Evaluation of immunoassay kits for aflatoxin determination in corn & rice
Evaluation of immunoassay kits for aflatoxin determination in corn & rice

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Bart Huybrechts
We would like to thank the kit manufacturers; Charm, Neogen, Tecna, R-Biopharm and Romerlabs for providing us with all the materials, tools, customer support and background information necessary to bring this assessment to a successful end.
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<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACN</td>
<td>Acetonitril</td>
</tr>
<tr>
<td>AFB1</td>
<td>Aflatoxin B1</td>
</tr>
<tr>
<td>AFB2</td>
<td>Aflatoxin B2</td>
</tr>
<tr>
<td>AFG1</td>
<td>Aflatoxin G1</td>
</tr>
<tr>
<td>AFG2</td>
<td>Aflatoxin G2</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>AOAC</td>
<td>Association of Analytical Communities (formerly &quot;Association of Official Analytical Chemists&quot;)</td>
</tr>
<tr>
<td>Av</td>
<td>Average</td>
</tr>
<tr>
<td>B</td>
<td>Absorbance</td>
</tr>
<tr>
<td>Bo</td>
<td>Absorbance blank</td>
</tr>
<tr>
<td>CCα</td>
<td>Decision limit</td>
</tr>
<tr>
<td>CCβ</td>
<td>Detection capability</td>
</tr>
<tr>
<td>CEN</td>
<td>European Committee for Standardization</td>
</tr>
<tr>
<td>CL</td>
<td>Confidence limit</td>
</tr>
<tr>
<td>CR%</td>
<td>Cross Reactivity %</td>
</tr>
<tr>
<td>CRM</td>
<td>Certified Reference Material</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of Variation (in %)</td>
</tr>
<tr>
<td>CA</td>
<td>Cyclopiazonic acid</td>
</tr>
<tr>
<td>DON</td>
<td>Deoxynivalenol</td>
</tr>
<tr>
<td>EC</td>
<td>European Commission</td>
</tr>
<tr>
<td>EFSA</td>
<td>European Food Safety Authority</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immuno Sorbent Assay</td>
</tr>
<tr>
<td>EMAN</td>
<td>European Mycotoxins Awareness Network</td>
</tr>
<tr>
<td>EU</td>
<td>European Union</td>
</tr>
<tr>
<td>FAVV-AFSCA</td>
<td>Federal Agency for the Safety of the Food Chain</td>
</tr>
<tr>
<td>FAO</td>
<td>Food and Agriculture Organisation</td>
</tr>
<tr>
<td>FAPAS</td>
<td>Food Analysis Performance Assessment Scheme</td>
</tr>
<tr>
<td>FB1</td>
<td>Fumonisin B1</td>
</tr>
<tr>
<td>FGIS</td>
<td>Federal Grain Inspection Service</td>
</tr>
<tr>
<td>(HP)LC-FL</td>
<td>Liquid Chromatography Fluorescence detection</td>
</tr>
<tr>
<td>FPIA</td>
<td>Fluorescence Polarization Immunoassay</td>
</tr>
<tr>
<td>FVO</td>
<td>Food and Veterinary Office</td>
</tr>
<tr>
<td>GIPSA</td>
<td>Grain Inspection, Packers and Stockyards Administration</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>IAC</td>
<td>Immunoaffinity clean-up</td>
</tr>
<tr>
<td>IC50</td>
<td>Half maximal inhibitory concentration</td>
</tr>
<tr>
<td>ICH</td>
<td>International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use</td>
</tr>
<tr>
<td>ISO</td>
<td>International Organization for Standardization</td>
</tr>
<tr>
<td>IQR</td>
<td>Inter Quartile Range</td>
</tr>
<tr>
<td>k</td>
<td>Coverage factor (usual = 2)</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>Liquid Chromatography with tandem in space mass spectrometry detection</td>
</tr>
<tr>
<td>LFD</td>
<td>Lateral Flow Device</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>N</td>
<td>Number</td>
</tr>
<tr>
<td>NS</td>
<td>Not specified</td>
</tr>
<tr>
<td>NR</td>
<td>Not relevant</td>
</tr>
<tr>
<td>NRL</td>
<td>National Reference Laboratory</td>
</tr>
<tr>
<td>OTA</td>
<td>Ochratoxin A</td>
</tr>
<tr>
<td>Q1</td>
<td>First quartile</td>
</tr>
<tr>
<td>Q2</td>
<td>Second quartile (median)</td>
</tr>
<tr>
<td>Q3</td>
<td>Third quartile</td>
</tr>
<tr>
<td>Re%</td>
<td>Recovery in %</td>
</tr>
<tr>
<td>SCF</td>
<td>Scientific Committee on Food</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>u</td>
<td>uncertainty</td>
</tr>
<tr>
<td>U(Ext)</td>
<td>Extended uncertainty</td>
</tr>
<tr>
<td>UK</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>US</td>
<td>United States of America</td>
</tr>
<tr>
<td>xₑ</td>
<td>Experimental mean (ng/g)</td>
</tr>
<tr>
<td>xᵣ</td>
<td>True referenced mean (ng/g)</td>
</tr>
<tr>
<td>ZEN</td>
<td>Zearalenon</td>
</tr>
</tbody>
</table>
Foreword & scope & background
1.1 Foreword

In Belgium, the Federal Agency for the Safety of the Food Chain (AFSCA-FAVV), aiming to preserve the safety of the food chain and the quality of food, is responsible for the official control of all feed and food products, either produced in Belgium or imported. As such, it is also the authority competent for monitoring mycotoxin contamination throughout the food chain. Each year, a monitoring program is established by the food agency specifying the number of samples, food and feed commodities, schedule, sampling method and the contaminants to be analyzed. From a practical point of view, samples are collected all over Belgium throughout the year. Analyses are carried out in approved and accredited laboratories for the purposes of the official control. A key requirement of any food safety management system is the process of demonstrating its continuous effectiveness by verification of the analytical methods. Given the potential application of the commercial rapid test kits as screening tools, it is important that the performance of these immunochemical techniques should, when possible, be evaluated against established reference methods to assess their reliability. In Belgium, AFSCA-FAVV has appointed CODA-CERVA, as national reference laboratory (NRL), to evaluate the fast mycotoxin test kits that are currently available on the market, based on currently available knowledge regarding applicability, reproducibility, precision, recovery, accuracy and cost level. This evaluation aimed to promote reliable fast mycotoxin measurement systems in Belgium. In 2008-2009 kits for deoxynivalenol were assessed\(^1\), in 2009-2010 kits for Ochratoxin A, this year kits for aflatoxin (B1 or/and total aflatoxin).

For the set-up of the evaluation protocol, a strategic group of experts was formed. This group included members of the AFSCA-FAVV, CODA-CERVA and several kit users. This network was extended to the manufacturers and allowed us to identify possible drawbacks of these kits. Figure 1 shows the Partnership developed for performing the evaluation work.

The evaluation was performed in three main steps: i) inventory of available rapid fast AFLATOXIN testing kits (ii) administrative evaluation based on questionnaires to be filled in by the kit producers and (iii) experimental evaluation of quantitative kits by checking some critical parameters (drawbacks).

\(^1\) http://www.var.fgov.be/
1.2 Scope

1.2.1 Objective and strategy of the inventory task

At the present immunoassay kits are available in three different formats; FPIA (fluorescence polarization immunoassay), ELISA (enzyme-linked immunosorbent assay) and LFD (lateral flow devices). Commercialized by several providers they are available for all legally regulated mycotoxins.

The inventory of fast test kits attempted to

- Identify all the existing kit providers active on the Belgian market
- Identify the available kits for aflatoxin (either Aflatoxin B1 or total aflatoxin) determination in corn & rice
- Establish collaboration with the identified kit providers

The inventory was based on the list of rapid mycotoxin testing kits available at the website “mycotoxins.org” of the European Mycotoxins Awareness Network (EMAN) project. Since the last version of the EMAN website was updated in 2003, the inventory was completed using the individual websites of the kit providers and personal contact (telephone calls, mailings, meetings with kit producers or distributors).

1.2.2 Objective and strategy of the administrative evaluation

Information was gathered on the available aflatoxin kits from manufacturers through two questionnaires, one for quantitative and one for qualitative aflatoxin kits. These were developed in close cooperation with the aforementioned expert group and were addressed by mailing to the kit manufacturers. Table 1 summarizes the most common parameters checked for quantitative or qualitative aflatoxin kits.

Table 1: Parameters for quantitative and qualitative kits²

<table>
<thead>
<tr>
<th>Quantitative kits</th>
<th>Qualitative kits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detection limit</td>
<td>Cut-off limit</td>
</tr>
<tr>
<td>Range and linearity</td>
<td>dichotomic value</td>
</tr>
<tr>
<td>Sensitivity and specificity</td>
<td>False positive and false negative rates</td>
</tr>
<tr>
<td>Measurement Uncertainty</td>
<td>Unreliability region</td>
</tr>
<tr>
<td>Accuracy: trueness, precision</td>
<td>Sensitivity and specificity</td>
</tr>
<tr>
<td>Selectivity: interferences</td>
<td>Selectivity: interferences</td>
</tr>
<tr>
<td>Ruggedness or robustness</td>
<td>Ruggedness or robustness</td>
</tr>
<tr>
<td>Formats and cost per kit</td>
<td>Formats and cost per kit</td>
</tr>
<tr>
<td>Quantity of materials and reagents needed</td>
<td>Quantity of materials and reagents needed</td>
</tr>
<tr>
<td>Protocol, throughput and time required for performing analysis</td>
<td>Protocol, throughput and time required for performing analysis</td>
</tr>
<tr>
<td>Scientific supports and quality assurance in manufacturing</td>
<td>Scientific supports and quality assurance in manufacturing</td>
</tr>
<tr>
<td>Easy-to-use¹ information according to the manufacturers</td>
<td>Easy-to-use¹ information according to the manufacturers</td>
</tr>
</tbody>
</table>

Note: for a quantitative method, sensitivity should be a numerical value that indicates how the response changes whenever there is a variation in the concentration of the analyte. However, this parameter will be evaluated in a different way if a qualitative method is used.

Regulation 1881/2006/EC³ (as amended by Regulation Nr. 165/2010⁴) lays down maximal limits for certain contaminants in specified foods, and as such determines the requirements with respect sensitivity of the analytical

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methods. Furthermore, Regulation 401/2006EC\(^3\) lays down methods for sampling and analysis, thereby defining some method performance criteria (i.e. minimum recovery rate, maximum reproducibility, restrictions with respect to the expanded analytical uncertainty). This is amended by regulation 178/2010\(^6\) regarding groundnuts (peanuts), other oilseeds, tree nuts, apricot kernels, liquorice and vegetable oil. Note that CEN-CR 13505:1999\(^7\) and Decision 2002/657/EC\(^8\) indicate the parameters that are relevant for screening and confirmation methods. Decision 2002/657/EC\(^8\) states that screening methods should be validated and that the ‘false compliant rate (false negatives) should be <5% at the level of interest without providing much detail on how to establish this. Fortunately, a complementary guideline has been published\(^9\). Essentially, both documents prescribe that in an initial validation, at least 20 samples spiked at the anticipated screening reporting level need to be analyzed and the target analyte(s) need to be detectable in at least 19 out of 20 samples.

For the approval of pre-export control on aflatoxins in peanuts from the U.S. Article 23 of Regulation (EC) No 882/2004\(^10\) provides that pre-export checks carried out by a third country on feed and food immediately prior to export to the Community with a view of verifying that the exported products comply with the Community requirements may be approved. Such an EC approval of pre-export controls has as consequence that these pre-export controls replace or reduce the documentary, identity and physical controls at import into the EC. An FVO inspection carried out in September 2006 concluded that the US have a well-defined control system for aflatoxin levels in peanuts and well performing approved laboratories. Therefore approval of pre-export control was granted by Commission Decision 2008/47/EC\(^11\) in December 2007. As these pre-export controls replace effectively and reliably the controls at import this should result in a significant decrease of controls at import. Special conditions governing certain foodstuffs imported from certain third countries due to contamination risks of these products by aflatoxins are laid down in Commission Regulation (EC) No 1152/2009\(^12\). In order to assist the competent authorities on the official control of aflatoxin contamination in food products which are subject to Commission Regulation (EC) 1152/2009, a guidance document "Guidance document for competent authorities for the control of compliance with EU legislation on aflatoxins"\(^13\) has been elaborated. This document is also applicable for the control of aflatoxins in food products not subject to the safeguard Regulation. A number of scientific opinions were also adopted: The Scientific Committee on Food (SCF) adopted on 23 September 1994 an opinion\(^14\) on toxicological safety of aflatoxins B1, B2, G1, G2 and M1. In addition, the European Food Safety Authority (EFSA) has adopted on 3 February 2004 an opinion\(^15\) related to aflatoxin B1 as undesirable substance in animal feed. EFSA adopted on 29 January 2007 an opinion\(^16\) related to the potential increase of consumer health risk by a possible increase of the existing maximum levels for aflatoxins in almonds, hazelnuts and pistachios and derived products. EFSA adopted on 16 June 2009 a statement\(^17\) related to the effects on public health of an increase of the levels for aflatoxin total from 4 µg/kg to 10 µg/kg for tree nuts other than almonds, hazelnuts and pistachios.
Table 2: Regulatory limits EU (ref. 3-4-6)

<table>
<thead>
<tr>
<th>Food product</th>
<th>Mycotoxin</th>
<th>Max Level (µg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Groundnuts to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs</td>
<td>Aflatoxin B1</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Total B1+B2+G1+G2</td>
<td>15</td>
</tr>
<tr>
<td>Nuts to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs</td>
<td>Aflatoxin B1</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Total B1+B2+G1+G2</td>
<td>10</td>
</tr>
<tr>
<td>Groundnuts, nuts and processed products thereof, intended for direct human consumption or use as Aflatoxin B1 an ingredient in foodstuffs</td>
<td>Aflatoxin B1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Total B1+B2+G1+G2</td>
<td>4</td>
</tr>
<tr>
<td>Dried fruit to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs</td>
<td>Aflatoxin B1</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Total B1+B2+G1+G2</td>
<td>10</td>
</tr>
<tr>
<td>Dried fruit and processed products thereof, intended for direct human consumption or use as an ingredient in foodstuffs</td>
<td>Aflatoxin B1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Total B1+B2+G1+G2</td>
<td>4</td>
</tr>
<tr>
<td>All cereals and all products derived from cereals, including processed cereal products</td>
<td>Aflatoxin B1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Total B1+B2+G1+G2</td>
<td>4</td>
</tr>
<tr>
<td>Maize or rice to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs</td>
<td>Aflatoxin B1</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Total B1+B2+G1+G2</td>
<td>10</td>
</tr>
<tr>
<td>Raw milk, heat-treated milk and milk for the manufacture of milk-based products and as defined by Council Directive 92/46/EEC)</td>
<td>Aflatoxin M1</td>
<td>0.05</td>
</tr>
<tr>
<td>Following species of spices: Capsicum spp. (dried fruits thereof, whole or ground, including chillies, chilli powder, cayenne, and paprika), Piper spp. (fruits thereof, including white and black pepper) Myristica fragrans (nutmeg) Zingiber officinale (ginger) Aflatoxin B1 Curcuma longa (turmeric)</td>
<td>Aflatoxin B1</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Total B1+B2+G1+G2</td>
<td>10</td>
</tr>
<tr>
<td>Processed cereal-based foods and baby foods for infants and young children</td>
<td>Aflatoxin B1</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>Total B1+B2+G1+G2</td>
<td>-</td>
</tr>
<tr>
<td>Infant formulae and follow-on formulae, including infant milk and follow-on milk</td>
<td>Aflatoxin M1</td>
<td>0.025</td>
</tr>
<tr>
<td>Dietary foods for special medical purposes intended specifically for infants</td>
<td>Aflatoxin B1</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>Total B1+B2+G1+G2</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Aflatoxin M1</td>
<td>0.025</td>
</tr>
</tbody>
</table>

1.2.3 Objective and strategy of the experimental evaluation

A research protocol has then been elaborated and discussed with the users’ committee. Several contacts have been made with the kits providers to obtain their formal consent in participating to this research. Analyses were performed by one analyst using the same lot of test kits in the same laboratory environment. The overall "ease of use" of the kit was also appreciated by laboratory workers during the experimental evaluation.
Evaluation of immunoassay kits for aflatoxin determination in corn & rice

According to Commission Decision 2002/657/EC the following parameters (Table 3) have to be validated for screening methods:

Table 3: Criteria to be verified according to 657/2002

<table>
<thead>
<tr>
<th>CCβ</th>
<th>CCα</th>
<th>Accuracy</th>
<th>Recovery</th>
<th>Precision</th>
<th>Specificity</th>
<th>Robustness</th>
</tr>
</thead>
<tbody>
<tr>
<td>Qualitative</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Quantitative</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

The main drawbacks identified, based on the literature, feedback from kit producers, experience of the members of the expert group and the experiences gained in the evaluation of the DON & OTA kits the past two years, the following problems were indicated:

1. Cross-reactivity
2. Accuracy
3. Recovery
4. Detection capability (CCβ)
5. Precision

The kits of the following manufacturers were assessed: Romerlabs, R-Biopharm, Tecna, Charm & Neogen.

1.3 Background

Mycotoxins are secondary metabolites of a range of filamentous fungi with deleterious effects on humans and animals, which can be found in agricultural commodities and animal foodstuffs. They are present in trace amounts so there is a need for highly sensitive and selective analytical methods for these natural toxins. Among various mycotoxins, aflatoxins have assumed significance due to their deleterious effects on human beings, poultry and livestock. The aflatoxin problem was first recognized in 1960, when there was severe outbreak of a disease referred as “Turkey X Disease” in UK, in which over 100,000 turkey poults died. The cause of the disease was shown due to toxins in peanut meal infected with Aspergillus flavus and the toxins were named “aflatoxins”. Food products contaminated with aflatoxins include cereal (maize, sorghum, pearl millet, rice, wheat), oilseeds (groundnut, soybean, sunflower, cotton), spices (chillies, black pepper, coriander, turmeric, zinger), tree nuts (almonds, pistachio, walnuts, coconut) and milk. Aflatoxins are potent toxic, carcinogenic, mutagenic, immunosuppressive agents, produced as secondary metabolites by the fungi Aspergillus flavus and Aspergillus parasiticus on a variety of food products. There are 18 different types of aflatoxins identified, major members are aflatoxin B1, B2, G1 and G2. Aflatoxin B1 (AFB1) is normally predominant in cultures as well as in food products. Pure AFB1 is pale-white to yellow crystalline, odorless solid. Aflatoxins are soluble in methanol, chloroform, acetone, acetonitrile. A. flavus typically produces AFB1 and AFB2, whereas Aspergillus. parasiticus produce AFG1 and AFG2 as well as AFB1 and AFB2. Aflatoxin M1 and M2 are major metabolites of aflatoxin B1 and B2 respectively, found in milk of animals that have consumed feed contaminated with aflatoxins. The aflatoxins display potency of toxicity, carcinogenicity, mutagenicity in the order of AFB1 > AFG1 > AFB2 > AFG2 as illustrated by their LD50.

values for day-old ducklings\textsuperscript{25, 26}. The aflatoxins fluoresce strongly in ultraviolet light (ca. 365 nm); B1 and B2 produce a blue fluorescence whereas G1 and G2 produce green fluorescence\textsuperscript{27}. According to FAO estimates, 25\% of the world food crops are affected by mycotoxins each year. And also crop loss due to aflatoxins contamination costs US producers more than $100 million per year on average including $ 26 million for peanuts ($69.34/ha)\textsuperscript{28}.

Analytical methods for rapid, sensitive, and accurate determination of these mycotoxins in unprocessed cereals and cereal-based products are highly needed in order to properly assess both the relevant risk of exposure and the relevant toxicological risk for humans and animals, as well as to ensure that regulatory levels (see table 1) fixed by the EU or other international organizations are met.

Testing for mycotoxins is conducted under many different circumstances and for a variety of reasons, which has led to a proliferation in the number of test methods. Selecting the appropriate method depends upon the intended use of the method. Factors such as the speed of the method, its accuracy, the skill level required for performing the assay, and the cost will all impact method selection. The methods basically fall into two major categories: those that can be conducted with minimal training in laboratories or in the field (screening assays), and those that must be conducted by more fully trained personnel in analytical laboratories. In general, fast and easy-to-use ELISA based aflatoxin screening kits are commercially available for all major types of aflatoxins. Quantification is predominantly done with LC-FL \textsuperscript{29}. Detection limits in the low ppt range can easily be achieved when iodine is added post-column to enhance method sensitivity. In addition, immunoaffinity sample clean-up has been shown to have a great potential to increase method specificity and sensitivity by selective enrichment and isolation of the target aflatoxins\textsuperscript{29}. A number of quantitative methods have been published to determine major aflatoxins and the structurally related

sterigmatocystin in food, milk, herbs, urine, and cigarette smoke. In this field, LC/MS seems to be just a minor alternative or confirmation technique for the already well established reliable and robust LC-FL methodology though it should be useful to confirm positive results and ELISA based screening analysis.

Table 4: Advantages and disadvantages of traditional and emerging methods for mycotoxin analysis

<table>
<thead>
<tr>
<th>Method</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gas Chromatography</td>
<td>Simultaneous analysis of mycotoxins, good sensitivity, may be automated</td>
<td>Expensive equipment, specialist expertise required, derivatization required,</td>
</tr>
<tr>
<td></td>
<td>(autosampler), provides confirmation (MS detector).</td>
<td>matrix interference problems, non-linear calibration curve, drifting response,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>carry-over effects from previous sample, variation in reproducibility and</td>
</tr>
<tr>
<td></td>
<td></td>
<td>precision</td>
</tr>
<tr>
<td>High Performance Liquid</td>
<td>Good sensitivity, good selectivity, good precision, may be automated</td>
<td>Expensive equipment, specialist expertise required, may require derivatization</td>
</tr>
<tr>
<td>Chromatography</td>
<td>(autosampler), short analysis times, official methods available.</td>
<td></td>
</tr>
<tr>
<td>Liquid Chromatography/Mass Spectrometry</td>
<td>Simultaneous analysis of mycotoxins, good sensitivity (LC/MS/MS), provides</td>
<td>Very expensive, specialist expertise required, sensitivity relies on ionization</td>
</tr>
<tr>
<td></td>
<td>confirmation, no derivatization required.</td>
<td>technique, matrix assisted calibration curve (for quantitative analysis)</td>
</tr>
<tr>
<td>Enzyme-Linked Immunosorbent Assay</td>
<td>Simple sample preparation, inexpensive equipment, high sensitivity, simultaneous</td>
<td>Cross-reactivity with related mycotoxins, matrix interference problems, possible</td>
</tr>
<tr>
<td></td>
<td>analysis of multiple samples, suitable for screening, limited use of organic</td>
<td>false positive/negative results, confirmatory LC analysis required.</td>
</tr>
<tr>
<td></td>
<td>solvents.</td>
<td></td>
</tr>
<tr>
<td>Lateral Flow Device/Dipstick</td>
<td>Rapid, no clean-up, no expensive equipment, easy to use, no specific training</td>
<td>Cross-reactivity with related mycotoxins, validation required for additional</td>
</tr>
<tr>
<td></td>
<td>required.</td>
<td>matrices.</td>
</tr>
<tr>
<td>Fluorescence Polarization</td>
<td>Rapid, no clean-up required</td>
<td>Cross-reactivity with related mycotoxins, matrix interference problems.</td>
</tr>
<tr>
<td>Immunoassay</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RI (Infrared) spectroscopy</td>
<td>Rapid, non-destructive measurement, no extraction or clean-up, easy operation.</td>
<td>Expensive equipment, calibration model must be validated, knowledge of</td>
</tr>
<tr>
<td></td>
<td></td>
<td>statistical methods, poor sensitivity</td>
</tr>
<tr>
<td>Biosensors</td>
<td>Rapid, no clean-up procedure</td>
<td>Cross-reactivity with related mycotoxins, extract clean-up needed to improve</td>
</tr>
<tr>
<td></td>
<td></td>
<td>sensitivity, variation in reproducibility and precision.</td>
</tr>
</tbody>
</table>

In all cases, obtaining a representative test sample of the overall lot is essential in order to ensure that the results of the test sample can be correctly ascribed to the lot. Sampling, subsampling, grinding, and extraction of commodities

take considerable attention and time. In fact, these steps often require more time than some of the rapid assays for detecting the toxins. Therefore, where possible, it is preferable to combine a rapid extraction technique with rapid assays in order to minimize the overall assay time. Extraction usually involves conventional procedures using acetone, chloroform and methanol or mixtures thereof. Adding water to the extraction solvent generally gives higher extraction efficiencies.

In spite of the striking importance of the quality of the commercial fast test kits for their effectiveness of mycotoxin control, transversal evaluation or comparison of several kits is also needed in order to check how far they could help to meet the ISO 17025 requirements. This report provides noticeable features of the inquiry as well as the overall outcome of the evaluation process as performed for AFLATOXIN commercial test kits. The kits evaluated were dedicated for cereal and cereal products because of its relevance to the official residue control in Belgium.

ELISA

Enzyme-Linked ImmunoSorbent Assay (ELISA) is an antibody-based assay that is commonly used to detect mycotoxins. A number of commercial ELISA kits are available for aflatoxins, deoxynivalenol, fumonisins, ochratoxin, HT2 & T2 and zearalenone. This is usually a competitive assay in which the mycotoxin of interest from a sample competes with a labeled mycotoxin for a limited number of specific antibody-binding sites. The greater the amount of toxin present in the sample, the lower the binding of the labeled toxin and the lower the signal generated by the assay. ELISA is one of the more affordable methods for detecting mycotoxins, and has the advantage that a large number of samples can be measured at once. Commercial 96-well assays and strip-tests are available for many mycotoxins.

ELISA techniques have been shown to be less accurate and less sensitive than conventional chromatographic assays. Very few correlations were found between the two types of techniques. In addition, false positive or false negative results often occurred with ELISA because of cross-reactions between molecules or interferences with the antibody reagents. They are thus considered to be suitable for qualitative assessment or for sample pre-screening but not for quantitative determination. It is also recommended to use the ELISA techniques only for the foods they were developed for.

Lateral flow devices

Immunochromatographic assays, also called “Lateral Flow Devices” are user-friendly formats requiring a very short time for results. Basically, a ligand that can be bound to a visually detectable solid support, such as dyed microspheres, can be qualitatively tested and in many cases even quantitatively. The most popular label for the lateral flow test are particles consisting of colloidal gold coated with the antibody which provide red-colored binding zones. Liquid sample is added to the sample pad, the liquid components of the assay move along the membrane by capillary flow to the absorbent pad at the end of the strip. When migrating, the sample suspends the gold particles and the mycotoxin, if present, binds to the particles. When the test line is reached, which is coated with the analyte of interest, the gold particles will bind with their antibody if they are still unbounded and color the line red. If the sample was contaminated, the gold particles will be already bounded and flush over the test line. Therefore, absence
of analyte results in red color for the test line. Additional chemicals or handling steps are, in contrast to ELISA tests, not required.

Lateral flow devices with colloidal gold labels have been studied for the most important mycotoxins, such as aflatoxins, DON, T-2, fumonisins, OTA and ZEN. They have the large advantage that no labor intensive and time consuming washing steps are necessary making it possible to use them in the field.
Inventory
2.1 Inventory results of providers of aflatoxin kits

Table 5 gives an overview of the kit manufactures that offer kits for aflatoxin (B1 or total aflatoxin, aflatoxin M1 is not included) determination. Of these manufacturers, 5 wished to participate (Table 6) in the evaluation.

Figure 3 gives an overview in function of: (1) type test, (2) B1 versus Total & (3) Qualitative versus Quantitative. It can be seen clearly that the market is dominated by ELISA kits with strip based tests (Lateral Flow Devices or dipsticks) following closely. Most of these kits are quantitative ones focusing on total aflatoxin. As quantitative kits dominate the market it was decided to focus on them, participants however were free to decide whether they send in a kit for AFB1 or for total aflatoxin. There was also no restriction set on the format that could be submitted for the experimental evaluation.
## Evaluation of immunoassay kits for determination of ochratoxin A in cereals

### Table 5: Overview of contacted manufacturers and their kits

<table>
<thead>
<tr>
<th>Producer</th>
<th>Kit Description</th>
<th>Format</th>
<th>Quant/Qual</th>
<th>Remark</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aflataxin</strong></td>
<td>Aflatoxin, ELISA Kit</td>
<td>ELISA</td>
<td>Quant</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Aflatxin, ELISA Kit Dipstick</td>
<td>ELISA</td>
<td>Quant</td>
<td></td>
</tr>
<tr>
<td><strong>Aukin</strong></td>
<td>Mycriteral</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Beacon</strong></td>
<td>Aflatoxin, Tube kit</td>
<td>ELISA</td>
<td>Quant</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Aflatoxin Plex kit</td>
<td>ELISA</td>
<td>Quant</td>
<td></td>
</tr>
<tr>
<td><strong>Charm Sciences</strong></td>
<td>ROSA® Fast AQ® (Quantitative)</td>
<td>LFD</td>
<td>Quant</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ROSA® AFQ (Quantitative)</td>
<td>LFD</td>
<td>Quant</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ROSA® aflatoxin P/N</td>
<td>LFD</td>
<td>Quant</td>
<td>methanol/ethanol</td>
</tr>
<tr>
<td></td>
<td>ROSA® Best aflatoxin P/N</td>
<td>LFD</td>
<td>Qual</td>
<td>methanol/ethanol</td>
</tr>
<tr>
<td><strong>Diac health</strong></td>
<td>Aflatoxin FPA Qualitative</td>
<td>FPIA</td>
<td>Qual</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Aflatoxin FPA 10 ng/g</td>
<td>FPIA</td>
<td>Qual</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Aflatoxin FPA Quantitative</td>
<td>FPIA</td>
<td>Qual</td>
<td></td>
</tr>
<tr>
<td><strong>Enzymologie</strong></td>
<td>QuickTox™ Kit for Aflatoxin - 20 ng/g, AS-101</td>
<td>Strip</td>
<td>Qual</td>
<td></td>
</tr>
<tr>
<td></td>
<td>QuickTox® Kit for Quickscan - Aflatoxin - AQ 109 BG</td>
<td>Strip</td>
<td>Qual</td>
<td></td>
</tr>
<tr>
<td><strong>Europroxima</strong></td>
<td>Aflatoxin B1 ELISA</td>
<td>ELISA</td>
<td>Quant</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Aflatoxin Total ELISA</td>
<td>ELISA</td>
<td>Quant</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Aflatoxin B1 FRT</td>
<td>ELISA</td>
<td>Quant</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Aflatoxin Total FRT</td>
<td>ELISA</td>
<td>Quant</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MycoMonitor™ Total Aflatoxin Assay</td>
<td>ELISA</td>
<td>Quant</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Aflatoxin (LOW MATRIX) Total</td>
<td>ELISA</td>
<td>Quant</td>
<td></td>
</tr>
<tr>
<td><strong>LCTech</strong></td>
<td>Aflatead</td>
<td>ELISA</td>
<td>Quant</td>
<td></td>
</tr>
<tr>
<td><strong>Neogen</strong></td>
<td>Agri-Screen for Aflatoxin</td>
<td>ELISA</td>
<td>Qual</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Veratox for Aflatoxin</td>
<td>ELISA</td>
<td>Quant</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Veratox for Aflatoxin AST (Aflatoxin Single Test)</td>
<td>ELISA</td>
<td>Quant</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Veratox for Aflatoxin HS (High Sensitivity)</td>
<td>ELISA</td>
<td>Quant</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NEOCOLUMN</td>
<td>ELISA</td>
<td>Qual</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reveal for Aflatoxin SQ Peanut</td>
<td>ELISA</td>
<td>Qual</td>
<td></td>
</tr>
<tr>
<td><strong>R-Biopharm</strong></td>
<td>AFLAPLATE</td>
<td>ELISA</td>
<td>Quant</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RIDASCREEN® Aflatoxin B1 30/15</td>
<td>ELISA</td>
<td>Quant</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RIDASCREEN® Aflatoxin Total</td>
<td>ELISA</td>
<td>Quant</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RIDASCREEN®FAST Aflatoxin</td>
<td>ELISA</td>
<td>Quant</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RIDASCREEN®FAST Aflatoxin B1</td>
<td>ELISA</td>
<td>Quant</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RIDASCREEN®FAST Aflatoxin SC</td>
<td>ELISA</td>
<td>Quant</td>
<td></td>
</tr>
<tr>
<td><strong>Romexlab</strong></td>
<td>FluoroQuant® Chemical Kits</td>
<td>LFD</td>
<td>Quant</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AgroQuant® Mycotoxin ELISA Kits</td>
<td>LFD</td>
<td>Quant</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AlfaCap™ Kit</td>
<td>ELISA</td>
<td>Qual</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AgroSpin® Aflatoxin Test Kit</td>
<td>LFD</td>
<td>Qual</td>
<td></td>
</tr>
<tr>
<td><strong>SDI</strong></td>
<td>Myco aflatoxin test strips</td>
<td>Strip</td>
<td>Qual</td>
<td></td>
</tr>
<tr>
<td><strong>Techna</strong></td>
<td>Celer AFA, quantitative, ELISA</td>
<td>ELISA</td>
<td>Quant</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Celer AFA B1, quantitative, ELISA</td>
<td>ELISA</td>
<td>Quant</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Proven AFA, quantitative, ELISA</td>
<td>ELISA</td>
<td>Quant</td>
<td></td>
</tr>
<tr>
<td><strong>Tramnia</strong></td>
<td>Aflatoxin B1</td>
<td>ELISA</td>
<td>Quant</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Aflatoxin B1 “sensitv”</td>
<td>ELISA</td>
<td>Quant</td>
<td></td>
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<tr>
<td></td>
<td>Aflatoxin B1 membrane test</td>
<td>Strip</td>
<td>Qual</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Aflatoxin Total membrane test</td>
<td>Strip</td>
<td>Qual</td>
<td></td>
</tr>
<tr>
<td><strong>Vicam</strong></td>
<td>Aflacheck</td>
<td>Strip</td>
<td>Qual</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Aflastain</td>
<td>ELISA</td>
<td>Qual</td>
<td></td>
</tr>
</tbody>
</table>

**Notes:**
- **Format:** ELISA (Enzyme-Linked Immunosorbent Assay), FPIA (Fluorescent Protein Assay), LFD (Lateral Flow Device), Strip, Quant (Quantitative), Qual (Qualitative).
- **Quant/Qual:** Quantitative/Qualitative.
- **Remark:** Details about the test's sensitivity, sample type, and additional remarks.
Table 6: Overview of kits involved in the experimental evaluation

<table>
<thead>
<tr>
<th>Kit</th>
<th>Manufacturer</th>
<th>Total Afla / B1</th>
<th>ELISA</th>
<th>LFD</th>
</tr>
</thead>
<tbody>
<tr>
<td>AgraQuant® Total Aflatoxin Assay 1/20</td>
<td>Romer Labs Singapore Pte. Ltd.</td>
<td>Total</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ROSA Aflatoxin Quantitative</td>
<td>Charm Sciences Inc</td>
<td></td>
<td></td>
<td>ν</td>
</tr>
<tr>
<td></td>
<td>659 Andover Street</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lawrence, MA USA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>01843-1032</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RIDASCREEN® Aflatoxin total</td>
<td>R-Biopharm AG, An der neuen Bergstr. 17, 64297 Darmstadt, Germany</td>
<td>Total</td>
<td></td>
<td>ν</td>
</tr>
<tr>
<td>Veratox® HS Total Aflatoxin</td>
<td>Neogen Corporation</td>
<td></td>
<td></td>
<td>ν</td>
</tr>
<tr>
<td></td>
<td>620 Lesher Place</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lansing, MI 48912</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(800) 234-5333 ext 4450</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Celer AFLA B1</td>
<td>Tecna S.r.l.</td>
<td></td>
<td></td>
<td>ν</td>
</tr>
<tr>
<td></td>
<td>Area Science park</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Padriciano Nr. 99</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>34149 Trieste</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Italy</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Administrative evaluation
3.1 Analytical performance criteria

All participants were asked to fill in a questionnaire in which data for recovery, accuracy, precision (intra-day & inter-day), reproducibility and some general information had to be reported.

Table 7 indicates how recovery, standard combined uncertainty and Extended Uncertainty are calculated.

Table 7: Calculation of parameters (for symbols, see list abbreviations)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Equation</th>
<th>Equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recovery</td>
<td>Equation 1</td>
<td>Rec% = (x_e/x_t) * 100</td>
</tr>
<tr>
<td>Uncertainty</td>
<td>Equation 2</td>
<td>u = √(CV^2 + bias^2)</td>
</tr>
<tr>
<td>Extended Uncertainty</td>
<td>Equation 3</td>
<td>U_(Ext) = k (&gt;2) * u</td>
</tr>
</tbody>
</table>

3.2 Intra-run precision, between-run precision & reproducibility

The data reported by the kit manufacturers for intra-run precision (same operator/same day/same batch, Table 8), between-run precision (different operator and/or different day, same batch, Table 9) and reproducibility (different operators and different days and different batches, Table 10) are reported.

According to document 401/2006 the recommended value for reproducibility should be calculated as by Horwitz with the maximum allowable value for reproducibility 2 times the value from Horwitz. The precision can be calculated as 0.66 * Horwitz. For 5ng/g this would generate an expected maximum reproducibility of 40%, a maximum allowable reproducibility of 80% and an expected precision of 25%.
### Table 8: Intra-run precision

<table>
<thead>
<tr>
<th>Level (ng/g)</th>
<th>Neogen</th>
<th>R-Biopharm</th>
<th>Romerlabs</th>
<th>Tecna</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Run</td>
<td>Run 1</td>
<td>Run 2</td>
<td>Run 3</td>
<td>Run 1</td>
</tr>
<tr>
<td>Uncertainty (%)</td>
<td>21</td>
<td>32</td>
<td>26</td>
<td>28</td>
</tr>
<tr>
<td>Extended Uncertainty ((\text{k}=2))</td>
<td>42</td>
<td>65</td>
<td>52</td>
<td>57</td>
</tr>
</tbody>
</table>

### Table 9: Between-run precision

<table>
<thead>
<tr>
<th>Level (ng/g)</th>
<th>Charm</th>
<th>Neogen</th>
<th>R-Biopharm</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.70</td>
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<td></td>
</tr>
<tr>
<td>5.00</td>
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<td></td>
</tr>
<tr>
<td>5.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Run</td>
<td>Scenario 1</td>
<td>Scenario 2</td>
<td>Scenario 3</td>
</tr>
<tr>
<td>Uncertainty (%)</td>
<td>28</td>
<td>27</td>
<td>28</td>
</tr>
<tr>
<td>Extended Uncertainty ((\text{k}=2))</td>
<td>57</td>
<td>53</td>
<td>56</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Level (ng/g)</th>
<th>9.20</th>
<th>10.00</th>
<th>15.00</th>
</tr>
</thead>
<tbody>
<tr>
<td>Run</td>
<td>Scenario 1</td>
<td>Scenario 2</td>
<td>Scenario 3</td>
</tr>
<tr>
<td>Uncertainty (%)</td>
<td>16</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td>Extended Uncertainty ((\text{k}=2))</td>
<td>33</td>
<td>50</td>
<td>46</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Level (ng/g)</th>
<th>21.00</th>
<th>20.00</th>
<th>50.00</th>
</tr>
</thead>
<tbody>
<tr>
<td>Run</td>
<td>Scenario 1</td>
<td>Scenario 2</td>
<td>Scenario 3</td>
</tr>
<tr>
<td>Uncertainty (%)</td>
<td>28</td>
<td>25</td>
<td>24</td>
</tr>
<tr>
<td>Extended Uncertainty ((\text{k}=2))</td>
<td>55</td>
<td>50</td>
<td>48</td>
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</table>
Table 9: Between-run precision (continued)

<table>
<thead>
<tr>
<th>Level (ng/g)</th>
<th>Romerlabs</th>
<th>Tecna</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Run</td>
<td>Scenario 1</td>
<td>Scenario 2</td>
</tr>
<tr>
<td>Uncertainty (%)</td>
<td>16</td>
<td>18</td>
</tr>
<tr>
<td>Extended Uncertainty (% k=2)</td>
<td>31</td>
<td>37</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Level (ng/g)</th>
<th>Romerlabs</th>
<th>Tecna</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Run</td>
<td>Scenario 1</td>
<td>Scenario 2</td>
</tr>
<tr>
<td>Uncertainty (%)</td>
<td>18</td>
<td>12</td>
</tr>
<tr>
<td>Extended Uncertainty (% k=2)</td>
<td>37</td>
<td>25</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Level (ng/g)</th>
<th>Romerlabs</th>
<th>Tecna</th>
</tr>
</thead>
<tbody>
<tr>
<td>60.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Run</td>
<td>Scenario 1</td>
<td>Scenario 2</td>
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<tr>
<td>Uncertainty (%)</td>
<td>17</td>
<td>26</td>
</tr>
<tr>
<td>Extended Uncertainty (% k=2)</td>
<td>35</td>
<td>52</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Level (ng/g)</th>
<th>Romerlabs</th>
<th>Tecna</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Run</td>
<td>Scenario 1</td>
<td>Scenario 2</td>
</tr>
<tr>
<td>Uncertainty (%)</td>
<td>26</td>
<td>26</td>
</tr>
<tr>
<td>Extended Uncertainty (% k=2)</td>
<td>52</td>
<td>52</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Level (ng/g)</th>
<th>Romerlabs</th>
<th>Tecna</th>
</tr>
</thead>
<tbody>
<tr>
<td>160.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Run</td>
<td>Scenario 1</td>
<td>Scenario 2</td>
</tr>
<tr>
<td>Uncertainty (%)</td>
<td>25</td>
<td>22</td>
</tr>
<tr>
<td>Extended Uncertainty (% k=2)</td>
<td>50</td>
<td>44</td>
</tr>
</tbody>
</table>

Table 10: Reproducibility

<table>
<thead>
<tr>
<th>Level (ng/g)</th>
<th>Neogen</th>
<th>Romerlabs</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Run</td>
<td>Scenario 1</td>
<td>Scenario 2</td>
</tr>
<tr>
<td>Uncertainty (%)</td>
<td>21</td>
<td>23</td>
</tr>
<tr>
<td>Extended Uncertainty (% k=2)</td>
<td>42</td>
<td>46</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Level (ng/g)</th>
<th>Neogen</th>
<th>Romerlabs</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Run</td>
<td>Scenario 1</td>
<td>Scenario 2</td>
</tr>
<tr>
<td>Uncertainty (%)</td>
<td>20</td>
<td>18</td>
</tr>
<tr>
<td>Extended Uncertainty (% k=2)</td>
<td>40</td>
<td>36</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Level (ng/g)</th>
<th>Neogen</th>
<th>Romerlabs</th>
</tr>
</thead>
<tbody>
<tr>
<td>15.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Run</td>
<td>Scenario 1</td>
<td>Scenario 2</td>
</tr>
<tr>
<td>Uncertainty (%)</td>
<td>23</td>
<td>18</td>
</tr>
<tr>
<td>Extended Uncertainty (% k=2)</td>
<td>47</td>
<td>35</td>
</tr>
</tbody>
</table>

3.3 Accuracy & Recovery

According to document 401/2006EC, recoveries should lay within 70 and 110% of the referenced values. From Table 11 & Table 12 it can be seen that in most cases the kits fulfill this requirement. Please note that Table 12 mentions the average recovery for all matrices and might disguise large differences in recovery efficiency for different matrices.
**Table 11: Reported accuracy**

<table>
<thead>
<tr>
<th></th>
<th>Conc 1</th>
<th>Conc 2</th>
<th>Conc 3</th>
<th>Conc 4</th>
<th>Conc 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Charm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corn Meal</td>
<td>Reference value (ng/g)</td>
<td>5</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean (ng/g)</td>
<td>6</td>
<td>25</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bias (%)</td>
<td>20%</td>
<td>25%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corn Flour</td>
<td>Reference value (ng/g)</td>
<td>5</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean (ng/g)</td>
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<td>22</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bias (%)</td>
<td>0%</td>
<td>10%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corn Soy Blend</td>
<td>Reference value (ng/g)</td>
<td>5</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean (ng/g)</td>
<td>6</td>
<td>23</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bias (%)</td>
<td>20%</td>
<td>15%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milled Rice</td>
<td>Reference value (ng/g)</td>
<td>5</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean (ng/g)</td>
<td>6</td>
<td>21</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bias (%)</td>
<td>20%</td>
<td>5%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neogen</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corn Flour</td>
<td>Reference value (ng/g)</td>
<td>3.3</td>
<td>15.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean (ng/g)</td>
<td>3.3</td>
<td>15.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bias (%)</td>
<td>-34%</td>
<td>-21%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corn Meal</td>
<td>Reference value (ng/g)</td>
<td>5</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean (ng/g)</td>
<td>5</td>
<td>23</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bias (%)</td>
<td>20%</td>
<td>15%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corn/Soy Blend</td>
<td>Reference value (ng/g)</td>
<td>4.2</td>
<td>18.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean (ng/g)</td>
<td>4.2</td>
<td>18.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bias (%)</td>
<td>-16%</td>
<td>-6.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R-Biopharm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corn</td>
<td>Reference value (ng/g)</td>
<td>4.5</td>
<td>14.3</td>
<td>31.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean (ng/g)</td>
<td>4.4</td>
<td>13.6</td>
<td>26.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bias (%)</td>
<td>-2%</td>
<td>-5%</td>
<td>-16%</td>
<td></td>
</tr>
<tr>
<td>Rice</td>
<td>Reference value (ng/g)</td>
<td>2.2</td>
<td>11.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean (ng/g)</td>
<td>1.97</td>
<td>8.52</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bias (%)</td>
<td>-10%</td>
<td>-26%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rice</td>
<td>Reference value (ng/g)</td>
<td>38.8</td>
<td>142</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Mean (ng/g)</td>
<td>35.6</td>
<td>168.6</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Bias (%)</td>
<td>8.20%</td>
<td>18.70%</td>
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</tr>
<tr>
<td>Romerlabs</td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Corn</td>
<td>Reference value (ng/g)</td>
<td>10</td>
<td>20</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean (ng/g)</td>
<td>11.6</td>
<td>21.1</td>
<td>31.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bias (%)</td>
<td>16%</td>
<td>5.50%</td>
<td>6%</td>
<td></td>
</tr>
<tr>
<td>Corn (FAPAS)</td>
<td>Reference value (ng/g)</td>
<td>1.3</td>
<td>3.9</td>
<td>4.7</td>
<td>4.7</td>
</tr>
<tr>
<td></td>
<td>Mean (ng/g)</td>
<td>1.5</td>
<td>4.1</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Bias (%)</td>
<td>13%</td>
<td>5%</td>
<td>6%</td>
<td>6%</td>
</tr>
<tr>
<td>Animal Feed (FAPAS)</td>
<td>Reference value (ng/g)</td>
<td>7.3</td>
<td>11.3</td>
<td>18.1</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>Mean (ng/g)</td>
<td>8.8</td>
<td>11.1</td>
<td>16.1</td>
<td>22.2</td>
</tr>
<tr>
<td></td>
<td>Bias (%)</td>
<td>17%</td>
<td>-2%</td>
<td>-12%</td>
<td>-4%</td>
</tr>
<tr>
<td>Tecna</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Nuts (FAPAS)</td>
<td>Reference value (ng/g)</td>
<td>1.5</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean (ng/g)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bias (%)</td>
<td>0%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Figs (FAPAS)</td>
<td>Reference value (ng/g)</td>
<td>4.4</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Mean (ng/g)</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bias (%)</td>
<td>12%</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

**Table 12: Reported average recovery for reference matrix**

<table>
<thead>
<tr>
<th></th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Charm</td>
<td>110</td>
</tr>
<tr>
<td>Neogen</td>
<td>99</td>
</tr>
<tr>
<td>R-Biopharm</td>
<td>78</td>
</tr>
<tr>
<td>Romerlabs</td>
<td>107</td>
</tr>
<tr>
<td>Tecna</td>
<td>109</td>
</tr>
</tbody>
</table>
3.4 Cross-reactivity

To achieve accurate results it is imperative that immune-assay kits do not suffer from a matrix effect caused by other components than the intended analyte reacting with the antibodies as this could result in false positives. All participants reported no cross-reactivity for other components than for the 4 aflatoxins (Table 13). The kits of Charm and Tecna which are intended for aflatoxin B1 only show some cross-reactivity for the other three aflatoxins.

Table 13: Reported cross-reactivity

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Charm</th>
<th>Neogen</th>
<th>R-Biopharm</th>
<th>Romerlabs</th>
<th>Tecna</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AFB1</td>
<td>AFB2</td>
<td>AFG1</td>
<td>AFG2</td>
<td></td>
</tr>
<tr>
<td>Charm</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Neogen</td>
<td>Corn</td>
<td>100%</td>
<td>31%</td>
<td>28%</td>
<td>3.8%</td>
</tr>
<tr>
<td>R-Biopharm</td>
<td>buffer</td>
<td>100%</td>
<td>48%</td>
<td>75%</td>
<td>18%</td>
</tr>
<tr>
<td>Romerlabs</td>
<td>Corn</td>
<td>100%</td>
<td>65%</td>
<td>70%</td>
<td>42%</td>
</tr>
<tr>
<td>Tecna</td>
<td>Methanol</td>
<td>100%</td>
<td>5%</td>
<td>19%</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

3.5 Matrix applicability

In Table 14 the matrices indicated by the kit manufacturers as the reference matrices are summarised, Table 15 summarises other possible matrices for which the kit can be used.

Table 14: Reference matrices

<table>
<thead>
<tr>
<th>Target main matrix</th>
<th>Charm</th>
<th>Neogen</th>
<th>R-Biopharm</th>
<th>Romerlabs</th>
<th>Tecna</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Barley</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oats</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corn</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Rice</td>
<td>X</td>
<td></td>
<td>X</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Table 15: Other possible matrices

<table>
<thead>
<tr>
<th>Supplier</th>
<th>Matrix</th>
</tr>
</thead>
<tbody>
<tr>
<td>Charm</td>
<td>Wheat, barley, oats, corn, rice, Basmati rice, broken/brewer’s rice, corn flour, corn germ meal, corn gluten meal, corn meal, corn screenings, corn/soy blend, cracked corn, Distillers grains, Dried Distillers Grains with Solubles, flaking corn grits, milled rice, millet, popcorn, rough rice, rye, sorghum, soybeans, wheat flour</td>
</tr>
<tr>
<td>Neogen</td>
<td>Corn, Ammoniated corn, ammoniated cottonseed meal, barley, beet pulp*, coconut, copra, corn, corn bran, corn grits, corn meal, corn germ meal, corn gluten meal, corn/soy blend, corn starch, cottonseed, cottonseed meal, figs, flaxseed meal, hominy, kamut, lentils, milo, oat fiber, peanut hulls, peanuts (raw), peanuts (roasted), peanut butter, peanut meal, pet food, popcorn, pumpkin seeds, quinoa, rice, rice bran, rice hulls, soy flour, soy germ meal, soy meal, sunflower meal, wheat and wheat midds. *</td>
</tr>
<tr>
<td>R-Biopharm*</td>
<td>Corn, rice</td>
</tr>
<tr>
<td>Romerlabs</td>
<td>Corn, Almond, barley, beer, canola oil, chickpea, chili, corn bran, corn gluten meal, corn meal, corn/soy blend, cotton seed, DDGs, feed, hazelnut, peanuts, petfood, pistachio, popcorn, raisin, rice, sesame, sorghum, soybean, walnuts, wheat</td>
</tr>
<tr>
<td>Tecna</td>
<td>Corn, Animal feed, nuts, dried fruits, corn germ</td>
</tr>
</tbody>
</table>

*) More matrices are available on costumer demand
3.6  Conclusions of the administrative evaluation

From the data provided by the manufacturers it can be seen that all the kits have the necessary sensitivity and selectivity to adequately detect positive samples; the relatively high variability however might hamper their use as a reference method. The most common used reference method, HPLC-FL with IAC achieves very good precision (reproducibility of <20%) but this method is fully standardised, uses labour intensive clean-up and expensive lab equipment that cannot be used in the field. LC-MS/MS makes the use of sample clean-up obsolete and, if an isotopically labelled standard is used, achieves repeatabilities rivalling those of HPLC-FL methods but suffers from the same main drawback; it cannot be used as a field screening method.
Experimental evaluation
4.1 Materials and methods

Kit manufacturers were given the choice to send in a kit for total aflatoxin determination or one dedicated solely for aflatoxin B1. As aflatoxin B1 is the most prevalent detected mycotoxin in Europe and, more important, is the most toxic one, aflatoxin B1 will be used to screen the total aflatoxin kits. Corn and rice were chosen as the model matrices; aflatoxins are not expected to be found on cereals grown in Belgium and are mainly detected on imported cereals as corn and rice. Tecna asked not to screen rice with their kit as at the time the evaluation started it was not yet validated for this matrix. As the MRL of focus 5 ng/g was chosen (the MRL in the EU for corn & rice (Table 2)). The final concentration used was corrected to 3.1 ng/g as the purchased reference solution was lower in concentration than expected.

Compared to the evaluation of last year two points were no longer evaluated namely the concentration accuracy of the standards and the cross-reactivity in matrix. The concentration accuracy was no longer verified for two reasons (1) it is the final result in quality control materials that is important (2) some manufacturers don’t use real mycotoxins as standard but an analogue which makes it very difficult to verify the concentration by fluorescence or LC-MS. Cross-reactivity was not repeated as it yielded no extra information.

4.1.1 Equipment and materials

4.1.1.1 Kits used

The kits assessed are summarised in Table 6.

4.1.1.2 Standards and reagents

Acetonitril and methanol (HPLC-MS grade) were purchased from Biosolve (Valkenswaard, The Netherlands). Deionised water was delivered by a Milli-Q system (Millipore, MA, USA). Certified reference solutions (Table 16) AFB2, AFG1, AFG2, OTA & FB1 were purchased from Biopure (Tulln, Austria). Crystalline AFB1 and sterigmatocystin were purchased from Biopure and dissolved in ACN, pure crystalline forms of cyclopiazonic acid were also purchased from Sigma-Aldrich (Bornem, Belgium).

<table>
<thead>
<tr>
<th>Provider</th>
<th>AFB2</th>
<th>AFG1</th>
<th>AFG2</th>
<th>OTA</th>
<th>Sterigmatocystin</th>
<th>Fumonisins B1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Provider</td>
<td>Biopure</td>
<td>Biopure</td>
<td>Biopure</td>
<td>Biopure</td>
<td>Biopure</td>
<td>Biopure</td>
</tr>
<tr>
<td>μg/ml</td>
<td>0.5</td>
<td>2</td>
<td>0.5</td>
<td>10</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Quant</td>
<td>5 ml</td>
<td>5 ml</td>
<td>5 ml</td>
<td>1 ml</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Solvent</td>
<td>ACN</td>
<td>ACN</td>
<td>ACN</td>
<td>ACN</td>
<td>ACN</td>
<td>50% ACN</td>
</tr>
</tbody>
</table>

A stock solution of 10 mg of cyclopiazonic acid in 100 ml ACN in a volumetric flask was prepared and stored in the freezer at <-15°C. Daily working dilutions were prepared by diluting the stock solutions in 70% MeOH to a concentration of 100 ng/ml. This solution was used to spike the blank matrix for the recovery experiments. Successive dilutions for direct application on the kit were made in the appropriate extraction solvent.
4.1.1.3 **Read-out**

For the read-out of the ELISA kits a microplate reader model 550 of BIO-RAD (Hercules, US) was used. The read-out of Charm’s LFD was done with the proprietary LFD reader of Charm.

4.1.1.4 **Blank matrix**

Two blank matrices, one corn and one rice, were used to assess the recovery. Both were purchased in a grocery store and were determined to be blank using an LC-MS/MS based method (publication pending). For spike experiments samples were weighted in appropriate HDPE extraction tubes and spiked with the 100 ng/ml working solution and left overnight to allow the solvent to evaporate.

4.1.1.5 **Accuracy**

Contaminated matrices (quality control test materials) namely maize (TO4138) and rice (T04151) from FAPAS were used to assess the accuracy (Table 17).

Table 17: FAPAS quality control test materials

<table>
<thead>
<tr>
<th>Component</th>
<th>Assigned value (µg/kg)</th>
<th>Satisfactory range (µg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rice (T04151)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Afla B1</td>
<td>1.93</td>
<td>1.08-2.78</td>
</tr>
<tr>
<td>Afla B2</td>
<td>0.99</td>
<td>0.55-1.42</td>
</tr>
<tr>
<td>Afla G1</td>
<td>1.56</td>
<td>0.87-2.24</td>
</tr>
<tr>
<td>Afla G2</td>
<td>0.60</td>
<td>0.39-1.00</td>
</tr>
<tr>
<td>Aflatoxin (Total)</td>
<td>5.09</td>
<td>2.85-7.32</td>
</tr>
<tr>
<td><strong>Corn (T04138)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Afla B1</td>
<td>1.87</td>
<td>1.05-2.70</td>
</tr>
<tr>
<td>Afla B2</td>
<td>0.51</td>
<td>0.28-0.73</td>
</tr>
<tr>
<td>Afla G1</td>
<td>0.96</td>
<td>0.54-1.38</td>
</tr>
<tr>
<td>Afla G2</td>
<td>0.52</td>
<td>0.29-0.75</td>
</tr>
<tr>
<td>Aflatoxin (Total)</td>
<td>3.79</td>
<td>2.12-5.46</td>
</tr>
</tbody>
</table>

4.2 **Work protocols**

4.2.1 **Cross-reactivity**

4.2.1.1 **Study design**

The stock solutions of AFB1, AFB2, AFG1, AFG2, OTA, Fumonisin B1, sterigmatocystin and cyclopiazonic acid were diluted using the appropriate extraction solvent (buffer) of the kit to obtain a calibration curve. This calibration curve has an equal number of points with comparable concentrations as the kit calibrants. Dose-response curves were constructed by plotting the theoretical concentration against the relative absorbance (B/Bo).
Concentrations on the abscissa are given in ng/g of components in the cereal, not the concentration in the solvent. This permits a comparison between different kits as they do not always use the same standard concentration.

4.2.1.2  Data handling and statistical analyses

4.2.1.2.1  ELISA kits

Dose-response curves were constructed by plotting the theoretical concentration against the relative absorbance (B/Bo). From this calibration curve the concentration at which 50% reduction in signal intensity occurs is calculated. In a typical log versus logit curve of an ELISA kit this corresponds to the intercept. The relative cross-reactivity in % (CR%) can be calculated as follows:

Equation 4  \[ CR\% = \frac{IC_{50}[AFB1]}{IC_{50}[\text{component}]} \times 100 \]

With:
- \( IC_{50}[AFB1] \): concentration of AFB1 required for a 50% signal reduction of the zero standard
- \( IC_{50}[\text{component}] \): concentration of specified component required for a 50% signal reduction of the zero standard

4.2.1.2.2  LFD

As the Charm LFD reader generates directly readings in concentration (ng/g), a different approach was used. Calibration curves were constructed, the cross-reactivity was calculated by comparing the slopes.

Equation 5  \[ CR\% = \frac{\text{Slope } [\text{component}]}{\text{Slope } [AFB1]} \times 100 \]

4.2.2  Accuracy

4.2.2.1  Study design

Although the determination of accuracy and precision is not demanded by 657/2002 it is our understanding from end-users there is an interest in comparing the performance of immuno-assays and other tests.

Contaminated matrices maize (TO4138) and rice (T04151) from FAPAS as summarized in Table 17 were used to assess the accuracy by analyzing 6 samples.

4.2.2.2  Data handling and statistical analyses

The following formula was used:

Equation 6  \[ x = \frac{x \pm (t \times sd)}{\sqrt{N}} \]

With:
- \( x \): experimental mean (ng/g)

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56 Skoog, West & Holler, Fundamentals of Analytical chemistry, 7th Edition
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\textit{x}: true value (ng/g)  
\textit{t}: statistic value at 95\% confidence level for levels of probability  
\textit{N}: number of replicate measurements

4.2.3 Precision

4.2.3.1 Study design

On three different days 6 aliquots of corn and rice were spiked at the MRL (e.g. 3.1 ng/g) and analyzed. So in total 18 samples were analyzed per kit over the course of three days.

4.2.3.2 Data handling and statistical analyses

Data were compared using ANOVA with the data analysis function of Excel. In its simplest form ANOVA provides a statistical test of whether or not the means of several groups are all equal, and therefore generalizes \textit{t}-test to more than two groups. Doing multiple two-sample \textit{t}-tests would result in an increased chance of committing a type I error. Data are represented using box plots. For this reason, ANOVAs are useful in comparing two, three or more means. More information on ANOVA and box plots can be found in ref. \textsuperscript{57}.

4.2.4 Detection capability (CC\textsubscript{\beta})

4.2.4.1 Study design

For screening tests the \textbeta\ error (i.e. false compliant rate) should be \textless 5\%\textsuperscript{58}. In the case of substances with an established regulatory limit, CC\textsubscript{\beta} is the concentration at which the method is able to detect permitted limit concentrations with a statistical certainty of 1−\textbeta. In this case, CC\textsubscript{\beta} must be less than or equal to the Regulatory Limit. The protocol followed is described in: “Guidelines for the validation of screening methods for residues of veterinary medicines”\textsuperscript{58}.

4.2.4.2 Data handling and statistical analyses

In short the following procedure is used: a blank matrix (corn or rice) was spiked 20 times at 0 * MRL, 20 times at 0.5 * MRL, 20 times at 1 * MRL, and 2 times 1.5 * MRL and 2 times 2* MRL (the latter two were included to obtain an indication of the cut-off level if the kit failed for the 3 former levels. Then the lowest response in the spiked

\textsuperscript{58} Guidelines for the validation of screening methods for residues of veterinary medicines (Initial validation and transfer), Community Reference Laboratories Residues (CRLs), 2010.
samples was selected. This is defined as the Cut-Off Level, provided that the lowest response for the spiked samples does not overlap with the highest response for the blank samples.

4.2.5 Recovery

4.2.5.1 Study design

For biochemical tests (e.g. ELISA), which can bind several analytes with varying cross-reactivity’s, initial validation must be sufficient to demonstrate that all of the analytes in question (included in the scope of the method) will be reliably extracted (if necessary) and detected.

Samples spiked at different levels (0 MRL, 0.5 MRL, 1 MRL, 1.5 MRL, 2 MRL), also used in the above described experiment, covering the expected working range of the kit, will be extracted (amount as indicated within the kit protocol), one in corn and one in rice.

Table 18: Recovery according to 401/2006

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Recommended value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Recovery aflatoxins (AFB1, AFB2, AFG1, AFG2)</strong></td>
<td></td>
</tr>
<tr>
<td>&lt; 1.0 µg/kg</td>
<td>50 t/m 120 %</td>
</tr>
<tr>
<td>1-10 µg/kg</td>
<td>70 t/m 110 %</td>
</tr>
<tr>
<td>&gt; 10 µg/kg</td>
<td>80 t/m 110 %</td>
</tr>
</tbody>
</table>

4.2.5.2 Data handling and statistical analyses

The recovery is calculated is graphically depicted in Figure 4. In short, recovery was calculated as the slope of the spiked concentration to the detected concentration, described by Vogelgesang et al. and should preferably between 70 and 110% for all components as set by Commission Regulation EC/401/2006. If the method does not suffer from interference the intercept should not be distinguishable from 0. To keep the uncertainty on both intercept and slope at 5% the bonferroni joint confidence interval was set at 1-α/2 with α = 5%.

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Evaluation of immunoassay kits for aflatoxin determination in corn & rice

4.2.6 Kit protocol

In Table 19, the extraction protocols of the four kits are schematised.

Table 19: Extraction protocols

<table>
<thead>
<tr>
<th></th>
<th>Charm</th>
<th>Neogen</th>
<th>R-Biopharm</th>
<th>Romerlabs</th>
<th>Tecna</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample (g)</td>
<td>10</td>
<td>10</td>
<td>5</td>
<td>20</td>
<td>5</td>
</tr>
<tr>
<td>Extractant</td>
<td>70% MeOH</td>
<td>70% MeOH</td>
<td>70% MeOH</td>
<td>70% MeOH</td>
<td>70% MeOH</td>
</tr>
<tr>
<td>Volume extractant (ml)</td>
<td>20</td>
<td>50</td>
<td>25</td>
<td>100</td>
<td>25</td>
</tr>
<tr>
<td>Other consumables</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NaCl</td>
</tr>
<tr>
<td>Clean-up</td>
<td>Filtration (0.45μm)</td>
<td>centrifugation</td>
<td>centrifugation</td>
<td>centrifugation</td>
<td>centrifugation</td>
</tr>
<tr>
<td>Incubator</td>
<td>Heater block specific to strip, 4 strips size, timer, 10 min, 45°C</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td></td>
</tr>
<tr>
<td>Reader</td>
<td>ROSA-M Reader</td>
<td>650 nm</td>
<td>450 nm</td>
<td>450nm with 630nm differential filter</td>
<td>450 nm</td>
</tr>
<tr>
<td>Extraction</td>
<td>shaker</td>
<td>shaker</td>
<td>shaker</td>
<td>shaker</td>
<td>shaker</td>
</tr>
</tbody>
</table>
4.3 Results

4.3.1 Accuracy

The graphical representation of the accuracy results is given in Figure 5. The results for the accuracy are represented in Figure 6 & Figure 7. The results from the kits are represented by the blue spots; the middle blue spot represents the average value obtained (for 9 points at three different days). The lower and upper blue spot represent the studentized confidence limits as described in 4.2.2.2. The solid red line represents the indicated reference value of the FAPAS quality control test material, the dotted red lines represent the satisfactory range as indicated by FAPAS (see Table 17). Both figures are divided in a left (for AFB1 dedicated kits, the red lines are for aflatoxin B1 solely in the quality control test material) and a right part (total aflatoxin kits, the red lines represent the certified values for total aflatoxin).

The kits dedicated to AFB1 generated results that lay within the acceptable range indicated by FAPAS. There seems to be a tendency to slightly overestimate the AFB1 content; this could be caused by some cross-reactivity with the other three aflatoxins present in the FAPAS quality control material. Only the kit of Neogen underestimates slightly the referenced value.
Evaluation of immunoassay kits for aflatoxin determination in corn & rice

**Figure 6:** Accuracy results for corn (T04138), results in ng/g

**Figure 7:** Accuracy results for rice (T04151), results in ng/g
4.3.2 Precision

Samples were analyzed as described in 4.2.3, the results are summarized in Figure 8 to Figure 12. ANOVA was used to verify whether the means generated in each day can be considered as equal e.g. given the experimental variation there is no statistical sound reason at a preset confidence level (5%) to assume the means are not equal (p<0.05). Table 20 summarizes the results of this exercise and confirms what a visual inspection of the boxplots indicates namely that, except for the Tecna kit, the inter-day variability is large enough to generate statistically significant day to day variations. The LFD from Charm also generated p-values > 0.05 (= means are statistically indistinguishable). When looking at the boxplots of the LFD (Figure 8) it is clear that the intra-day variability is relatively high, making it impossible for the ANOVA test to detect inter-day differences. It is possible that this variability of the Charm kit is due to the fact that the dedicated LFD reader only generates integer digits and at the low levels used here this will easily result in a high apparent variability (see also discussion 4.3.4). Note also that in Figure 8 the kit generated almost consistently 4 as result for rice on day 3 hence there seems to be no boxplot.

<table>
<thead>
<tr>
<th>Table 20: Results (p-values) of ANOVA test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn</td>
</tr>
<tr>
<td>Charm</td>
</tr>
<tr>
<td>R-Biopharm</td>
</tr>
<tr>
<td>Neogen</td>
</tr>
<tr>
<td>Romerlabs</td>
</tr>
<tr>
<td>Tecna</td>
</tr>
</tbody>
</table>
Evaluation of immunoassay kits for aflatoxin determination in corn & rice

Figure 8: Precision results for Charm, results in ng/g

Figure 9: Precision results for Neogen, results in ng/g
Evaluation of immunoassay kits for aflatoxin determination in corn & rice

Figure 10: Precision results for R-Biopharm, results in ng/g

<table>
<thead>
<tr>
<th>Labels</th>
<th>Corn 1</th>
<th>Corn 2</th>
<th>Corn 3</th>
<th>Rice 1</th>
<th>Rice 2</th>
<th>Rice 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Min</td>
<td>2.18</td>
<td>2.94</td>
<td>2.70</td>
<td>2.80</td>
<td>3.26</td>
<td>2.93</td>
</tr>
<tr>
<td>Q1</td>
<td>2.23</td>
<td>2.99</td>
<td>2.93</td>
<td>3.22</td>
<td>3.63</td>
<td>3.97</td>
</tr>
<tr>
<td>Median</td>
<td>2.26</td>
<td>3.15</td>
<td>3.18</td>
<td>3.40</td>
<td>3.64</td>
<td>4.64</td>
</tr>
<tr>
<td>Max</td>
<td>2.57</td>
<td>3.57</td>
<td>3.42</td>
<td>4.00</td>
<td>3.87</td>
<td>5.46</td>
</tr>
<tr>
<td>IQR</td>
<td>0.17</td>
<td>0.33</td>
<td>0.37</td>
<td>0.51</td>
<td>0.03</td>
<td>0.91</td>
</tr>
<tr>
<td>Upper Outliers</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>1.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Lower Outliers</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Figure 11: Precision results for Romerlabs, results in ng/g

<table>
<thead>
<tr>
<th>Labels</th>
<th>Corn 1</th>
<th>Corn 2</th>
<th>Corn 3</th>
<th>Rice 1</th>
<th>Rice 2</th>
<th>Rice 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Min</td>
<td>2.97</td>
<td>2.60</td>
<td>4.41</td>
<td>3.22</td>
<td>2.91</td>
<td>2.93</td>
</tr>
<tr>
<td>Q1</td>
<td>3.42</td>
<td>3.01</td>
<td>4.77</td>
<td>3.71</td>
<td>3.04</td>
<td>3.23</td>
</tr>
<tr>
<td>Median</td>
<td>3.50</td>
<td>3.12</td>
<td>4.97</td>
<td>4.30</td>
<td>3.10</td>
<td>3.33</td>
</tr>
<tr>
<td>Q3</td>
<td>3.58</td>
<td>3.31</td>
<td>5.19</td>
<td>4.49</td>
<td>3.30</td>
<td>3.43</td>
</tr>
<tr>
<td>Max</td>
<td>3.64</td>
<td>3.54</td>
<td>6.44</td>
<td>4.60</td>
<td>4.17</td>
<td>3.47</td>
</tr>
<tr>
<td>IQR</td>
<td>0.16</td>
<td>0.30</td>
<td>0.42</td>
<td>0.79</td>
<td>0.26</td>
<td>0.20</td>
</tr>
<tr>
<td>Upper Outliers</td>
<td>0.00</td>
<td>0.00</td>
<td>1.00</td>
<td>0.00</td>
<td>1.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Lower Outliers</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>1.00</td>
</tr>
</tbody>
</table>
4.3.3 Recovery

The results of the recovery experiments are summarized in Figure 13 to Figure 16. The x-axis represents the kit identity, the y-axis the value of the intercept (Figure 13 & Figure 14) or slope (Figure 15 & Figure 16). The vertical lines belonging to each kit represents the 97.5% bonferroni confidence limit. For the intercept this should enclose 0, the slope should lie between 70% and 110%.

From Figure 13 & Figure 14 it can be seen that most kits don’t seem to suffer from any noticeable matrix effect, only the kit of Romerlabs gave a slight increased response for a blank. When looking at the slope the kit of Tecna gave a higher recovery for corn while the kit of R-Biopharm gave an increased recovery for rice. The higher recovery of Tecna might be explained by the fact that we centrifuged the samples before applying them to the kit instead of filtering them, which often gives cleaner extracts, as the manufacturer recommends in the manual. This decision was made as we had to process 64 samples on one day do determine both the recovery and the CCβ and this turned out to be not feasible with filtration.

From a food safety point of view, false negative results due to a low recovery are not acceptable. Nearly all kits fulfill this central requirement and are therefore fit-for-purpose.
Evaluation of immunoassay kits for aflatoxin determination in corn & rice

Figure 13: Intercept for corn

Figure 14: Intercept for rice
Evaluation of immunoassay kits for aflatoxin determination in corn & rice

Figure 15: Slope for corn

Figure 16: Slope for rice
4.3.4 Detection capability (CCβ)

For an immuno-assay to be used routinely as a high throughput screening method it is imperative that it can differentiate in a sufficiently reliable (e.g. $\beta<0.05$) manner between blank samples and samples that are considered to be positive. No specific protocol exists for mycotoxin dedicated immuno-assays, ref 58 gives a Guideline for the determination of CCβ of screening methods for residues of veterinary medicines and was deemed a good starting point to determine the CCβ for mycotoxin tests. In short a blank matrix has to be spiked at 0.5 * MRL (or if the kit doesn’t achieve this level, at 1 * MRL) and analysed in 20-fold together with a 20-fold analysis of the blank matrix. If the lowest signal of the spiked sample is lower than the highest level of the blank it has to be concluded that the kits is unable to differentiate between a negative and a positive sample. Figure 17 to Figure 25 represent the obtained data and are summarised in Table 21. The kit of R-Biopharm & Neogen differentiates 0 and 1.6 ng/g in both rice and corn, Tecna detects reliably 1.6 ng/g in corn, Romerlabs 1.6 in corn and 3.1 in rice. The kit of Charm works well in rice but struggles with corn. As stated earlier it is our opinion that this is due to some part to the fact this reader only generates integer digits with possible increased errors due to rounding.

<table>
<thead>
<tr>
<th></th>
<th>Corn (ng/g)</th>
<th>Rice (ng/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Charm</td>
<td>4.7</td>
<td>3.1</td>
</tr>
<tr>
<td>Neogen</td>
<td>1.6</td>
<td>1.6</td>
</tr>
<tr>
<td>R-Biopharm</td>
<td>1.6</td>
<td>1.6</td>
</tr>
<tr>
<td>Romerlabs</td>
<td>1.6</td>
<td>3.1</td>
</tr>
<tr>
<td>Tecna</td>
<td>1.6</td>
<td>NA</td>
</tr>
</tbody>
</table>

Figure 17: CCβ for Charm in corn
Evaluation of immunoassay kits for aflatoxin determination in corn & rice

Figure 18: CCβ for Charm in rice

Figure 19: CCβ for R-Biopharm in corn
Evaluation of immunoassay kits for aflatoxin determination in corn & rice

Figure 20: CCβ for R-Biopharm in rice

Figure 21: CCβ for Neogen in corn
Figure 22: CCβ for Neogen in rice

Figure 23: CCβ for Romerlabs in corn
Evaluation of immunoassay kits for aflatoxin determination in corn & rice

Figure 24: CCβ for Romerlabs in rice

Figure 25: CCβ for Tecna in corn
4.3.5 Cross-reactivity

The cross-reactivity (specificity) of the anti-bodies for other possible interferents that are often found in samples contaminated with aflatoxins was checked using solvent based calibration curves. The results are represented in Figure 26 to Figure 30 and are summarized in Table 22. The x-axis represents the amount of analyte in equivalents ng/g solid sample while the y-axis represents the results indicated by the FLD reader (Figure 26) or the standardized dose response for the ELISA kits. The result for FB1 is not indicated on the graphs as this component was spiked at a level 50 times higher than that of AFB1 (which one could expect in real life), for all kits no cross-reactivity was detected.

None of the kits reacted to any of the components other than the aflatoxins, besides AFB1 all kits reacted to the other 3 aflatoxins which given their structural similarity might be expected. The discriminating capability for the different aflatoxins between AFB1 dedicated and total aflatoxin kits is not that large.

Table 22: Cross-reactivity for 4 aflatoxins

<table>
<thead>
<tr>
<th></th>
<th>R-Biopharm</th>
<th>Neogen</th>
<th>Romerlabs</th>
<th>Tecna</th>
<th>Charm</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFB1</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>AFB2</td>
<td>55%</td>
<td>64%</td>
<td>74%</td>
<td>37%</td>
<td>50%</td>
</tr>
<tr>
<td>AFG1</td>
<td>81%</td>
<td>76%</td>
<td>64%</td>
<td>51%</td>
<td>33%</td>
</tr>
<tr>
<td>AFG2</td>
<td>35%</td>
<td>68%</td>
<td>40%</td>
<td>30%</td>
<td>17%</td>
</tr>
</tbody>
</table>

Figure 26: Cross-reactivity for Charm
Evaluation of immunoassay kits for aflatoxin determination in corn & rice

Figure 27: Cross-reactivity for R-Biopharm

Figure 28: Cross-reactivity for Neogen
Figure 29: Cross-reactivity for Romerlabs

Figure 30: Cross-reactivity for Tecna
4.4 Conclusions of the experimental evaluation

The results we obtained concord well with the data kindly provided by the kit manufacturers. The cross-reactivity, recovery & detection capability experiments clearly show that these tests possess the selectivity and sensitivity to adequately screen samples and differentiate positive from negative samples. Reassuringly, all extraction protocols (often a significant source of error) are clearly adequate for the matrices investigated here. The day to day variability on the other hand is somewhat troublesome, a point that was also discussed in the administrative evaluation.
Conclusion and future prospects
If a general conclusion has to be drawn from this exercise it essentially doesn’t differ from the conclusions drawn the past two years for the evaluation of DON and OTA kits: immuno-assays are very powerful screening tools to assess a large number of samples in a very short timescale with a minimum of effort, costly labour hours or expensive lab equipment. These kits, especially the strip type kits, are the preferred tool for in-field screening.

It is advisable to analyse samples flagged by immune-assays as positive by a more reliable (but much more expensive) reference method.

The 4 ELISA kits are very competitive in terms of detection capability and sensitivity. The LFD of Charm struggled a little bit more with corn but its strong point lies in its user friendliness: no calibration curve is needed which makes it a true “field” test.