therefore, groups to be compared were defined as reported in Table 3. The irrelevance of the period of production (and consequently, or partially consequently, of animal feeding) observed on Italian cheese samples could be explained by the fact that aflatoxin producing fungi also affects crops in the field. Nevertheless, the main limitation in making this analysis is the uncertainty of attribution of samples. In fact, some samples were accompanied by exhaustive information (period of production, animal feeding), however for most of them information was incomplete or unavailable. In these

cases, attribution to groups was assumed on the basis of generic information regarding the type of cheese, the expiry date and the similarity to other samples. Therefore, results on this factors cannot be considered as conclusive and would need further investigation. In fact, Taikarimi and co-workers observed that the season of production is relevant in determining aflatoxin M1 in cheese and demonstrated that cheese produced in winter are more contaminated than those produced in summer (Tajkarimi et al., 2008). Accordingly, Fallah et al. (2011) observed that samples produced in winter-spring are more contaminated than those produced in summer and autumn.

As in the case of samples from cows' milk, a further statistical analysis should be conducted by separately isolating the two categories of industrial and small-scale manufacturing samples and re-run statistical tests on the remaining categories (season of production and maturation) to highlight eventual significant differences which may have been hidden by the non-random distribution of samples between groups. However, the number of samples in each category and groups would become non-representative, therefore it would be interesting to increase the number of analysed samples to achieve more conclusive results.

Despite the high incidence of AFM1 at detectable concentrations all samples were contaminated beyond the admissible limit (250 ng/kg), except for 1 hard cheese, which still complied with legal limits (because MRL for hard cheese has been raised to 450 ng/kg in Italy since 2004). It is likely that the screening of milk (by control organisms or, most likely, by internal audit) is in general adequate to also secure the safety of cheeses, as undertaken by those countries that established admissible limits in milk and not in other dairy products.

4. Conclusions

Some of the inconsistencies highlighted by surveys conducted over the past five years could be clarified in light of these results, namely by separate samples according to the origin of the milk and to the type of manufacturing. For example, Virdis and co-workers found low positivity in Italian cheeses in 2008 compared to our survey, however it is justifiable since their study regarded specifically goats' cheese, which showed itself to be less contaminated than that of cows also in this study. The same is true for the work carried out by Gurbay et al (2006). On the other hand, authors who found high levels of contamination analysed cheese samples exclusively from cows' milk (Tekinsen & Eken, 2008; Dashti, et al., 2009; Yapar et al., 2008; Fallah et al. 2009) or samples produced at least partially from cows' milk (Ardic et al., 2009). Oliveira and co-workers reported a distribution of contamination levels which is in good accordance with that observed in the present study. More controversial are the results shown by Er et al. (2010) and Amer & Ardic (2009). The latter reported low contamination levels; however, few details regarding the type of samples are stated in the text. Er et al. showed very low incidences of AFM1 contamination in cheeses made from cows' milk (Er et al., 2010), which is in contradiction with all other published studies.

In general, most works were limited to reporting the occurrence of the toxin and the level of contamination, without correlating this information with any characteristics of the analysed samples. Therefore, conclusions were partial and related to specific circumstances and did not permit authors to generalise their observations. The reported findings of the study conducted in a one-year survey on various types of cheese in Italy and their correlation to some of the factors which could influence aflatoxin M1 presence in cheese allowed the identification of some relevant factors (milk origin, manufacturing type) and to rationalise

the results of the study and also preceding observations. The statistical approach is promising; however, further investigations on already identified factors, together with attempts to widen the number of considered factors, would occur.

From the point of view of the risk to consumers posed by AFM1 intake with cheese, the assumption seems verified that control strategies to limit AFB1 in feed and AFM1 in milk are an adequate protection for consumer health. Nevertheless, data representing the occurrence of aflatoxin M1 in cheese belonging to those countries which represent the principal cheese producers and consumers (United States, France, Germany) would be of great interest to further support this conclusion and to procure reliable suggestions to those who have legislative responsibility on this matter. Finally, it has been demonstrated once more that immunochemical methods of analysis, associated with rapid and simple treatments of samples, allow large screening surveys to be completed, thus providing researchers with a lot of information. The advantage of having readily available data should be, however, counterbalanced by appropriate methods of data management to achieve meaningful conclusions.

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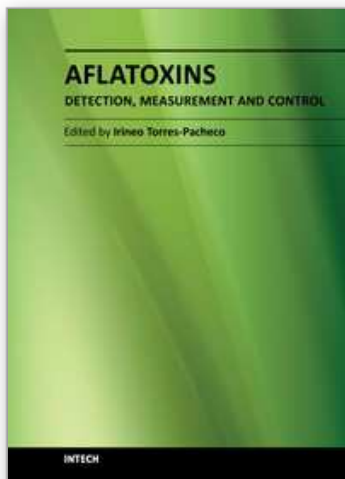
Aflatoxins - Detection, Measurement and Control

Edited by Dr Irineo Torres-Pacheco

This book is divided into three sections. The section called Aflatoxin Contamination discusses the importance that this subject has for a country like the case of China and mentions examples that illustrate the ubiquity of aflatoxins in various commodities The section Measurement and Analysis, describes the concept of measurement and analysis of aflatoxins from a historical prespective, the legal, and the state of the art in methodologies and techniques. Finally the section entitled Approaches for Prevention and Control of Aflatoxins on Crops and on Different Foods, describes actions to prevent and mitigate the genotoxic effect of one of the most conspicuous aflatoxins, AFB1. In turn, it points out interventions to reduce identified aflatoxin-induced illness at agricultural, dietary and strategies that can control aflatoxin. Besides the preventive management, several approaches have been employed, including physical, chemical biological treatments and solvent extraction to detoxify AF in contaminated feeds and feedstuffs.

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