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Occurrence of aflatoxin M1 in milk samples from Italy analysed by online-SPE UHPLC-MS/MS

Luca Campone^{a,b}, Anna Lisa Piccinelli^b, Rita Celano^b, Imma Pagano^b, Rosa Di Sanzo^a,
Sonia Carabetta^a, Mariateresa Russo^a and Luca Rastrelli^b

^aDepartment of Agriculture, Università Mediterranea di Reggio Calabria, Reggio Calabria, Italy; ^bDepartment of Pharmacy, Università degli studi di Salerno, Fisciano, Italy

CONTACT Luca Campone lcampone@unisa.it

ABSTRACT

The occurrence of aflatoxin M1 in 69 milk samples collected in a south region of Italy in 2016 was evaluated. The samples were analysed using an automated method based on online SPE coupled with UHPLC tandem mass spectrometry. After a salt induced liquid–liquid extraction with acetonitrile to remove protein from milk, the extract was diluted with water and analysed using an automated online SPE MS/MS method. Among the analysed samples no one had AFM1 higher than the legally allowable limits whereas 71.4% of the other analysed samples were above the LOD of the method. The highest contamination level of AFM1 was found in pasteurised milk (44.39 ng kg⁻¹). The results show the worrying and widespread of AFM1 contamination, highlighting the necessity of monitoring studies in order to evaluate the reduction of the maximum legal limit.

1. Introduction

In nature there are many environmental pollutants, both inorganic such as heavy metals (Arukwe et al. 2014; Salvo et al. 2014; Naccari et al. 2015; Bua et al. 2016; Salvo et al. 2016; Di Bella et al. 2015; Albergamo et al. 2017; Graci et al. 2017) and organic contaminants as pesticide that can be found in foods (Cicero et al. 2017). Another class of highly toxic biological contaminants is mycotoxins (Bhat et al. 2010). Mycotoxins are a heterogeneous group of chemical substances, produced by some fungal such as *Aspergillus*, *Penicillium* and *Fusarium* species, which can contaminate a wide range of food products. Mycotoxins have been detected in a wide range of commodities, including cereals, spices, wine, coffee and animal

feeding stuff (O'Brien and Dietrich 2005; Shephard 2009; Imperato et al. 2011; Di Stefano et al. 2014, 2015). Among the various feedstuffs susceptible of mycotoxins contamination, cereals and silage are the major source of contamination (Scudamore et al. 1998) Aflatoxin B1 (AFB1) is the most widespread aflatoxin in animal feed with respect to the other aflatoxins.

AFB1 is considered to be a human carcinogen and for its high toxicity the International Agency for Research in Cancer (IARC) classified AFB1 into group 1 (IARC 1993, 2002). After the ingestion AFB1 is converted to AFM1 by hydroxylation at the tertiary carbon of the difuran ring system, metabolized by cytochrome P450 enzyme system in the liver (Fallah et al. 2011). In the liver into reactive epoxide intermediate or hydroxylated metabolites the 4-hydroxy derivative of AFB1, known as aflatoxin M1 (AFM1) and mainly excreted in urine and milk of dairy animal. AFM1 is cytotoxic, as demonstrated in human hepatocytes *in vitro* and its acute toxicity in several species is similar to that of AFB1. In animal study, the acute and short-term toxicity of AFM1 was similar to or slightly less than that of AFB1. AFM1 can also cause DNA damage, gene mutation, chromosomal anomalies and cell transformation in mammalian cells *in vitro* (Prandini et al. 2009). Although, the toxicity of AFM1 is lower than AFB1, it has an acute toxicity comparable to AFB1 and is considered a potential carcinogen for animals and humans. The consume of milk and dairy products contaminated with AFM1 may have negative health impact for consumers, particularly for infants and children that are more exposed to AFM1 ingestion because milk is the major constituent of their diet (Campone et al. 2016). Aflatoxin M1 is a very stable toxin in which neither, storage or processing such as pasteurisation and UHT treatment, can destroys this toxin (Yousef et al. 1989; Govaris et al. 2001). For this high toxicity and for its stability to the milk process, legal limit for AFM1 in milk and milk products have been established in many countries by national regulatory authorities. The European Union fixed at 50 ng kg⁻¹ the limit of AFM1 in milk (Communities, 1881/2006). Therefore, the presence of AFM1 in milk is considered to be undesirable. The European Union have regulated the maximum limits of 50 and 25 ng kg⁻¹ for consumable milk and infant formulae, respectively, aiming to reduce human exposure of the AFM1 to the lowest achievable level.

Due to its low maximum levels permitted, accurate and sensitive methods for the analysis of AFM1 in milk and milk products are increasingly required. In order to analyse AFM1 in milk and derivate products, several samples preparation strategies have been developed including solid phase extraction (SPE) (Wang et al. 2012), dispersive liquid–liquid micro extraction (Campone et al. 2013), and immunoaffinity columns (IAC) (Dragacci et al. 2001).

Although IA columns are time and solvent consuming and require the use of disposable cartridge, are usually preferred to other samples preparation technique since provide more accurate result and lower quantification limit. Regarding the instrumental determination of AFM1 different methods have been employed, most frequently used are high-pressure liquid chromatography techniques (HPLC) coupled to fluorescence detector (FLD) (Andrade et al. 2013) and triple-quadrupole (Campone et al. 2016). The present study reports the application of an automated on-line SPE method

previously developed for the analysis of AFM1 in milk (Campone et al. 2016). The method has been applied to analysed 69 milk samples (43 pasteurised and 26 UHT) collected in different local stores located in the south of Italy in 2016.

2. Results and discussion

2.1. Method validation

The developed method was validated according to EU guidelines for the analysis of mycotoxins in food (Communities C. of the E 2006) for the following parameters: selectivity, linearity, recovery, intraday precision, detection and quantification limits. The selectivity of method was experimentally evaluated monitoring the MS chromatograms in several blank samples of milk samples. The absence of any co-eluted compounds and interfering peaks in the retention time of target analyte indicated that no matrix compounds that could give a false positive results are extracted highlighting the were reported in Table 1 high selectivity of online SPE clean-up. The linearity was evaluated by the construction of calibration curve in the working range of 5–200 ng kg⁻¹ for AFM1 used 5 calibration levels each injected in triplicate. The correlation between peak areas and mycotoxins concentration was determined by linear regression accepting R₂ value above 0.999 for all analytes ($y = 1E + 07x + 181,67$), indicating a linear response in studied range. Recovery experiments were carried out by spiking a non-contaminate UHT milk sample at three different levels (25, 50 and 100 ng kg⁻¹) using appropriate volume of stock solution. The AFM1 recoveries of spiked samples were calculated using the solvent calibration curve and the results were reported in Table 1.

The intra-day precision of the method was evaluated by analysing six independent milk samples ($n = 3$), spiked at concentration of 50 ng kg⁻¹ for AFM1. The value, expressed as relative standard deviation (RSD%), was about less than 10%. The method sensitivity was calculated experimentally by the analysis of spiked blank samples at the signal-to-noise ratio (S/N) ratio of 3 and 10 for LOD and LOQ, respectively and the calculated limit of detection and quantification for AFM1 were reported in Table 1. The results of validation study show that online SPE clean up fullfil the EU regulation (Communities C. of the E 2006) regarding the performance criteria of official methods for the analysis of mycotoxins in foodstuffs.

2.2. Occurrence of aflatoxins M1

The value of AFM1contamination were summarised in Table 2. Samples with mycotoxin levels above the LOD have been considered positive. Regarding the pasteurised milks samples analysed in this study AFM1 were detected in 32 samples ranged from 0.72 up to 5.36 ng kg⁻¹ among all positive samples, no one milk sample exceed the maximum level of 50 ng kg⁻¹ set by European regulations in liquid milk. The maximum level of AFM1 in the positive samples was 44.39 ng kg⁻¹, just a little bit below the European 's legal limit of 50 ng kg⁻¹. The incidence of AFM1 in pasteurised milk was 72.7% with the absence of positive sample above the EU limit. The occurrence of AMF1 in the 26 analysed UHT milk samples is reported in Table 2. The AFM1 was detected (>LOD) in 15 samples, ranged from 0.72 to 1.57 ng kg⁻¹ with a 20.3% rate of contamination. Among the positive UHT samples, no samples exceeding the AFM1 legal limit content. This study revealed that even if just one samples exceeded the limit of contamination set for EU countries, a high proportion, up to 31.4% of analysed milk consumed in Italy was contaminated with AFM1 with an average concentration of 2.74 ng kg⁻¹.

3. Experimental

See Supplementary materials for Milk samples, Chemicals and Reagents, Samples preparation, AFM1 Mass spectrometry analysis, AFM1 Mass spectrometry analysis and Performances of the Method.

4. Conclusions

Several studies in different countries have demonstrated that milk is a food with a high rate of AFM1 contamination. For these reasons milk could be considered an important source of mycotoxin M1 for human exposure especially for children where milk is the main constituent of their diets. In this paper, the occurrence of mycotoxins AFM1 in milk has been monitored using an online SPE-UHPLC-MS method. The results showed that no sample exceeded the maximum level set by the European regulations for AFM1 in milk but the contamination rate above the legal limit was quite high (31.4%). This study underlines the importance of periodic milk's monitoring in order to reduce as much as possible milk's contamination avoiding very dangerous public health problems.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Table 1. Analytical performance of online SPE method.

Sample	LOD	LOQ	Recovery \pm RSD ^a (n = 3)		
	ng kg ⁻¹		Level (ng kg ⁻¹)		
			25	50	100
UHT milk	0.7	2.3	98 \pm 8	89 \pm 5	102 \pm 7
Pasteurised milk	0.8	2.6	110 \pm 6	95 \pm 3	100 \pm 5

^aRSD: relative standard deviation.

Table 2. Number of analysed samples and contamination range of AFM1 collected in the year 2016.

	Aflatoxin M1					Average concentration ng kg ⁻¹
	Number of samples	Contaminated samples			Contamination range	
		n.d.	<LOQ	>LOQ	ng kg ⁻¹	
UHT milk	26	8	12	6	0.71–3.63	1.64
Pasteurised milk	43	11	20	13	0.85–44.39	3.45
Total	69	19	32	19		
Total %		27.5	46.4	27.5		

n.d.: <LOD.