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Aflatoxin measurements: How did HPLC methods evolve in the last 20 years?

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Abstract

The Institute for Reference Materials and Measurements (IRMM) has provided high quality certified reference materials for decades. Certified reference materials for mycotoxin analysis have been available from the IRMM since 1985. Since then IRMM characterises, in general, reference materials by the means of interlaboratory studies with expert laboratories. Such measurement exercises are based on routinely used analytical procedures in the laboratory, but are driving the performance of them to the best achievable quality under the given circumstances. Reference materials for aflatoxin measurements have been certified by such studies in the years 1991, 1993, 2008 and 2009. After two decades it is time to look back, reflect and ask some questions. The questions are: How did HPLC methods for the analysis of aflatoxins evolve in the last 20 years? Is there a trend towards a preferred methodology? Does this evolution reflect improvements due to the introduction of standardised methods? Is there an improvement of performance parameters such as repeatability, reproducibility and measurement uncertainty? To answer those questions the data of six interlaboratory studies, performed in the last 20 years for the certification of aflatoxins in peanut meal, peanut butter and animal feed, have been re-evaluated. It is concluded that there is a trend towards a preferred methodology, which reflects developments in standardised methods. But no significant improvement in repeatability, reproducibility and measurement uncertainty, could be detected in expert laboratories for mycotoxin analysis.

Keywords: aflatoxins, peanut, groundnut, animal feed, certified reference material

Introduction

The discovery of aflatoxins and the identification of them as the causing agent of the turkey X diseases in the 1960s boosted mycotoxin research (Sargeant 1961, Nesbitt 1962). Aflatoxins have always stayed at the centre of attention due to their toxicity and prevalence. They occur in a wide variety of food and feed stuff of plant origin.

Many countries introduced legal limits for the aflatoxin content in food and feed to protect consumers and farm animals. These limits exist either for aflatoxin B₁ or for the sum of aflatoxin B₁, B₂, G₁ and G₂ and vary between commodities and their intended use. A survey undertaken by the Food and Agricultural Organisation of the United Nations (FAO) in 2002/2003 showed that the most frequent limits for aflatoxin B₁ in food are 2 and 5 µg/kg, respectively (Van Egmond 2004). The Rapid Alert System for Food and Feed (RASFF) of the European Union reported 902 notifications regarding aflatoxins in 2008, corresponding to around 30 % of all notifications of the RASFF system (European Commission 2009). This underlines the need for continuous monitoring of food and feed for contamination with aflatoxins. For the monitoring, accurate methods and appropriate tools for quality assurance are needed, including certified reference materials for calibration and internal as well as external quality control. Therefore the Institute for Reference Materials and Measurements (IRMM) has produced certified reference materials for mycotoxin analysis since 1985. Currently peanut butter, peanut meal and animal feed materials as well as calibrators are available for aflatoxin B₁, B₂, G₁ and G₂ analysis.

Recently published reviews (Reiter 2009, Shephard 2009) provide an overview of currently used strategies for sample clean-up and methods applied for aflatoxin analysis. They also mention briefly standardised methods. Currently there are several

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standardised methods published for the analysis of aflatoxins in various matrices by the International Organization for Standardization (ISO), the European Committee for Standardization (CEN) and by the AOAC International. Honma et. al. published a comparison of 10 proficiency tests performed between 1978 and 2003 based on the percentage of laboratories with a satisfactory results for various mycotoxins (Honman 2004).

This paper focuses on the development of the performance of HPLC methods over the last 20 years. Exemplarily, three interlaboratory certification studies for peanut meal, peanut butter and animal feed conducted nearly 20 years ago will be compared with certification studies for the same matrices performed in the last two years. The data of all six studies have been re-evaluated and the methodology used will be compared. The final goal was to detect trends in methodology and if possible a link to improvements of method performance.

Interlaboratory studies

The six studies selected are very similar in design. Only details regarding the parts of the studies concerning the aflatoxin B₁ analysis are given. Most of the studies also included the aflatoxins B₂, G₁ and G₂. Full details can be found in the corresponding certification reports (Buttinger 2008a, Buttinger 2008b, Buttinger 2009, Gilbert 1991, Van Egmond 1994). The measurement results of the studies in 1991 and 1993 are traceable to a published extinction coefficient of aflatoxin B₁, whereas the results of the studies in 2008 and 2009 are traceable to the SI via a certified calibrant (ERM-AC059). All final results were corrected by the respective recovery rate. Laboratories have reported individual results for all measurements.

Peanut butter 1991 (Gilbert et al., 1991)

The nine participating laboratories were provided with ampoules of the common calibrant in chloroform and two candidate materials, RM385 and RM401. The material RM401 was an intended blank material. The laboratories had to carry out the following measurements repeated, on each of three days: a three to four point calibration, a duplicate recovery experiment by spiking RM 401 at a level of 5 µg/kg aflatoxin B₁, a duplicate measurement of RM385.

The results of one laboratory were discarded as the variation of the recovery rate from day to day was too high (more than 10 % absolute). The methods used by the laboratories can be split into three groups. One group was using chloroform for extraction following a draft official method of the European Community for animal feed from 1991 (Van Egmond 1991) (4 laboratories) including a two step clean-up on SepPak[®] Florisil[®] and SepPak[®] C18 columns. The other group was using acetonitrile for extraction and immunoaffinity columns for clean-up (2 laboratories), whereas the third group was using methanolic extraction solutions (2 laboratories) with either silica gel column clean-up or no clean-up at all. All laboratories applied reversed phase HPLC with fluorescence detection for the final determination. All laboratories but one used post-column halogenisation for derivatisation. One laboratory performed derivatisation with trifluoroacetic acid. The achieved recovery rates in this study and the percentage of accepted results are summarized in Table 1.

Peanut butter 2008 (Buttinger et al 2008b)

Eight laboratories, with proven measurement capability and demonstrated experience in aflatoxin analysis, were provided with ampoules of a certified calibrant (ERM-

AC057) in acetonitrile, and two candidate materials, BCR-385R and BCR-401R. The material BCR-401R was an intended blank material. The laboratories had to carry out the following measurements repeated, on each of three days: a five point calibration, a triplicate recovery experiment by spiking BCR-401R at a level of 0.5 µg/kg aflatoxin B₁, a duplicate measurement of BCR-385R.

The results were subjected to technical evaluation and checked for compliance with performance criteria for recovery rate laid down in European Commission Regulation 401/2006 (European Commission 2006). Recovery rates at a level of 0.5 µg/kg are satisfactory between 50 % and 120 %. If a laboratory failed to fulfil that criterion on more than one day, all results of that laboratory were rejected. Otherwise only the results of the affected day were rejected. This led to the exclusion of the data from one day of two laboratories (one was below 50 %, one above 120 %). Furthermore the results underwent a Cochran test to check for outlying laboratory variances. Results with outlying variances have been rejected based on the assumption that similar methods of analysis were used and an outlying variance indicates poor repeatability and hence suboptimal control of method performance. This criterion led to the rejection of the results of one laboratory.

The methods employed all immunoaffinity column clean-up and reversed phase HPLC with fluorescence detection and post-column halogenation. One laboratory used chloroform for extraction, four methanol/water mixtures and two acetonitrile/water mixtures. The achieved recovery rates in this study and the percentage of accepted results are summarized in Table 1.

Peanut meal 1993 (Van Egmond et al. 1994)

Seventeen laboratories, which had to prove in a preliminary study, that they could achieve linear and stable four point calibrations, recovery rates between 70 and 110% at a level of 50, 100 and 150 µg/kg aflatoxin B₁ in peanut meal, blank values for RM 262 below 2 µg/kg and consistent recovery rates, were provided with ampoules of common calibrants in chloroform, and two candidate materials, RM 262 and RM 263. The material RM 262 was an intended blank material. The laboratories had to carry out the following measurements repeated, on each of three days: a four point calibration, a duplicate recovery experiment by spiking RM 262 at a level of 100 µg/kg aflatoxin B₁, a duplicate measurement of RM 263. Measurements could also be performed on two days but then triplicate measurement of RM 263 had to be performed.

Two laboratories withdraw one result. For evaluation of the remaining results the following acceptance levels were used: recovery range 70 % to 110 %, additionally the recovery rate achieved had to be within the established range of the specific laboratory, recovery rates should be consistent between days of this study and repeatability of two recovery experiments on one day CV ≤ 15 %. This led to the rejection of the complete datasets of six laboratories.

Ten laboratories used chloroform as extraction solvent. Of these ten, five laboratories employed a two step clean-up with SepPak[®] Florisil[®] and SepPak[®] C18 columns, two laboratories used a single step clean-up with SepPak[®] Florisil[®] columns and three a single step clean-up with silica gel columns. All ten laboratories used reversed phase HPLC with fluorescence detection and post-column halogenation except one, which

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3 preferred derivatisation with trifluoroacetic acid. One laboratory extracted with an
4 acetonitrile/water mixture and performed the clean-up with an immunoaffinity column
5 before determining the aflatoxin with reversed phase HPLC with fluorescence
6 detection and post-column halogenation. The achieved recovery rates in this study
7 and the percentage of accepted results are summarized in Table 1
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15 16 17 *Peanut meal 2008 (Buttinger et al. 2008a)*

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19 This study was carried out in combination with the above mentioned peanut butter
20 study of 2008 by the same laboratories. The laboratories were provided with
21 ampoules of a certified calibrant (ERM-AC057) in acetonitrile, one candidate
22 material, BCR-263R, and the certified reference material BCR-262. The later is
23 certified as a blank material (aflatoxin B₁ below 3 µg/kg with a level of confidence of
24 95 %). The laboratories had to carry out the following measurements repeated, on
25 each of three days: a five point calibration, a triplicate recovery experiments by
26 spiking BCR-262R at a level of 3 µg/kg aflatoxin B₁, a duplicate measurement of
27 BCR-263R.
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43 The results were subjected to technical evaluation and checked for compliance with
44 performance criteria for recovery rate laid down in European Commission Regulation
45 401/2006 (European Commission 2006). Recovery rates at a level of 3 µg/kg are
46 satisfactory between 70 % and 110 %. Rejection of data was based on the same
47 criteria as for the peanut butter study of 2008. This led to the exclusion of all results
48 from three laboratories (all with recovery rates above 110 % on at least two days). All
49 remaining data passed the Cochran criterion.
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The methods employed all immunoaffinity column clean-up and reversed phase HPLC with fluorescence detection and post-column halogenisation. One laboratory used chloroform for extraction, three methanol/water mixtures and one an acetonitrile/water mixture. The achieved recovery rates in this study and the percentage of accepted results are summarized in Table 1.

Animal feed 1993 (Van Egmond et al. 1994)

This study was carried out in combination with the peanut meal study of 1993. The study was performed by the same laboratories fulfilling similar criteria. They had to achieve linear and stable four point calibrations, recovery rates between 70 and 110% at a level of 10 µg/kg aflatoxin B₁ in animal feed, blank values for RM 375 below 1 µg/kg and consistent recovery rates. They were provided with ampoules of common calibrants in chloroform, and two candidate materials, RM 375 and RM 376. The material RM 375 was an intended blank material. The laboratories had to carry out the following measurements repeated, on each of two days: a four point calibration, a duplicate recovery experiment by spiking RM 375 at a level of 10 µg/kg aflatoxin B₁ and a triplicate measurement of RM 376.

Two laboratories withdraw one result each. The evaluation criteria were the same as for the peanut meal study of 1993. This led to the rejection of the complete result of three laboratories. Twelve laboratories used chloroform as extraction solvent. Of these twelve, ten laboratories employed a two step clean-up with SepPak[®] Florisil[®] and SepPak[®] C18 columns, one laboratory used a two step clean-up with liquid/liquid extraction and C18 SPE and one a single step clean-up with silica gel column. Eleven of those laboratories used reversed phase HPLC with fluorescence detection and post-

column halogenation except one, which preferred derivatisation with trifluoroacetic acid. One of those laboratories employed two dimensional TLC on silical gel plates.

One laboratory extracted with an acetonitrile/water mixture and performed the clean-up with an immunoaffinity column before determining the aflatoxin with reversed phase HPLC with fluorescence detection and post-column halogenation. One laboratory extracted with a methanol/hexane mixture and performed the clean-up with Zn precipitation, liquid/liquid extraction and cellulose column before determining the aflatoxin with reversed phase HPLC with fluorescence detection and post-column halogenation. The achieved recovery rates in this study and the percentage of accepted results are summarized in Table 1.

Animal feed 2009

Nine laboratories, with proven measurement capability and demonstrated experience in aflatoxin analysis, were provided with ampoules of a certified calibrant (ERM-AC057) in acetonitrile, and two candidate materials, ERM-BE375 and ERM-BE376. The low level material ERM-BE375 was used for the determination of the recovery. The laboratories had to carry out the following measurements repeated, on each of three days: a five point calibration, a triplicate recovery experiments by spiking ERM-BE375 at a level of 15 µg/kg aflatoxin B₁ and a duplicate measurement of ERM-BE376.

The results were subjected to technical evaluation and checked for compliance with performance criteria for recovery rate laid down in European Commission Regulation 401/2006 (European Commission 2006). Recovery rates at a level of 15 µg/kg are satisfactory between 80 % and 110 %. Rejection of data was based on the same

criteria as for the peanut butter study of 2008. This led to the exclusion of the complete data of two laboratories (one below 80 % on three days, the other below 80 % on one day and above 110 % on another) and from data of one day of another laboratory based on the recovery rate criterion. None of the laboratories failed the Cochran test.

The methods employed all immunoaffinity column clean-up and reversed phase HPLC with fluorescence detection and post-column halogenisation. Two laboratories used chloroform for extraction, two methanol/water mixtures, two acetonitrile/water mixtures and one an acetone/water mixture.

Results and discussion

The results of all studies are summarized with some statistical data in Table 1 and visualised in Figures 1, 2 and 3.

Peanut butter studies

The reproducibility (RSD_R) for both studies is the same, but the repeatability (RSD_r) is about 2.5 times higher in the study of 2008 compared to the study of 1991. The limit of detection (LOD) of the participating laboratories has improved by a factor of 3 to 5. Comparing the means of means of the studies, it is obvious that the value of 2008 is about four times lower than that of 1991. This might be seen, at the first glance, as an explanation for the higher value for the RSD_r . But looking carefully and taking the improvement of LOD into account it becomes apparent that the ratio mean of means / LOD has not changed. The recovery rates reported in 1991 are all below 100 % and the variation is much smaller than for the recovery rates reported in 2008.

Peanut meal studies

The RSD_r in the study of 2008 is about 30 % better than in the study of 1993, whereas the RSD_R is comparable. The LOD of the participating laboratories improved by a factor of 10. The mean of means of the study in 2008 is about 2.5 times lower than that of 1993. The recovery rates reported in 1993 and in 2008 are very similar.

Animal feed studies

The RSD_R and RSD_r are for both studies comparable. The LOD of the participating laboratories improved by a factor of 10.. The mean of means of the studies of 2009 is about 30 % higher than that of 1993. The recovery rates reported in 1993 and in 2008 are in very similar range..

Conclusions

Combining the outcome of all six studies, the improvements seen in method performance of HPLC methods over the last 20 years seem rather marginal. Achievable RSD_R are between 8 % and 14 % and RSD_r are around 7 % for all studies, independent of the year they were conducted. So no improvement can be seen here. The only proven progress is an increased sensitivity by a factor of 5 to 10. It is not clear if this is due to better instrumentation, e.g. fluorescence detectors with increased sensitivity or HPLC systems with lower noise, or due to a better clean-up procedure or a combination of both.

A clear trend in the analytical methodology for HPLC methods can be seen. In the past, extraction with chloroform and a two step clean-up procedure were preferred. This methodology has been replaced by a less labour intensive one, namely single step clean-up with immunoaffinity columns and extraction with aqueous mixture of methanol or acetonitrile, which are more compatible with antibodies and the environment.

This methodology might be less labour intensive, nevertheless it seems to be far from simple as still around one quarter of the submitted result of, already pre-selected, laboratories had to be rejected due to poor quality. This is about the same rejection rate as for the old studies.

In summary it can be concluded that HPLC methods for aflatoxin analysis have become less labour intensive and therefore cheaper and they have increased their sensitivity. Nevertheless looking at the RSD_R and the RSD_r measurement uncertainty has not improved at all. The main focus of HPLC method development seems to have been on economic aspects (speed, costs, complexity) whereas metrological points like measurement trueness and precision have been neglected. Which leads to the questions: Are the HPLC methods fit for purpose and so do not need any further improvement on measurement uncertainty? Is the aim to develop HPLC methods which are done in seconds by untrained personnel, but deliver results not any more reliable then decades ago? Or would it be preferable to have perhaps not so simple methods but with a low measurement uncertainty and a high trueness?

The first question can be answered easily for official controls as the method performance criteria are stated in legislation (European Commission 2006). The recommended value for RSD_R is derived from the Horwitz equation and the maximum value is twice this value. RSD_r is calculated as RSD_R times 0.66. This corresponds to recommended values from 33 to 64 % for the RSD_R for the mass fractions of interest (0.1 to 8 $\mu\text{g/kg}$) and from 22 to 42 % for the RSD_r . This clearly indicates the fitness for purpose of these HPLC methods. It raises on the other hand the question of the stringency of the laid down criteria.

The second question might be answered that due to the facilitation of the HPLC methods a higher number of laboratories is able to perform accurate measurements. It also has to be mentioned that the conclusions are drawn on the results of a group of laboratories, which does not reflect the performance of single laboratories. It must be stated that individual laboratories with optimised methods can achieve results with higher precision than the look at the selected group suggests.

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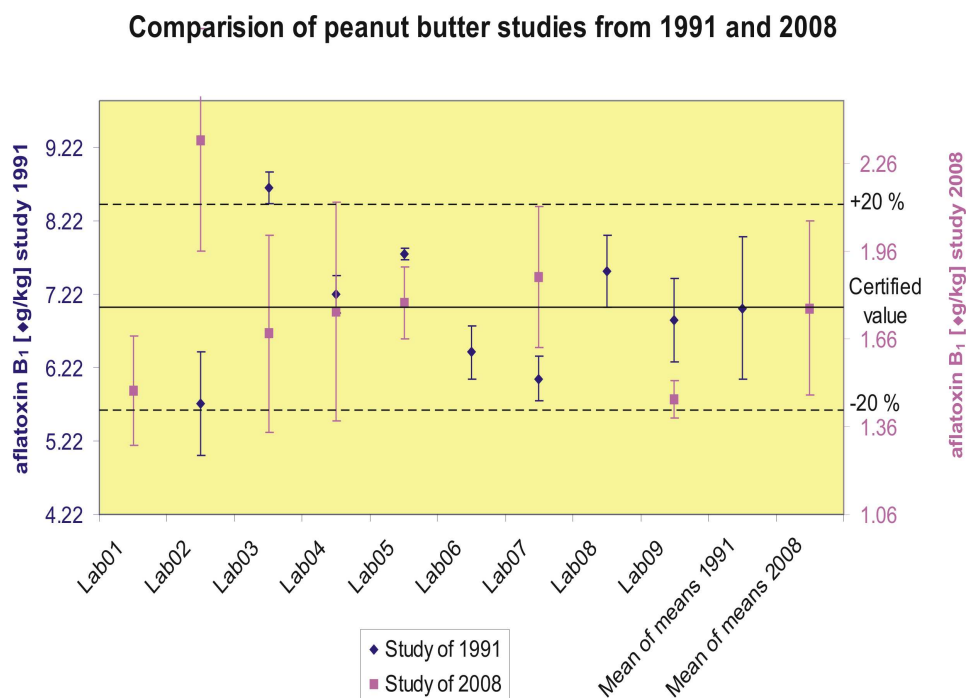


Figure 1. Comparison of the characterisation studies for peanut butter BCR-385 (1991) and BCR-385R (2008), laboratory means \pm 1 standard deviation (absolute scales for the two y-axis are different)

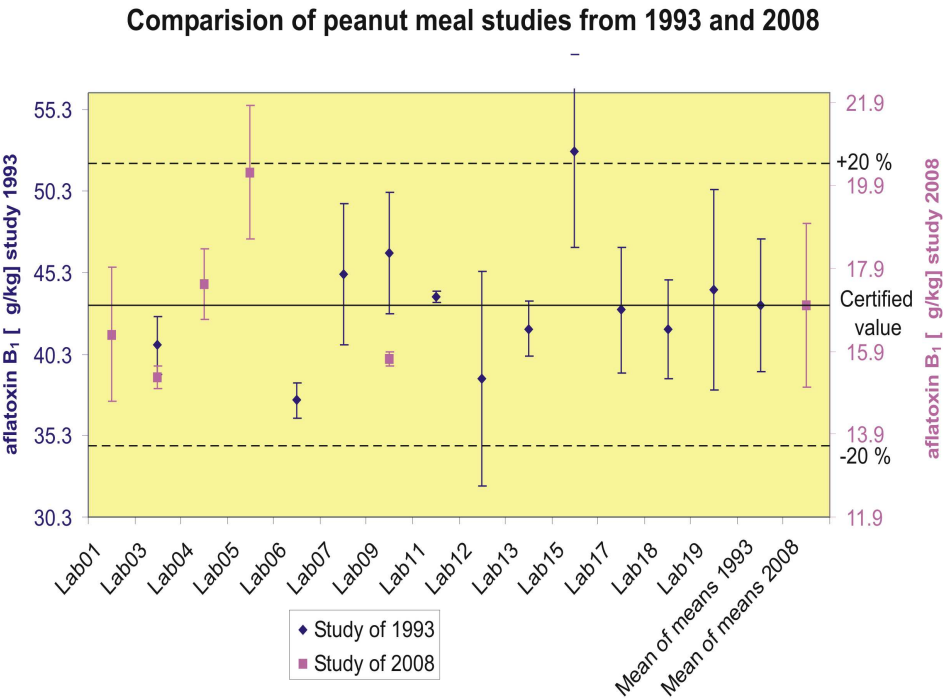


Figure 2. Comparison of the characterisation studies for peanut meal BCR-263 (1993) and BCR-263R (2008), laboratory means \pm 1 standard deviation (absolute scales for the two y-axis are different)

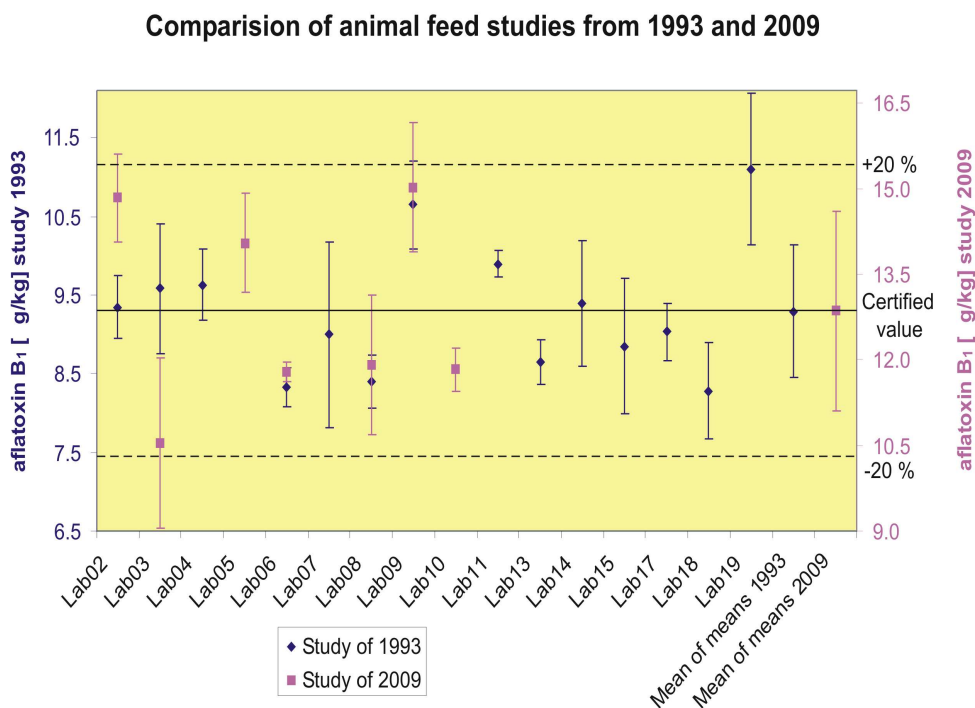


Figure 3. Comparison of the characterisation studies for peanut butter BCR-376 (1999) and ERM-BE376 (2009), laboratory means \pm 1 standard deviation (absolute scales for the two y-axis are different)

	Peanut butter		Peanut meal		Animal feed	
Aflatoxin B ₁	1991	2008	1993	2008	1993	2009
Mean of means [$\mu\text{g/kg}$]	7.04	1.77	43.3	17.1	9.3	12.9
RSD of mean of means	13.7 %	16.8 %	9.5 %	11.6 %	9.0 %	13.7 %
Number of laboratories	8	7	11	5	14	7
Reproducibility, RSD _R	13.5 %	13.5 %	8.6 %	11.3 %	8.6 %	13.5 %
Repeatability, RSD _r	6.0 %	15.2 %	9.5 %	6.4 %	7.0 %	7.7 %
Accepted results	89 %	79 %	63 %	62 %	80 %	74 %
Achieved recovery rates	59 – 93 %	70 – 116 %	72 – 106 %	74 – 107 %	71 – 98 %	82 – 105 %

Table 1. Summarised statistical data of the various studies