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Title: **A survey of AFM₁ contamination in row cow milk produced in region of Kumanovo**

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A survey of AFM₁ contamination in row cow milk produced in region of Kumanovo

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Abstract

Aflatoxins (AFs) are type of mycotoxins produced by certain *Aspergillus* species. In the livers of cows, the aflatoxin B₁ (AFB₁) ingested with contaminated food is metabolized in carcinogenic aflatoxin M₁ (AFM₁) which is then excreted in the milk and poses a serious risk to the health of the consumers. In January of 2019, twenty five (25) samples of milk were collected from local farms in the surrounding area of Kumanovo, Republic of North Macedonia. The samples were analysed in the Centre for Public Health, Kumanovo. A method MKC EN ISO 14501:2007 for analysis of AFM₁ by HPLC with fluorescence detection was applied. This method is selective, precise and applicative. The final analytical result has shown AFM₁ below the LOD in any of the analyzed samples. Due to the well-known fact that AFM₁ is toxic, i.e. carcinogenic and the fact that milk is widely used as staple food, continuous development as well as introduction of new, more sensitive methods for detection of AFM₁ are required.

Keywords: Aflatoxin M₁, HPLC-FLD, milk

Introduction

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Mycotoxins are secondary metabolites of certain fungi that can be found as contaminants in food and animal feed. Aflatoxins (AFs) are a type of mycotoxins. They are the main secondary metabolites of certain *Aspergillus* species, such as *Aspergillus flavus*, *Aspergillus parasiticus* and rarely *Aspergillus nomius* (Bennett and Klich, 2003). There are four types of AFs: B₁, B₂, G₁ and G₂, of which AFB₁ is the most toxic and carcinogenic one (Gourama and Bullerman, 1995). When cows ingest animal feed contaminated with AFB₁, 4-hydroxy derivative of AFB₁ (AFBM₁) is produced in the liver via CYP450 enzymes which is then eliminated through milk (Kuilman et al., 2000). Many researchers have proven that AFBM₁ is less toxic and carcinogenic than AFB₁ (Hsieh et al., 1984). Due to this, the International Agency for Research on Cancer (IARC) reclassified it in Group 2B (possibly carcinogenic to humans) hepatic carcinogens (IARC Monographs Vol. 82, 2002). The aflatoxin M₁ is relatively stable during the process of pasteurization, preparation and storage of dairy products. The presence of AFBM₁ in milk poses a serious risk to human health, given that milk is used as staple food. According to the European Commission, the maximum allowed concentration of AFBM₁ in milk is 0.05 µg/kg (Commission Regulations (EC) No. 1881, 2006).

The purpose of this research was to analyse the presence and concentration of AFBM₁ in milk samples taken from local farms in the surrounding area of Kumanovo, Republic of North Macedonia. HPLC technique was used according to the ISO method recommended for determination of AFBM₁ in milk.

Materials and methods

Chemicals

HPLC grade reagents (acetonitrile, water) and chemicals were purchased from Merck (Darmstadt, Germany). For clean-up/purification, immune-affinity columns Aflaprep M1 Glasgow, Scotland were used. Stock solution aflatoxin M₁ in acetonitrile with a concentration of 0.5 µg/mL, (Romer Labs Diagnostic GmgH Austria) was used as a standard.

Methods

Qualitative and quantitative analyses of AFM₁ was carried out using the method MKC EN ISO 14501:007 (Milk and milk powder - determination of aflatoxin M₁ content - clean up by immune-affinity chromatography and determination by high-performance liquid chromatography).

Milk samples

Twenty five (25) milk samples (500 mL each sample) were collected in five consecutive days (five samples per day) from different ten (10) farms that supply the dairy factory Buchen Kozjak, Kumanovo with milk. The samples were collected in accordance with regulations, without exposure to sunlight and in sterile containers (ISO 707/IDF 50, 2008). The samples were delivered to the Centre for Public Health, Kumanovo where they were immediately analysed according to the MKC EN ISO 14501:2007 method, at room temperature and without exposure to sunlight.

Sample preparation

The samples were defatted when 100 mL of each sample of milk was heated in a water bath at temperatures from 35 to 37 °C and centrifuged at 2000 x g for 15 minutes. The fat accumulated onto the surface of the test tube was mechanically removed and the milk sample was filtered through filter paper. 50 mL of the filtered sample was passed through an aflatoxin immune-affinity column M₁. After the entire milk volume has been passed through, the column was washed with 10 mL water (GRADE 1). The aflatoxin M₁ was eluted using 2 mL 10% acetonitrile solution. This eluate was collected in vials and immediately analysed using HPLC.

In each lot of samples prepared for analysis, one sample was spiked with 500 µL 0.005 µg/mL standard working solution for AFM₁.

HPLC conditions

HPLC analysis was performed with Agilent Technologies 1260 Series chromatographic system equipped with vacuum degasser G4225A, Binary Pump G1312B, Autosampler G1329E, Column Compartment G1316C. Aflatoxin M₁ was separated on ZORBAX Eclipse Plus C18 Column 4.6 x 100 mm, 3.5 µm at room temperature. The mobile phase was a mixture of water and acetonitrile (25:75, V/V).

The flow rate was 1 mL/min and the injection volume was 50 μ L. The detection was carried out at $\lambda_{\text{ex}} = 365\text{nm}$ and $\lambda_{\text{em}} = 435\text{nm}$. Data were acquired using Agilent life Sciences OpenLAB CDS ChemStation software.

Identification of AFM₁ based on retention time

To test for carryover, a mobile phase blank was injected. As shown in Fig. 1 the chromatogram of this blank showed no detectable peak.

Fig. 1

Fig. 2 shows the chromatogram of aflatoxin working standard solution with concentration of 0.2 ng/mL and retention time 5.257 min).

Fig. 2

Chromatographic repeatability was confirmed via replicate injection of working standard solution with concentration of 0.1 ng/mL of AFM₁, demonstrating exceptional reproducibility. Analyses of working standard solutions were performed in a concentration range of 0.05-0.4 ng/mL and aflatoxin M₁ was identified based on retention time. Concentrations of working standard solutions and the corresponding retention time for aflatoxin M₁ are shown in Table 1. The relative standard deviation %RSD of the retention time for M₁ was 0.13% demonstrating exceptional reproducibility. The limit of detection (LOD) based upon signal to noise S/N was 0.02 ng/mL and the limit of quantitation (LOQ) based S/N was calculated to be 0.05 ng/mL.

Table 1

Standard solution preparation and calibration

With several dilutions of stock standard solution, the working standard solutions of AFM₁ with concentrations of 0.05, 0.10, 0.20, 0.3, 0.4 ng/mL were prepared and subsequently were used to obtain the calibration/standard curve as described by the following regression equation: $Y=0.64042297X+0.003654$, where Y is the peak area

and X is the corresponding concentration of AFM₁ (Fig. 3). The linearity of the standard curve over the range studied is presented in Fig. 3. The coefficient of regression (R^2) was 0.99975.

Fig. 3

Results and discussion

The analysis of all twenty five samples has shown that AFM₁ was not detected in any of the milk samples. Because there is no significant difference between chromatograms from each sample (it was not detected M₁ peak) on Fig. 4 (a-e), is represented one chromatogram from daily analysis.

Fig. 4

The analyses were performed in January when the highest concentration of aflatoxin M₁ was expected as a result of positive conditions for mold spreading and feeding circumstances (Tajkarimi et al., 2017). In order to prove the accuracy of the method and the results, one milk sample was spiked with known amounts of working standard solution of AFM₁. The obtained peak was identified as AFM₁ based on retention time (Fig. 5). Overall the recovery of 124% is very good considering the rater complexed matrix for sample extractions and very low concentration.

Fig. 5

The applied method for determination of aflatoxin M₁ is fast, simple, selective and suitable for routine application (MKC EN ISO 14501:2007).

The obtained results are encouraging, but continued monitoring is suggested. In addition, several studies have been conducted to determine the contamination of milk with AFM₁ (Table 2). The incidence of AFM₁ observed in the presented study by other authors was higher (Bilandzic et al., 2015; Bilandzic et al., 2016; Dimitrieska-Stojkovic et al., 2013; Dimitreska et al., 2014; Godic Tarkar et al., 2008; Jajic et al., 2018;

Markaki et al., 1997; Polovinski et al., 2009; Polovnski-Horvatovic et al., 2016; Skrbic et al., 2014). Presented results may be attributed to the differences in the regions, the origin of animal feed, season of milk collection and the different AFM₁ analytical methods for determination.

Table 2

Conclusion

The method applied to determine the AFM₁ using HPLC with fluorescence detection is sensitive, specific and accurate. However, it is of general interest to continuously develop new methods for determination of AFM₁ in milk. It was also revealed that in any of analyzed samples AFM₁ was lower than LOD. Even though the results from the survey are encouraging, when it comes to the mycotoxicological quality of the milk available on our market, a legal obligation of continuing monitoring is obvious, in order to prevent possible poisonings, especially in children as the most vulnerable group in society whose diet consists mostly of milk.

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Резиме

Испитувања на AFM₁ во сурово млеко произведено во Кумановскиот регионИва Фуруновиќ-Димитријевски^{1*}, Сузана Ангелова², Кристина Шутевска³Татјана Каdifкова Пановска³¹ПЗУ Аптека Илинден, Доне Божинев 18, 1300 Куманово,

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Клучни зборови: Афлатоксин M₁, HPLC-FLD, млеко

Афлатоксини (AFs) се микотоксини, продукти на одредени *Aspergillus* видови. Во црниот дроб на крава, афлатоксин B₁ (AFB₁) внесен со контаминирана храна се метаболизира во карциноген афлатоксин M₁ (AFM₁) кој се излучува во млекото и претставува сериозен ризик по здравјето на потрошувачите. Во јануари 2019-тата година 25 примероци на млеко се земени од локални фарми во околината на Куманово, Република Северна Македонија. Примероците се испратени за анализа во Центарот за Јавно Здравје во Куманово. За анализа на AFM₁ е применет HPLC метод со флуоресцентен детектор. Методот е селективен, прецизен и апликативен. Добиениот аналитички резултат покажа AFM₁ под LOD во сите испитувани примероците. Заради познатата токсичност односно карциногеност на AFM₁ и широката употреба на млекото во секојдневната исхрана на луѓето, потребно е континуирано развивање и воведување на нови поосетливи методи за детекција на AFM₁.

Table 1. Working standard solutions and corresponding retention time for aflatoxin M₁

Concentration of working standard solution (ng/mL)	Retention time
0.05	5.256
0.1	5.252
0.2	5.257
0.3	5.269
0.4	5.264

Table 2. The incidence of milk contamination with AFM₁ in the Balkan Peninsula

Location contamination	References	Method of detection	Sample size	Percentage of contamination	Percentage of >50ng/L
Serbia	Skrbic et al., 2014	HPLC	50	76	76
Serbia	Polovinski- Horvatovic et al., 2009	HPLC	65	27.7	0
Macedonia	Dimitreska-Stojkovic et al., 2014	HPLC	3407	6.2	0
Croatia	Bilandzic et al., 2016	HPLC	3543	26.6	9.32
Serbia	Jajic et al., 2018	HPLC	423	80.9	49.1
Greece	Markaki et al., 1997	HPLC	81	77.78	0
Slovenia	Godic Tarkar et al., 2008	HPLC	40	10	10
Macedonia	Dimitrieska-Stojkovic et al., 2013	HPLC	3635	31.8	2.9
Serbia	Polovinski- Horvatovic et al., 2016	HPLC	38	36.84	63.16
Croatia	Bilandzic et al., 2015	HPLC	548	100	0

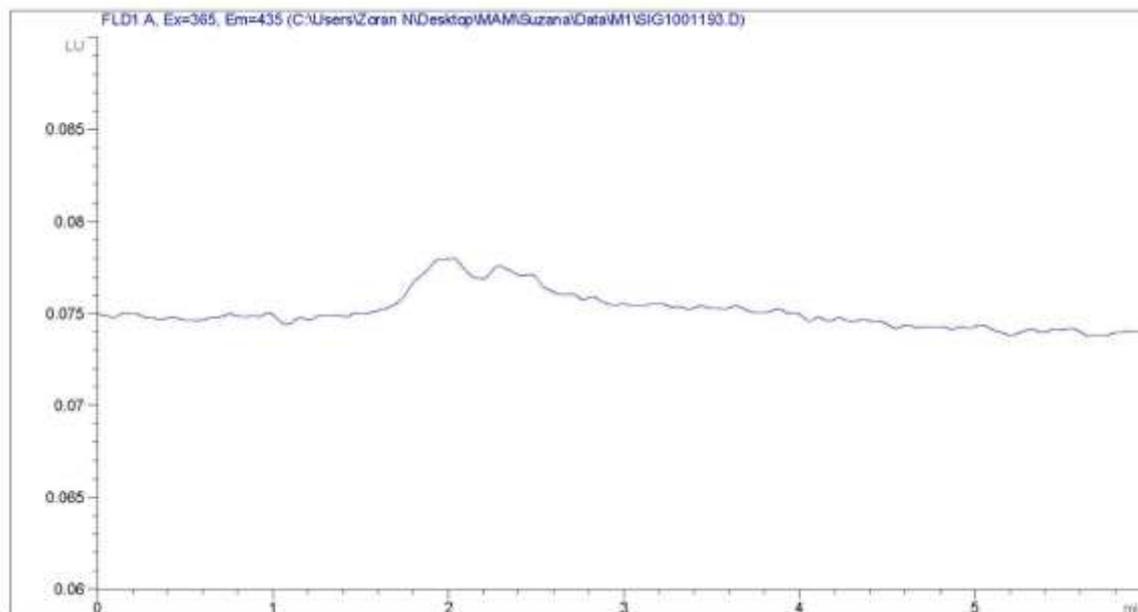


Fig. 1. Chromatogram of blank.

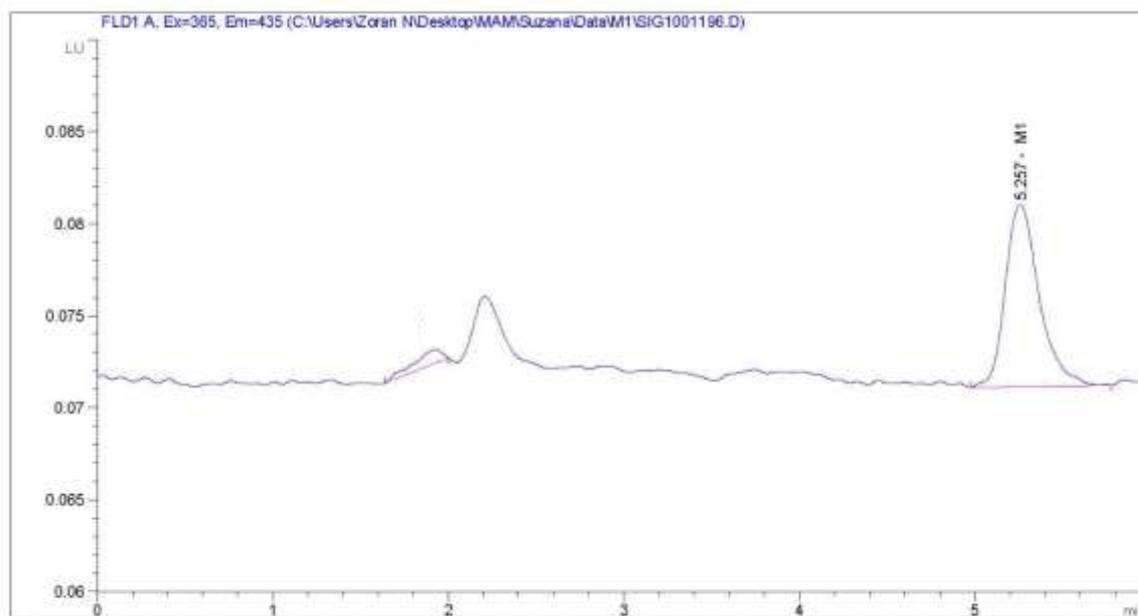


Fig. 2. Chromatogram with peak M_1 (working standard solution of aflatoxin M_1 with concentration of 0.2 ng/mL).

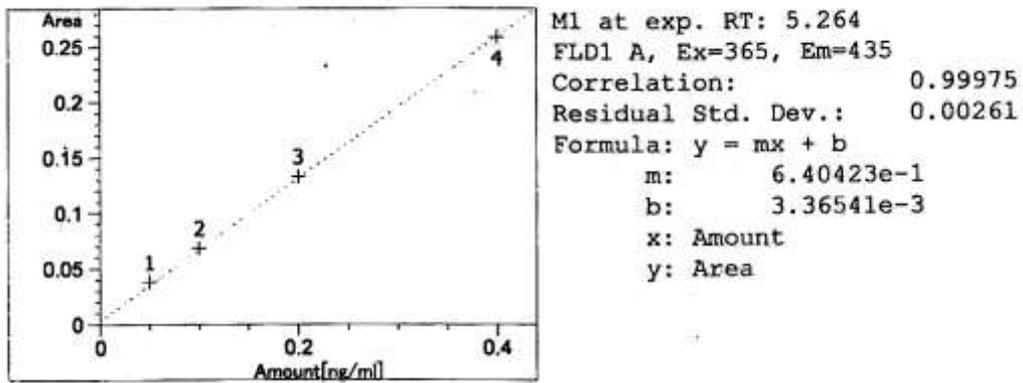


Fig. 3. Calibration curve of standard solution of aflatoxin M₁ by high-performance liquid chromatography analysis.

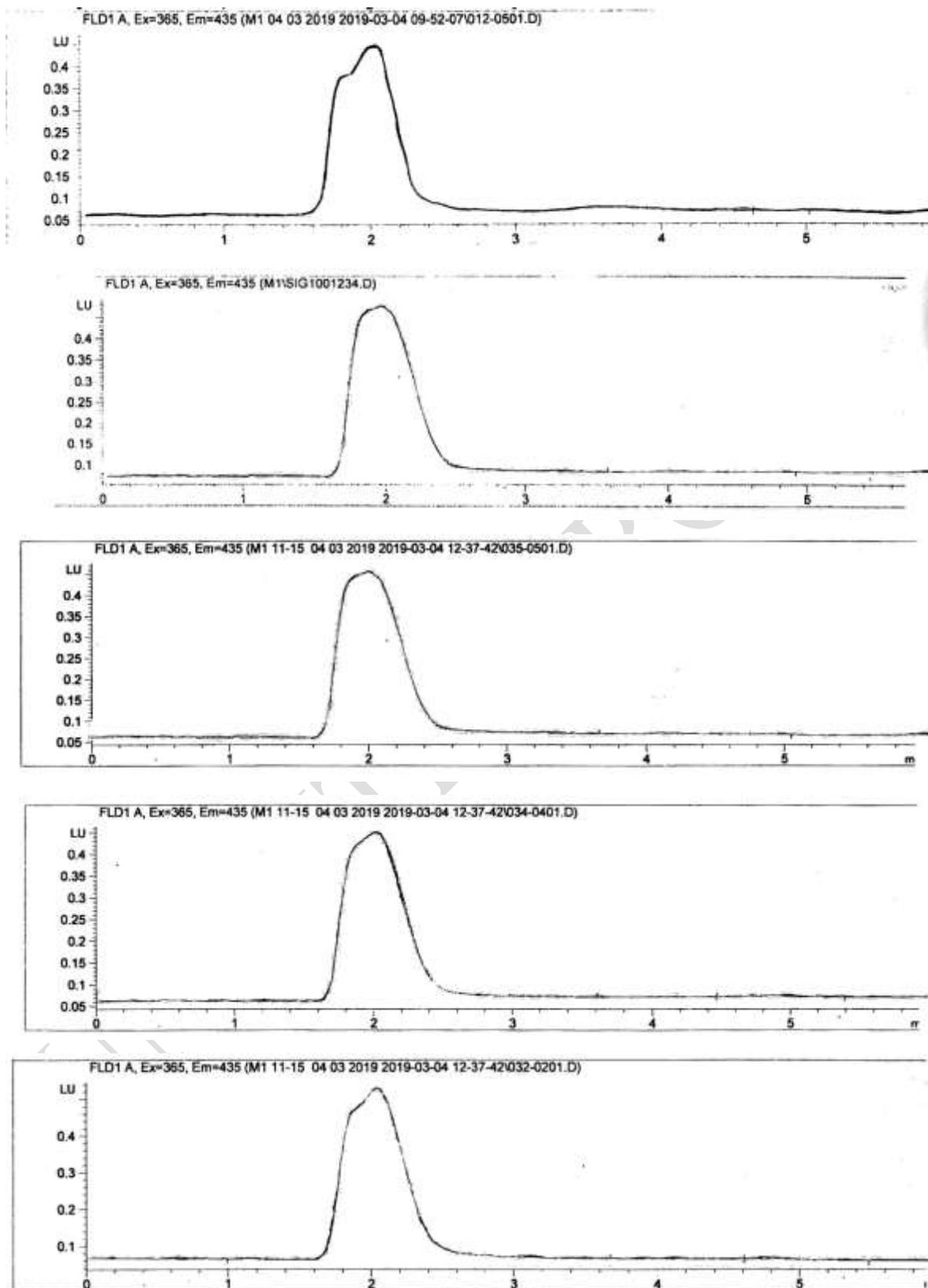


Fig. 4. Chromatograms of milk samples (a, b, c, d, e corresponding to day 1, 2, 3, 4 and 5).

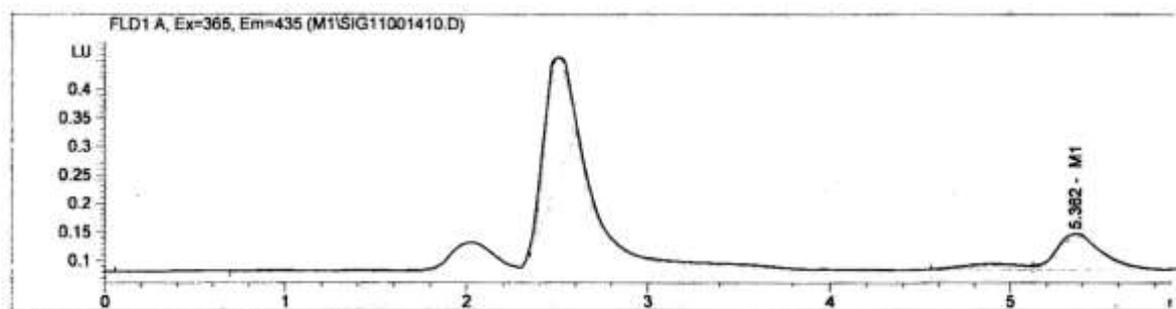


Fig. 5. Chromatogram of a spiked milk sample.