Evaluation of Aflatoxin M1, enrichment factor in different cow milk cheese hardness category

Ivan Pecorelli,1 Raffaella Branciari,2 Rossana Roila,2 David Ranucci,2 Rita Bihl,1 Mariska van Asselt,3 Andrea Valiani2

1Istituto Zooprofilattico Sperimentale dell’Umbria e delle Marche “Togo Rosati”, Perugia, Italy; 2Department of Veterinary Medicine, University of Perugia, Italy; 3Department of Applied Research, Aeres University of Applied Sciences Dronten, the Netherlands

Abstract

Aflatoxin M1 (AFM1) is a hepatocarcinogenic and genotoxic derivative of aflatoxin B1, excreted into milk after ingestion of feed contaminated by Aspergillus genus fungi. Because of the important role of dairy products, especially cow cheese, in the human diet, there is great concern about the presence of AFM1, in this food category. EC Regulation No. 1881/2006 establishes the importance of the enrichment factor (EF), an essential parameter that must be defined in order to evaluate the maximum level of the toxin in cheese aiming to ensure that cheese has been produced from compliant milk. The Italian Ministry of Health has established two provisional AFM1, EFs (5.5 and 3.0) to be applied to as many cheese categories (hard and soft), defined according to the moisture content on a fat free basis (MFFB) classification. Two experimental productions of Primosale and Fior di Latte cheese, both belonging to the soft cheese category, showed an EF of 4.1 and 2.9 respectively. Data in literature also suggest that the EF attribution based on the current categorization may need reconsideration.

Introduction

Aflatoxins (AFs) are secondary metabolites, toxic to superior animals including humans, produced mainly by genus Aspergillus fungi, in particular A. flavus and A. parasiticus. These harmful molecules can be present in forages and feedstuffs as well as in food such as nuts, dried fruit, spices and cereals as a consequence of fungal contamination occurred before and after harvest (Campagnolo et al., 2016; Pecorelli et al., 2018, 2019). Human exposure to such contaminants through food consumption must be as limited as possible because of their proven genotoxic and carcinogenic activities. Among all AFs, B1 (AFB1) it is the most widespread in food products and among the most powerful in terms of genotoxicity and carcinogenicity (IARC, 2002). Due to the phenomenon referred to as carry-over, aflatoxin M1 (AFM1), a hydroxylated metabolite of AFB1, is usually found in the milk of lactating animals fed with contaminated feed. The International Agency for Research on Cancer has recently reassessed the toxicity of AFM1, upgrading it from Group 2B (possible carcinogens for humans) to Group 1, therefore classifying it as a certain carcinogen for humans, as has already been established for AFB1 (Pecorelli et al., 2019). Owing to its toxicity and in order to minimize the risk for consumers, the European Commission (EC) established a maximum level for AFM1, in raw milk, heat-treated milk, and milk for the manufacture of dairy products at 0.050 μg/kg (Regulation EC No. 1881/2006); while no limit was set for the presence of this toxin in dairy products. During the stages of the production process of dairy products, AFM1 usually concentrates at a ratio, defined trough the enrichment factor (EF), due to its preferential binding to milk caseins (Pecorelli et al., 2018).

According to Article 2 of Regulation (EC) No. 1881/2006 (European Commission, 2006), the EF is an important parameter that must be established to evaluate the maximum level of contaminants in dried, diluted, processed, and composed foodstuffs, aiming to ensure that cheese is being produced from compliant milk. In the persisting absence of specific maximum levels for AFM1 in dairy products and aiming to pursue public health protection, the Italian Ministry of Health established provisional AFM1 EFs of 5.5 for “hard cheese” and 3.0 for “soft cheese” (Italian Ministry of Health, 2013 opinion N. 13). The Decision of December 18th 1996 (European Commission) categorized the cheeses belonging to these two groups by the moisture content on a fat free basis (MFFB) parameter, identifying five different cheese subcategories: soft (MFFB ≥ 68%), semi-soft (68% > MFFB ≥ 62%), semi-hard (62% > MFFB ≥ 55%), hard (55% > MFFB ≥ 47%), and very hard (MFFB < 47%). Later the Italian Ministry of Health specified in a note (Italian Ministry of Health 2017 DGISAN note L4) that the “hard cheese” group to which EF 5.5 is applied, includes the semi-hard, hard, and very hard cheese categories, while the soft cheese group, characterized by an EF of 3.0, includes the hardness categories of soft and semi-soft cheeses. Data collected from numerous studies in the literature, concerning the definition of specific EFs during different cheese productions (Cattaneo et al., 2008; Fernandes et al., 2012; Cavallarin et al., 2014; Pietri et al., 2016; Pecorelli et al., 2018, 2019), suggest that the division of cheeses into the abovementioned two wide categories based on MFFB and the consequent attribution of the suggested EF, may not be very accurate due to experimental evidences showing that cheeses belonging to the same hardness category are characterized by different EFs.

The aim of the present study was to evaluate the EF of two soft cheeses and to study the correct definition of EF in different cow cheese categories.
Materials and Methods

Naturally contaminated AFM1 milk sampling

Milk samples were collected from cattle farms located in the Umbria Region (Central Italy) during 2016; samples were subsequently analyzed for AFM1, determination by the Istituto Zooprofilattico Sperimentale of Umbria and Marche regions (IZSUM), as reported by Pecorelli et al. (2019). Among all milk samples analyzed, contaminated samples were chosen for the production of Primosale and Fior di Latte cheese.

Manufacture of Primosale cheese

The cheesemaking of Primosale was carried out in the experimental pilot plant of the IZSUM with an aliquot (55 kg) of AFM1 naturally contaminated milk. Raw milk was heated at 37°C, and a mixed-strain starter culture (Lactobacillus lactis subsp. helveticus and Streptococcus thermophilus; Fermenti lattici, Laboratorio Prodro, Piacenza, Italy) was added. Coagulation occurred at 37°C with the addition of liquid calf rennet and was completed in about 40 min. The resultant curd was cut into hazelnut-sized granules and pressed into cylindrical forms. To facilitate whey draining, the forms were maintained at 30°C until pH reached the value of 5.2 (Branciari et al., 2014). The curd was then dry-salted and placed at 4°C. Six cheeses, of approximately 1 kg each (a total of 6 kg), were obtained from 55 kg of bovine whole milk.

Manufacture of Fior di Latte cheese

The Fior di Latte cheese was manufactured in an industrial plant from AFM1 naturally contaminated cow milk, pasteurized at 72°C for 15s. Acidification was carried out with natural starter culture and stretching of the curd was performed over 10 min at 86°C. The obtained products were transferred to the cooling vat with water at 8–10 °C, allowed to chill and firm before the Fior di Latte cheese was mechanically packaged (Roila et al., 2019).

Cheese composition

The chemical composition of Primosale and Fior di Latte cheese was determined. Fat, protein and moisture contents in cheese samples were determined by near-infrared transmission spectroscopy (FoodScan Lab, Foss Electric) (Pecorelli et al., 2018).

Evaluation of the cheese category

The two cheeses were classified according to firmness, following the criteria of the (EC) Decision of December 18th 1996 (European Commission, 1997). The classification of the hardness category was based on the moisture content on a fat-free basis (MFFB).

\[
\text{MFFB (\%)} = \left(\frac{\text{weight of water content}}{\text{total weight - weight of fat content}}\right) \times 100
\]

Evaluation of the EF

The EF of AFM1 was obtained using the equation reported below:

\[
\text{EF} = \frac{[\text{AFM}_1]_{\text{cheese}} (\mu g/kg)}{[\text{AFM}_1]_{\text{milk}} (\mu g/kg)}
\]

AFM1 analysis

Milk and cheese samples were assessed for AFM1 content, using the validated methods described by Pecorelli et al. (2018, 2019). Briefly, 50 g of milk were centrifuged to remove fat and applied to the Immunoaffinity column (IAC). AFM1 was eluted from the column by acetonitrile: methanol (6:4; v/v) and water in sequence.

For AFM1 analysis 20 g of cheese were used for extraction by MeOH 60% (50 mL) and then filtered through a glass fiber and centrifuged. The extract (30 mL) was diluted by PBS pH 7.4 (30 mL) before purification by IAC. AFM1 was eluted by 1 mL of MeOH and 1 mL of water applied in sequence over the IAC.

AFM1 instrumental analysis was carried out using a Shimadzu Nexera X2 UPLC–FLD apparatus (Shimadzu, Kyoto, Japan), using gradient elution conditions, as previously reported by Pecorelli et al. (2018). Briefly, for chromatographic separation of AFM1, a Kinetex C18 (50x2 mm; 5µm) column from Phenomenex (Torrance, CA, USA) was used. Analyte elution was achieved using a ternary gradient composed of water, ACN and MeOH at a flow rate of 0.8 mL/min. Fluorescence detection was used to identify λex = 360 nm; λem = 440 nm and quantify AFM1. LOD and LOQ were calculated according to Miller and Miller (2010). Furthermore analytical performances of the method in terms of recovery and standard deviation (SD) were assessed for both milk and cheese. Every unknown sample (milk or cheese) was analyzed twice (to assess for repeatability). In the same batch a blank and a spike quality control sample were included to evaluate recovery. According to EU legislation (Regulation (EC) No. 401/2006, European Commission), concentration of AFM1 was corrected by recovery in all samples.

Results and Discussion

The physical–chemical characteristics of Fior di Latte and Primosale cheeses are reported in Table 1. The cheese composition was 21.94 and 18.55% protein, 22.91 and 21.61% fat, 48.59 and 58.10% moisture for Primosale and Fior di Latte respectively.

The two cheeses produced in this study were classified according to their firmness as semi-soft and soft cheese with a MFFB between 66 and 63% for Primosale and ranging from 73 to 75% in Fior di Latte cheese (Table 2). The characteristics of Primo Sale cheese as well as those of Fior di Latte (Tables 1 and 2) are comparable to those reported in the literature for this type of cow milk cheeses (Lucera et al., 2014; Cavallarin et al., 2016; Roila et al., 2019).

The amount of AFM1 transferred in cheese was approximately 44% of the total amount of the toxin present in the milk for Primosale and 45% for Fior di Latte. Regarding the quantity of AFM1, found in the cheese, some authors registered a higher percentage of AFM1 transferred in whey than curd during cheese production (Pietri et al., 2016). On the contrary, other authors reported higher percentage of the toxin in cheese due, mainly, to its semi-polar characteristics and high affinity for the casein fraction of milk (Brackett, et al., 1982; Blanco et al., 1988; Yousef & Marth, 1989). These different results in literature are probably due to the differences in manufacture process, analytical techniques, type and degree of contamination, cheese type and cheese chemical composition (Wiseman and Marth, 1983; Blanco et al., 1988; López, et al., 2001). A crucial factor likely to influence the result is that the use of naturally contaminated milk in experimental cheesemaking can reproduce faithfully the real behaviour of AFM1.

The EF of Primosale and Fior di Latte cheese, calculated as the ratio of the toxin concentration in cheese to that in milk, was

| Table 1. Chemical composition of Primosale and Fior di Latte cheese. |
| --- |
| **Primosale, Mean ± SD** | **Fior di Latte, Mean ± SD** |
| Fat (%) | 22.91±1.88 | 21.61±1.12 |
| Protein (%) | 21.94±1.20 | 18.55±0.05 |
| Moisture (%) | 48.59±3.29 | 58.1±1.52 |
4.1 and 2.9 respectively. Yousef & Marth, (1989) state that in soft cheeses the AFM1 is 2.5 and 3.3 times higher than that in the milk from which they were made. According to Yousef and Marth (1989), Cattaneo et al. (2008) and Wiseman and Marth (1983) for Crescenza and Bakers cheese made with naturally contaminated milk, an EF of 2.55 and 2.97 respectively was defined. These values are comparable to those obtained in this study for Fior di Latte cheese, while their results were lower than those obtained for Primosale. An explanation for these differences can be the MFFB value, in fact Crescenza and Bakers cheese both belong to soft cheeses category as Fior di Latte (MFFB value ≥68%) while Primo Sale belongs to the semi-soft category (MFFB value between 68 and 62%).

Govaris et al. (2001), and Oruc et al. (2006) referred higher EF values for two soft cheeses, Telmes with EF of 4.4 and white pickled with EF of 4.0 respectively, even though they had a MFFB value ≥ 68%. Nevertheless, these two studies were carried out with AFM1 spiked samples that may show different behaviour compared to naturally contaminated specimens as mentioned above.

The EF value obtained by Brackett and Marth (1982a) for Cheddar cheese is similar to that obtained in the present study for Primosale; both dairy products can be ascribed to semi-soft cheese category.

The Italian Ministry of Health specified in a note (DGISAN note 1.4.cc 8.9/2010/6, 21-11-2017) that the soft cheeses group, characterized by an EF of 3.0, should include the hardness categories of soft and semi-soft cheeses. Data obtained in this study suggest that considering soft and semi-soft cheeses together may not be accurate and suggest that the definition of EF of soft cheeses may need to be confirmed by a larger amount of experimental data. Regarding the EF of hard categories, the above mentioned note grouped the cheeses with MFFB between 47 and 62% in one unique category to which an EF of 5.5 should be applied. The information obtained by the results on soft cheeses, induces us to emphasize the importance of finding correct definition of EF for all cheeses categories. For this purpose an analysis of data present in literature was carried out and the MFFB, as well as the EF values for cheeses of different hardness, were examined (Table 3). Pecorelli et al. (2019) showed that the AFM1 concentration in Caciotta cheese, a semi-hard cow milk ripened cheese with a MFFB value of 56%, was about 5-fold higher than in contaminated milk, which was close to that proposed by the Italian Ministry of Health for hard cheeses. Oruc et al. (2007) reported lower EF value in Kashar cheese, probably because the authors employed AFM1 spiked milk instead of a naturally contaminated one, for this reason the percentage of toxin bound to the protein might have been lower compared to naturally contaminated milk (Pecorelli et al., 2018). The EF value of semi-hard cheese like Caciotta is similar to that of Gouda (Table 3; Sakuma et al., 2016), a hard cheese with a MFFB value of 54, and was lower than 5.8 reported for Parmesan, a very hard cheese ripened for 10 months (Brackett and Marth, 1982b), characterized by a MFFB < 47%. Considering another typical Italian hard and long maturing cheese, such as Grana Padano (Manetta et al., 2009), the EF was slightly lower than 5.8 obtained for AFM1, naturally contaminated Parmesan. Nevertheless, the author referred that in the analysis of the AFM1 in cheese, the mean recovery was below the minimum recommended (70–110%) by the Commission Regulation (EC) N. 401/2006 (Annex II, Commission of European Communities, 2006) about mycotoxins in foodstuffs, probably due to the interactions AFM1 - matrix components.

In conclusion, this study stresses the importance of establishing the correct definition of EF in the different cow cheese categories with the purpose to guarantee public health protection and fair dairy products trade. Data from the present study together with data obtained from different studies reported in literature concerning the definition of specific EFs, suggest that the grouping of cheeses into two wide categories (hard and soft cheeses), based on MFFB, and the consequent attribution of the sug-

| Table 2. Concentration of AFM1 in milk and in cheese and enrichment factor (EF). |
|-----------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Sample                      | Milk            | Cheese          | EF              | MFFB            |
|                             | AFM1 [µg/kg]    | Total AFM1 (µg)| AFM1 [µg/kg]    | Total AFM1 (µg)| EF              | MFFB            |
| Primosale                   |                 |                 |                 |                 |                 |                 |
| Batch 1                     | 0.079           | 4.34            | 0.324           | 1.91            | 4.10            | 63%             |
| Batch 2                     | 0.051           | 2.81            | 0.207           | 1.25            | 4.01            | 66%             |
| mean±SD                     | 0.06±0.06       |                 |                 |                 |                 |                 |
| Fior di Latte               |                 |                 |                 |                 |                 |                 |
| Batch 1                     | 0.029           | 145             | 0.081           | 52.65           | 2.79            | 75%             |
| Batch 2                     | 0.029           | 145             | 0.086           | 55.90           | 2.97            | 73%             |
| mean±SD                     | 0.028±0.012     |                 |                 |                 |                 |                 |

Table 3. Cheese hardness category, MFFB% (moisture content on a fat free basis) and AFM1 EF (enrichment factor).

| Hardness category       | Type of cheese | MFFB % | EF    | Source                   |
|-------------------------|----------------|--------|-------|--------------------------|
| Very hard (MFFB < 47%)  | Parmesan       | 42     | 5.80  | Brackett and Marth, 1982 |
|                         | Parmesan       |        | 5.90  | Pietri et al., 2016      |
| Hard (55 % > MFFB ≥ 47%)| Gouda           | 54     | 5.01  | Sakuma et al., 2016      |
| Semi-hard (62% > MFFB ≥ 55%)| Caciotta     | 56     | 5.16  | Pecorelli et al., 2019   |
| Semi-soft (68% > MFFB ≥ 62%)| Primosale   | 65     | 4.06  | This work                |
|                          | Cheddar        | 66     | 4.30  | Bracket and Marth        |
| Soft (MFFB ≥ 68%)        | Fior di Latte  | 74     | 2.90  | This work                |
|                         | Crescenza      | 80     | 2.55  | Cattaneo et al., 2008    |
|                         | Bakers Cheese  | 84     | 2.97  | Wiseman and Marth, 1983  |
gested EF, may not be accurate because the experimental evidences show that soft and semi-soft, as well as semi-hard and very-hard cheese categories, may present different results addressing for different categorization.

References

Blanco JL, Dominguez L, GómezLucia E, Garayzabal JFF, Goyache J, Suárez G, 1988. Behavior of aflatoxin during the manufacture, ripening and storage of Manchego-type cheese. J Food Sci 53:1373–88.

Brackett RE, Marth EH, 1982a. Association of aflatoxin M, with casein. Z Lebensm Unters Forsch 174:439–441.

Brackett RE, Marth EH, 1982b. Fate of aflatoxin M, in parmesan and mozzarella cheese. J Food Prot 45:597–600.

Brackett RE, Applebaum RS, Wiseman DW, Marth EH, 1982. Fate of aflatoxin M, in Brick and Limburger-like cheese. J Food Prot 45:553–6.

Branciari R, Mugnotti L, Ranucci D, Miraglia D, Valiani A, Acuti G, Selvaggini R, Trabalza-Marinucci M, 2014. Influence of manufacturing procedure on the compositional and sensory properties of n-3 fatty acid-enriched pecorino cheese. J Dairy Res 81:455-61.

Campagnollo FB, Ganev KC, Khaneghah AM, Portela JB, Cruz AG, Granato D, Corrassin CH, Oliveira CA, Sant’Ana AS, 2016. The occurrence and effect of unit operations for dairy products processing on the fate of aflatoxin M1: a review. Food Control 68:310–29.

Cattaneo TMP, Monti L, Panarelli EV, Francolino S, Bertuzzi T, Pietri T, 2008. Fate of aflatoxin M1 during production and storage of Crescenza cheese. Ital J Food Sci 20:463-70.

Cavallarin L, Antonazzi S, Giaccone D, Tabacco E, Borreani G, 2014. Transfer of aflatoxin M1 from milk to ripened cheese in three Italian traditional production methods. Food Control 38:174-7.

European Commission. 1997. Commission Decision of 18 December 1996 laying down provisions for the implementation of Council Directive 96/16/EC on statistical surveys of milk and milk products (97/80/EC). In: Official Journal L 24/26, 25/01/1997.

European Commission. 2006. Commission Regulation (EC)No. 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. In: Official Journal L364/5, 20/12/2006.

European Commission. 2006. Commission regulation (EC) No. 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. In: Official Journal L70/12, 09/03/2006.

Fernandes AM Corrêa B, Rosim RE, Kobashigawa E., and Oliveira CAF, 2012. Distribution and stability of aflatoxin M, during processing and storage of Minas Frescal cheese. Food Control 24:104–108.

Gavras, A., V. Roussi, P. A. Koidis, and N. A. Botsoglou. 2001. Distribution and stability of aflatoxin M1 during processing, ripening and storage of Telemes cheese. Food Addit. Contam. 18:437–443.

International Agency for Research on Cancer (IARC), 2002. Monographs on the evaluation of carcinogenic risks to humans: Some traditional herbal medicines, some mycotoxins, naphthalene and styrene, vol. 82, p. 1–556. IARC Press, Lyon, France.

Italian Ministry of Health CNSA 2013 opinion N. 13, 10-06-2013. Problematiche Aflatossina M1 nei formaggi – Applicabilità dei coefficienti di trasformazione in equivalente latte (All. 2, D.M. 31 luglio 2003 del Ministero delle Politiche Agricole e Forestali).

Italian Ministry of Health 2017 DGISAN note I.4 ee. 8.9/2010/6, 21-11-2017. Fattori di concentrazione per l’aflatossina M1 nei formaggi; criteri oggettivi per la classificazione dei formaggi; art.2 del regolamento (CE) 1881/2006.

López C, Ramos L, Ramadán S, Bulacio L, Perez J, 2001. Distribution of aflatoxin M1 in cheese obtained from artificially contaminated milk. Food Control 18:437–51.

Lucera A, Mastromatteo M, Conte A, Zambrini AV, Facciola M, Del Nobile MA, 2014. Effect of active coating on microbiological and sensory properties of fresh mozzarella cheese. Food Packag Shelf Life 1: 25-9.

Manetta AC, Giammarco M, Di Giuseppe L, Fusaro I, Gramenzi A, Formigoni A, Vignola G, Lamberti L, 2009. Distribution of aflatoxin M1 during production, ripening and storage of Telemes cheese. Food Addit Contam 26:104–108.

Oruc HH, Cibik R, Yilmaz E, Yilmaz E, 2007. Fate of aflatoxin M, in Kashar cheese. J Food Saf 27:82-90.

Oruc HH, Cibik R, Yilmaz E, and Kalkanli O, 2006. Distribution and stability of aflatoxin M1 during processing and ripening of traditional white pickled cheese. Food Addit Contam 23:190-5.

Pecorelli I, Branciari R, Ortenzi R, M, Ciriaci M., Checcarelli S, Roila R, Capotorti A, Spaccini G, Valiani A, 2018. Evaluation of the concentration factor of aflatoxin M1 in a semi-hard Pecorino cheese obtained from naturally contaminated milk. Food Control 85:194-8.

Pecorelli I, Branciari R, Roila R, Bibi R, Ranucci D, Onofri A, Valiani A, 2019. Evaluation of Aflatoxin M1 Enrichment Factor in Semihard Cow’s Milk Cheese and Correlation with Cheese Yield. J Food Prot 82:1176-82.

Pietri A, Mulazzi A, Piva G, Bertuzzi T, 2016. Fate of aflatoxin M1 during production and storage of parmesan cheese. Food Control 60:478-83.

Roila R, Valiani A, Ranucci D, Ortenzi R, Servili M, Veneziani G, Branciari R, 2019. Antimicrobial efficacy of a polyphenolic extract from olive oil by-product against “Fior di latte” cheese spoilage bacteria. Int J Food Microbiol 295:49-53.

Sakuma H, Kamata Y, Sugita-Konishi Y, Yoshida T, Kobayashi M, Igoshi K, Kawakami H, 2016. Enrichment factors of aflatoxin M1 in dairy products for making naturally and artificially aflatoxin M1 contaminated milk. Japan J Food Chem Safety 23:118-25.

Wiseman DW, Marth EH, 1983. Behavior of aflatoxin M1 during manufacture and storage of Queso Blanco and Bakers’ cheese. J Food Prot 46:910-3.

Yousef AE, Marth EH, 1989. Stability and degradation of aflatoxin M1. In H. P. Van Egmon (Ed.), Mycotoxins in dairy products (pp. 127e161). London and New York: Elsevier Applied Science.