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Validation of an Analytical Method to Determine the Content of Fumonisins in Baby Food, Breakfast Cereals and Animal Feed

Report on the Collaborative Trial "Determination of Fumonisins B_1 and B_2 in Baby Food, Breakfast Cereals and Animal Feed by Immunoaffinity Column Clean-up with High Performance Liquid Chromatography and Fluorimetric Detection"

J. Stroka, A. Breidbach, K. Bouten, K. Kroeger, C. Mischke

EUR 24501 EN - 2010

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JRC 59960

EUR 24501 EN
ISBN 978-92-79-16493-4
ISSN 1018-5593
DOI 10.2787/29057

Luxembourg: Publications Office of the European Union

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VALIDATION OF AN ANALYTICAL METHOD TO DETERMINE THE CONTENT OF FUMONISINS IN BABY FOOD, BREAKFAST CEREALS AND ANIMAL FEED

REPORT ON THE COLLABORATIVE TRIAL

Determination of Fumonisins B_1 and B_2 in Baby Food, Breakfast Cereals and Animal Feed by Immunoaffinity Column Clean-up with High Performance Liquid Chromatography and Fluorimetric Detection

Collaborative Study

ADMINISTRATIVE ARRANGEMENT
No B5-1000/02/000566
BETWEEN DG Health and Consumer Protection (DG SANCO)
AND
THE JOINT RESEARCH CENTRE (JRC)

J. Stroka A. Breidbach, K. Bouten, K. Kroeger, C. Mischke

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Abstract

An inter-laboratory comparison was carried out to evaluate the effectiveness of a method based on immunoaffinity column clean-up followed by derivatisation and high performance liquid chromatography with fluorimetric quantification (HPLC-FL). The method was tested for the determination of Fumonisins B_1 and B_2 (FB_1 & FB_2) in baby food, breakfast cereals and animal feed to monitor compliance with limits according to Regulation 1881/2006/EC and Recommendation 576/2006/EC. The test portion of the sample was extracted with methanol:water. The sample extract was filtered, diluted, passed over an immunoaffinity column for clean-up and evaporated. The redissolved and purified eluate was separated and determined by reverse-phase high performance liquid chromatography (HPLC) and fluorescence detection after the fumonisins had been derivatised to their fluorescent isoindols in the presence of o-phthaldehyde and a thiol-coupling reagent with either pre- or post-column derivatisation.

Baby food, breakfast cereal and animal feed samples, both blank and naturally contaminated with FB_1 and FB_2, were sent to 40 laboratories from 19 EU Member States, and a laboratory in Uruguay. For recovery determination extra test portions of the blank samples were to be spiked by the participants at levels of 135 µg/kg for the sum of FB_1 and FB_2 in baby food, 400 µg/kg in breakfast cereals, and 3700 µg/kg in animal feed. All samples were sent as blinded duplicates.

Mean recoveries were calculated as 71 % for baby food, and 87 % for breakfast cereals. Based on results for the spiked and naturally contaminated samples the relative standard deviations for reproducibility (RSD_R) in baby food were 31 % at a spiked level of 135 µg/kg, 44 % at a natural contamination level of 267 µg/kg, and 33 % at a natural contamination level of 501 µg/kg. For breakfast cereal these figures were 15 % at a spiked level of 400 µg/kg, and 33 % at a natural contamination level of 1034 µg/kg. The values for RSD_r in those materials ranged from 5 to 29 % in baby food and 12 to 14 % in breakfast cereal.

For animal feed the recovery was 68 % and RSD_R values were 84 % at a spiked level of 3700 µg/kg, and for naturally contaminated samples 68 % at 2730 µg/kg, 88 % at 3695 µg/kg, and 49 % at 10037 µg/kg. The values for RSD_r values ranged from 6 to 49 %.

European Commission Regulation 401/2006/EC lays down performance criteria that must be met by a method to determine fumonisin FB_1 and FB_2 in food. These criteria have been met by this method for the baby food and the breakfast cereal, whereas for animal feed the determined performance criteria failed to comply with legal requirements.

Introduction

The accurate determination of mycotoxins in food and feed matrices for which EU legislative limits apply, require robust and reliable analytical techniques. Previous collaborative study projects with other mycotoxins (1, 2, 3, 4, 5) have shown that it is possible to achieve fit-for-purpose performance characteristics, in particular low limits of detection, provided suitable methodology is available. Due to the complexity of food and feed matrices, particular care has to be taken during test material preparation (blending of relevant matrix constituents and extensive homogenisation) and in demonstrating inter-unit homogeneity before undertaking the study.

Methods for fumonisins have been subject to method validation studies in the past and the methodologies used involved either solid-phase extraction or immunoaffinity clean-up to purify the sample extracts, followed by a chromatographic separation and the transformation into fluorescent derivatives prior detection. The method commonly used is based on pre-column derivatiatisation with o-phthaldehyde as this procedure does not require any additional equipment or difficult to handle reagents, while the chromatographic separation of the fumonisin derivatives is thought to be no challenge. One disadvantage of pre-column derivatisation is however that the formed derivatives are not stable and thus the analysis of longer sequences of samples requires either strict time programmed procedures, manual injections, or special derivatisation techniques, such as on-column derivatisation.

An alternative to pre-column derivatisation is post column derivatisation, which forms equivalent derivatives, but can easily run larger sequences of samples. Post column derivatisation requires some additional equipment (mainly one auxiliary pump) and has also been described in the literature.

During method development at IRMM it was found that both methods offer their own advantages and can in most cases equally compare with respect to the requirements by EU legislation for method performance and working range.

Test materials for the collaborative study

For this inter-laboratory comparison exercise the following products were purchased from local food supermarkets/animal feed warehouses: various brands of cereal based baby food, breakfast cereals, various kinds of animal feed (e.g. pig and cattle feed) and maize. Naturally contaminated processed breakfast cereals from pilot experiments (extrudates) have been kindly provided by Keith Scudamore (KAS MYCOTOXINS, United Kingdom) and Dr. Robin Guy (CCFRA, United Kingdom).

For the collaborative trial, powdered baby food and animal feed was produced by the Institute for Reference Materials and Measurements, Geel (IRMM), which belongs to the European Commission's Joint Research Centre, from the materials listed below. In the case of animal feed several different types of feed were blended, after they were confirmed to be free of Fumonisins (<50 µg/kg). For the Fumonisin-free baby food, all commercial baby food samples tested were found to be free from Fumonisins (<20 µg/kg). Therefore a mixture of the different baby food powders was prepared.

To obtain naturally contaminated baby food test materials, mixes of the blank baby food, with a contaminated corn extrudate were blended to achieve the desired levels. In the same manner contaminated breakfast cereals were prepared. For animal feed test materials, blank animal feed ingredients were blended with contaminated maize to achieve the desired levels. The composition of the test materials is given in Table 1 to Table 3.

Table 1: Composition of baby food test materials

Test Material	Ingredient	Amount (kg)	Composition[1]
blank	Baby Bircher-Müesli	2 kg	oat flakes, wholemeal wheat flour, 21 % dried fruits (banana & apple), wholemeal rye flour, sorghum flakes, wholemeal barley flour, vitamins
	Corn flakes	2 kg	91% corn, sugar, salt, malt of barley, vitamins
	Honey Loops	3 kg	57 % wholemeal flour of several crops (oat, wheat, barley, rye), 4 % sugar and honey, vitamins, iron, colorants
	Corn flakes	3 kg	98 % corn, sugar, malt of barley, vitamins, iron, salt
	Cereal based Baby Food	4 kg	wheat, oat, rice, sorghum, barley, corn, rye, vitamin B1
Level 1	blank	Ratio to achieve target level of 200 µg/kg	See "blank"
	Corn flakes		Extruded maize product from pilot plant with 2000 µg/kg Fumonisins
Level 2	blank	Ratio to achieve target level of 300 µg/kg	See "blank"
	Corn pellets		Extruded maize product from pilot plant with 2000 µg/kg Fumonisins
Level 3	blank	Ratio to achieve target level of 600 µg/kg	See "blank"
	Corn pellets		Extruded maize product from pilot plant with 2000 µg/kg Fumonisins

[1] In decreasing amounts.

Table 2: Composition of breakfast cereal test materials

Test Material	Ingredient[2]	Amount (kg)	Composition[3]
blank	Corn flakes	10 kg	91% corn, sugar, salt, malt of barley, vitamins
Level 1	blank	Ratio to achieve target level of 500 µg/kg	See "blank"
	Corn pellets		Extruded maize product from pilot plant with 2000 µg/kg Fumonisins
Level 2	blank	Ratio to achieve target level of 800 µg/kg	See "blank"
	Corn pellets		Extruded maize product from pilot plant with 2000 µg/kg Fumonisins
Level 3	blank	Ratio to achieve target level of 1200 µg/kg	See "blank"
	Corn pellets		Extruded maize product from pilot plant with 2000 µg/kg Fumonisins

[2] Materials for the blank were all free of fumonisins.
[3] In decreasing amounts.

Table 3: Composition of animal feed test materials

Test Material	Ingredient[4]	Amount (kg)	Composition[5]
blank	Rabbit feed	4 kg of	cereals, seeds, crop by-products, vegetables, minerals
	Horse feed	5 kg of	oat, barley flakes, flour pellets, corn flakes, pea flakes, fibres, oil, molasses
	Pig feed	5 kg of	peas, roasted soy, wheat, barley, tapioca, cabbage seeds, animal grease, corn, salt
Level 1	blank	Ratio to achieve target level of 600 µg/kg	See blank
	Maize		Maize with a Fumonisin content (FB_1 & FB_2) of ~4500 µg/kg
Level 2	blank	Ratio to achieve target level of 1200 µg/kg	See "blank"
	Maize		See "level 4"
Level 3	blank	Ratio to achieve target level of 5 mg/kg	See "blank"
	Maize		See "level 4"
Level 4	Maize	-	Maize with a Fumonisin content (FB_1 & FB_2) of ~12 mg/kg

Whole grains were first milled with a Romer RAS® mill prior to blending. All other materials were blended directly in a modified concrete mixer for 30 minutes. After blending the whole lot was milled with a Retsch centrifugal mill (Model ZM 100) with a sieve of 3 mm. This milled material was thereafter again mixed in the concrete mixer for 30 minutes and milled, for a second time, down to a particle size of < 1 mm in the centrifugal mill. Finally, the twice mixed and milled material was mixed again in the concrete mixer for 2 – 3 hours and then milled a final time to achieve a particle size of < 0.5 mm. The approx. 30 g test material were subsequently filled into 50 mL containers (and stored frozen at –18° C. The filled containers were kept at this temperature until analysis for homogeneity or dispatch for collaborative trial testing.

In order to achieve a reasonable significance of the collaborative trial, while not exceeding a just and reasonable workload (number of samples analysed in duplicates) for the participants, especially with respect to the reporting time, not all produced materials were included in the collaborative trail. The selection of test materials and

[4] Materials for the blank were all free of fumonisins.

levels was made in a way that still allowed the validation of all three matrices (foods for infants and young children "baby food", breakfast cereals and animal feed) with the minimum amount of test samples to be analysed by each participant.

[5] In decreasing amounts.

Homogeneity of the Test Materials and In-House Method Performance

According to generally accepted procedures for homogeneity testing, every 10^{th} sample had been taken from the sequence during packing. These selected test materials were analysed for the content of FB1 and FB2 with HPLC and post column derivatisation prior fluorimetric quantification.

The number of the first container from which the sampling started was randomly selected for each material. Sample were split and analysed either by Rijksinstituut voor Volksgezondheid en Milieu, Bilthoven (RIVM) and IRMM for homogeneity. The obtained data were subjected to one-way analysis of variance (ANOVA). The outcome of the statistical evaluation showed that in all cases the bottled test material was sufficiently homogeneous.

Organisation of the collaborative study

The instructions for participants in the inter-laboratory comparison are given in the Annex of this report. The pool of interested participants for this study grew to an extent, that a single collaborative trial including all participants and covering all three matrices (animal feed, breakfast cereal, and baby food) would have resulted in an unnecessary large number of analyses and data. As a result the pool of participants was split into two groups, analysing either baby food or animal feed for the trial.
In addition, not all laboratories reported to be able to use post column derivatisation, which was identified at IRMM to be the method of choice, and a fraction of participants were free to chose between the post or a pre-column derivatisation step.

A total of 41 collaborators from 20 different countries were invited to participate in the collaborative trial. These collaborators represented a cross-section of government, food control, university and food industry affiliations. The names and addresses of the participants that eventually took part in the trial are given in Table 4.

Table 4: List of participants in the inter-laboratory comparison exercise that returned results for the determination of Fumonisins.

Participant	Institution
M.-P.Herry	Laboratoire du Ministère de l'Economie, des Finances et de l'Industrie (MINEFI) de Rennes
A.Solyakov	(SVA) The National Vetinary Institute
I.Bujara	SGS,Consumer Testing Services,
H.Wisniewska-Dmytrow	National Veterinary Research Institute
Y.Vojsova	State Veterinary and Food Institute Bratislava
J.Rosmus	State Veterinary Institute Prague
S. Friis-Wandall; A. Iversen	The Danish Plant Directorate
J.Petrová	ÚKZÚZ Praha
J.Vancutsem	Federaal Agentschap voor de Veiligheid van de Voedselketen (FAVV)
B.Brand	Staatliches Veterinäruntersuchungsamt Arnsberg
J.Dömsödi	National Institute for Agricultural Quality Control
M.Bartosik	ARVALIS - Institut du végétal, Station Expérimentale
M. Clinckspoor T. Hamoir	ERC NV Environmental Research Center
P. Cuhra	Czech Agriculture and Food Inspection Authority
R. Le Bouquin	LAREAL -Laboratoire de Recherche Alimentaire-Talhouët
J. Postupolski	National Institute of Hygiene;Department of Food Research
S. Tkacikova	State Veterinary and Food Institute Košice
K. van Schalm	MasterLab BV
U. Lauber	Chemisches und Veterinäruntersuchungsamt
K. Nuotio	Tullilaboratorio - Finnish Customs Laboratory
M. Solfrizzo	CNR Institute of Sciences of Food production
C. Brera	Istituto Superiore di Sanità, Centro Nazionale per la Qualità degli Alimenti e per I Rischi Alimentari
P. A. Burdaspal	Centro Nacional de Alimentación Agencia Española de Seguridad Alimentaria
J.-Y. Michelet	Scientific Institute of Public Health - Louis Pasteur – Section Foodstuffs
S. Patel	RHM Technology Premier Foods
P. González	SILLIKER Ibérica
A. Raditschnig	AGES Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH
H. Klaffke	Bundesinstitut für Risikobewertung BfR
U. Meister	IGV Institut für Getreideverarbeitung GmbH
J. Danier	Technische Universität München (TUM), Zentralinstitut für Ernährungs- und Lebensmittelforschung (ZIEL),
H. Thiele	Hessisches Landeslabor
J. Keegan	Public Analyst's Laboratory
D. Theodosis	Food Chemistry LGC Limited
A. M. Thim	National Food Administration Research and Development Department
G. Tavčar Kalcher	University of Ljubljana, Veterinary Faculty, National Veterinary Institute,
S. Guffogg	Lincolne, Sutton & Wood Ltd.
M.-E. Esteves	Laboratorio Central de Qualidade Alimentar
J. Melo	DIN SA
E. Ioannou-Kakouri	State General Laboratory
T. de Rijk	RIKILT, Wageningen
J. Cea	Technological Laboratory of Uruguay

For the collaborative trial each participant received:

1. For food analysis ten coded sample containers (blind duplicates at five concentration levels) plus four ‘blank’- labelled ones (two for baby food and two for breakfast cereals) for spiking. For animal feed analysis eight coded sample containers (blind duplicates at four concentration levels) plus two ‘blank’- labelled ones for spiking.
2. One amber vial marked ‘Fumonisin calibrant’ containing fumonisins, which was to be employed as the calibrant solution, as described in the method.
3. For food analysis four vials for spiking experiments. For animal feed analysis two vials for spiking experiments.
4. For food analysis eighteen and for feed analysis fourteen immunoaffinity columns for fumonisins.
5. A copy of the collaborative study method.
6. A copy of the spiking protocol.
7. Chromatograms of analysed materials containing fumonisin.
8. A ‘Collaborative Study Materials Receipt’ form.
9. Report forms.
10. A results questionnaire.

Each participant was required to prepare one extract from each container and perform the analysis by HPLC. Additionally each participant was required to spike materials indicated as ‘Blank’ using the provided ‘Spike Solutions’.

Method of analysis

The method of analysis that was used in this study can be found in the Annex.

Statistical analysis of results

In some cases data were excluded from the statistical analysis because of non-compliances. This was the case for laboratories which did not follow critical areas of the protocol or which reported no or only one result for a pair of blind duplicates.

Precision estimates were obtained using a one-way ANOVA approach according to the IUPAC Harmonised Protocol (6, 7). Details of the average analyte concentration, the standard deviations for repeatability (RSD_r) and reproducibility (RSD_R), the number of statistical outlier laboratories, the HORRAT ratio and the percentage recovery are presented in Tables 6, 8, and 10. The collaborative trial results were also examined using Cochran's and Grubbs' tests ($p<0.025$) for evidence of outliers (6). Pairs of results that were identified as outliers are indicated with shaded background in Tables 5, 7, and 9.

Results and Discussion

Baby food:

Table 5 lists the summed-up mass fractions of the reported values for FB_1 & FB_2 toxins by laboratory, each row representing one laboratory identified by a code, and the columns representing the different materials. Cells show mass fractions as reported, where no value was reported the cell is empty. Cells shaded in gray indicates exclusion from the statistical evaluation, light gray for non-compliance, dark gray for being an outlying result (Grubbs and/or Cochran test).

Table 6 lists the performance parameters of the tested method for baby food. The mean of the reported results for the blank material indicates a contamination of 23.4 µg/kg for the sum of the two analytes. However, the associated relative reproducibility standard deviation of 128 % indicates that this value cannot be quantified with any confidence.

Since for recovery determination the blank material was spiked the reported values for the spike have been corrected for the individual values reported in the blank material. The resulting mean apparent recovery is then 96 µg/kg of 135 µg/kg, or 71 %, for baby food. The Horwitz ratios of 1.3 and 1.8 for the spiked and highly

contaminated materials, respectively, demonstrate acceptable performance. The recovery and the values for the relative standard deviations of repeatability and reproducibility are within the limits set in Commission Regulation 401/2006.

Table 5: Individual results of fumonisin (sum of FB1 and FB2) in baby food

Lab	Deriv[1]	Spike[2]		Blank		Medium[3]		High[3]	
201[4]	Pre	111.1	124.3	0.0	0.0	352.4	302.7	455.7	652.5
202	Pre	109.5	105.1	0.0	0.0	292.4	287.7	33.0	533.8
205	Pre	93.5	94.0	6.0	6.0	265.2	319.1	479.6	573.1
211	Pre	23.1	32.6	0.0	0.0	0.0	274.4	325.2	433.4
212	Pre	0.0	0.0	0.0	0.0	46.0	83.0	318.9	197.3
301	Post	258.1	190.9	74.1	82.8	312.2	301.8	177.0	487.3
302	Pre	144.8	137.2	0.0	0.0	347.4	325.6	630.0	627.6
303	Pre	122.2	117.3	36.1	34.5	66.6	339.2	583.5	601.5
304	Post					319.4	379.3		604.7
306	Post	0.0	0.0	0.0	0.0	340.0	308.2		647.1
307	Post	0.0	0.0	0.0	0.0	0.0	207.4	351.2	239.5
308	Post	31.1	48.9	45.2	22.1	112.5	206.0	335.7	426.8
309	Post	135.5	131.7	0.0	34.3	0.0	17.6	82.5	0.0
313	Pre	130.7	127.4	28.9	23.1	207.2	292.3	465.6	423.1
314	Post	114.4	114.4	10.7	4.4	313.2	334.7	569.5	551.0
316	Post	139.8	142.3	66.2	61.7	156.2	168.8	572.3	518.6
317	Post	147.5	153.4	68.6	58.5	496.1	467.4	829.8	796.7

[1] Pre- or post-column derivatization
[2] Fortified Material
[3] Naturally contaminated material
[4] Shading: light gray – non-compliant, dark gray – outlying result

Table 6: Performance parameters for the sum of FB_1 & FB_2 in baby food

Level	Mean	N	nc	outl	n	r	s_r	RSD_r	R	s_R	RSD_R	HoR
Blank	23.4	17	3	2	12	9.4	3.4	14	83.5	29.8	128	4.5
Medium	267.2	17	2	0	15	216.3	77.2	29	332.3	118.7	44	2.3
High	501.3	17	4	1	12	245.8	87.8	18	457.3	163.3	33	1.8
Recovery at 135 µg/kg	96.0	17	5	1	11	12.4	4.4	5	82.0	29.3	31	1.3

Legend: Mean –mean mass fraction [µg/kg]; N – number of labs; nc – non-compliant laboratories; outl. – outlying laboratories; n – number of laboratories used for statistics; r – repeatability [µg/kg], s_r – repeatability standard deviation [µg/kg], RSD_r – relative standard deviation under repeatability conditions [%]; R, s_R, RSD_R– the respective values for reproducibility, HoR_{mod} – the HorRat value for reproducibility

Breakfast cereals:

All the results for the cereal mix are listed in Table 7 for which the same conventions apply as for Table 5.

Table 7: Individual results of fumonisin in breakfast cereals

Lab	Deriv[1]	Spike[2]		Blank		High[3]	
201[4]	Pre	397.2	423.5	10.6	15.6	341.9	1259.1
202	Pre	370.9	391.2	0.0	0.0	1109.5	1074.8
205	Pre	380.7	295.6	6.0	6.0	1155.4	1282.2
211	Pre	340.2	200.2	0.0	0.0	1279.6	1240.6
212	Pre	59.4	125.4	0.0	0.0	731.1	589.6
301	Post	449.6	472.2	126.2	109.5	1461.9	1149.6
302	Pre	401.5	379.1	45.8	47.4	1086.6	1096.0
303	Pre	337.6	386.3	38.5	41.9	1297.1	1246.1
304	Post	324.7	398.1			1244.6	1074.6
306	Post	0.0	0.0	0.0	0.0	1275.9	1014.3
307	Post	219.6	0.0	0.0	0.0	580.4	490.0
308	Post	85.6	100.9	37.8	48.9	842.0	617.8
309	Post	0.0	68.1	60.6	6.9	98.0	455.9
313	Pre	398.5	314.7	24.4	17.0	850.0	1001.1
314	Post	369.4	369.2	14.6	2.1	780.6	947.4
316	Post	328.4	336.4	0.0	0.0	1000.1	1037.4
317	Post	440.5	557.2	125.3	66.2	1657.5	1558.4

[1] Pre- or post-column derivatization
[2] Fortified Material
[3] Naturally contaminated material
[4] Shading: light gray – non-compliant, dark gray – outlying result

Table 8: Performance parameters for the sum of FB_1 & FB_2 in the breakfast cereal

Level	Mean	N	nc	outl	n	r	s_r	RSD_r	R	s_R	RSD_R	HoR
Blank	16.2	17	3	3	11	11.5	4.1	25	52.9	18.9	117	3.9
High	1034.5	17	2	1	14	338.1	120.7	12	953.7	340.6	33	2.1
Recovery at 400 µg/kg	347.4	17	4	2	11	136.2	48.6	14	142.7	51.0	15	0.8

Legend: Mean –mean mass fraction [µg/kg]; N – number of labs; nc – non-compliant laboratories; outl. – outlying laboratories; n – number of laboratories used for statistics; r – repeatability [µg/kg], s_r – repeatability standard deviation [µg/kg], RSD_r – relative standard deviation under repeatability conditions [%]; R, s_R, RSD_R– the respective values for reproducibility, HoR– the HorRat value for reproducibility

Table 8 lists the performance parameters of the tested method for the breakfast cereal. Again, as for the baby food blank material, the mean of the reported results indicates a contamination of 16.2 µg/kg with an associated relative reproducibility standard deviation of 117 %. This means no quantification is possible at this contamination level. The mean recovery after correction for the blank results is 87 % (347.4 µg/kg of 400 µg/kg). The performance for the spiked materials shows a HoR of

0.8 and for the highly contaminated it is just above the limit of 2. The same is true with regards to the limits set in Commission Regulation 401/2006.

Animal feed

All the results for the animal feed mix are listed in Table 9 for which the same conventions apply as for Table 5.

Table 9: Individual results of Fumonisins in animal feed.

Lab	Deriv[1]	Spike[2]		Blank		Low[3]		Medium[3]		High[3]	
102[4]	Pre	3031	2898	78	76	2375	2378	4412	4503	16228	16311
103	Pre	4067	3759	572	825	3057	2954	1045	5651	16742	2921
104	Pre	5502	6866	0	0	4871	4883	9855	9866	35779	25175
105	Pre	828	491	82	87	1843	409	2288	3405	12342	5589
106	Pre	5544	4710	2158	7979	3526	3438	6839	7334	17799	17293
107	Pre	918	919	0	0	860	692	1449	1937	8516	7226
108	Pre	3231	733	0	0	569	674	1649	814	7495	14921
112	Pre	17	1862	13	19	4475	4969	2073	2091	6562	5593

[1] Pre- or post-column derivatization
[2] Fortified Material
[3] Naturally contaminated material
[4] Shading: light gray – non-compliant, dark gray – outlying result

Table 10 lists the performance parameters of the tested method for animal feed. The mean of the reported results for the blank material indicates a contamination of 29.7 µg/kg for the sum of the two analytes. However, as before the associated relative reproducibility standard deviation of 136 % indicates that this value cannot be quantified with acceptable reliability.

Since for recovery determination the blank material was spiked the reported values for the spike have been corrected for the values reported in the blank material. The resulting mean apparent recovery is 68 % (2508.6 µg/kg of 3700 µg/kg) for animal feed. The high Horwitz ratios for every level indicate insufficient performance.

Table 10: Performance parameters for the sum of FB_1 & FB_2 in the animal feed

Level	Mean	N	nc	outl	n	r	s_r	RSD_r	R	s_R	RSD_R	HoR
Blank	29.7	8	1	1	6	6.8	2.4	8	113.0	40.4	136	5.0
Low	2729.9	8	1	1	6	438.2	156.5	6	5178.7	1849.5	68	4.9
Medium	3695.1	8	1	1	6	1196.6	427.4	12	9068.5	3238.8	88	6.7
High	10037.1	8	1	1	6	13868.1	4952.9	49	13893.1	4961.8	49	4.4
Recovery at 3700 µg/kg	2508.6	8	1	0	7	2563.1	915.4	36	5927.7	2117.0	84	6.1

Legend: Mean –mean mass fraction [µg/kg]; N – number of labs; nc – non-compliant laboratories; outl. – outlying laboratories; n – number of laboratories used for statistics; r – repeatability [µg/kg], s_r – repeatability standard deviation [µg/kg], RSD_r – relative standard deviation under repeatability conditions [%]; R, s_R, RSD_R– the respective values for reproducibility, HoR – the HorRat value for reproducibility

Table 11: Method performance parameters obtained in the collaborative trials

Method	Matrix	Level[6] µg/kg	Obtained parameter			Acceptable performance[7]
			RSD_r %	RSD_R %	Recovery %	
HPLC-FL with post- and pre-column derivatisation	Baby food	23	14	128	-	NO
		267	29	44	-	YES
		501	18	33	-	YES
		96	5	31	71	YES
	Breakfast cereals	16	25	117	-	NO
		1034	12	33	-	NO
		347	14	15	87	YES
	Animal feed	30	8	136	-	NO
		2730	6	68	-	NO
		3695	12	88	-	NO
		10037	49	49	-	NO
		2509	36	84	68	NO

Materials for which no recovery data is given (marked with '–') were naturally contaminated.

[6] Mean level as reported in the collaborative trial.

[7] A qualification is considered to be granted, when the minimum performance parameters set in Regulation 2006/401/EC are obtained (this was also applied for feed).

Conclusions

The results of this inter-laboratory comparison show precision characteristics which fulfil the criteria (RSD_r, RSD_R and recovery) as given in Regulation 401/2006 (1) only for the baby food material and the spiked breakfast cereal. For the highly contaminated breakfast cereal the results of this collaborative trial do not comply with legal requirements (1). Taking into account the same method performance requirements for animal feed, all animal feed materials showed apparently unacceptable method performance.

The JRC will transform this method into CEN format and will submit it to CEN TC 275/WG 5 for discussion and possible adoption as EN standard.

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European Commission

EUR 24501 EN – Joint Research Centre – Institute for Reference Materials and Measurements
Title: Validation of an Analytical Method to Determine the Content of Fumonisins in Baby Food, Breakfast Cereals and Animal Feed
Authors: J. Stroka, A. Breidbach, K. Bouten, K. Kroeger, C. Mischke
Luxembourg: Publications Office of the European Union
2010 – 20 pp. – 21.0 x 29.7 cm
EUR – Scientific and Technical Research series – ISSN 1018-5593
ISBN 978-92-79-16493-4
DOI 10.2787/29057

Abstract
An inter-laboratory comparison was carried out to evaluate the effectiveness of a method based on immunoaffinity column clean-up followed by derivatisation and high performance liquid chromatography with fluorimetric quantification (HPLC-FL). The method was tested for the determination of Fumonisins B1 and B2 (FB1 & FB2) in baby food, breakfast cereals and animal feed to monitor compliance with limits according to Regulation 1881/2006/EC and Recommendation 576/2006/EC. The test portion of the sample was extracted with methanol:water. The sample extract was filtered, diluted, passed over an immunoaffinity column for clean-up and evaporated. The redissolved and purified eluate was separated and determined by reverse-phase high performance liquid chromatography (HPLC) and fluorescence detection after the fumonisins had been derivatised to their fluorescent isoindols in the presence of o-phthaldehyde and a thiol-coupling reagent with either pre- or postcolumn derivatisation.

Baby food, breakfast cereal and animal feed samples, both blank and naturally contaminated with FB1 and FB2, were sent to 40 laboratories from 19 EU Member States, and a laboratory in Uruguay. For recovery determination extra test portions of the blank samples were to be spiked by the participants at levels of 135 µg/kg for the sum of FB1 and FB2 in baby food, 400 µg/kg in breakfast cereals, and 3700 µg/kg in animal feed. All samples were sent as blinded duplicates.

Mean recoveries were calculated as 71 % for baby food, and 87 % for breakfast cereals. Based on results for the spiked and naturally contaminated samples the relative standard deviations for reproducibility (RSDR) in baby food were 31 % at a spiked level of 135 µg/kg, 44 % at a natural contamination level of 267 µg/kg, and 33 % at a natural contamination level of 501 µg/kg. For breakfast cereal these figures were 15 % at a spiked level of 400 µg/kg, and 33 % at a natural contamination level of 1034 µg/kg. The values for RSDr in those materials ranged from 5 to 29 % in baby food and 12 to 14 % in breakfast cereal.

For animal feed the recovery was 68 % and RSDR values were 84 % at a spiked level of 3700 µg/kg, and for naturally contaminated samples 68 % at 2730 µg/kg, 88 % at 3695 µg/kg, and 49 % at 10037 µg/kg. The values for RSDr values ranged from 6 to 49 %.

European Commission Regulation 401/2006/EC lays down performance criteria that must be met by a method to determine fumonisin FB1 and FB2 in food. These criteria have been met by this method for the baby food and the breakfast cereal, whereas for animal feed the determined performance criteria failed to comply with legal requirements.

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LA-NA-24501-EN-C