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Evaluation and comparison of Mycotoxin (AFM1) in imported cow milk with Kurdistan milk available in Sulaimani markets by using High performance liquid chromatography

Dereen Omer Ramzi[1] and Ahmed Yaseen Hamadameen[2*]

[1]Department of Basic Sciences, College of Veterinary Medicine, University of Sulaimani, Sulaimani New, Street 27,Sulaimani City, Kurdistan Region, Northern Iraq

[2]Department of Microbiology, College of Veterinary Medicine, University of Sulaimani, Sulaimani New, Street 27,Sulaimani City, Kurdistan Region, Northern Iraq

Correspondening Author: Ahmad.Hamadamin@univsul.ed.iq

ABSTRACT

This work determined the quality of milk regarding AFM_1 residue in four types of milk. An highlighted and sensitive method of toxin extraction applied called solid phase extraction (SPE) with using liquid chromatography which was HPLC- fluo-rescence detector (FLD) to determine of aflatoxin M_1 (AFM_1) in pasteurized liquid cow milk and produced fresh raw milk(cow milk). The detection limits of this method were 0.5µg/kg in liquid fresh and pasteurized packaged milk. The rehabilitation achieved in all drink form was between 85.4 and 96.9%, and the RSDs were between 0.8 and 3.6%. In addition, the level of AFM_1 contamination settled in 27 samples from all the evaluated samples; however, AFM_1 residue in contaminated samples were not more than FDA international legal limit (0.5µg/kg) while, AFM_1 in 11 samples were exceed than CR international limits(0.05µg/kg), the rest 33 samples were totally free from AFM_1contamination. OASIS HLB and IAC also used with HPLC to detect AFM1 residue and the optimization of OASIS HLB cartridge were examined to minimize interferences and pre-concentrate analyse. The work totally evaluated three types of milk regarding AFM1 which are available in Sulaimani markets, and a couple of cleaning up technique applied. Finally, all the results statistically analyzed by (Simple *t* test and ANOVA) SPSS-version 18 program.

Keywords: Aflatoxin M1; packaged milk; raw milk; SPE extraction; HPLC

INTRODUCTION

Mycotoxins generally are toxic metabolises produced by fungi growing on a variety of food stuffs including that of animal feeds and affected animals through ingestion contaminated feeds and build-up in the animals circulatory system, liver and other tissues such as adipose and muscle; hence, passes up the food chain(Turner *et al*, 2009). Aflatoxins and Ochratoxins are two common toxins found in milk and the four major AFT are B1, B2, G1, and G2 expected to be in animal products which determined based on their fluorescence and mobility during thin-layer chromatography (Herzallah, 2009). They are heat stable and high in resistance; therefore, threat to human health through ingestion animal products such as milk, cheese, meat and eggs. Ingestion of infected milk or other animal products causes liver damage, cancer, mental impairment, abdominal pain, vomiting, convulsions, pulmonary edema, coma and death. (Sani *et al* 2013) Group of hepatocarcino-genic bisdihydrofurano metabolites are closely related to the aflatoxin and its formed by specific Aspergillus species particularly by specific strains Aspergillus flavus and Aspergillus parasiticus (Richard, 2007). AflatoxinM1 (AFM_1) is a metabolite of aflatoxin hydroxilation which present in the milk products and its reached when cattle consumed contaminated grass and/or ration. Another source is AFB_1, which can provide 0.3- 6.2% of total AFM_1 in milk (Creppy, 2002).

During sterilization, milk-based products, milk storage and pasteurization, the AFM_1 is stable (Dutton *et al*, 2012) whereas consumption of AFM1, even on a small absorption, there is a major threat to community health, particularly for children who are great consumers of milk and its by-products.

Therefore, AFM_1 globally audit and its manage the level of AFM_1 present in milk products. According to the U.S Food and Drug Administration, AFM1 residue milk have no more than 0.5 microgram / kg (Food and Drug Administration, 1996). Indeed, European Union (EU) has settled AFM_1 as 0.05 µg/kg in milk used for adult intake (Commission Regulation, 2001). For the child's milk and its by-product, this amount must be less than 0.025 microgram / kg (Commission Regulation, 2004). In Iraq, there is no specific Iraqi regulation for AFM1 in milk; hence, the EU regulation has been established and applied for authorisation the toxin. For all these reasons, it is crucial to explore the most accurate as well as simple and inexpensive method to determine AFM1 in raw as well as packaged milk. There is many techniques to determine AFM1 in milk such as Enzyme-linked immunosorbent assay (ELISA), which is the existence of a rapid method of screening AFM1, 0.01 microgram/L sensitivity (Kim *et al*., 2000; Rodriguez-Velasco, *et al*, 2003).

Basically the purpose of quantitative analysis including the most popular technique is liquid chromatography (LC), that is typical or reverse phase separation, mainly followed the fluorescence detection. LC mixed with mass spectrometry (MS) also attracted much consideration for its benefits in curing out to identify and determine of analytes at traceelevation (Lee *et al*, 2009). Determining AFM1, a clean-up or enhancement method is generally essential which is Carbograph-4, C18, Multi-functional clean-up column and immune-affinity column (IAC), have desirable purification consequence for AFM1 clean-up of product of milk (Cavaliere *et al*., 2006; Chen, *et al*., 2005 and Manetta *et al*., 2005). Certainly, immune-affinity column is the most classical clean up technique which can select and isolate of analyte from a complex materials; whereas, OASIS HLB sorbent has been established as an adequate method, for polar and non-polar admixture with perfect retention (Fontanals *et al*., 2005). In 20015, Kok-konen's groups extracted AFM1 from cheese, and determined named mycotoxinn in mould cheeses with liquid chromatography tandem mass spectrometry, in which the permit of quantification for AFM_1 was 0.6 µg/kg (Kokkonen, *et al*, 2005). OASIS HLB cartridges have also been used to clean up of mycotoxins in cow milk by HPLC mingled with mass spectrometry. The restoration for AFM1 achieved in this study was more than 80% in the concentration range 0.02-1 µg/L (Sørensen and Elbæk, 2005). In 2011, Beltrán's groups also tried to use OASIS HLB cartridges clean up AFM_1 in cereals in which heavy signal suppression was attended, which make it unattainable to evaluate AFM_1 assuredly; subsequently the matrix effect formed by analogize admixture compounds (Beltrán *etal*., 2011). Although, the usage of OASISHLB cartridge connected with HPLC and FLD, reprivatisation for determination of AFM1 cannot be performed in all types of liquid, and powder milk and still no perfect and highly sensitive method has been improved.

The objective of this work is to determine and evaluate the level of residual AFM_1 in pasteurised imported cow milk with comparing to fresh raw milk are available in Sulaimani markets to confirm that how far they are acceptable in terms of quality and suitability for human consumption regarding the level of AFM1. Moreover, to evaluate the optimization and validation of the OASIS HLB cartridges and IAC clean up technique.

MATERIAL AND METHODS

Collection of Samples

Total of 60 sample of liquid milk were collected in 22 January to 03 February 2015, which involved 15 sample of fresh raw milk and 45 samples of three different types of pasteurised imported packaged milk (Bgah, Ichim, and Nada). All the samples were stored at room temperature before analysis.

Chemicals and Reagents

All chemicals that used in this project were in high performance liquid chromatography grade; chromatographic grade methanol and acetonitrile were purchased from Fisher Scientific (New Jersey, USA). Some other reagents were purchased from Sigma (Sigma–Aldrich, Steinhem, Germany). Immunoaffinity columns were supplied by Vicam (Watertown, MA, USA). OASIS HLB (500 mg, 6 cc) cartridges were achieved from Merck (Darmstadt, Germany).Standard solutions of AFM1 (10 µg/mL in acetonitrile) was purchased from Sigma Chemical (St Louis, MO, USA). AFM1standard were protected from light by putting into amber bottles as AFM1 is relatively light degradation and 0.2-4.0 µg/L working solution was prepared for each injection daily.

High Performance Liquid Chromatography

To follow the (Sørensen, *et al*, 2005) method, the sample extracts are kept at 5–10 ^{o}C until analysis. A volume sample is injected into the chromatographic system and the column flow rate regulated in 600 µl /min and the column temperature kept. The probe voltage also regulated in 4200V in negative ion mode and 5000V in positive ion mode. The SHIMADZU

Prominence HPLC with LC-20AT Solvent Delivery System was equipped with a SIL-20A auto-sampler and RF-10AXL fluorescence detector was applied to separate and determine AFM_1. Isolation was performed on a C18 reverse phase column (250×4.6 mm, ID = 5 µm, Diamonsil®). The mobile phases A and B were acetonitrile and ultrapure water, respectively. Flow rate was held at 1 µL/min and the gradient conditions were as follows: 0-25 min, 25% A; 15-20 min, 30-00% A; 20-30 min, 80% A; 26-28 min, 80-30% A; 28-38 min, 27% A. The temperature of column was controlled at 35 ^{o}C. Injection volume was 50 µL. AFM_1 were detected by FLD and the wavelengths for excitation and emission were 365 and 435 nm, respectively.

Sample pretreatment

By following (Wang *et al*, 2012) method, the procedure was performed as follow. AL All the liquid milk samples were accurately weighed (50 ± 0.1 g) into 100 mL centrifuge glass tube. After centrifugation for 15 min (4000 rpm), fat layer was separated and the supernatant was collected in to 20 mL tube. The supernatant solution (20 mL) was applied to an OASIS HLB cartridge which had previously been conditioned with 5 mL acetonitrile and 5 mL water, successively. The column was washed with 10 mL 20% acetonitrile aqueous solution. The AFM_1 were eluted with 5 mL acetonitrile and the eluate was separated and evaporated to dryness using stream nitrogen. According to (Lee *et al*., 2009) method, the residue was reconstituted with 1.0 mL of 20% aqueous acetonitrile and the achieved solution was forced through a PTFE syringe filter (pore size 0.45 µm). Finally, the cleanup of AFM_1 in milk carried out by using IAC.

RESULTS AND DISCUSSION

Method optimization

On the mode of 250 mm C18 reverse phase column HPLC analysis of AFM_1. Solid phase extraction method was used which includes elution, adsorption, and washing can increase the recovery and the interface, this work compared the optimization of clean up by OASIS HLB and IAC to purify the toxin which are non-expensive method. If these cleanup methods are compared with immunosorbent cartridge which is another common method to clean up AFM1, the cleanup methods are much less in price and so much easier to be applied. In this work, The OASIS HLB cartridge adsorption efficiency initially evaluated from the retrieval values of 10 micro-grams/Liter, and at small flow rate the standard solutions inserted into the cartridge. The outcome presented that the recovery of the AFM_1 was 93.7 ± 1.8%, which indicated that there is no highlighted losses happened during the extraction technique. The admixture of acetonitrile/water at essential wash solvent in different proportions was evaluated to select. A little bit of recovery, when the amount proposed is higher than 30% of acetonitrile. Acetonitrile to keep the maximum amount of analyte retention of about 10% according to the (Beltrán *et al*., 2011). The work presented that the ideal outcome was achieved when concentration of acetonitrile was 30%. Acetonitrile and methanol were tested at various volumes of elution (1, 2, 5, 10 and 15 mL), correspondingly, this is for evaluation of tenor of elution solvent to achieve greater efficiency in the investigation. The primal result was achieved with 5 mL of acetonitrile (91.7 ± 1.8%) and 5 mL of methanol (82.3±2.5%) and the latter was selected as a elution solvent. The acceptable state of extraction based on OASIS HLB cartridges which were showed in (Table1). Corresponding spiked samples which were spiked with 0.050 µg/L or 0.250 µg/kg of AFM1, were determined to check potential interferences in all the blank samples which were include in fresh raw milk (cow milk) and the 3 types of imported milk. All the chromatograms presented in Fig. 1and 2 presented well separated AFM1 peaks and provided desirable optimization condition in both cleanup techniques. The retention time of AFM1 was approximately 8.1 ± 0.2 min. In addition, there is no peak (see arrows in Fig1) in blank samples at the same time; this could be due to the fact that, no components in samples have relatively same retention time as AFM1 to give false positive in four type milk samples. Regarding the cleanup efficacy of OASIS HLB cartridge and IAC columns, there is relatively similar purity peaks were observed in both cartridge, in spite of the fact that tiny differentiation in on the quantitative of AFM_1 been observed.

Method performance

The determination limit of this method is more sensitive than derivative technique of mass spectrometry revealed in previous experiments (Cavaliere *et al*., 2006; Manetta *et al*., 2005). While, Technology presented requires simple laboratory equipment, and detection limit is well below the legal limit Iraq's recent international standards. Hence, this advanced method is applicable for the determination of AFM_1 in imported packaged liquid milk and raw milk. In this experiment, the standard value by following the of 0.10 µg/L to3.00 µg/L was designed and used to detect AFM1 in samples.

Table 1. Optimized parameters for OASIS HLB and IAC

	OASIS HLB	IAC
Adsorption efficiency	93.7%	92.3%
Elution solvent	5 mL acetonitrile	1.5 m acetonitrile methanol (2.2 ± 3, v/v)
Washing solvent	10 mL 30% acetonitrile	10 mL water
Extraction time	21 min	35.0 min

Table 2. Comparison of recoveries in different matrixes cleaned up by IAC or OASIS HLB column (n = 2)

Milk Sample	Spiked (µg/kg)	**Cleaned up by OASIS HLB**		**Cleaned up by IAC**	
		Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Raw milk (Cow milk)	0.010	88.9	4.2	92.6	3.4
	0.150	92.7	3.2	94.8	4.8
Bgah milk	0.100	81.3	4.4	86.4	2.8
	0.025	94.6	2.2	92.5	4.7
Ichim milk	0.250	91.2	3.6	94.6	2.4
	0.050	85.9	2.8	98.4	3.6
Nada milk	0.050	92.5	2.4	90.6	2.6
	0.100	81.6	3.2	88.2	4.1

Table 3. Chromatograms raw data presenting the real AFM_1 in four types of milk samples

Sample type	Raw milk (Cow milk)		*Bgah milk		*Ichim milk		*Nada milk	
	AFM1 µg/kg		**AFM1 µg/kg**		**AFM1 µg/kg**		**AFM1 µg/kg**	
Sample No.	Cleaned up by OASIS HLB	Cleaned up by IAC	Cleaned up by OASIS HLB	Cleaned up by IAC	Cleaned up by OASIS HLB	Cleaned up by IAC	Cleaned up by OASIS HLB	Cleaned up by IAC
1	0.013	0.019	nq	nq	nq	nq	0.008	0.065
2	nq	nq	nq	nq	nq	nq	nq	nq
3	nq	nq	0.093	0.08	nq	nq	nq	nq
4	nq	nq	nq	nq	0.0012	0.0015	nq	nq
5	0.055	0.042	0.011	0.014	nq	nq	nq	nq
6	nq	nq	nq	nq	0.078	0.09	0.098	0.069
7	0.015	0.09	0.03	0.12	nq	nq	0.009	0.003
8	0.013	0.039	0.06	0.044	0.076	0.043	nq	nq
9	0.15	0.01	0.02	0.06	0.0071	0.012	0.0012	0.064
10	0.011	0.014	nq	nq	nq	nq	nq	nq
11	0.014	0.033	0.019	0.034	nq	nq	nq	nq
12	nq	nq	nq	nq	nq	nq	0.016	0.011
13	nq	nq	nq	nq	nq	nq	0.006	0.0017
14	0.012	0.018	0.018	0.0094	nq	nq	nq	nq
15	nq	nq	nq	nq	0.008	0.002	0.0089	0.013

**Bgah, *Ichim,*Nada, are the trade names of 3 types of (Iranian, Turkish and Saudi Arabia) milk respectively*

**nq=non quantified*

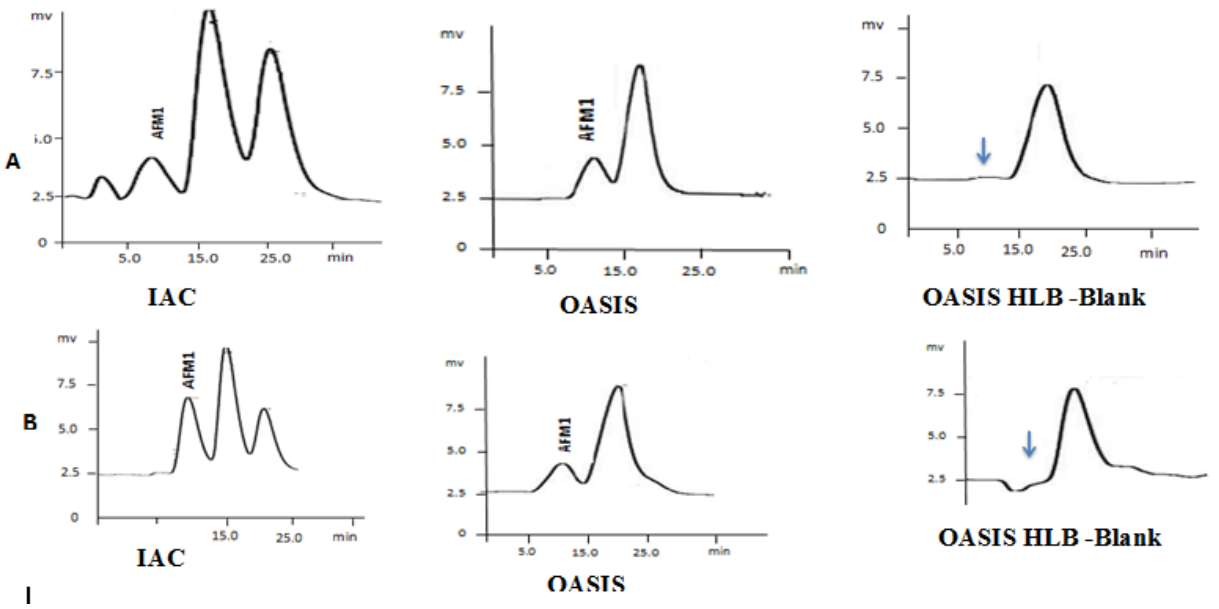

Fig. 1. HPLC-FLD chromatograms for AFM1 in different milk samples cleaned up by IAC and OASIS HLB column: (A) raw milk spiked at 0.050 µg/kg; (B) pasteurized packaged milk (Bgah), spiked at 0.250 µg/kg

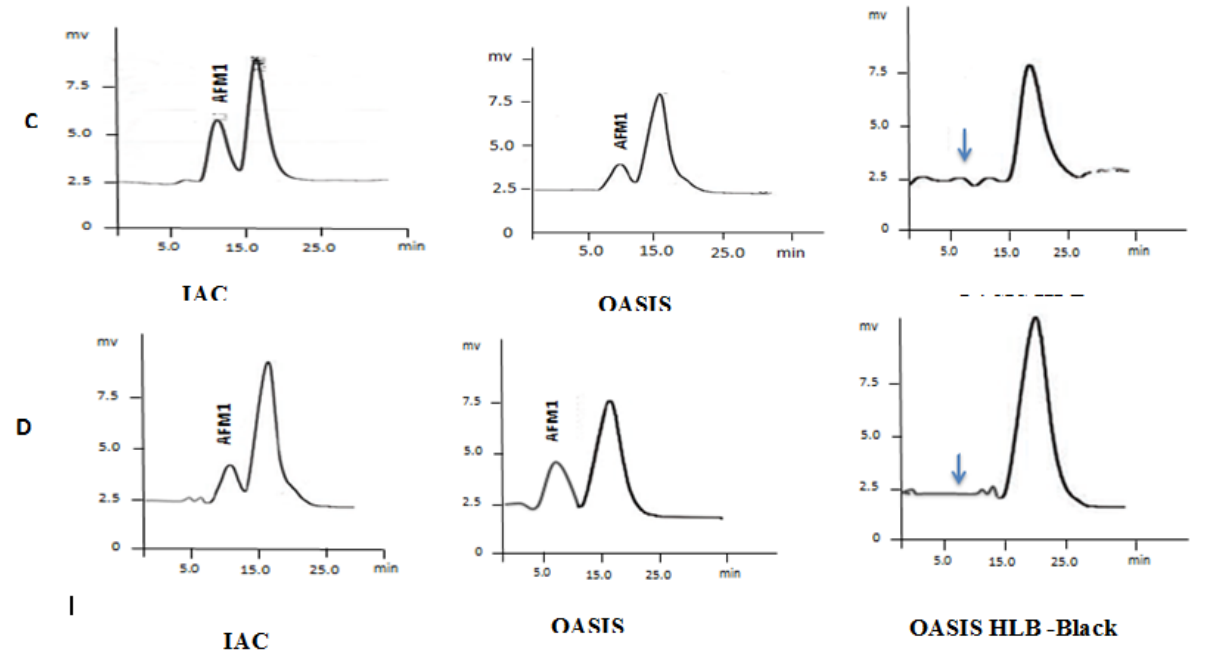

Fig. 2. HPLC-FLD chromatograms for AFM1 in different milk samples cleaned up by IAC and OASIS HLB column: (C) pasteurized packaged milk (Ichim) spiked at 0.250 µg/kg; (D) pasteurized packaged milk (Nada) spiked at 0.250 µg/kg

The linear equation was ("y" = 16,93x - 1332, and" y" = area, "x" = concentration), and the correlation coefficient (R^2) was 0.974. The limits of detection (LOD) of AFM_1 for raw milk and pasteurized milk were 0.050µg/kg and 0.037µg/kg for the limits of quantitation (LOQ). The retrievals and repeatability of Aflatoxin M_1 from packaged pasteurized liquid milk and raw milk and at different levels were attained by (OASIS HLB) column and the detailed data of recoveries conducted which is considered as practical and reliable for detection of AFM_1 from milk. Furthermore, the IAC clean-up column was used as a differentiation to assess the optimization of the method and the results presented that statistically there is no significant difference(p value=0.26). between the two methods.(see table 2).

Application to samples

As it has been shown in table 3, the level of AFM_1 residue fresh raw milk and imported milk was various regarding milk types (fresh and pasteurised packaged milk), also different regarding the milk trade names. The number of positive samples for AFM_1 residue in 15 samples was 8 (53.4%). Furthermore, out of 15 sample of imported milk (pasteurised with ultra-high-temperature (UHT), in Bgah type, 7 (46.6%) were positive, and Ichim was 5 (33.3%) and Nada was also 7(46.6%), respectively. Hence, out of 60 samples of raw and pasteurised milk, there was only 27(45%) of samples contained AFM_1. One more result was that, 53.4%, of raw milk contained AFM_1 residue; whereas, out of 45 pasteurised imported milk samples, only 42% of milk samples contained AFM_1 residue. Moreover, out of all 27 positives, the the values of concentration do not exceed the international law and regulation (0.5micro-g per kilogram) regarding FDA regulation. While, out of all 27 positives, 11 samples contained exceed AFM_1 regarding RC limitation (0.05µg/kg).Fortunately, in Iraq, the FDA regulation is used by ministry of health; hence, the level of contaminationAFM_1 does not seem to be in threatened for public health (see table 3). Finally, both clean-up methods by (IAC) and (OASIS HLB) attained comparatively similar consistent outcomes. Statistically the (t test) results presented that, there is no significant difference between residual AFM1 value in all the milk samples versus to international limit(0.5µg/kg) (p value=0.085).One more interesting (ANOVA) result was that, no significant difference between the level of AFM1 residue in all milk samples cleaned by OASIS HLB versus IAC(p value=0.26).Statistically there was only one significant difference resulted which was between the level of the AFM1 residue between raw milk (Cow milk) and Ichim milk(p value 0.043).

CONCLUSION

High Performance Liquid Chromatography considered a sensitive method to detect components in food and beverages especially if the samples extracted by using one of liquid and solid phase extraction method. In this work, OASIS HLB and IAC used with HPLC to detect AFM1residue in liquid milk and the (OASIS HLB) cartridge were examined optimization to minimize interference and pre-concentration analysis. This performed to reach extreme purification. The technique presented advisable results regarding accuracy, and detected tiny particle of toxin in liquid milk.regarding the results, pasteurised packed milk which is imported in Iran(Pgah),Turkya(Ichim) and Saudi Arabia (Nada) presented safe condition in terms of AFM1 residue. Although, fresh raw milk raw milk in Sulaimani presented AFM_1, the level still would be in safe to public health.

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REFERENCES

1. Beltrán, E., M. Ibánez, J.V. Sancho, M.A. Cortés, V. Yusà and F. Hernández. 2011.UHPLC eMS/MS highly sensitive determination of aflatoxins, the aflatoxin metabolite M_1 and ochratoxin A in baby food and milk. Food Chem. 126: 737-744.
2. Cavaliere, C., P. Foglia, E. Pastorini, R. Samperi and A. Laganà. 2006. Liquid chro-matography/tandem mass spectrometric confirmatory method for determining aflatoxin M_1 in cow milk: comparison between electrospray and atmospheric pressure photoionization sources. J. Chrom. 110: 69-78.
3. Chen, C. Y., W.J. Li and K.Y. Peng. 2005. Determination of aflatoxin M_1 in milk and milk powder using high-flow solid phase extraction and liquid chromatogra-phyetandem mass spectrometry. J. Agri. Food Chem. 53: 8474-8480.
4. Commission Regulation (EC). 2000. Food residue regulations. Off. J. Eur. Com. 077: 0001-0013.

5. Commission Regulation (EC). 2004. No. 683/2004 of 13 April 2004 amending Regulation Official. J. Eur. Com. (EC). 466: 0003-0005.
6. Creppy, E.E. 2002. Update of survey, regulation and toxic effects of mycotoxins in Europe. Toxicol. Lett. .27: 19-28.
7. Dutton, M.F., M. Mwanza, S. de Kock and L.D. Khilosia 2012. Mycotoxins in South African foods: a case study on aflatoxin M 1 in milk. Mycotoxin Res. 28: 17-23.
8. Fontanals, N., M. Galià, P.A.G. Cormack, R.M. Marcè, D.C. Sherrington and F. Borrull. 2005. Evaluation of a new hypercrosslinked polymer as a sorbent for solid-phase extraction of polar compounds. J.Chrom. 1075: 51-56.
9. Herzallah, S.M. 2009. Determination of aflatoxins in eggs, milk, meat and meat products using HPLC fluorescent and UV detectors. Food Chem. 114: 1141-1146.
10. Kim, E. K., D.H. Shon, D. Ryu, J.W. Park, H.J. Hwang and Y.B. Kim. 2000. Occurrence of aflatoxin M_1 in Korean dairy products determined by ELISA and HPLC. Food Add. Con.17: 59-64.
11. Kokkonen, M., M. Jestoi and A. Rizzo. 2005. Determination of selected mycotoxins in mould cheeses with liquid chromatography coupled to tandem with mass spectrometry. Food Add. Con. 22: 449-456.
12. Lee, J. E., B.M. Kwak, J.H. Ahn and T.H. Jeon. 2009.Occurrence of aflatoxin M_1 in raw milk in South Korea using an immunoaffinity column and liquid chroma-tography. Food Con. 20: 136-138.
13. Manetta, A.C., L.D. Giuseppe, M. Giammarco, I. Fusaro, A. Simonella, L. Di Giuseppe, M. Giammarco, I.A. Fusaro, A. Gramenzi and A. Formigoni. 2005. High-performance liquid chromatography with post-column deri-vatisation and fluorescence detection for sensitive determination of aflatoxin M_1 in milk and cheese. J.Chromat. 1083: 219-222.
14. Richard, J.L. 2007. Some major mycotoxins and their mycotoxicoses--an overview. International journal of food microbiology. 119: 3-10.
15. Rodriguez-Velasco, M. L., M.M. Calonge-Delso and D. Ordonez-Escudero, D. 2003. ELISA and HPLC determination of the occurrence of aflatoxin M_1 in raw cow's milk. Food Add. Cont. 20:276-280.
16. Sani, A.M. and H. Nikpooyan. 2013. Determination of aflatoxin M1 in milk by high-performance liquid chromatography in Mashhad (north east of Iran). Toxicol. Indus. Health. 29: 334-338.
17. Sørensen, L.K. and T.H. Elbæk. 2005. Determination of mycotoxins in bovine milk by liquid chromatography tandem mass spectrometry. J. Chromat.. 820:183-196.
18. Turner, N.W., S. Subrahmanyam and S.A. Piletsky, S.A. 2009. Analytical methods for determination of mycotoxins: A review. Anal. Chim. Acta.632: 168-180.
19. Food and Drug Administration (FDA).1996. Compliance Policy Guides. Washington, DC: FDA.
20. Wang Y, X. Liu, C. Xiao, Z. Wang, J. Wang, H. Xiao, L. Cui, Q. Xiang, T. Yue. 2012. HPLC determination of aflatoxin M1 in liquid milk and milk powder using solid phase extraction on OASIS HLB. Food Cont.28:131-134.