Determination of the toxic metabolite aflatoxin M1 in raw, pasteurized and ultra-pasteurized milks comparing the ELISA Technique and High Performance Liquid Chromatography.

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Determinación del metabolito tóxico aflatoxina M1 en leches crudas, pasteurizadas y ultrapasteurizadas comparando la Técnica ELISA y Cromatografía Líquida de Alta Resolución

Determinació del metabòlit tòxic aflatoxina M1 en llets crues, pasteuritzades i ultrapasteuritzades comparant la tècnica ELISA i la cromatografia líquida d'alt rendiment.

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ABSTRACT

Aflatoxins are very potent toxic metabolites with carcinogenic, teratogenic, mutagenic, hepatotoxic and immunosuppressive effects, which constitute a potential risk to human health, so they should be considered as a latent problem that requires continuous examination in order to ensure food quality.

This study was carried out with the objective of quantitatively determining the content of Aflatoxin M1 in three types of raw, pasteurized and ultrapasteurized milk consumed in the city of Cuenca-Ecuador, through the comparative analysis of the High Performance Liquid Chromatography HPLC Techniques, after an extraction process in immunoaffinity columns and the ELISA Technique.

Eighty-four samples were analyzed in three types of milk: raw, pasteurized and ultra-pasteurized, with the ELISA technique, none exceeded the detection limit of Aflatoxin M1(125 ppt or 0.125 ppb), established both by the national INEN: 9-10 in force and the parameters established by the Food and Agriculture Organization of the United Nations, Food and Drug Administration of the United States (0.5ppb) and with the High Performance Liquid Chromatography technique, 16 positive samples were found that were between the limit of detection and quantification (0.09 - 0.18 ppb.), obtaining a prevalence of 19%, so that the High Performance Liquid Chromatography technique is more sensitive in the quantitative detection of the toxic metabolite Aflatoxin M1 in milk.

Key words: aflatoxin, mycotoxin, mutagenicity, teratogenicity, carcinogenicity

RESUMEN

Las aflatoxinas son metabolitos tóxicos muy potentes con efectos cancerígenos, teratogénicos, mutagénicos, hepatotóxicos e inmunosupresores, que constituyen un riesgo potencial para la salud humana, por lo que deben considerarse como un problema latente que requiere un examen continuo para garantizar la calidad de los alimentos.

Este estudio se realizó con el objetivo de determinar cuantitativamente el contenido de Aflatoxina M1 en tres

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tipos de leche cruda, pasteurizada y ultrapasteurizada consumida en la ciudad de Cuenca-Ecuador, mediante el análisis comparativo de las Técnicas de Cromatografía Líquida de Alta Resolución HPLC, luego de un proceso de extracción en columnas de inmunoafinidad y la Técnica ELISA.

Se analizaron ochenta y cuatro muestras en tres tipos de leche: cruda, pasteurizada y ultrapasteurizada, con la técnica ELISA ninguna superó el límite de detección de Aflatoxina M1 (125 ppt o 0.125 ppb), establecido ambos por el INEN nacional: 9- 10 vigentes y los parámetros establecidos por la Organización de las Naciones Unidas para la Alimentación y la Agricultura, Administración de Alimentos y Medicamentos de Estados Unidos (0.5ppb) y con la técnica de Cromatografía Líquida de Alta Resolución se encontraron 16 muestras positivas que se encontraban entre el límite de detección y cuantificación (0.09 - 0.18 ppb.), obteniendo una prevalencia del 19%, por lo que la técnica de Cromatografía Líquida de Alta Resolución es más sensible en la detección cuantitativa del metabolito tóxico Aflatoxina M1 en leche.

Palabras claves: aflatoxina, micotoxina, mutagenicidad, teratogenicidad, carcinogenicidad

RESUM:

Les aflatoxines són metabòlits tòxics molt potents amb efectes cancerígens, teratogènics, mutagènics, hepatotòxics i immunosupressors, que constitueixen un risc potencial per a la salut humana, per la qual cosa s'han de considerar com un problema latent que requereix un examen continuat per garantir la qualitat dels aliments.

Aquest estudi s'ha realitzat amb l'objectiu de determinar quantitativament el contingut d'aflatoxina M1 en tres tipus de llet crua, pasteuritzada i ultrapasteuritzada consumides a la ciutat de Conca-Equador, mitjançant l'anàlisi comparativa de les Tècniques HPLC de cromatografia líquida d'alt rendiment, després d'un estudi. procés d'extracció en columnes d'immunoafinitat i la tècnica ELISA.

S'han analitzat vuitanta-quatre mostres en tres tipus de llet: crua, pasteuritzada i ultrapasteuritzada, amb la tècnica ELISA, cap ha superat el límit de detecció d'Aflatoxina M1 (125 ppt o 0,125 ppb), establert ambdós per l'INEN nacional: 9- 10 vigents i els paràmetres establerts per l'Organització de les Nacions Unides per a l'Agricultura i l'Alimentació, Food and Drug Administration dels Estats Units (0,5 ppb) i amb la tècnica de cromatografia líquida d'alt rendiment, es van trobar 16 mostres positives que es trobaven entre el límit de detecció i quantificació (0,09 - 0,18 ppb.), obtenint una prevalença del 19%, de manera que la tècnica de cromatografia líquida d'alt rendiment és més sensible en la detecció quantitativa del metabòlit tòxic Aflatoxina M1 a la llet.

Paraules clau: aflatoxina, micotoxina, mutagenicitat, teratogenicitat, carcinogenicitat

INTRODUCTION

Mycotoxins are toxic substances produced in the secondary metabolism of fungi, which appear as natural contaminants in food when climatic conditions are favorable and are associated with the development of multiple carcinogenic, mutagenic and teratogenic pathologies in both humans and animals.

Aflatoxins are a type of mycotoxins *that are considered the most toxic among them,* only a few species of the genus *Aspergillus and Penicillium* are able to synthesize *this kind of* mycotoxins and even within the same species of fungus, only certain strains have this ability.

These molds develop at temperature ranges between 40°C and 45°C, while the toxins can be produced from 11°C to 35°C, the optimum conditions for production being 22°C and 80 - 90% relative humidity.

Aflatoxins have received more attention than any other type of mycotoxins because they have been shown to have a potent immunosuppressive and carcinogenic effect in laboratory animals and humans (Carmean Fernández & Repetto Jiménez, 2012).

Aflatoxin AFM_1 is a heat-resistant metabolite, so is not destroyed by sterilization, demonstrating experimentally its hepatotoxicity and mutagenicity. and according to the International Agency for Research on Cancer this substance is included in group 2B type because it is considered as a possible carcinogenic metabolite for humans (FAO/WHO 1999).

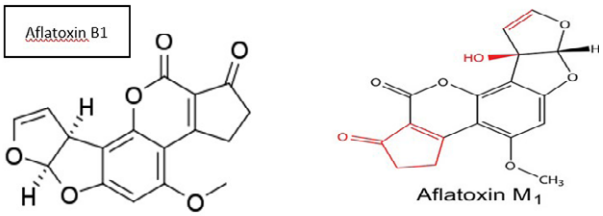

Figure 1: Chemical Structure of AFB1 - AFM1
Source: Smith JE, Henderson RS (1991).

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Aflatoxin B1 (AFB1), is a mycotoxin that can be found in feed consumed by dairy cattle, also inside the organism of the animal that consumed feed contaminated with AFB1 (ELIKA, 2013).

The excretion of aflatoxin B1 occurs 50% through the mammal's bile, already metabolized, and 15 -25% through unmetabolized urine. Cows' metabolism can bio transform AFB1 into AFM1 within 12 to 24 hours, and even after 6 hours AFM1 residues can appear in milk intended for human consumption (Gimeno, 2005).

The presence of AFM1 in milk is transitory, reaching a maximum two days after consumption of the contaminated food and disappearing two to 5 daysafter the feed is withdrawn (Gimeno, 2005).

AFM_1 found in milk is not degraded in the processing of dairy products, therefore, it contaminates the final

product, which puts the consuming population at risk (Torres, Aparicio, & Garcia, 2014).

Chemically, aflatoxins are a group of non-protein organic compounds, of low molecular weight, whose basic structure is a furan ring attached to a coumarin core to which a cycle pentanone is added in the case of AFB_1. AFM1 is a 4-hydroxylated derivative of AFB1.

Food contamination by Aflatoxin M1 is continuously monitored worldwide by agencies such as the FDA and FAO. However, no studies of AFM1 in milk have been reported in Ecuador.

In this work, the presence of Aflatoxin M1 was investigated for the toxicological risk it represents in three types of raw, pasteurized and ultra-pasteurized milk, which are mass consumption foods, comparing the concentrations with the values established for the current National Standard INEN, which allows knowing its safety or food risk. The quantitative techniques used were ELISA (enzyme-linked immunosorbent assay) and HPLC (High Performance Liquid Chromatography).

MATERIALS AND METHODS

The criteria for the selection of the different brands of milk tested in this work was to choose the most demanded by the population.

For the quantification of Aflatoxin M1, the competitive enzyme immunoassay (ELISA) technique was used, for which RIDASCREEN FAST AFLATOXIN Kits were used, whose supplier is R-Biopharm AG, Darmstadt, Germany. For aflatoxin M1 in milk, the detection limit is of < 125 ppt. and with the most sensitive technique such as high performance liquid chromatography (HPLC) the detection and quantification limit (0.09 µg/L - 0.18 µg/L) whose specifications are detailed below:

ELISA TECHNIQUE RIDASCREEN®FAST AFLATOXIN M_1

The basis of the test is the antigen-antibody reaction. The microtiter wells are coated with capture antibodies directed against anti-aflatoxin M1 antibodies. Aflatoxin M1 standards or sample solutions, aflatoxin M1 enzyme conjugate and anti-aflatoxin M1 antibodies are added. Free aflatoxin M1 and aflatoxin M1 enzyme conjugate compete for the aflatoxin M1 antibody binding sites (this is a competitive enzyme immunoassay). At the same time, the anti-aflatoxin M1 antibodies are also bound by the immobilized capture antibodies. Any unbound enzyme conjugate is then removed in a washing step. Substrate/chromogen is added to the wells, bound enzyme conjugate converts the chromogen into a blue product. The addition of the stop solution leads to a color change from blue to yellow. The measurement is made photometrically at 450 nm. The absorbance is inversely proportional to the aflatoxin M1 concentration in the sample. (Art. No.: R5812)

Figure 2 *shows the equipment where the AFM1 quantification was performed at a wavelength of 450 nm.*

Cell holder

HPLC HIGH PERFORMANCE LIQUID CHROMATOGRAPHY:

The high-performance liquid chromatograph HPLC model Agilent 1200 series (Agilent, USA) was used, equipped with a quaternary pump, an autosampler with thermostat and coupled to a DAD thermostat and coupled to a DAD detector. The separation was carried out by means of a ZORBAX Eclipse a ZORBAX Eclipse Plus C18 column (4.6 x 250 mm, 5 µm) (Agilent, USA) at a wavelength of 200 to 700 nm (Figure 3).

Figure 3 *HPLC high performance chromatography equipment.*

High performance liquid chromatography (HPLC) consists of a stationary phase (Agilent ZORBAX C18 reversed-phase chromatographic column 4.6 mm X 250 mm, particle size 5 µm with octadecyl silica gel packing) and a mobile phase (90 ml (60%) HPLC grade acetonitrile, 90 ml (60%) HPLC grade methanol, 400 ml (20%) HPLC water.

AFM1 is extracted and cleared by passing the sample through an immunoaffinity column (IAC). The column contains specific monoclonal antibodies covalently bound to a solid support material. As the sample passes through the column, the antibodies selectively bind with any AFM1 (antigen) contained in the sample to give an antibody-antigen complex.

The column is washed, and the bound toxin is released by the antibody after elution from the column with methanol/acetonitrile, filtering through a 0.45 µm pore diameter membrane and degassing which acts as a sample carrier. The components of the

mixture interact differently with the stationary phase and the mobile phase. In this way, the components pass through the stationary phase at different speeds and are separated. Subsequently, they pass to a detector that generates a signal that will depend on the concentration and type of compound (Chalco, 2014).

TECHNIQUE FOR AFM1 EXTRACTION

AFM1 is extracted and clarified by passing the sample through a Zorbax Eclipse Plus C18 immunoaffinity column (IAC) (4.6 x 250 mm, 5 um) (Agilent, USA) Merck. The column contains specific monoclonal antibodies covalently attached to a solid support material. As the sample passes through the column, the antibodies selectively bind to any AFM1 (antigen) contained in the sample to form an antibody-antigen complex.

The column is washed and bound toxin is released from the antibody after elution from the column with methanol/acetonitrile.

SAMPLE PREPARATION:

Milk samples should be kept refrigerated at 10°C until analysis.

Bring the samples to room temperature 12 hours prior to analysis, measure 20 ml of milk sample and place in water bath at 37°C for 10 minutes. Then, centrifuge at 4000 rpm for 15 minutes at 4°C and remove the fat layer. After this, filter through a Whatman N°4 filter paper and finally collect the sample in conical tubes of 50 ml and measure the final volume.

CLEAN UP

1. Temper the immunoaffinity columns to room temperature and condition with 3 ml of pre-washed phosphate-buffered saline (PBS).
2. Pass the sample slowly through the column at a flow rate of 2-3 ml/min. A slow constant pressure is essential for the capture of the toxin by the antibody. Avoid allowing to dry.
3. Wash the column by passing 10 ml of phosphate buffer saline (PBS) through the column at a flow rate of approximately 5 ml per minute.
4. Place 10 ml of HPLC water through the column at a flow rate of approximately 5 ml per minute.

ELUTION

5. Add 1.5 ml of methanol: acetonitrile Me OH: ACN (2:3 v/v). Slowly pass it through the column at a flow rate of approximately 2 - 3 ml/min.

To ensure complete removal of the bound toxin from the antibody it is recommended that the acetonitrile: methanol solution be left in contact with the column for 30 seconds during the elution process. Then backflushing is performed three times.

RESULTS AND DISCUSSION:

The results of all samples indicate that the quantified concentrations are below the detection limits of the ELISA technique by competition (< 125p.p.t or 0.125p.p.b) which is below the INEN NTE 9-10 (0.5 ug/L), and international FAO (0.5ug/L) and FDA 0.5 p.p.b. standards for aflatoxin M1.

RESULTS OF AFLATOXIN M1 DETERMINATION IN MILK BY HPLC

Table 1 shows that, of the 84 milk samples analyzed, 16 samples were positive for AFM1 between the limit of detection and quantification (0.09 µg/L - 0.18 µg/L) with a prevalence of 19% (16/84), but only in two samples it was possible to quantify the AFM1 concentration (0.18 µg/L in both samples).

Table 1: *Table of results for the 16 samples between the limit of detection and limit of quantification.*

Sample Code	AFM_1		Correction Recovery (%R=70)
	Area	TR	
NE1.1	0,054	4,167	*0,12*
NE1.2	0,066	4,201	*0,15*
NE1.3	0,075	4,204	*0,17*
NE2.1	0,049	4,238	*0,10*
NE2.3	0,046	4,255	*0,10*
ND1.1	0,044	*4,166*	*0,09*
ND1.3	0.047	*4,208*	*0,10*
ND2.1	0.054	*4,286*	*0,12*
LD2.1	0.056	*4,078*	*0,12*
LD2.2	0.048	*4,081*	*0,10*
LD2.3	0.051	*4,086*	*0,11*
PE2.2.1	0.078	*4,089*	*0,18*
PE2.2.2	0,071	*4,097*	*0,16*
PE.2.2.3	0,079	*4,101*	*0,18*
LD2.2.1	0,046	4,092	*0,10*
LD2.2.2	0,045	4,097	*0,09*

Figure 4 shows in the chromatogram that a whole milk sample was found above the quantification limit with an AFM1 concentration of 0.18 µg/L, with a peak with a retention time of 4.078

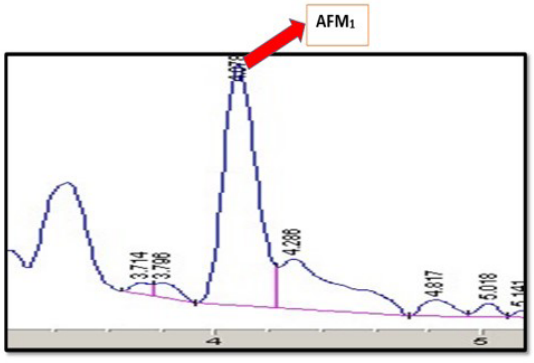

Figure 4. *Milk Entera UHT HPLC Chromatogram of de AFM1*

PEAK	RETENTION TIME	AREA	Metabolite
1	3.714	$1.61503\ e^{-3}$	
2	3.796	$2.45881e^{-3}$	
3	**4.078**	**$5.63456e^{-2}$**	**AFM1**
4	4.286	$2.21054e^{-2}$	
5	4.817	$3.67087e^{-3}$	

The quantifiable AFM1 values were lower than the concentration allowed by INEN NTE 9-10: 2012 (0.5 µg/L) and FDA (0.5 ppb), therefore, the different kinds of milk studied in this research could be considered suitable for human consumption because they comply with the established standards. In similar investigations, Perez and collaborators in Mexico, using the HPLC technique in raw, ultra-pasteurized and organic milk, observed higher prevalences of up to 59% and in all cases values above the maximum limit proposed by the European Union were found. In Brazil, Fernandez, Soares & Fagundes using the same HPLC technique determined higher prevalences of 36.7% in raw milk but only one sample showed values above the tolerance limit adopted in that country. Another study using HPLC by Gutiérrez, Vega & Pérez found that 23.3% of the organic milk samples analyzed exceeded the maximum residue limit proposed by Mexican regulations, while 62.7% were above the maximum limit of the European Union.

In Ecuador, using the ELISA technique, Uguña Rosas concluded that the values obtained in raw, pasteurized and ultra-pasteurized milk were lower than the Aflatoxin M1 concentration value allowed by the Ecuadorian INEN standard and the international FDA regulation.

Some of these studies differ from our research work since they present AFM1 values that are above the values established by the European Union and the regulations corresponding to each country. With respect to the 16/84 study, they were positive, but do not exceed the values established by the Ecuadorian INEN regulations and the international FDA regulations.

AFM1 levels obtained in different studies show wide differences, this may be due to various factors such as feeding regimes, animal breed, milk production, environmental conditions, among others.

CONCLUSIONS:

Aflatoxin levels found by competitive ELISA Technique are below the detection limits of the technique (< 125ppt or 0.125ppb) and are also below the values allowed by INEN NTE 9-10 (0.5 µg/l) and international FAO (0.5µg/l) and FDA 0.5 ppb. of Aflatoxin M1 content in milk.

The levels of AFM1 found in raw, pasteurized and ultra-pasteurized milk sold in the city of Cuenca-Ecuador and analyzed by the HPLC technique are below the limits established by the national INEN NTE 9-10:2012 and international FDA regulations.

The HPLC technique is more sensitive in the determination of Aflatoxin M1 in milk.

From this work it can be said that the consumption of milk marketed in Ecuador seems to be safe with respect to the concentration of AFM1.

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