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Milk and milk powder – Determination of aflatoxin M_1 content – Clean-up by immunoaffinity chromatography and determination by high-performane liquid chromatography

WD stage

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Forewords

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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This document was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products* and the International Dairy Federation (IDF). It is being published jointly by ISO and IDF.

IDF (the International Dairy Federation) is a non-profit private sector organization representing the interests of various stakeholders in dairying at the global level. IDF members are organized in National Committees, which are national associations composed of representatives of dairy-related national interest groups including dairy farmers, dairy processing industry, dairy suppliers, academics and governments/food control authorities.

ISO and IDF collaborate closely on all matters of standardization relating to methods of analysis and sampling for milk and milk products. Since 2001, ISO and IDF jointly publish their International Standards using the logos and reference numbers of both organizations.

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This document was prepared by the IDF *Standing Committee on Analytical Methods for Composition* and the ISO Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*. It is being published jointly by ISO and IDF.

The work was carried out by the IDF-ISO Action Team on A12 of the *Standing Committee on Analytical Methods for Composition* under the aegis of its project leader xxxxxxxx.

Milk and milk powder – Determination of aflatoxin M_1 content – Clean-up by immunoaffinity chromatography and determination by high-performane liquid chromatography

1 Scope

This International Standard specifies a method for the determination of aflatoxin M_1 content in milk and milk powder. The lowest level of validation is 0,08 µg/kg for whole milk powder i.e. 0,008 µg/l for reconstituted liquid milk. The limit of detection (LOD) = 0,05 µg/kg for milk powder and LOD = 0,005 µg/kg for liquid milk. The limit of quantification(LOQ) = 0,1 µg/kg for milk powder and LOQ = 0,01 µg/kg for liquid milk.

Commented [MdV1]: Requested by end users: lowest level of validation is not identical with LOQ- the LOQs given here are based on 3 independent SLV conducted after edition of this version

The method is also applicable to low fat milk, skimmed milk, low fat milk powder and skimmed milk powder.

CAUTION 1 – The method described in this protocol requires the use of solutions of aflatoxin M_1. Aflatoxins are carcinogenic to humans. Attention is drawn to the statement made by the International Agency for Research on Cancer (WHO) [1, 2].

CAUTION 2 – Protect the laboratory in which the analyses are performed adequately from daylight and keep aflatoxin standard solutions protected from light, e.g. by using aluminium foil.

CAUTION 3 – The use of non-acid-washed glassware (e.g. tubes, vials, flasks, beakers, syringes) for aqueous aflatoxin solutions may cause loss of aflatoxin.
Moreover, brand new laboratory glassware, before coming into contact with aqueous solutions of aflatoxin, should be soaked in dilute acid (e.g. sulfuric acid, 2 mol/l) for several hours, then rinsed well with distilled water to remove all traces of acid (check to ensure pH is in the range 6 to 8).

CAUTION 4 – Use decontamination procedures for laboratory wastes such as solid compounds, solutions in organic solvents, aequeous solutions and spills, and for glassware coming into contact with carcinogenic materials. Suitable decontamination procedures have been developed and validated by the International Agency for Research on Cancer (WHO) [1, 2].

2 Normative references

There are no normative references in this document.

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

3.1
Aflatoxin M_1 content
Concentration (in µg/l) or mass fraction (in µg/kg) of substances determined by the procedure specified in this International Standard.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— IEC Electropedia: available at http://www.electropedia.org/

— ISO Online browsing platform: available at https://www.iso.org/obp

4 Principle

Aflatoxin M_1 is extracted by passing the test portion through an immunoaffinity column that contains specific antibodies bound onto a solid support material.

As the sample passes through the column, the antibodies are selectively bound with any aflatoxin M_1 (antigen) present and form an antibody-antigen complex. All other components of the sample matrix are washed off the column with water. Then aflatoxin M_1 is eluted from the column and the eluate is collected. The amount of aflatoxin M_1 present in this eluate is determined by means of high-performance liquid chromatography (HPLC) coupled with fluorimetric detection.

5 Reagents

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade and distilled or demineralized water or water of equivalent purity.

5.1 Immunoaffinity column

The immunoaffinity column shall contain antibodies against aflatoxin M_1. The column shall have a maximum capacity of not less than 100 ng of aflatoxin M_1 (which corresponds to 2 µg/l when a volume of 50 ml of a test portion is applied). It shall give a recovery of not less than 80 % for aflatoxin M_1 when a standard solution containing 4 ng of toxin is applied (which corresponds to 80 ng/l when a volume of 50 ml of sample is applied). Any immunoaffinity column meeting the performance specifications mentioned above can be used. [1]

The performance of the columns shall be checked regularly and at least once for every batch of columns (see the procedure in 5.1.1 and 5.1.2).

5.1.1 Capacity check

Dilute 1,0 ml of aflatoxin M_1 standard stock solution (5.4.2) to 50 ml with water. Mix well and apply the whole volume to the immunoaffinity column carefully following the recommendations given by the manufacturer for the use of columns. Wash the column and elute the toxin. Determine the amount of aflatoxin M_1 eluted from the column by HPLC after preparing a suitable dilution of the final eluate.

Calculate the capacity for the aflatoxin. Compare the result with the requirements given in 5.1.

5.1.2 Recovery check

Use a pipette (6.4) to dilute 0,8 ml of aflatoxin M_1 standard working solution of 0,005 µg/ml (5.4.3) to ~~10~~ 50 ml with water. Mix well and apply the whole volume to the immunoaffinity column carefully following the recommendations given by the manufacturer for the use of columns. Wash the column and elute the toxin. Determine the amount of aflatoxin M_1 eluted from the column by HPLC after preparing a suitable dilution of the final eluate.

Commented [MdV2]: Dilution from 10 to 50 improves the repeatability of the transfer to the column

Calculate the recovery for the aflatoxin. Compare the result with the requirements given in 5.1. The concentration shall not be less than 0,064 µg/l. Recovery checks can also be conducted with commercially available reference materials.

[1] Examples of immuno affinity columns: Afla Test P (Vicam®), Aflaprep® M (R-Biopharm), similar products are also available from Romer Labs®, Bioo Scientific® and Neogen®, these products are examples of suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO and/or IDF of these products.

5.2 Acetonitrile, pure, HPLC grade.

5.2.1 Acetonitrile solution, 25 % in H_20.

Add 250 ml of acetonitrile (5.2) to 750 ml of water and mix. Other volumes in the same proportion may be used. Degas the solution (eluent) before using it.

5.2.2 Acetonitrile solution, 10 % in H_20.

Add 100 ml of acetonitrile (5.2) to 900 ml of water and mix. Other volumes in the same proportion may be used. Degas the solution (eluent) before using it.

5.3 Nitrogen gas.

5.4 Aflatoxin M_1 standard solutions.

5.4.1 Aflatoxin M_1 standard calibration solution, (ρ = 10 µg/ml aflatoxin M_1 in acetonitrile).

Prepare an aflatoxin M_1 standard calibration solution by dissolving aflatoxin M_1 ($C_{17}H_{12}O_7$) in acetonitrile (5.2) to give a nominal concentration of 10 µg/ml. Determine the actual aflatoxins M_1 concentration by measurement of the absorbance at the maximum absorption wavelength of the solution as follows.

Use the spectrophotometer (6.13) to record the absorbance of the aflatoxin M_1 standard calibration solution against acetonitrile (5.2) as blank at wavelengths between 330 nm and 370 nm. Measure the absorbance, A, at its maximum absorption wavelength, λ_{max}, which is close to 350 nm.

Calculate the concentration, c_1, expressed in micrograms per millilitre, by using Formula (1):

$$c_1 = A \times M \times \frac{100}{d \times \varepsilon} \quad (1)$$

where

A is the numerical value of the absorbance at λ_{max};

M is the molar mass, in grams per mole, of aflatoxin M_1 (M = 328 g/mol);

d is the optical path length, in centimetres (d = 1 cm);

ε is the numerical value of the absorption coefficient, in square metres per mole, of the toxin in acetonitrile (ε = 1 985 $m^2 \cdot mol^{-1}$).

Alternatively, certified reference materials are available commercially (e.g. BCR-423 10 µg/ml aflatoxin M_1 in chloroform).

5.4.2 Aflatoxin M_1 standard stock solution, (ρ = 0,1 µg/ml aflatoxin M_1 in acetonitrile).

After checking its concentration, dilute the aflatoxin M_1 standard calibration solution (5.4.1) with 25 % acetonitrile (5.2) to an aflatoxin M_1 standard stock solution of 0,1 µg/ml. The standard stock solution shall be well-stoppered and wrapped in aluminium foil to protect it from light.

Store the aflatoxin M_1 standard stock solution in a refrigerator at a temperature between 1 °C and 5 °C in the dark. Under these conditions the stock solution is stable for at least two months. If the standard

stock solution is more than two months old, determine the aflatoxin M_1 concentration before use. If there is any change, discard the solution and prepare a fresh standard stock solution.

5.4.3 Aflatoxin M_1 standard working solutions, (ρ = 0,005 µg/ml aflatoxin M_1 in a mixture of 9 parts per volume of water and 1 part per volume of acetonitrile).

Before preparing the aflatoxin M_1 standard working solutions, allow the standard stock solution (5.4.2) to attain ambient temperature. Prepare the standard working solutions on the day of use.

Dilute the aflatoxin M_1 standard stock solution (5.4.2) with the 10 % acetonitrile solution (5.4.2) to an aflatoxin M_1 concentration of 0,005 µg/ml.

Remove aliquots of the diluted standard stock solution to prepare a series of 5 standard working solutions containing, for example, 0,05 ng/ml, 0,10 ng/ml, 0,20 ng/ml, ~~and~~ 0,40 ng/ml, and 0,80 ng/ml of aflatoxin M_1 by diluting with the 10 % acetonitrile solution (5.2.2). Other final dilutions may be chosen, depending on the injection loop volume.

Commented [MdV3]: 6 calibration points are recommended therefore 1 additional concentration level was added in this section

6 Apparatus

Usual laboratory equipment and, in particular, the following.

6.1 Disposable syringes, of capacities 10 ml and 50 ml.

6.2 Vacuum system [e.g. Büchner flask, Vac-Elut system [2] or peristaltic pump].

6.3 Centrifuge, capable of producing a radial acceleration of at least 2 000*g*.

6.4 Pipettes, of capacities 1,0 ml, 2,0 ml and 50,0 ml or suitable autopipette.

6.5 Glass beakers, of capacity 250 ml.

6.6 One-mark volumetric flask, of capacity 100 ml.

6.7 Water baths, capable of operating at 30 °C ± 2 °C, at between 35 °C and 37 °C and 50 °C ± 5 °C.

6.8 Filter paper, Whatman No. 4 [2] or equivalent.

6.9 Graduated conical glass tubes, with ground glass neck and stopper of capacities 5 ml, 10 ml and 20 ml.

6.10 HPLC apparatus, equipped with a pulse-free pump, capable of producing a constant volume flow rate of about 1 ml/min, and an injector system, with a fixed or variable injection volume loop, capable of injecting volumes of 20 µl to 500 µl.

6.11 Reversed phase HPLC analytical column, with 3 µm or 5 µm octadecyl silica packing and a guard column filled with reverse phase material.

[2] The Vac-Elut system and Whatman are examples of suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO or IDF of these products.

6.12 Fluorescence detector, capable of providing about 365 nm excitation and ~~435~~ 425 nm emission wavelengths and of detecting (signal to noise ratio: 5) aflatoxin M_1 when 0,02 ng is injected under appropriate chromatographic conditions. A typical emission spectrum for excitation at 366 nm is given in Annex B.

~~**6.13 Strip chart recorder,** with a printer or plotter, or electronic integrator or computer-based data processing system.~~

Commented [MdV4]: lab instruments are now equipped with instruments connected to PCs

~~6.14~~6.13 **Spectrophotometer,** capable of measuring at wavelengths from 200 nm to 400 nm, with quartz face cells of optical pathlength 1 cm.

~~6.15~~6.14 **Analytical balance,** capable of weighing to the nearest 0,01 g.

7 Sampling

A representative sample should have been sent to the laboratory. It should not have been damaged or changed during transport or storage. Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in ISO 707 | IDF 50 [3].

8 Procedure

Carry out the procedure with daylight excluded, as far as possible.

Notice that the procedures ~~for reconstituting milk powder, for centrifugation,~~ for loading the sample on to the affinity columns, for washing the column and elution will vary slightly between column manufacturers. Follow precisely, therefore, the specific instructions supplied with the columns.

Commented [MdV5]: Reconstitution is not linked to IAC column but rather to the sample. Therefore the project team suggests that this confusing sentence will be removed

8.1 Preparation of test samples

8.1.1 Milk

Warm the test sample in the water bath (6.7) to between 35 °C and 37 °C. Either filter the sample through filter paper(s) (6.8) using several filters, if necessary, or centrifuge it at a radial acceleration of at least 2 000*g* for 15 min. Collect at least 50 ml of the thus prepared skimmed milk sample. Continue as specified in 8.3.

8.1.2 Milk powder

Weigh, to the nearest 0,01 g, 10 g of test sample into a 250 ml beaker (6.5). Add 50 ml water, prewarmed in the water bath (6.7) to 50 °C, in small amounts to the test sample. Mix, using a stirring rod, until a homogeneous mixture is obtained.

If the test sample does not become completely suspended, place the beaker in a water bath (6.7) set at 50 °C for at least 30 min. Stir the mixture frequently.

Allow the test solution to cool to between 20 °C and 25 °C. Then, quantitatively transfer the test solution to a 100 ml one-mark volumetric flask (6.6) using small amounts of water. Dilute to the 100 ml mark with water. Filter enough of the reconstituted sample through filter paper(s) (6.8) or centrifuge it at a radial acceleration of at least 2 000*g* for 15 min. Collect at least 50 ml of the prepared milk powder sample. Continue as specified in 8.3.

8.2 Immunoaffinity column preparation

Attach the barrel of a 50 ml disposable syringe (6.1) to the top of an immunoaffinity column (5.1). Connect the immunoaffinity column to the vacuum system (6.2).

8.3 ~~Extraction and purification of samples~~Test sample extraction

Add 50 ml of the prepared test sample (8.1.1 or 8.1.2) into the 50 ml syringe barrel (6.1). Allow it to pass through the immunoaffinity column at a rate of 2 ml/min to 3 ml/min while controlling the volume flow by using the vacuum system (6.2).

Replace the 50 ml syringe barrel by a clean 10 ml syringe barrel. Wash the column with 2 x 10 ml water by allowing it to pass through the column at a steady volume flow rate. Blow the column to completely dry it after washing.

Commented [MdV6]: End users have shown that this improves reproducibility

Disconnect the column from the vacuum system. Elute aflatoxin M_1 slowly from the column by passing 4 ml pure acetonitrile (5.2) in about 60 s through the column using a 10 ml syringe. Control the volume flow rate by means of the syringe plunger.

Collect the eluate in a conical tube (6.9). Reduce the eluate to ~~a volume, V_e, of between 20 µl and 500 µl,~~dryness by placing the tube in the water bath (6.7) set at ~~30~~ 50 °C and blowing a gentle stream of nitrogen (5.3) over it.

Commented [MdV7]: losses during evaporation to dryness have not been observed by the end users even when evaporating at 50°C

~~WARNING — Losses may occur when evaporating the eluate to complete dryness.~~

Make up to a final eluate volume, $V_f = 10V_e$, i.e. 500 µl to 5 000 µl, with water (see NOTE).

Alternatively 25 ml of samples can be applied to the immunoaffinity column and the final reconstituion volume decreased to 200 µl.

NOTE If the acetonitrile content of the injected extract containing aflatoxin M_1 exceeds the 10 % (volume fraction) limit, peak broadening will occur on the HPLC chromatogram. However, a water content of over 90 % (volume fraction) has no influence on the peak-shape [4].

8.4 High performance liquid chromatography

8.4.1 Pump setting

Pump the eluent (5.2.1) at a constant flow rate through the HPLC column. Depending on the type of column used, adjust the acetonitrile/water ratio of the HPLC eluent (5.2.1), if necessary, to ensure an optimal separation of the aflatoxin M_1 from other extract components.

NOTE The flow rate of the eluent (5.2.1) also depends on the column (6.11) used. As guidelines for conventional columns with a length of approximately 25 cm: internal diameter about 4,6 mm, the optimal flow rate is approximately 1 ml/min; with internal diameter about 3 mm, the optimal flow rate is approximately 0,5 ml/min and typical elution conditions isocratic eluents (mixture of 80 parts per volume of water, 12 parts per volume of isopropanol and 8 parts per volume of acetonitrile or a mixture of 1 000 parts per volume of water,275 parts per volume of methanol and 275 parts per volume of acetonitrile) depending on the column .

It is advisable to ascertain optimal conditions by using a sample extract (preferably free from aflatoxin M_1) which is injected separately and in combination with an aflatoxin M_1 standard working solution (5.4.3).

8.4.2 Chromatographic performance

Check the stability of the chromatographic system by repeatedly injecting a fixed amount of aflatoxin M_1 standard working solution (5.4.3) until stable peak areas or heights are achieved. Consecutive injections shall not differ more than 5 % in peak area or peak height.

The responses in retention time of the aflatoxin M_1 peaks depend on the temperature. Therefore, to compensate for drift in the detection system, inject a fixed amount of aflatoxin M_1 standard working solution (5.4.3) at regular intervals. If needed, the result for the standard working solution used can be corrected for the observed drift.

8.4.3 Calibration curve of aflatoxin M_1

Inject, in sequence, suitable volumes of the standard working solutions (5.4.3) containing 0,05 ng, 0,10 ng, 0,20 ng, ~~and~~ 0,40 ng and 0,80 ng of aflatoxin M_1 into the HPLC apparatus via the injection loop. Prepare a calibration graph by plotting the obtained peak area or peak height for each standard working solution against the mass of aflatoxin M_1 injected.

8.4.4 Analysis of the purified extracts and injection scheme

Inject a similar volume of the eluate (8.3) to that used for the standard working solutions (8.4.3) into the HPLC apparatus via the injection loop. Separate aflatoxin M_1 present, using the same conditions as for the standard solutions. Perform the injection of standards and sample extracts according to a specified injection scheme.

When a series of sample eluates is to be injected one after the other, it is recommended that an aflatoxin M_1 standard working solution is injected after every five injections of sample eluates.

Determine the area or height of the aflatoxin M_1 peak of the sample eluate. Calculate, from the calibration graph, the mass, in nanograms, of aflatoxin M_1 in the sample extract.

If the peak area or peak height of aflatoxin M_1 in the sample eluate is greater than that of the highest standard solution, dilute the eluate quantitatively with water. Re-inject the diluted extract into the HPLC apparatus as described above.

9 Calculation and expression of results

9.1 Milk

9.1.1 Calculation

Calculate the aflatoxin M_1 content, as a concentration, of the test sample, *c*, in micrograms per litre, by using Formula (2):

$$c = m_a \times \left(\frac{V_f}{V_i}\right) \times \left(\frac{1}{V_t}\right) \quad (2)$$

where

m_a is the mass, in nanograms, of aflatoxin M_1 corresponding to the area or height of the aflatoxin M_1 peak of the sample eluate;

V_i is the volume, in microlitres, of the test sample eluate injected;

V_f is the final volume, in microlitres, of the test sample eluate;

V_t is the volume, in millilitres, of the prepared test solution passing through the column.

9.1.2 Expression of results

Express the test results to 3 decimal places for liquid milk.

9.2 Milk powder

9.2.1 Calculation

Calculate the aflatoxin M_1 content, as a mass fraction, of the test sample, w, in micrograms per kilogram, by using Formula (3):

$$w = m_a \times \left(\frac{V_f}{V_i}\right) \times \left(\frac{1}{m_t}\right) \times f \qquad (3)$$

where

m_t is the mass, in grams, of the test sample present in 50 ml of the prepared test sample (7.3);

f is the dilution factor of the test sample (for undiluted solutions, $f = 1$).

9.2.2 Expression of results

Express the test results to ~~3~~ 2 decimal places for milk powders.

Commented [MdV8]: Powders are approx. 10x more concentrated than liquid milk .- therefore the 3rd decimal is not significant when calculating back from a reconstituted milk to the powder

10 Precision

10.1 Interlaboratory test

The values for repeatability and reproducibility derived from the interlaboratory test were determined in accordance with ISO 5725-1 [5] and ISO 5725-2 [6]. Details of the test are summarized in Annex A.

The values obtained may not be applicable to concentration ranges and matrices other than those given.

10.2 Repeatability

The absolute difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, will in not more than 5 % of cases be greater than the values given in Table A.1.

10.3 Reproducibility

The absolute difference between two independent single test results, obtained using the same method on identical test material in different laboratories with different operators using different equipment, will in not more than 5 % of cases be greater than the values given in Table A.1.

11 Test report

The test report shall specify:

a) all information required for the complete identification of the sample;

b) the sampling method used, if known;

c) the test method used, together with reference to this International Standard;

d) all operating details not specified in this International Standard, or regarded as optional, together with details of any incident that may have influenced the result(s);

e) the test result(s) obtained, and, if the repeatability has been checked, the final quoted result(s) obtained.

Annex A
(normative)

Results of an interlaboratory trial

An international collaborative test [7] involving 16 laboratories was carried out on test samples of milk powder having: a fat mass fraction of 1 % ("low fat" test samples 1 and 3); and a fat mass fraction of 28 % ("high fat" test samples 2, 4, and 5). The latter samples were remainders of batches of milk powders used to prepare reference materials [8], hence the aflatoxin M_1 contents were known.

The contamination levels varied from 0,08 µg/kg to 0,6 µg/kg, i.e. 0,008 µg/l to 0,060 µg/l for reconstituted milk.

The results obtained were subjected to statistical analysis in accordance with ISO 5725-1 [6] and ISO 5725-2 [7] to give the precision data shown in Table A.1.

Table A.1 — Precision data

Sample	1	2	3	4	5
Labs participating [a]	12	14	13	11	14
Mean, w (µg/kg)	0,081	0,150	0,080	0,202	0,580
Standard deviation of repeatability, s_r (µg/kg)	0,008	0,022	0,005	0,010	0,073
Repeatability value, $r = 2{,}8s_r$, µg/kg	0,023	0,060	0,015	0,027	0,203
Coefficient of variation of repeatability, CV(r), %	9,9	14,0	6,8	4,7	12,5
Standard deviation of reproducibility, s_R, µg/kg	0,019	0,035	0,015	0,022	0,110
Reproducibility value, $R = 2{,}8s_R$, µg/kg	0,052	0,098	0,041	0,061	0,310
Coefficient of variation of reproducibility, CV(R), %	23	22,7	18,3	10,8	19,1
[a] Labs were eliminated using the Cochran and/or Grubbs outliers test.					

Annex B
(informative)

Example of an emission spectrum of aflatoxin M_1

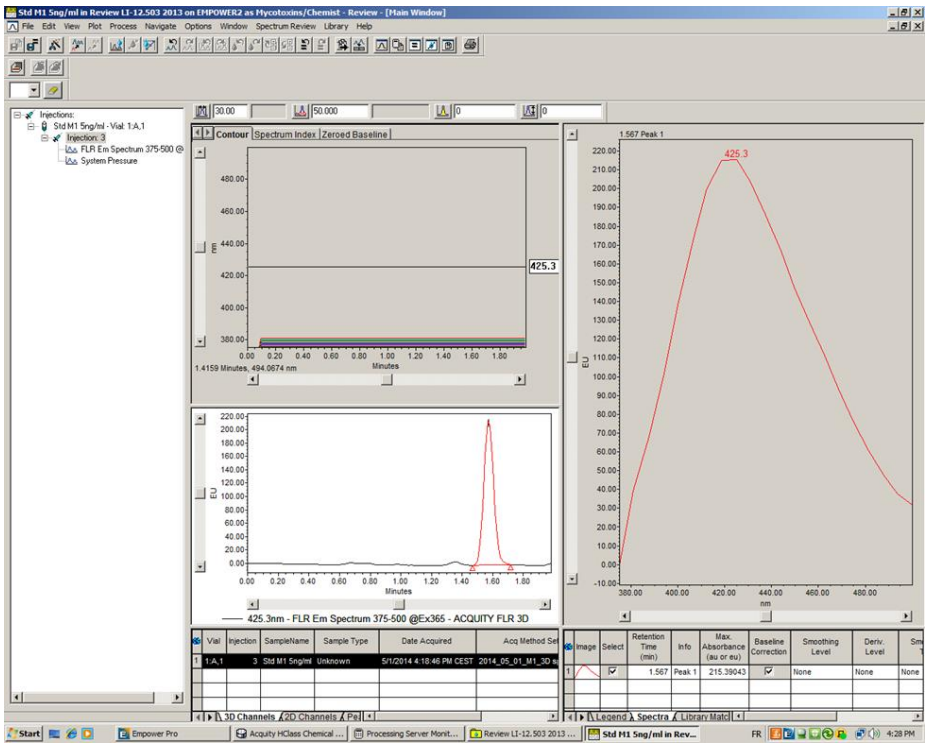

Figure A.1 — Example of an emission spectrum of aflatoxin M_1

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