# Evaluation of Aflatoxin $M_1$ enrichment factor in different cow milk cheese hardness category

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## Abstract

Aflatoxin $M_1$ ($AFM_1$) is a hepatocarcinogenic and genotoxic derivative of aflatoxin $B_1$ 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 $AFM_1$ 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 $AFM_1$ 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 feedstuff as well as in food such as nuts, dried fruit, spices and cereals as a consequence of fungal contamination occurred before and after harvest (Campagnollo *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, $B_1$ ($AFB_1$) 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 $M_1$ ($AFM_1$), an hydroxylated metabolite of $AFB_1$, 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 $AFM_1$ 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 $AFB_1$ (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 $AFM_1$ 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, $AFM_1$ 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 foodtuffs, aiming to ensure that cheese is being produced from compliant milk. In the persisting absence of specific maximum levels for $AFM_1$ in dairy products and aiming to pursue public health protection, the Italian Ministry of Health established provisional $AFM_1$ 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 I.4) 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.

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## 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 $AFM_1$ 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 $AFM_1$ 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 Prodor, 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 $AFM_1$ 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).

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

### Evaluation of the EF

The EF of $AFM_1$ was obtained using the equation reported below:

$$EF = [AFM_1]_{cheese}\ (\mu g/kg)/[AFM_1]_{milk}\ (\mu g/kg)$$

### AFM1 analysis

Milk and cheese samples were assessed for $AFM_1$ 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). $AFM_1$ was eluted from the column by acetonitrile: methanol (6:4; v/v) and water in sequence.

For $AFM_1$ 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. $AFM_1$ was eluted by 1 mL of MeOH and 1 mL of water applied in sequence over the IAC.

$AFM_1$ 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 $AFM_1$, 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 ($\lambda_{ex}$ = 360 nm; $\lambda_{em}$ = 440 nm) and quantitate $AFM_1$. 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 asses 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 $AFM_1$ 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 $AFM_1$ transferred in cheese was approximatively 44% of the total amount of the toxin present in the milk for Primosale and 45% for Fior di Latte. Regarding the quantity of $AFM_1$ found in the cheese, some authors registered a higher percentage of $AFM_1$ 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 $AFM_1$.

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 $AFM_1$ 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, Telemes 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 $AFM_1$ 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 I.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 toghether 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 cheses 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 $AFM_1$ 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 $AFM_1$ 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), carachterized 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 $AFM_1$ naturally contaminated Parmesan. Nevertheless, the author referred that in the analysis of the $AFM_1$ 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 $AFM_1$ - 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 helath 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 |
|---|---|---|---|---|---|---|
| | $AFM_1$ [µg/kg] | Total $AFM_1$ (µg) | $AFM_1$ [µg/kg] | Total AFM1 (µg) | | |
| 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 | | | | | 4.06±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 | | | | | 2.88±0.12 | |

**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.

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