BS EN 16006:2011

BSI Standards Publication

Animal feeding stuffs — Determination of the Sum of Fumonisin B1 & B2 in compound animal feed with immunoaffinity clean-up and RP-HPLC with fluorescence detection after pre- or post-column derivatisation

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National foreword

This British Standard is the UK implementation of EN 16006:2011.

The UK participation in its preparation was entrusted to Technical Committee AW/10, Animal feeding stuffs.

A list of organizations represented on this committee can be obtained on request to its secretary.

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ISBN 978 0 580 66993 4

ICS 65.120

Compliance with a British Standard cannot confer immunity from legal obligations.

This British Standard was published under the authority of the Standards Policy and Strategy Committee on 31 August 2011.

Amendments issued since publication

Date	Text affected

EUROPEAN STANDARD

NORME EUROPÉENNE

EUROPÄISCHE NORM

EN 16006

August 2011

ICS 65.120

English Version

Animal feeding stuffs - Determination of the Sum of Fumonisin B1 & B2 in compound animal feed with immunoaffinity clean-up and RP-HPLC with fluorescence detection after pre- or post-column derivatisation

Aliments pour animaux - Dosage de la somme des fumonisines B1 et B2 dans les aliments pour animaux avec purification par immuno-affinité et RP-HPLC avec détection par fluorescence après dérivation pré- ou post-colonne

Futtermittel - Bestimmung der Summe der Fumonisine B1 und B2 in Mischfutter durch Reinigung an einer Immunoaffinitätssäule und RP-HPLC mit Fluoreszenzdetektion nach Vor- oder Nachsäulenderivatisierung

This European Standard was approved by CEN on 25 June 2011.

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EUROPÄISCHES KOMITEE FÜR NORMUNG

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Ref. No. EN 16006:2011: E

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Foreword

This document (EN 16006:2011) has been prepared by Technical Committee CEN/TC 327 “Animal feeding stuffs”, the secretariat of which is held by NEN.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by February 2012, and conflicting national standards shall be withdrawn at the latest by February 2012.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. CEN [and/or CENELEC] shall not be held responsible for identifying any or all such patent rights.

This document has been prepared under a mandate given to CEN by the European Commission and the European Free Trade Association.

WARNING — The use of this protocol can involve hazardous materials, operations and equipment. This protocol does not purport to address all the safety problems associated with its use. It is the responsibility of the user of this protocol to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.

WARNING — Fumonisins are toxic. Gloves and safety glasses should be worn at all times and all standard and sample preparation stages should be carried out in a fume cupboard.

According to the CEN/CENELEC Internal Regulations, the national standards organizations of the following countries are bound to implement this European Standard: Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden, Switzerland and the United Kingdom.

1 Scope

This European Standard is applicable to the determination of Fumonisin B_1 & B_2 (FB_1 & FB_2) in compound animal feed at levels starting from 3 mg/kg up to 16 mg/kg.

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

EN ISO 835, *Laboratory glassware — Graduated pipettes (ISO 835:2007)*

EN ISO 1042, *Laboratory glassware — One-mark volumetric flasks (ISO 1042:1998)*

EN ISO 3696, *Water for analytical laboratory use — Specification and test methods (ISO 3696:1987)*

3 Principle

FB_1 and FB_2 are extracted from the test material with a solution of 50% methanol in phosphate-buffered saline (PBS). The extract is then diluted with PBS and cleaned up using immunoaffinity columns (IAC). FB_1 and FB_2 are eluted from the IAC using methanol and then water. After volume adjustment, the eluate is directly injected into the HPLC and FB_1 and FB_2 are detected by their fluorescence after either pre- or post column derivatisation.

4 Reagents and materials

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade. Solvents shall be of HPLC or better quality and only double-distilled water or water of at least grade 2 as defined in EN ISO 3696 shall be used.

4.1 Double distilled or deionised water (EN ISO 3696)

4.2 Methanol, CH_3OH

WARNING — Methanol is hazardous and handling should be carried out inside a fume cupboard. Appropriate safety equipment (lab coat, goggles, gloves) should be worn.

4.3 Acetonitrile, CH_3CN

WARNING — Acetonitrile is hazardous and handling should be carried out inside a fume cupboard. Appropriate safety equipment (lab coat, goggles, gloves) should be worn.

4.4 Potassium chloride, KCl

4.5 Sodium chloride, NaCl

4.6 Disodium hydrogenphosphate dodecahydrate, Na_2HPO_4*12 H_2O

4.7 Disodium tetraborate decahydrate, $Na_2B_4O_7$ *10H_2O

4.8 Sodium carbonate, Na_2CO_3.

4.9 Boric acid, H_3BO_3

4.10 Potassium sulphate, K_2SO_4

4.11 NAC, N-Acetyl-L-Cystein, $C_5H_9NO_3S$

4.12 OPA, o- Phthalaldehyde, $C_6H_4(CHO)_2$

4.13 BME, β-Mercaptoethanol, $HOCH_2CH_2SH$

4.14 Formic Acid, HCO_2H, 98%-100%

WARNING — Concentrated Formic acid is hazardous and handling should be carried out inside a fume cupboard. Appropriate safety equipment (lab coat, goggles, gloves) should be worn.

4.15 PBS concentrate, Phosphate buffered saline concentrate

Dissolve the following in 1 800 ml of water (4.1):

— 4 g KCl (4.4);

— 160 g NaCl (4.5);

— 72 g Na_2HPO_4*12 H_2O (4.6).

Adjust to pH 7,4 with 10 mol/l HCl and make up to 2 000 ml.

4.16 PBS Ready to use

Dilute 100 ml of PBS concentrate (4.15) to 1 000 ml with water (4.1),

or

PBS tablets, Phosphate buffered saline tablets

one tablet dissolved in 200 ml of water (4.1) yields 0,01 mol/l phosphate buffer, 0,002 7 mol/l potassium chloride and 0,137 mol/l sodium chloride, pH 7,4, at 25°C (e.g. Sigma P4417).

4.17 Diluent

Mix 50 parts per volume methanol (4.2) with 50 parts per volume water (4.1).

4.18 Extraction solvent

Mix 50 parts per volume methanol (4.2) with 50 parts per volume of PBS (4.16).

4.19 Reaction buffer

4.19.1 Post-column derivatisation (0,006 mol/l OPA, 0,006 mol/l NAC, 0,384 mol/l sodium carbonate, 0,216 mol/l boric acid and 0,108 mol/l potassium sulphate):

- Dissolve 40,7 g sodium carbonate (4.8), 13,4 g boric acid (4.9) and 18,8 g potassium sulphate (4.10) per 1,0 l of water (4.1);
- stir for 10 min;
- add 800 mg of OPA (4.12) per 1,0 l of the above solution;
- add 1 g of NAC (4.11) per 1,0 l of the above solution;
- stir for 10 min;
- sonicate for 15 min;
- stir for 10 min;
- sonicate again for 15 min and
- filter the solution through a 0,45 µm nylon filter (5.17).

Proper dissolution of the OPA is very important!

The reaction buffer should not be changed within a sequence of HPLC runs.

Prepare fresh for every sequence of HPLC runs.

4.19.2 Pre-column derivatisation (0,05 mol/l OPA, 0,12 mol/l BME, 0,08 mol/L disodium tetraborate, 16,7% methanol):

- Dissolve 40 mg OPA (4.12) in 1,0 ml methanol (4.2);
- mix until completely dissolved;
- add 5,0 ml of a 0,1 mol/l solution of disodium tetraborate decahydrate (3,8 g / 100 ml; 4.7);
- mix thoroughly;
- add 50 µl of BME (4.13), and
- mix thoroughly.

Alternatively:

- Phthaldialdehyde Reagent.

4.20 FB_1 & FB_2 stock solution:

- A certified solution of Fumonisin FB_1 and FB_2 of ca. 50 µg/ml each in an appropriate solvent. Take exact concentration from certificate;

or

- Separate certified solutions of Fumonisin FB_1 and FB_2 in appropriate solvents that will be mixed such that a stock solution containing ca. 50 µg/ml of each is obtained. Calculate exact concentrations from certificates and dilutions.

NOTE 1 The above solutions (4.20) may also be prepared gravimetrically by accurately weighing the dry substance and the solvent used to dissolve it. Accurately measuring the volume of the solvent is also allowed.

NOTE 2 The above solutions may be stored for up to six months at below -18°C in the dark.

4.21 FB_1 & FB_2 diluted stock solution for calibration

- Add 160 µl of the FB_1 & FB_2 stock solution (4.20) to a 2 ml volumetric flask (5.13), and

- Make up to mark (2,0 ml) with diluent (50% methanol, 4.17).

This will result in 2,0 ml of a solution containing ca. 4 µg/ml FB1 & FB2 each in mostly methanol /water (50/50, v/v).

4.22 Calibration solutions

From the diluted stock solution for calibration (4.21) prepare five levels of calibration solutions by adding the volumes of diluted stock solution listed in the following table to a volumetric flask (5.13) of the indicated volume and make up to the mark with diluent (4.17).

Calculate the concentrations of FB_1 & FB_2 for the different calibration levels by dividing the certified or calculated concentrations of the stock solution (4.20) by the final dilution stated below. Should you observe saturation of the detector signal at the highest calibration level dilute 250 µl of diluted stock solution into 2,0 ml for a final dilution of 100.

Table 1 — Recommended calibration solutions (4.22) for the determination of the sum of Fumonisin B1 & B2

Calibrant	Diluted stock solution (4.21) (µl)	Volumetric flask (5.13) (ml)	Final dilution of stock solution (4.20)	Approx. concentration of FB1 & FB2 each (ng/ml)
1	50	20,0	5 000	10
2	125	10,0	1 000	50
3	125	5,0	500	100
4	500	2,0	50	1 000
5	1 000	2,0	25	2 000

These calibration levels are recommendations and may be adjusted to the individual needs. The exact concentrations of the calibration levels should be calculated based on the final dilution and the exact concentration of the stock solution (4.20).

NOTE The above solutions may be stored for up to 5 days at 6 °C ± 2°C in the dark.

4.23 IAC (Immunoaffinity column)

The immunoaffinity columns must contain a stationary phase with immobilized monoclonal antibodies specific to, at least, Fumonisin B_1 and B_2. To be suitable for this method they must meet the requirements stated below:

An aliquot of more than 5 ml of an extract of a fumonisin-free representative compound animal feed material is spiked with FB_1 & FB_2 in equal parts at either 920 (high) ng/ml or 110 (low) ng/ml for the sum of both. Then dilute 5,0 ml of that spiked extract to a total volume of 50,0 ml (see 6.2).

Following the procedures described in 6.3 and 6.4 this will result in expected concentrations in the injection solutions of either 460 ng/ml or 55 ng/ml for the sum of FB_1 & FB_2.

After measuring (Clause 7) these solutions the observed concentrations of FB_1 & FB_2 can be calculated with Equation (1) and Equation (2) of Clause 8. Dividing the sum of the observed concentrations of FB_1 & FB_2 by the expected concentrations will result in the yield of the immunoaffinity columns.

These yields must be 99 % ± 18 % (U, k=2) at the high level and 118 % ± 18 % (U, k=2) at the low level.

The above column test should be performed for each level on at least three randomly selected columns of every new batch of immunoaffinity columns which will be used. Should the tested batch not meet the above requirements either a new batch which does should be obtained or the conditions described in 6.3 need to be adjusted such that the requirements are met (the user instructions supplied with the columns are a good starting point).

Any changes to the clean-up procedures will necessitate a revalidation of the clean-up and all subsequent steps (chromatography).

5 Apparatus

Usual laboratory equipment and, in particular, the following:

5.1 Mill

5.2 Tumble mixer

Creates a folding motion of the material through, for instance, a rotating drum with internal fins and paddels or moving a closed container head-over-heels.

5.3 Vortex mixer

5.4 Laboratory shaker

5.5 250 ml flasks with screw caps

5.6 Graduated cylinders, 5 ml, 50 ml, 1 000 ml and 2 000 ml

5.7 Graduated pipettes (Class A, EN ISO 835) 2 ml, 10 ml and 50 ml

5.8 Analytical balance (d= 0,01g)

5.9 Glass micro fibre filter, binder-free with ca. 2 µm pore size

5.10 Filter funnel, of appropriate size

5.11 Auto sampler vials, of appropriate size with caps

5.12 Reservoirs for IACs

Of appropriate size with adapter for connection to top of IACs.

5.13 Volumetric flasks (Class A, EN ISO 1042) 2 ml, 5 ml, 10 ml and 20 ml

5.14 Gastight glass syringes and/or direct displacement pipettors

Capable of precisely dispensing the following volumes: 5 µl, 50 µl, 125 µl, 160 µl, 500 µl, and 1 000 µl.

5.15 Support stand for immunoaffinity columns, of appropriate size

5.16 HPLC instrumentation, comprising the following:

5.16.1 Solvent delivery system

Capable of generating a binary gradient with sufficient precision at the required pressures.

5.16.2 Auto sampler

Capable of injecting sufficient volumes of injection solution with sufficient repeatability and, for pre-column derivatization, capable of mixing reagent and sample solution before injection.

5.16.3 Chromatographic column

Any column which provides symmetric peak (peak asymmetry factor 0,9< A_s <1,4 at 10% of full height), sufficient retention (k > 2), and resolution (R_s >1) for FB_1 & $FB_{2..}$

5.16.4 Fluorescence detector

Capable of providing the required excitation and emission wavelengths and equipped with a flow cell of appropriate size.

5.16.5 Post-column derivatisation system (not necessary if pre-column derivatisation is used)

Either a commercial unit or self-assembled. If self-assembled the following is needed:

- **Reagent pump:** capable of delivering a constant pulsation-free flow of the derivatisation reagent against the required pressures.

- **Peek tubing:** of the outer diameter required by the HPLC system in use and varying inner diameters, e.g. 1/16"OD and 0,04"ID, 0,02"ID, 0,01"ID, and/ or 0,005" ID.

- **Mixing Tee:** small internal volume PEEK.

5.17 Nylon filter 0,45 µm

6 Sample preparation

6.1 Sample preparation

It is important that the laboratory receives a sample which is truly representative and has not been damaged or changed during transport or storage. Samples should be taken and prepared in accordance with European legislation where applicable [1]. Samples should be finely ground and thoroughly mixed using a mill (5.1) and a tumble mixer (5.2) or another process that has been shown to give complete homogenisation before a test portion is removed for analysis.

In all instances: If the sample has been frozen allow it to thaw completely before sampling. Mix the sample thoroughly before removing an analytical test portion.

NOTE The materials for the interlaboratory study [3] were milled to a particle size of ca. 0,5 mm.

6.2 Extraction of FB_1 & FB_2

- Weigh 20,0 g of the test sample into a large enough container with lid, e.g. 250 ml flask (5.5);

- add 200 ml of extraction solvent (4.18), cap, and shake vigorously by hand, so that the material disperses evenly;

- put on a shaker (5.4) for 120 min; choose speed such that the material is mixed well without collecting in the top of the flask;

- allow the extracted sample to settle after shaking;

- of the supernatant take 5,0 ml and dilute with PBS (4.16) to a total volume of 50,0 ml and mix;

- prepare a filter funnel (5.10) with a glass micro fibre filter (5.9), and

- filter the diluted supernatant of the extracted sample into a new flask (5.5).

The diluted filtered extract may be stored at 4ºC to 10ºC overnight.

In case of a highly contaminated material above 10 000 µg/kg (see Clause 8), take 10,0 ml of the stored filtered diluted extract and dilute again with PBS (4.16) to a total volume of 50,0 ml and mix.

6.3 Clean up

- Take one IAC (immunoaffinity column; 4.23) per extract;
- attach a reservoir (5.12), do not empty storage solution from column;
- to the reservoir add 25,0 ml of the filtered diluted extract (6.2);
- open the column outlet;
- allow everything to pass slowly through the column (flow rate should be one drop per second to two drops per second);
- after the extract has passed completely, wash the IAC with 10 ml of PBS (4.16);
- pass air through the IAC (e.g. using a properly fitted large syringe) in order to expel excess PBS;
- place a 5 ml volumetric flask (5.13) or a 5 ml graduated cylinder (5.6) underneath the IAC and add 5 x 500 µl of methanol (4.2) to the IAC (add next aliquot only after previous has completely passed);
- collect all the eluate in the volumetric flask (5.13) or graduated cylinder (5.6);
- add 2,0 ml of water (4.1) to the IAC after all of the methanol (4.2) has passed through the column;
- continue to collect the eluate in the same volumetric flask or graduated cylinder, and
- carefully pass air through the column in order to collect most of the applied water (4.1).

6.4 Test solution

- For pre-column derivatisation: make up the content of the volumetric flask or graduated cylinder to the 5 ml mark with water (4.1);
- For post-column derivatisation: add 5 µl of formic acid (4.14) and make up the content of the volumetric flask or graduated cylinder to the 5 ml mark with water (4.1);
- Mix the content of the volumetric flask or graduated cylinder and transfer an aliquot to an autosampler vial (5.11).

This test solution may be stored at 4ºC to 10ºC for up to two days.

6.5 Spiking procedure

To determine recovery spike a fumonisin-free representative compound animal feed material with FB_1 & FB_2 stock solution (4.20) or a dilution thereof. The spiking level should be appropriate and the concentration of the solution used such that not more than 1 ml is added. Leave the spiked sample to stand for a period of 30 min to ensure evaporation of the solvent.

7 Measurements

7.1 HPLC operating conditions

7.1.1 General

Below we provide recommendations for the operating conditions that work well with the equipment listed in 5.16. More likely than not you will have to make adjustments if you are using different equipment to obtain appropriate resolution and retention (5.16.3). These adjustments might comprise, but are not limited to the following: injector program, injection volumes, percentage of organic modifier in isocratic or gradient mode, the flow rate and/or the column temperature.

7.1.2 Pre-column derivatisation

Using the equipment outlined in 5.16, the following conditions have shown to produce satisfying results:

a) Auto sampler injector program:

   1) aspire 20 µl pre-column reaction buffer (4.19.2);

   2) aspire 40 µl test solution (6.4);

   3) aspire 20 µl pre-column reaction buffer (4.19.2);

   4) mix 20 times, and

   5) inject all.

The above can be done manually (adjusting the total volume while maintaining the relative volumes if necessary) if it is ascertained that the solution is injected within 3 minutes after mixing. It is also important that the time period between mixing and injecting is the same for all test and calibration solutions.

b) Injection volume: 80 µl;

c) Column temperature: 40ºC;

d) Flow: 1,0 ml/ min;

e) Fluorescence detector: Excitation λ: 335 nm; Emission λ: 440 nm (it should be checked if these are local maxima for the fluorescence detector in use);

f) Mobile phase:

   1) A: 0,5% formic acid (4.14) in water (4.1);

   2) B: 0,5% formic acid (4.14) in methanol (4.2).

g) Gradient settings (HPLC dwell volume 0,8 ml) see Table 2.

Table 2 — Gradient settings for the pre-column derivatisation

Time (min)	B (%)
0	69,5
14	79
14,01	100
17,01	100
17,02	69,5
20	69,5

Instruments with different dwell volume will need adjustment of the gradient to achieve the same separation as shown in Annex A. The aim should be an apparent capacity factor at elution for FB_1 of $k > 3$.

7.1.3 Post-column derivatisation

Instructions for self-assembled system (5.16.5):

The flow path to the chromatographic column (5.16.3) is unchanged from normal operation. The outlet of the column is connected to one of the outside ports of a mixing Tee (see 5.16.5). The tubing from column to mixing Tee should be as short as possible.

The other outside port of the mixing Tee is connected to the outlet of a pump (see 5.16.5) delivering the reagent flow. This connection should be made of a long piece of 0,005" ID PEEK tubing (see 5.16.5) so that a sufficient back pressure is created for the reagent pump to work properly. It is of utmost importance that the reagent flow is delivered pulsation-free. A slight pulsation can be minimized be introducing a large damping volume between the pump and the back pressure creating PEEK tubing. Large ID PEEK tubing can serve this purpose.

The remaining centre port of the mixing Tee is connected through a reagent loop to the fluorescence detector. The length, and therefore the volume, of this reagent loop is a balance between retaining the resolution of the chromatographic column (short) and achieving complete reaction (long). The internal diameter is of lesser importance. If chosen too small excessive back pressure will be created. Satisfying results were achieved with a 2,5 m length of 0,02" ID PEEK tubing.

Using the equipment outlined in 5.16, the following conditions have shown to produce satisfying results:

a) Injection volume: 50 µl

b) Column temperature: 45°C

c) Flow : 1,2 ml/ min (mobile phase); 0,45 ml/min (post-column reagent (4.19.1))

d) Fluorescence detector: Excitation λ: 335 nm; Emission λ: 440 nm (it should be checked if these are local maxima for the fluorescence detector in use).

e) Mobile phase:

1) A: 0,1% formic acid (4.14) in water (4.1);

2) B: 0,1% formic acid (4.14) in acetonitrile (4.3).

Gradient settings: see Table 3.

Table 3 — Gradient settings for the post column-derivatisation

Time (min)	B (%)
0	34
13	34
13,01	95
16	95
16,01	34
19	34

This separation is isocratic but to avoid accumulation of matrix components a step-up to 95% B is included. The percentage of organic modifier should be adjusted such that the capacity factor for FB1 will be $k > 2$.

7.2 Determination of fumonisins in test solutions

Inject aliquots of the test solutions (6.4) into the chromatograph using the same conditions as used for the calibration solutions (4.22).

7.3 Batch (Sequence) composition

Always start a batch of measurements with a reagent blank to check the system. Subsequently inject the calibration solutions. Before the injection of the first test solution the reagent blank should be injected to prove that there is no carry-over of analytes. The test solutions should be run in duplicate and reruns of the calibration solutions should be interspersed in regular intervals. The frequency of these calibration reruns depend on the stability of your chromatographic system.

7.4 Calibration

Plot the signals (peak area or height) of all the measured calibration solutions against the corresponding concentrations for FB_1 and, separately, for FB_2. Do not use means of the multiple injections. With linear regression estimate slope and intercept of each of the two calibration functions (FB_1 & FB_2). Check for significance of the intercept and for linearity (use e.g. a residuals vs. fitted-values plot).

7.5 Peak identification

Identify the Fumonisin B_1 & B_2 peaks in the test solution by comparing the retention times with those of the closest calibration solution in the batch. The signal (peak area or height) of FB_1 or FB_2 in the test solution must fall within the calibration range. If the FB_1 and/or FB_2 signal in the test solution exceeds the signals of the

highest calibration solution the test solution shall be diluted with diluent (4.17) to bring it within calibration range, and be reanalysed. The dilution factor must be incorporated into all subsequent calculations.

8 Determination of concentrations

Using the estimated slopes and intercepts (if significant, otherwise use zero) from linear regression (7.4) calculate the concentrations of FB_1 ($c_{T(FB1)}$) and FB_2 ($c_{T(FB2)}$) in the test solutions (6.4) from the mean signal of the duplicate injections as follows:

$$c_{T(FB1)} = \frac{\text{signal}_{FB1} - \text{intercept}_{FB1}}{\text{slope}_{FB1}} \text{ (ng/ml)} \quad (1)$$

$$c_{T(FB2)} = \frac{\overline{\text{signal}}_{FB2} - \text{intercept}_{FB2}}{\text{slope}_{FB2}} \quad (2)$$

If the test solution was diluted because of a signal above the calibration range (7.5) multiply the calculated concentrations of FB_1 ($c_{T(FB1)}$) and FB_2 ($c_{T(FB2)}$) with the dilution factor.

To calculate the mass fractions (w_{SMP}) of the analytes in the original materials use the following equation:

$$w_{SMP} = \frac{c_T \times V_5 \times V_3 \times V_1}{V_4 \times V_2 \times m_{SMP}} \text{ (ng/g or µg/kg)} \quad (3)$$

where

c_T is the calculated concentration (ng/ml) of either FB_1 (1) or FB_2 (2), possibly corrected for dilution;

m_{SMP} is the weight (g) of the test material used for extraction (20,0 g);

V_1 is the total volume (ml) of the extraction solvent (200,0 ml);

V_2 is the volume (ml) of the aliquot of the filtered raw extract used for dilution (5,0 ml);

V_3 is the total volume (ml) of the diluted filtered raw extract (50,0 ml);

V_4 is the volume (ml) of the aliquot of the diluted filtered raw extract applied to the IAC (25,0 ml);

V_5 is the total volume (ml) of the test solution (5,0 ml).

If the weight of the test material and the volumes described herein before are kept the above Equation (3) can be simplified to:

$$w_{SMP} = c_T \times 20 \text{ (µg/kg)} \quad (4)$$

Should the result of Equation (4) be larger than 10 000 µg/kg or if it is known beforehand that the contamination level might exceed that value clean-up the respective diluted filtered extract (see 6.1) using an additional dilution (additional dilution factor 50/10 = 5). The simplified equation will then be:

$$w_{SMP} = c_T \times 20 \times 5 = c_T \times 100 \text{ (µg/kg)} \quad (5)$$

Carry out the above calculations for FB_1 and FB_2. The sum of both will then be calculated as follows:

$$w_{SMP} = w_{SMP,FB1} + w_{SMP,FB2} \text{ (µg/kg)} \quad (6)$$

9 Precision

9.1 Interlaboratory study

Details of an interlaboratory study on the precision of the method are shown in [3]. The values derived from this interlaboratory study may not be applicable to concentration ranges and/or matrices other than those given.

9.2 Repeatability

The absolute difference between two single test results found on identical test materials by one operator using the same apparatus within the shortest feasible time interval will exceed the repeatability limit *r* in not more than 5% of the cases.

Table 4 — Precision data repeatability

$\overline{x}$ = 3 110 µg/kg	*r* = 229 µg/kg
$\overline{x}$ = 5 000 µg/kg	*r* = 282 µg/kg
$\overline{x}$ = 5 600 µg/kg	*r* = 826 µg/kg
$\overline{x}$ = 8 200 µg/kg	*r* = 809 µg/kg
$\overline{x}$ = 16 000 µg/kg	*r* = 1 120 µg/kg

The relationship between *r* and x can be sufficiently approximated by the following function:

$$r = 2.8 \times 0.031 \times \overline{x} \quad (7)$$

meaning that the relative repeatability standard deviation is constant over the working range.

9.3 Reproducibility

The absolute difference between two single test results on identical test materials reported by two laboratories will exceed the reproducibility limit *R* in not more than 5% of the cases.

Table 5 — Precision data reproducibility

$\overline{x}$ = 3 110 µg/kg	R = 1 650 µg/kg
$\overline{x}$ = 5 000 µg/kg	R = 2 140 µg/kg
$\overline{x}$ = 5 600 µg/kg	R = 2 420 µg/kg
$\overline{x}$ = 8 200 µg/kg	R = 3 450 µg/kg
$\overline{x}$ = 16 000 µg/kg	R = 8 980 µg/kg

The relationship between R and x can be sufficiently approximated by the following function:

$$R = 2.8 \times 0.18 \times \overline{x} \tag{8}$$

meaning that the relative reproducibility standard deviation is constant over the working range.

10 Test report

The test report shall contain the following data:

a) information necessary for the identification of the sample (kind of sample, origin of sample, designation);

b) a reference to this European Standard;

c) the date and type of sampling procedure (if known);

d) the date of receipt;

e) the date of test;

f) the test results and the units in which they have been expressed;

g) whether the repeatability has been verified;

h) particular points observed in the course of the test;

i) operations not specified in the method or regarded as optional, which might have affected the results.

Annex A
(informative)

Precision data

The following data were obtained in an interlaboratory study [3] according to AOAC Guidelines for collaborative study procedures to validate characteristics of a method of analysis [2].

Table A.1 — Precision data

Sample	**Animal feed**	**Animal feed**	**Animal feed**	**Animal feed**	**Animal feed**
Year of inter-laboratory study	2008	2008	2008	2008	2008
Number of laboratories	16	16	16	16	16
Number of outliers (laboratories)	0	0	0	0	0
Number of non-compliant laboratories	4	4	4	4	4
Number of accepted results	12	12	12	12	12
Mean value $\overline{x}$ (µg/kg)	3 110	5 000	5 600	8 200	16 000
Repeatability standard deviation s_r (µg/kg)	81,9	101	295	289	398
Repeatability relative standard deviation RSD_r (%)	2,6	2,0	5,3	3,5	2,5
Repeatability limit r [a] (µg/kg)	229	282	826	809	1 120
Reproducibility standard deviation s_R (µg/kg)	589	766	863	1 230	3 210
Reproducibility relative standard deviation RSD_R (%)	19	15	15	15	20
$HORRAT_R$	1,4	1,2	1,3	1,3	1,9
Reproducibility limit R [b] (µg/kg)	1 650	2 140	2 420	3 450	8 980
Recovery (%)	n.a [c]	69	n.a. [c]	79	n.a. [c]

[a] – $r = 2{,}8 \times s_r$
[b] – $R = 2{,}8 \times s_R$
[c] – not applicable

Table A.2 — Material composition

Test Material	Ingredient	Parts	Principal components
Blank	Rabbit feed	4	cereals, seeds, crop by-products, vegetables, minerals
	Horse feed	5	oat, barley flakes, flour pellets, corn flakes, pea flakes, fibres, oil, molasses
	Pig feed	5	peas, roasted soy, wheat, barley, tapioca, cabbage seeds, animal grease, corn, salt
Level B	Blank	4	See "Blank".
	Maize	1	Naturally contaminated Maize
Level C	Blank	2	See "Blank".
	Maize	1	Naturally contaminated Maize
Level SH	Maize	1	Naturally contaminated Maize

The following table lists the precision data computed after correcting the reported values of each laboratory with the apparent mean recovery of the respective laboratory. The table has been added purely for informative purposes.

Table A.3 — Precision data after recovery correction

Sample	Animal feed	Animal feed	Animal feed	Animal feed	Animal feed
Year of inter-laboratory study	2008	2008	2008	2008	2008
Number of laboratories	16	16	16	16	16
Number of outliers (laboratories)	0	0	0	0	0
Number of non-compliant laboratories	4	4	4	4	4
Number of accepted results	12	12	12	12	12
Mean value x (µg/kg)	4 160	6 760	7 610	11 100	21 700
Repeatability standard deviation s_r (µg/kg)	113	148	389	397	543
Repeatability relative standard deviation RSD_r (%)	2,7	2,2	5,1	3,6	2,5
Repeatability limit r [a] (µg/kg)	315	413	1 090	1 110	1 520
Reproducibility standard deviation s_R (µg/kg)	814	816	1 670	1 740	5 560
Reproducibility relative standard deviation RSD_R (%)	20	12	22	16	26
$HORRAT_R$	1,5	1,0	1,9	1,4	1,5
Reproducibility limit R [b] (µg/kg)	2 280	2 290	4 660	4 880	15 600
Recovery (%)	n.a. [c]	100	n.a. [c]	100	n.a. [c]

[a] – $r = 2{,}8 \times s_r$
[b] – $R = 2{,}8 \times s_R$
[c] – not applicable

Annex B
(informative)

Examples of a chromatogram

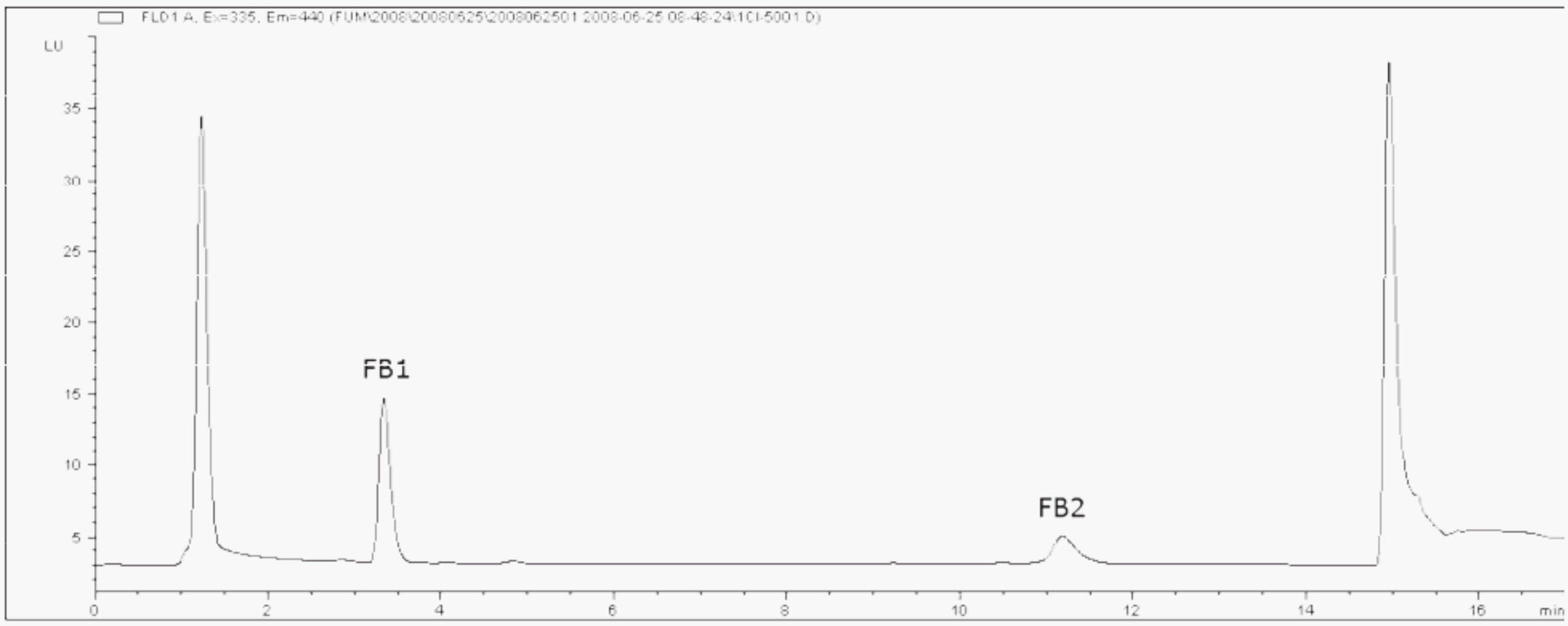

Figure B.1 — Example chromatogram with post-column derivatization and the conditions described herein before

Animal feed blank material spiked at a level of ca. 5 mg/kg for the sum of FB_1 & FB_2. IAC eluate was dried down and reconstituted with 0,5 ml mobile phase.

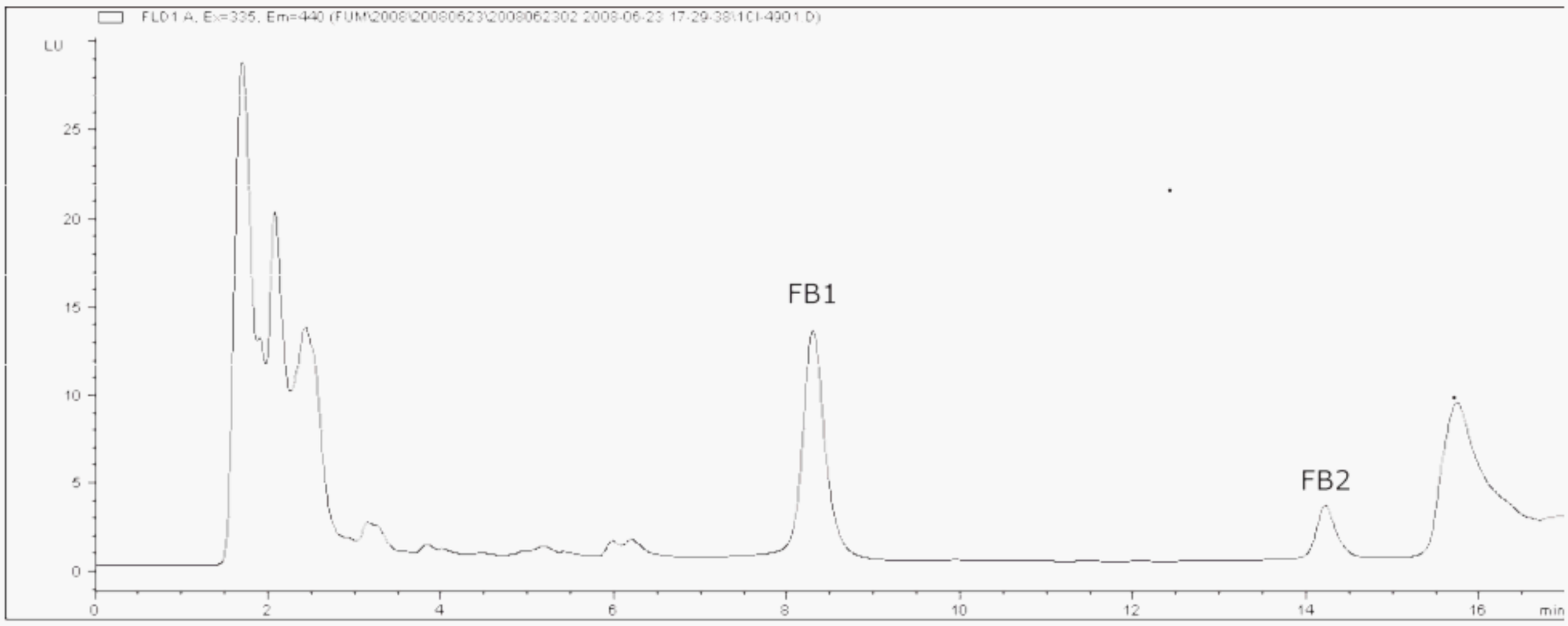

Figure B.2 — Example chromatograms with pre-column derivatisation and the conditions described herein before

Animal feed blank material spiked at a level of ca. 5 mg/kg for the sum of FB_1 & FB_2. IAC eluate was dried down and reconstituted with 0,5 ml mobile phase.

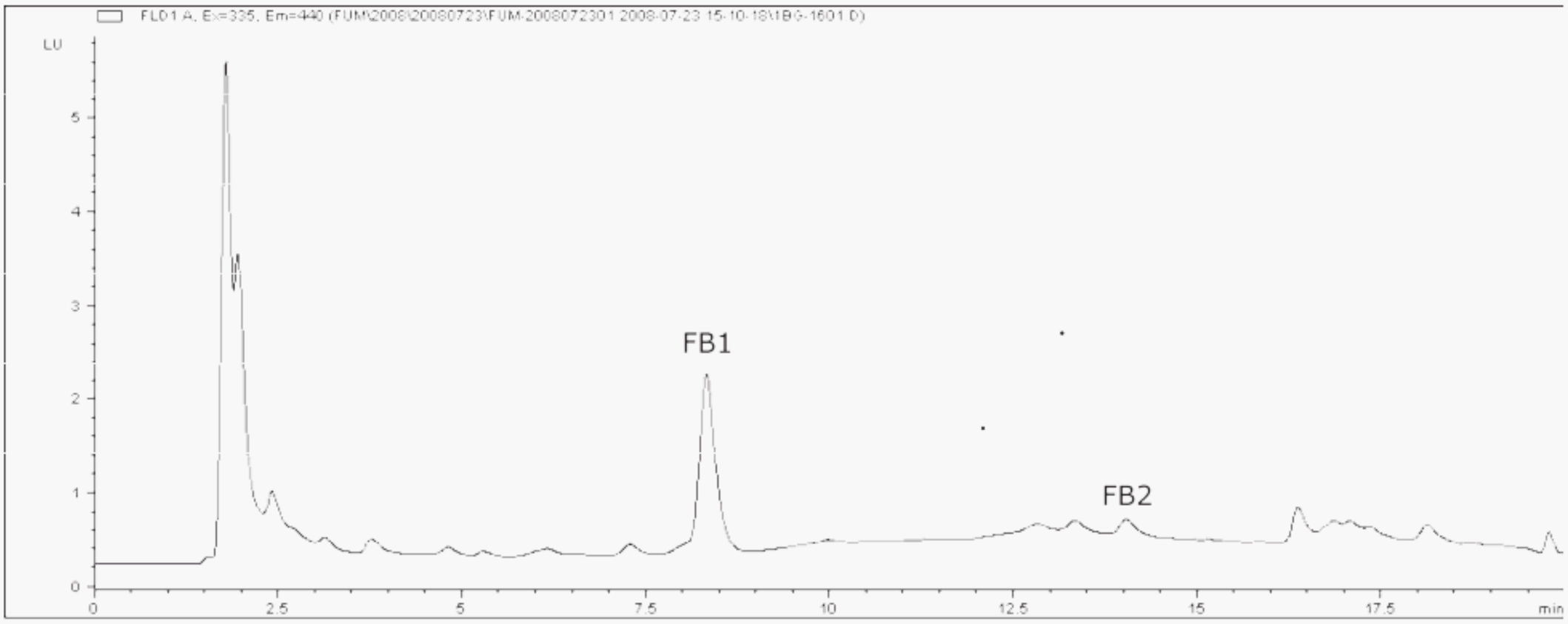

Figure B.3 Example chromatograms with pre-column derivatisation and the conditions described herein before

Animal feed material naturally contaminated at a level of ca. 6 mg/kg for the sum of FB_1 & FB_2. IAC eluate was not dried down.

Bibliography

[1] *Commission Regulation (EC) No 152/2009 of 27 January 2009 laying down the methods of sampling and analysis for the official control of feed, Official Journal of the European Union, 26.2.2009, L54, p. 1-130.*

[2] *AOAC International 1995, AOAC Official Methods Program, p. 23-51*

[3] *REPORT OF THE FOLLOW-UP COLLABORATIVE STUDY Determination of the sm of Fumonisin B1 and B2 in Compound Animal Feed and Maize by Immunoaffinity Column Clean-up and High Performance Liquid Chromatography with Fluorometric Detection, Breidbach, A., Bouten, K., Kroeger, K., Stroka,J.. European Commission - Scientific and Technical research series, EUR 23941 EN, ISSN 1018-5593. http://publications.jrc.ec.europa.eu/repository/handle/111111111/11727*

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