AflaCLEAN™ M1 SMART

SMART Immunoaffinity Column for Aflatoxin M1 Analysis

User Manual

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1. Introduction

Aflatoxins are naturally occurring mycotoxins, which are highly toxic for humans and animals. The aflatoxins are produced by mold (e. g. *A*spergillus *fla*vus) as primary contaminants in various food and feed stuffs.
Aflatoxin M1 (Figure 1) is a direct derivative from aflatoxin B1, which is taken up by animal and converted to aflatoxin M1, which could be found in milk, dairy products, meat and egg.

Due to the toxic effects on humans, the European Community (EC) has set maximum levels in several matrices (MCL;Table 1) in the Commission Regulation (EC) No 1881/2006 and amended by Commission Regulation (EU) 165/2010.

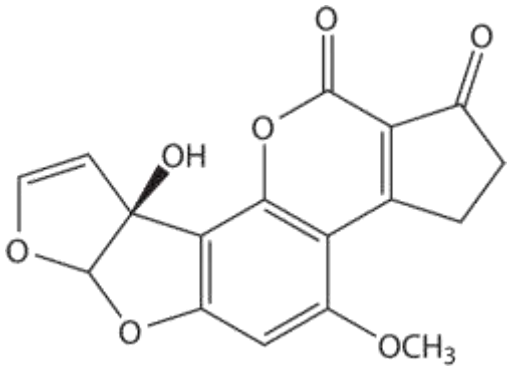


Figure 1: Structure of Aflatoxin M1.

Table 1: Maximum levels for aflatoxins in corresponding matrices as regulated by the European Community

Matrix	HW B1 [µg/kg]	HW Σ B1/B2/G1/G2	HW M1 [µg/kg]
Groundnuts to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs	8.0	15.0	
Nuts to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs	5.0	10.0	
Groundnuts and nuts and processed products thereof, intended for direct human consumption or us as an ingredient in foodstuffs	2.0	4.0	
Dried fruit to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs	5.0	10.0	
Dried fruit and products thereof, intended for direct human consumption or use as an ingredient in foodstuffs	2.0	4.0	
All cereals and all products derived from cereals, including processed cereal products, with the exception of foodstuffs listed in *	2.0	4.0	
* Maize to be subjected to sorting or other physical treatment before human consumption or use as an ingredient in foodstuffs	5.0	10.0	
Raw milk, heat-treated milk and milk for the manufacture of milk-based products			0.05
Following species of spices: *Capsicum ssp.* (dried fruits thereof, whole or ground, including chilli powder, cayenne, and paprika) *Piper ssp.* (fruits thereof, including white and black pepper) *Myristica fragrans* (nutmeg) *Zingiber officinale* (ginger) *Curcuma longa* (turmeric)	5.0	10.0	
* Processed cereal-based foods and baby foods for infants and young children	0.1		
Infant formulae and follow-on formulae, including infant milk and follow-on milk			0.025
* Dietary foods for special medical purposes intended specifically for infants	0.1		

2. Information about AflaCLEAN M1 SMART:

The immunoaffinity column AflaCLEAN M1 SMART has been developed for high throughput sample preparation within the food analysis via HPLC with fluorescence detection and LC-MS, respectively.
The monoclonal antibody has been specifically designed for this field of application and thus guarantees best results, even with difficult matrices.
Although dedicated for milk analysis, most dairy products can be analyzed with very good results. The working mode is based on the principles of immunoaffinity.
The material within the columns is covered with antibodies which are directed against M1 aflatoxin. Upon application of an extract onto the column, the aflatoxins are retained, whereas the remaining matrix components pass through the column.
After a washing step to remove matrix interferences, the aflatoxins can be eluted quantitatively from the column with methanol and subsequently measured with HPLC.
The column bed consists of a soft-gel, which is covered by a storage buffer (contains a preservative).

The following recoveries of a standard will be obtained when the provided LCTech protocols are applied.

Aflatoxin M1: ≥ 80 %

The maximum loading capacity is 100 ng aflatoxin M1 absolute and may be lower for the other toxins.
The minimum shelf life of the columns is 12 months at a storage temperature of 4 °C to 8 °C. Higher or lower temperatures will make the columns unfit for use!
The cross-reactivity against aflatoxin M2 or B/G aflatoxins is observed, but could be lower.

Since the antibodies as well as the immunoaffinity columns are produced within LCTech, numerous in-house quality tests over the whole production process guarantee a persistent quality.

The final release of the columns for sale is done with the aflatoxin challenge test described here:

0.8 ng Aflatoxin M1 diluted in phosphate buffered saline (PBS) are applied onto the immunoaffinity column by flow rate of 1.0 mL/min.
The column is washed with 2 mL water and dried by flushing air through it.
The toxins are eluted by 0.4 mL methanol, which is loaded onto the column and need to be incubated for 5 minutes within the bed material to completely denature the antibody.

Exemplary recovery rates of various matrices are shown in the following:

Table 2: Various matrices with recoveries.

Matrix	Matrix load [g]	Recovery (%)
Milk	10	96
Raw milk	10	90
Milk powder	1.0	98

3. Reagents

In the following the chemicals needed are listed and explicitly described.
Depending on the extraction and clean-up procedure applied, one or the other chemical might not be necessary. All chemicals used must be of analytical grade; especially the suitability for fluorescence measurement must be assured!

- Acetonitrile p. a.
- n-Hexane p. a.
- Methanol p. a.
- NaCl p. a.
- Na2HPO4 x 12 H2O p. a.
- NaH2PO4 x 1 H2O p. a.
- Methylene chloride
- diatomaceous earth
- Water p. a.
- Extraction solution (Standard Procedure); methanol/water(4/1, v/v)
- PBS (5x) buffer stock solution pH 7.2
- 50.14 g Na2HPO4 x 12 H2O are dissolved in 700 mL of water
- 9.66 g NaH2PO4 x 1 H2O are dissolved in 350 mL of water. The second solution is added to the first one as long as a pH of 7.2 is adjusted. 42.5 g of sodium chloride are added
- PBS (1x) working solution pH 7.2
- 200 mL PBS (5x) buffer stock solution pH 7.2 is diluted with 800 mL of water.
- HPLC eluent water/methanol/acetonitrile (60/30/15, v/v/v)

4. Apparatus

AflaCLEAN M1 SMART (LCTech P/N 14246; 100 pieces/package) According to the amount and texture of the material to be homogenized, appropriate tools should be used, such as kitchen mixers, lab mills, mortar with pestle, homogenizers or others

- Ultraturrax or stirrer for extraction
- Evaporator (for cheese samples)
- Beakers 150 mL, 100 mL
- Plaited filters with funnel
- Single-use syringes 10 mL (PP)
- Glass fiber filters (Whatman GF/A)
- Lab stands with clamp (e.g. three-finger clamp, LCTech P/N 10081)
- Sample preparation vials with screw cap and sealing ring for AflaCLEAN M1 (LCTech P/N 10896)
- Stopper with female Luer adapter for 3 mL columns
- Pipettes 10 mL
- Pipettes 1.0 mL
- Measuring flask 2 mL
- Lab tissues
- HPLC system consisting of autosampler with injector, pump, column oven, and fluorescence detector; PC with chromatography software
- HPLC column for the aflatoxin analysis from LCTech (P/N 10522) with guard column cartridge holder (P/N 10750) and guard cartridge (P/N 10523)

5. Extraction and Cleanup with AflaCLEAN M1 SMART Columns

5.1. Extraction of Milk / Raw Milk or UHT Milk According to the Official AOAC Method 2000.08:

100 mL milk is pre-warmed to room temperature.
The milk is stirred to disperse the fat in the milk. The milk is centrifuged at 2000 x g to separate fat from the residual milk. The thin fat layer is discarded. The milk is filtered through a plaited filter or glass fiber filter to remove any precipitations in the milk.

The immunoaffinity column is adapted to room temperature and opened.
A maximum of 10 mL milk is diluted by adding 10 mL PBS buffer and applied onto the immunoaffinity column with a maximum flow rate of 1.0 mL/min.

To wash the column, the sample reservoir is rinsed with 4 mL water, this solution is applied onto the column. The washing efficiency could be increased by applying the washing solution stepwise (e. g. two times 2 mL).

The residual water is now removed by a gentle gas stream or vacuum.

Now 0.2 mL methanol is applied onto the open column. Please wait until the methanol has reached the lower Luer exit of the column. The column is immediately closed and incubated for 5 minutes, to thoroughly break the analyte-antibody-binding. After the column is reopened, the eluate is collected and again eluted with 0.2 mL.

Dilute or concentrate eluate to your requirements and measure by HPLC.

5.2. Extraction of Milk Powder According to the EN ISO 14501:2007:

10 gram milk powder is put into a 250 mL beaker, add 50 mL water, pre-warmed to 50 °C, and stir to solve the test sample. In case that the sample is not solved completely the material could be warmed to 50°C and the material is stirred frequently to solve completely.

Allow the test solution to cool to between 20 °C and 25 °C.

Quantitatively transfer the test solution to a 100 mL volumetric flask using small amounts of water. Dilute to the 100 mL mark with water.

Filter enough of the reconstituted sample through filter paper or glass fiber filter or centrifuge it with at least 2 000 x g. Collect a minimum of 50 mL of the prepared milk powder sample.

A 10 mL aliquot is diluted by adding 10 mL PBS buffer and applied onto the immunoaffinity column, which need to be adapted to room temperature. The maximum flow rate of 1.0 mL/min should not be exceeded.

To wash the column, the sample reservoir is rinsed with 4 mL water, this solution is applied onto the column.

The washing efficiency could be increased by applying the washing solution stepwise (e. g. two times 2 mL).

The Residual water is now removed by a gentle gas stream or vacuum.

Now 0.2 mL methanol is applied onto the open column. Please wait until the methanol has reached the lower Luer exit of the column. The column is immediately closed and incubated for 5 minutes, to thoroughly break the analyte-antibody-binding. After the column is reopened, the eluate is collected and again eluted with 0.2 mL.

Dilute or concentrate eluate to your requirements and measure by HPLC.

5.3. Extraction of Aflatoxin M1 from Cheese:

A sample of 10 g of cheese was cut into small pieces and blended for 2 min at high speed with 80 mL of methylene chloride and 7 g of diatomaceous earth.

After washing further with 40 mL methylene chloride, the mixture was filtered (glass fiber filter) and pressed to released maximum amount of filtrate.

The filtrates were combined and evaporated in a rotary evaporator at 40 ºC.

The residue was dissolved in 1 mL methanol, 30 mL water and 50 mL n-hexane and transferred to a separating funnel. The water phase (lower layer) is collected.

The n-hexane phase was then washed twice with 10 mL water and the water phase was also collected. Subsequently both water phases were homogenized.

20 mL are applied onto the immunoaffinity column AflaCLEAN M1 SMART.

To wash the column, the sample reservoir is rinsed with two times 2 mL water, this solution is applied onto the column.

The Residual water is now removed by a gentle gas stream or vacuum.

Now 0.4 mL methanol are given onto the open column. Please wait until the methanol has reached the lower Luer exit of the column.

The column is immediately closed and incubated for 5 minutes, to thoroughly break the analyte-antibody-binding.

After the column is reopened, the eluate is collected and by a gentle flush of air residual methanol is removed from the column bed and combined with the eluate.

6. HPLC Analysis

For the aflatoxin analysis an isocratic HPLC is required. Thus the analysis can be run without expensive eluent and temperature gradients, respectively. Independently thereof it is advantageous to use a fluorescence detector model which is state-of-the-art.

The gain of sensitivity can be used in such a way that as a rule no labor intensive steps in the sample preparation such as the concentration of the eluate from the column are necessary.

Furthermore lower injection volumes can be used, which improve the chromatographic separation due to a decreased amount of interfering substances, and it leaves the opportunity to inject higher volumes in case of a lack of sensitivity. The achievable limits of detection are in the low ppt level and depend on the system and sample preparation.

The following conditions and settings, respectively, can be used as a first indication:

- Eluent: Water/methanol/acetonitrile 60/30/15 (v/v)
- Flow rate 1.2 mL/min
- Injection volume 10 – 100 µL
- HPLC Column 150 x 4.6 mm; RP C18 (LCTech P/N 10522)
- with guard column 8 x 4 mm (P/N 10523; holder P/N 10750)
- Column temperature 36 °C
- Fluorescence detector Ex.: 365 nm; Em.: 435 nm

7. Important Parameters and Troubleshooting

As with every procedure, sources of errors or possibilities of errors may occur during the aflatoxin analysis. These are starting with the sampling and end with the evaluation of the obtained chromatographic data. For the sampling please refer to the corresponding legal regulations. In case of a malfunction of the HPLC system contact the service of the respective supplier.

In the following some possible reasons or parameter shall be discussed, which may lead to suboptimal results for the AflaCLEAN M1 SMART column.

- **Weighing, Concentration, Calculation Errors and Mistake**
  First of all, the materials used have to be checked. Were new solutions or chemicals used? Are the used chemicals indeed the correct ones? Are the used measuring devices correct? Was a new protocol applied or were new calculations conducted? Did a new coworker do the job?

- **AflaCLEAN M1 SMART- Shelf Life and Storage**
  Were the AflaCLEAN M1 SMART columns stored at the temperature (4 °C to 8 °C) indicated and has the shelf life not expired?

- **Homogenization and Extraction**
  Was the spiked sample sufficiently homogenized and extracted? Was the correct procedure chosen for the sample? For many matrices various procedures can be applied. As a rule all samples with a high concentration of matrix compounds (e.g. fat, essential oils, aromas, and so on) should be processed with procedure for cheese

- **Formation of Phases**
  Has the correct lower phase been used for the further processing? This is important in all procedures especially for the extraction of cheese.

- **Clogging, Matrix Load/Initial Weight, Filtration and Pressure**
  A plugging of the column may have various reasons. The most common error is loading the column with too a high matrix load to improve the sensitivity or using too low volumes of extracting solution per amount of matrix.

  As a rule the columns should not be loaded with more than 10 g matrix per column (milk) or
  1 g milk powder or 2 g matrix equivalents for cheese samples. If higher initial weights are required, this has to be checked individually;
  owing to circumstances a lower flow rate must be expected.

  In all cases, independently of the application of a gentle vacuum or positive pressure, a flow rate of 1.0 mL/min must not be exceeded.
  Otherwise the soft gel is compressed too much and the performance will decrease.

  In any case it is advisable to filter the extract that has been filtered with a plaited filter already with a 0.2 µm syringe filter or glass fiber filter prior to loading it onto the column.

For all samples that still lead to a clogging, despite obeying all recommendations mentioned above, the sample weight must be reduced or the amount of extraction solution per sample must be increased.

In general resulting losses of sensitivity can be compensated by increasing the injection volume or a previous concentration.

- **Alcohol and Salt Concentration, pH Value**
  Due to their protein nature, the antibodies react sensitive to high alcohol and salt concentrations, respectively or inappropriate pH values. All deviations hereof will lead to an untimely elution or the loss of bonding towards the antibody, respectively, which in all cases will result in no or low recoveries. The salt content as well as the pH should be kept at physiological conditions.

- **Flow Rate and Running to Dryness**
  The maximum flow rate of the columns is 1.0 mL/min. Higher rates lead to lowered recoveries due to an increased pressure and insufficient time for retention of the sample. Running to dryness of the column has to be avoided, except during the drying step
  after the washing!

- **Elution, Holding Time**
  The eluting agent is pure methanol, which cleaves the antibody-analyte bond. To ensure this process, a holding time of 5 min must be kept after the first addition of methanol, so it can act on the column. Eluting too fast or too early will not cleave all bond and result in lowered recoveries. To remove the analyte quantitatively from the column, it is recommended to elute at least with two times of 0.2 mL methanol!

  A previous slight drying of the column with 10 mL of air from a syringe or a gentle vacuum from a SPE apparatus is recommended for the removal of residual water.

- **Analyte Concentration and Sample Volume**
  The AflaCLEAN M1 SMART column can be loaded with up to 100 ng Aflatoxin M1; A concentration of 0.2 ng M1/mL sample volume must not be exceeded to ensure a proper binding of the analyte to the antibody. In order to get an optimal result, it is advisable to dilute the crude extract in case of high results.

- **Concentration and Injection Volume**
  In most of the cases recent HPLC systems with the corresponding fluorescence or mass detectors will have no sensitivity problems in the aflatoxin analysis.

  Should a higher sensitivity be requested anyway, and the detector is already set to its limit, the matrix amount should not be increased initially, but a concentration step after the elution from the AflaCLEAN M1 SMART column taken into account. At this a significant increase in sensitivity is obtained.

  If low injection volumes are applied, higher volumes will increase the sensitivity. Basically it is an advantage, when the injected sample and the mobile phase of the eluent have the same composition!

  This is especially important when the injection volume is increased.

  Using the LCTech HPLC analytical column for the aflatoxin analysis, an injection volume of 100 µL should not be exceeded! Only if all previously mentioned measures were not successful, an increase of the matrix amount should be taken into consideration!

8. Literature

1) AOAC official method 2000.08 Aflatoxin M1 in liquid milk immunoaffinity column by liquid chromatography first action 2000

2) J. EN ISO 14501

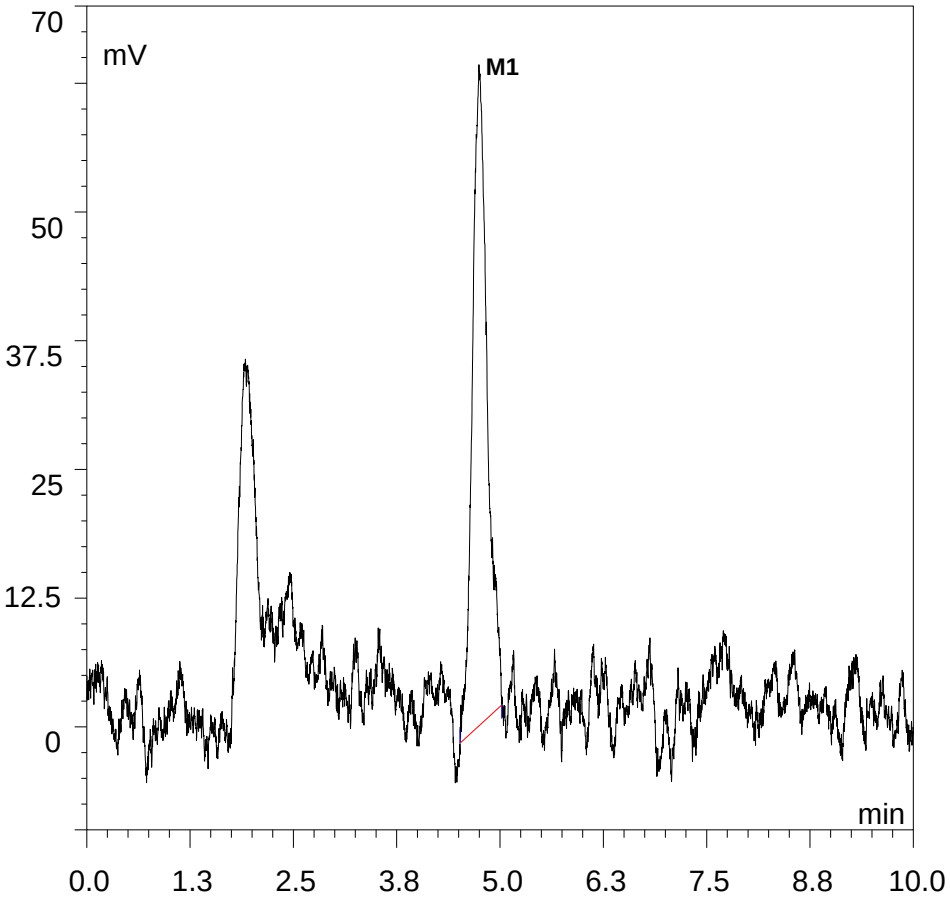
   European norm for extraction and analysis of aflatoxin M1 in milk and milk powder

3) A. EC 165/2010
   COMMISSION REGULATION (EU) No 165/2010 of 26 February 2010 amending Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards aflatoxins

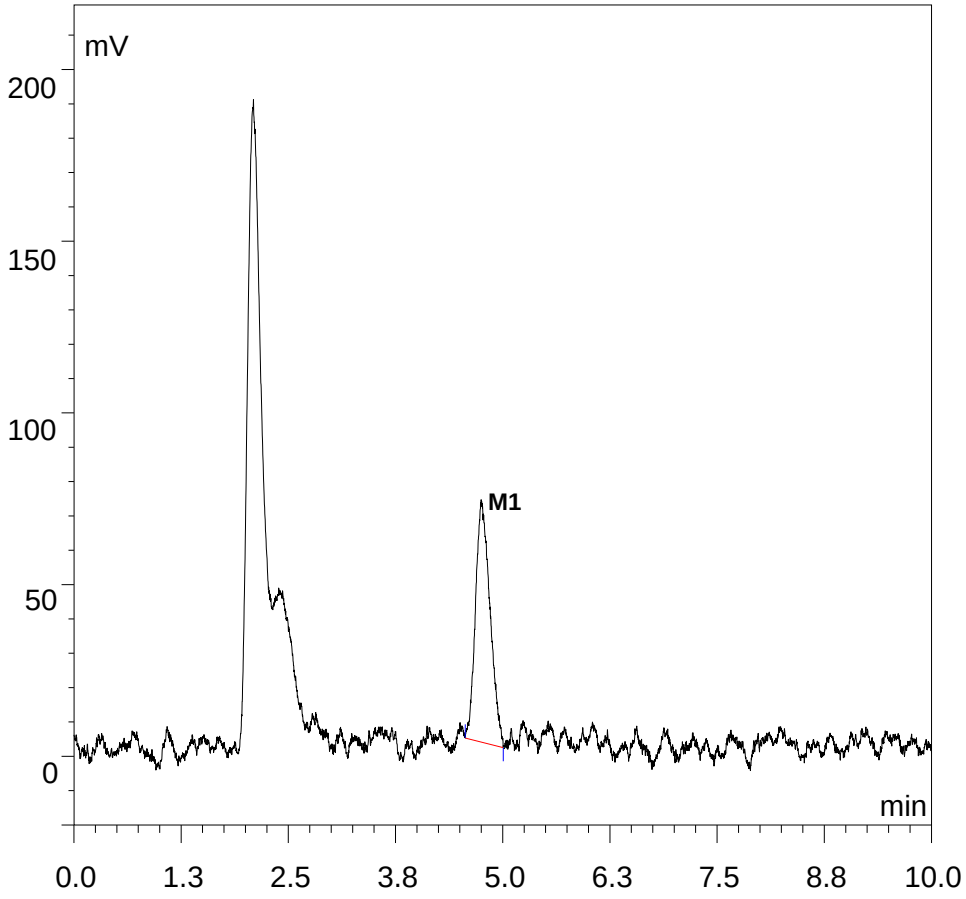
4) H. Marina Martins 2007
   Aflatoxin M1 determination in cheese by immunoaffinity column clean-up coupled to high-performance liquid chromatography

9. Annex

Chromatography of 0.8 ng/ 400 µL aflatoxin M1 standard.

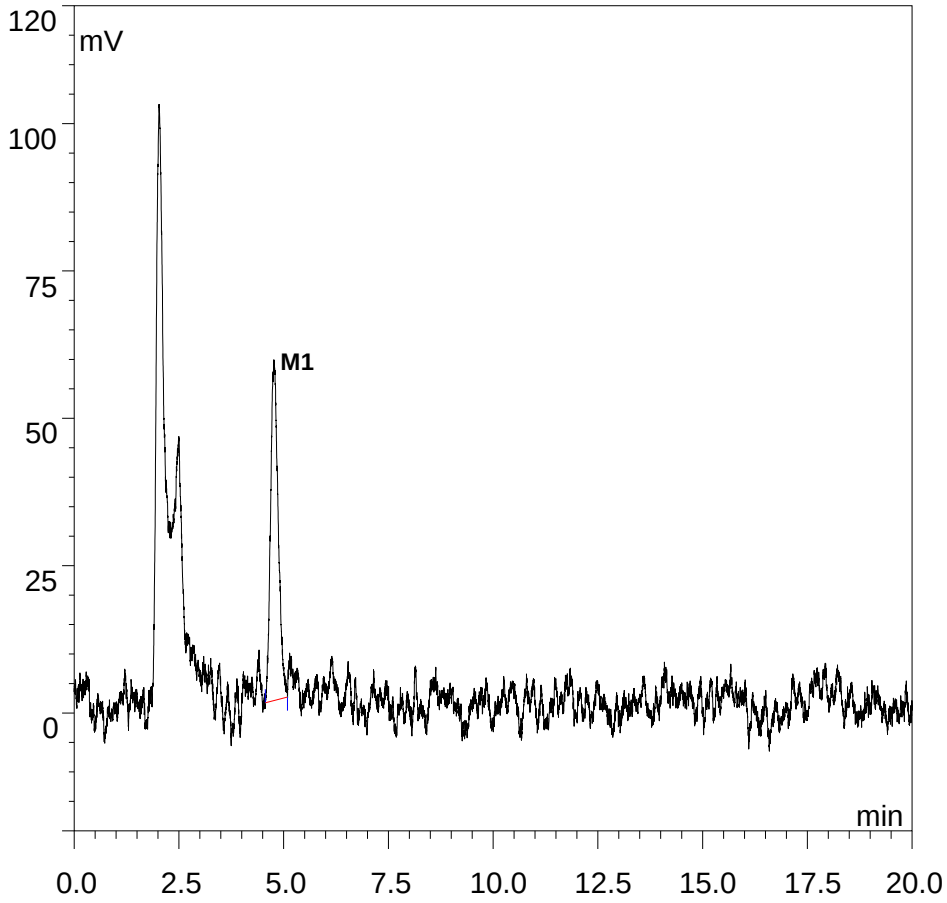


Chromatography of aflatoxin M1 0.8ng in 10mL milk UHT (80 ppt) extracted according to protocol for UHT and raw milk:

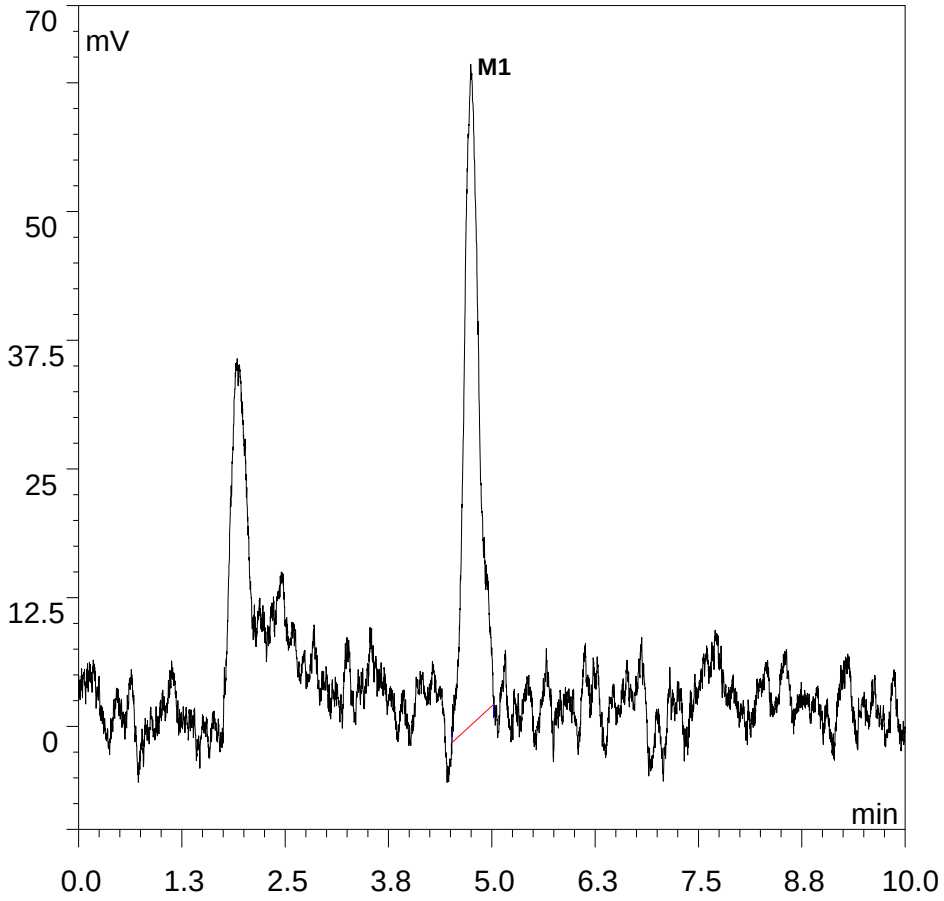


Raw milk extracted according to the official AOAC 2000.08 method:

10 mL milk are loaded onto the immunoaffinity column spiked with 0.8 ng aflatoxin M1 (represents 80 ppt).



Chromatography of aflatoxin M1 extracted from milk powder.
Milk powder sample was spiked with 80ppt, extracted and diluted according to the described protocol.



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1.6	alle	Handbuch neu formatiert	JU	10.01.2019
1.8	Alle	Neues CD angewandt	AM	02.05.2023
1.9	5	Neue Haltbarkeit: 12 statt 9 Monate	SU	08.01.2024