



CODA-CERVA

Veterinary and Agrochemical Research Centre

A photograph of a laboratory setting. A person wearing a blue nitrile glove is using a pipette to transfer liquid into a multi-well plate. The background shows a laboratory bench with various glassware and equipment.

Evaluation of immunoassay kits for ochratoxin A determination in cereals



AFSCA-FAVV

Federal Agency for the Safety of the Food Chain

CODA-CERVA

VETERINARY AND AGROCHEMICAL RESEARCH CENTRE

CHEMICAL SAFETY OF THE FOOD CHAIN

UNIT OF TOXINS AND NATURAL COMPONENTS

NATIONAL REFERENCE LABORATORY ON MYCOTOXINS

Evaluation of immunoassay kits for ochratoxin A determination in cereals

June 2010

Bart Huybrechts & Emmanuel Tangni

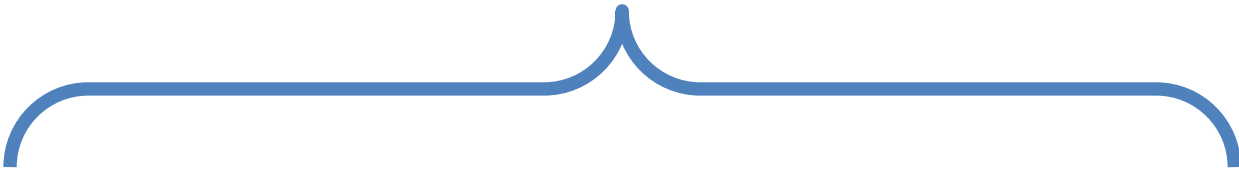
Correspondance to:

Dr. Ir. Bart Huybrechts
Leuvensesteenweg 17
B-3080 Tervuren
Belgium

Tel: +32 02 769 22 76

Fax: + 32 2 769 23 05

Bart.Huybrechts@var.fgov.be



We would like to thank the kit manufacturers; Aokin, Charm, Europroxima, LCTech, 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.




Table of Contents

1. Foreword & scope & background.....	1-1
1.1 Foreword.....	1-1
1.2 Scope.....	1-2
1.2.1 Objective and strategy of the inventory task	1-2
1.2.2 Objective and strategy of the administrative evaluation.....	1-2
1.2.3 Objective and strategy of the experimental evaluation.....	1-3
1.3 Background.....	1-3
2. Inventory.....	2-9
2.1 Inventory results of providers of OTA kits.....	2-11
3. Administrative evaluation.....	3-13
3.1 Quantitative kits.....	3-15
3.1.1 Overview of the kits	3-15
3.1.2 Quality assurance.....	3-15
3.1.3 International approval frames	3-16
3.1.4 Characterisation by appropriate analytical performance criteria	3-16
3.1.4.1 Calibration requirements.....	3-17
3.1.4.2 Matrix applicability	3-17
3.1.4.3 Limit of detection & quantification	3-18
3.1.4.4 Recovery.....	3-19
3.1.4.5 Accuracy.....	3-20
3.1.4.5.1 Accuracy and measurement uncertainties.....	3-21
3.1.4.5.2 Confirmation (ELISA/LFD versus reference values).....	3-21
3.1.4.5.2.1 Charm Science	3-22
3.1.4.5.2.1.1 Descriptive comparison.....	3-22
3.1.4.5.2.1.2 Ordinary linear regression	3-22
3.1.4.5.2.1.3 Conclusion	3-23
3.1.4.5.2.2 R-Biopharm	3-23
3.1.4.5.2.2.1 Descriptive comparison.....	3-23
3.1.4.5.2.2.2 Ordinary linear regression	3-24
3.1.4.5.2.2.3 Conclusion	3-25
3.1.4.6 Intra-run repeatability, inter-run repeatability and reproducibility.....	3-25
3.1.4.6.1 Intra-run repeatability (same batch/ operator).....	3-25
3.1.4.6.2 Inter-run repeatability (same batch/ different operator).....	3-26
3.1.4.6.3 Reproducibility (different batch/ different operator).....	3-27
3.1.4.7 Cross-reactivity.....	3-29
3.1.5 Materials, time requirements and estimated costs.....	3-29
3.2 Qualitative kits	3-30
3.2.1 Overview of the kits.....	3-30
3.2.2 Quality assurance.....	3-30
3.2.3 International approval frames	3-31
3.2.4 Characterisation by appropriate analytical performance criteria	3-31
3.2.4.1 Calibration requirements.....	3-32
3.2.4.2 Determination of unreliability region.....	3-32
3.2.4.3 Precision, sensitivity and specificity	3-32
3.2.4.4 Relative accuracy after confirmation of screening materials against reference method.....	3-33
3.2.4.5 Cross-reactivity.....	3-34
3.2.5 Materials, time requirements and estimated costs.....	3-34
3.3 Conclusions of the administrative evaluation.....	3-35
4. Experimental evaluation	4-37
4.1 Materials and methods.....	4-39
4.1.1 Kits used	4-39
4.1.2 Standards and reagents.....	4-39
4.1.3 Equipment and materials.....	4-40
4.1.3.1 Read-out.....	4-40
4.1.3.2 Matrix effect.....	4-40
4.1.3.3 Accuracy.....	4-40
4.1.3.4 Recovery.....	4-40
4.1.3.5 Standard solutions.....	4-40
4.1.4 Work packages	4-40
4.1.4.1 Work package I: Concentration accuracy of standards	4-40
4.1.4.1.1 Study design.....	4-40
4.1.4.1.2 Data handling and statistical analyses.....	4-41

4.1.4.2	Work package II: Cross reactivity.....	4-41
4.1.4.2.1	Cross reactivity in solvent.....	4-41
4.1.4.2.1.1	Study design.....	4-41
4.1.4.2.1.2	Data handling and statistical analyses.....	4-41
4.1.4.2.1.2.1	Elisa kits.....	4-41
4.1.4.2.1.2.2	LFD.....	4-41
4.1.4.2.2	Cross reactivity in matrix.....	4-41
4.1.4.2.2.1	Study design.....	4-41
4.1.4.2.2.2	Data handling and statistical analyses.....	4-42
4.1.4.3	Work package III: Matrix effect.....	4-42
4.1.4.3.1	Study design.....	4-42
4.1.4.3.2	Data handling and statistical analyses.....	4-42
4.1.4.3.2.1.1	Elisa kits.....	4-42
4.1.4.3.2.1.2	LFD.....	4-44
4.1.4.4	Work package IV: Accuracy.....	4-44
4.1.4.4.1	Study design.....	4-44
4.1.4.4.2	Data handling and statistical analyses.....	4-44
4.1.4.5	Work package V: Blank sample analysis.....	4-44
4.1.4.5.1	Study design.....	4-44
4.1.4.5.2	Data handling and statistical analyses.....	4-44
4.1.5	Extraction protocols.....	4-45
4.2	ROSA OCHRATOXIN GRAIN by CHARM.....	4-46
4.2.1	Concentration accuracy of standards.....	4-46
4.2.2	Cross-reactivity.....	4-46
4.2.2.1	Solvent.....	4-46
4.2.2.2	Matrix.....	4-47
4.2.3	Matrix effect.....	4-48
4.2.4	Accuracy.....	4-49
4.2.5	Blank sample analysis.....	4-49
4.2.6	Conclusions.....	4-50
4.3	RIDASCREEN® Ochratoxin A 30/15 by R-Biopharm.....	4-51
4.3.1	Concentration accuracy of standards.....	4-51
4.3.2	Cross-reactivity.....	4-51
4.3.2.1	Solvent.....	4-51
4.3.2.2	Matrix.....	4-52
4.3.3	Matrix effect.....	4-53
4.3.4	Accuracy.....	4-53
4.3.5	Blank sample analysis.....	4-54
4.3.6	Conclusions.....	4-54
4.4	AgraQuant® Ochratoxin by Romerlabs.....	4-55
4.4.1	Concentration accuracy of standards.....	4-55
4.4.2	Cross-reactivity.....	4-55
4.4.2.1	Solvent.....	4-55
4.4.2.2	Matrix.....	4-56
4.4.3	Matrix effect.....	4-57
4.4.4	Accuracy.....	4-57
4.4.5	Blank sample analysis.....	4-58
4.4.6	Conclusions.....	4-58
4.5	OCHRATOXIN-A ELISA by Europroxima.....	4-59
4.5.1	Concentration accuracy of standards.....	4-59
4.5.2	Cross-reactivity.....	4-59
4.5.2.1	Solvent.....	4-59
4.5.2.2	Matrix.....	4-60
4.5.3	Matrix effect.....	4-60
4.5.4	Accuracy.....	4-61
4.5.5	Blank sample analysis.....	4-62
4.5.6	Conclusions.....	4-62
4.6	Conclusions of the experimental evaluation.....	4-63
5.	Conclusion and future prospects.....	5-65

List of Figures

Figure 1: Partnership.....	1-1
Figure 2: Different forms of ochratoxin and analogues.....	1-4
Figure 3: Open form of OTA.....	1-4
Figure 4: Descriptive comparison of data in relative values obtained via Charm.....	3-22
Figure 5: Linear fit of measured OTA concentration versus reference value for Charm.....	3-23
Figure 6: Descriptive comparison of data in relative values obtained via R-Biopharm.....	3-24
Figure 7: Linear fit of measured OTA concentration versus reference value for R-Biopharm.....	3-24
Figure 8: Standard curve of OTA, OTB, OT α , Citrinin, Zearalenone, AFLA B1, Coumarin, 4-HydroxyCoumarin and L-phenylalanine (n=1) ..	4-46
Figure 9: Standard curves of ochratoxin A (ng/g) in wheat.....	4-48
Figure 10: Standard curves of ochratoxin A (ng/g) in winter barley versus control (proprietary buffer).....	4-48
Figure 11: Standard curves of ochratoxin A (ng/g) in oats.....	4-48
Figure 12: Standard curve of OTA, OTB, OT α , Citrinin, Zearalenone, AFLA B1, Coumarin, 4-HydroxyCoumarin and L-phenylalanine (n=2)	4-52
Figure 13: Standard curve of OTA, OTB, OT α , Citrinin, Zearalenone, AFLA B1, Coumarin, 4-HydroxyCoumarin and L-phenylalanine (n=2)	4-56
Figure 14: Standard curve of OTA, OTB, OT α , Citrinin, Zearalenone, AFLA B1, Coumarin, 4-HydroxyCoumarin and L-phenylalanine (n=2)	4-60

List of Tables

Table 1: Parameters for quantitative and qualitative kits.....	1-2
Table 2: Different structures of ochratoxine.....	1-4
Table 3: Overview of contacted manufacturers and their kits.....	2-11
Table 4: Overview of kits involved in the administrative evaluation.....	2-12
Table 5: Overview of kits involved in the experimental evaluation.....	2-12
Table 6: Parameters for quantitative kits.....	3-15
Table 7: Quality assurance.....	3-15
Table 8: Guidelines used for performing the validation.....	3-16
Table 9: Brief definition of various validation parameters.....	3-16
Table 10: Calculation of parameters.....	3-16
Table 11: Extend of agreement based on Lin's concordance coefficient.....	3-17
Table 12: Evaluation of the calibration curves.....	3-17
Table 13: Reference Matrices.....	3-17
Table 14: Applicable matrices.....	3-18
Table 15: LOD and LOQ for different matrices.....	3-18
Table 16: Calculation of LOD and LOQ.....	3-19
Table 17: Recovery.....	3-19
Table 18: Accuracy and measurement uncertainties.....	3-21
Table 19: Summary of the ordinary linear regression for Charm.....	3-23
Table 20: Summary of the ordinary linear regression for R-Biopharm.....	3-25
Table 21: Intra-run repeatability.....	3-26
Table 22: Inter-run repeatability.....	3-27
Table 23: Reproducibility.....	3-28
Table 24: Cross-reactivity in%.....	3-29
Table 25: Materials, time requirements and estimated costs.....	3-29
Table 26: Parameters for quantitative kits.....	3-30
Table 27: References.....	3-30
Table 28: Standards provided for verifying the test.....	3-30
Table 29: Guidelines used for performing the validation.....	3-31
Table 30: Standards.....	3-32
Table 31: Unreliability region.....	3-32
Table 32: False positives/negatives & true positives/negatives rates.....	3-32
Table 33: Calculation of false positives/negatives & true positives/negatives.....	3-33
Table 34: Contingency table.....	3-33
Table 35: Calculation of Relative Accuracy, Relative Sensitivity and Relative Specificity.....	3-33
Table 36: Relative accuracy, relative sensitivity and relative specificity.....	3-33
Table 37: Cross-reactivity in%.....	3-34
Table 38: Materials, time requirements and estimated costs.....	3-34
Table 39: Summary of kits.....	4-39
Table 40: Standard solutions.....	4-39
Table 41: Example of dilution scheme for matrix affect (calibration curve with 4 points).....	4-42
Table 42: Absorbance of the calibration curve in extraction solvent (A), undiluted matrix (B) and diluted matrix (C).....	4-43
Table 43: Extraction protocols.....	4-45
Table 44: Cross-reactivity in solvent.....	4-47

Table 45: Cross-reactivity in matrix.....	4-47
Table 46: Matrix effect.....	4-48
Table 47: Accuracy on two reference materials	4-49
Table 48: Minimum detectable limit.....	4-49
Table 49: Accuracy of calibrants.....	4-51
Table 50: Cross-reactivity in solvent	4-51
Table 51: Cross-reactivity in matrix.....	4-52
Table 52: Matrix-effect calculated as relative (IC_{50} ng/g) extinction.....	4-53
Table 53: Matrix-effect calculated as percentage color reduction to the control (%CCR)	4-53
Table 54: Accuracy on two reference materials	4-53
Table 55: Detection limit for three matrices	4-54
Table 56: Accuracy of calibrants.....	4-55
Table 57: Cross-reactivity in solvent	4-55
Table 58: Cross-reactivity in matrix.....	4-56
Table 59: Matrix-effect calculated as relative (IC_{50}) extinction.....	4-57
Table 60: Matrix-effect calculated as percentage color reduction to the control (%CCR)	4-57
Table 61: Accuracy on two reference materials	4-57
Table 62: Detection limit for three matrices	4-58
Table 63: Accuracy of calibrants.....	4-59
Table 64: Cross-reactivity in solvent	4-59
Table 65: Cross-reactivity in matrix.....	4-60
Table 66: Matrix-effect calculated as relative (IC_{50}) extinction.....	4-61
Table 67: Matrix-effect calculated as percentage color reduction to the control (%CCR)	4-61
Table 68: Accuracy on two reference materials	4-61
Table 69: Detection limit for three matrices	4-62

List of abbreviations and symbols

AC	: Relative accuracy in %	LOQ	: Limit of quantification
ACN	: Acetonitril	M	: Molar concentration
A_{control}	: Absorbance of control (pure solvent)	MeOH	: Methanol
Afla B1	: Aflatoxin B1	MS	: Mass spectrometry
AOAC International	: Association of Analytical Communities (formerly “Association of Official Analytical Chemists”)	N	: Number
Av	: Average	NS	: Not specified
B	: Absorbance	NR	: Not relevant
Bo	: Absorbance blank	NRL	: National Reference Laboratory
%CCR	: Percentage Control Color Reduction	OTA	: Ochratoxin A
CEN	: European Committee for Standardization	OTα	: Ochratoxin α
CIT	: Citrinin	OTB	: Ochratoxin B
CL	: Confidence limit	Phe	: L-phenylalanine
Cou	: Coumarin	Q1	: First quartile
CR	: Cross-reactivity in %	Q2	: Second quartile (median)
CRM	: Certified Reference Material	Q3	: Third quartile
CV	: Coefficient of Variation (in %) (equals relative standard deviation)	q_c	: Lin’s concordance coefficient
Dev	: Deviation in ng/g calculated as $X_{true} - X_{experimental}$	R²	: Coefficient of determination
Dev%	: Deviation in % calculated as $100 * (X_{true} - X_{experimental}) / X_{true}$	Rec	: Recovery in %
DF	: Degrees of freedom	SD	: Standard deviation
DON	: Deoxynivalenol	SE	: Relative Sensitivity in %
EC	: European Commission	SP	: Relative Specificity in %
ELISA	: Enzyme Linked Immuno Sorbent Assay	SPE	: Solid phase extraction
EMAN	: European Mycotoxins Awareness Network	Std	: Standard
EU	: European Union	SQR	: Square root mean
AFSCA- FAVV	: Federal Agency for the Safety of the Food Chain	t(95)	: statistic value at 95% confidence level for levels of probability
FN	: False negative	TN	: True negative
FGIS	: Federal Grain Inspection Service	TP	: True positive
FP	: False positive	u_(bias)	: Weighted average bias
FLD	: Fluorescence Light Detection	u_(comb)	: Standard combined uncertainty
FPIA	: Fluorescence Polarization Immunoassay	U_(comb)	: Expanded uncertainty
FTRT™	: Flow Through Rapid Test (trademark of Europroxima)	U_(95%)	: 95% confidence interval
GIPSA	: Grain Inspection, Packers and Stockyards Administration	UCL	: Upper Confidence Limit
HPLC	: High performance liquid chromatography	UPLC™	: Ultra Performance Liquid Chromatography™
IAC	: Immunoaffinity clean-up	USDA	: United States Department of Agriculture
IC₅₀	: Half maximal inhibitory concentration	UT	: Ultraturax™
ICH	: International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use	x_e	: Experimental mean (ng/g)
ISO	: International Organization for Standardization	x_t	: True (reference) value
4-HC	: 4-HydroxyCoumarin	ZEA	: Zearalenone
k	: Coverage factor		
LCL	: Lower Confidence Limit		
LFD	: Lateral Flow Device		
LOD	: Limit of detection		

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, repeatability, recovery, accuracy and cost level. This evaluation aimed to promote reliable fast mycotoxin measurement systems in Belgium. In 2008-2009 kits for deoxynivalenol were assessed¹, this year the evaluation was repeated for ochratoxin A.

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. For the present evaluation the focus was set on a “storage” mycotoxin: ochratoxin A. Cereals, and related products, were clearly the most interesting matrices for the Belgian market.

The evaluation was performed in three main steps: i) inventory of available rapid fast OTA 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).

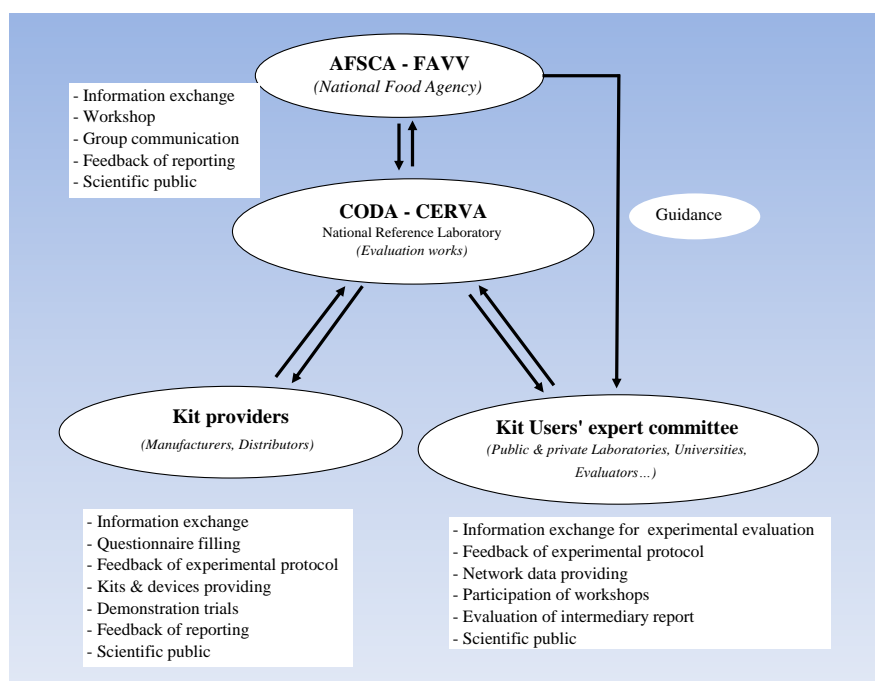


Figure 1: Partnership

¹ <http://www.var.fgov.be/>






1.2 Scope

1.2.1 Objective and strategy of the inventory task

At the present moment 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 OTA determination in cereal and cereal products
-  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 OTA kits from manufacturers through two questionnaires, one for quantitative and one for qualitative OTA 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 OTA kits.

Table 1: Parameters for quantitative and qualitative kits²

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

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.

For quantitative kits, the information was checked against the Association of Official Analytical Chemists (AOAC) requirements, the Grain Inspection, Packers and Stockyards Administration (GIPSA) of the United States Department of Agriculture (USDA/GIPSA/FGIS) directives and the EU legislation.

Regulation 1881/2006/EC³ (as amended by both Regulations 1126/2007/EC⁴ and 105/2010/EU⁵) lays down maximal limits for certain contaminants – including ochratoxin A – in specified foods, and as such determines the





² E. Trullols *Trends in Anal. Chem.* 23, 2004, 137



requirements with respect sensitivity of the analytical methods. Furthermore, Regulation 401/2006/EC¹⁰ (as amended by Regulation 178/2010/EU⁹) 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). The European Food Safety Authority (EFSA) has adopted an opinion on ochratoxin A in food on April 4th 2006⁷ and June 4th 2010⁸. The Commission is currently considering the need to introduce maximum limits for ochratoxin A in other foods not currently included in the legislation. As for qualitative kits, they were checked against the AOAC requirements and the USDA/GIPSA/FGIS directives 9181.2⁹ as well as Regulation 401/2006/EC¹⁰. Note that CEN-CR 13505:1999¹¹ and Decision 2002/657/EC¹² indicate the parameters that are relevant for screening and confirmation methods. Decision 2002/657/EC¹² 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 published¹³. 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.

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. 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 kits last year, the following problems were indicated:

-  Cross-reactivity^{49 50 51}
-  Matrix effect^{49 50 51}
-  Accuracy^{14 15 16 17}
-  Limit of detection^{14 15 16 17}

The kits of the following manufacturers were assessed: Romerlabs, Charm, EuroProxima and R-Biopharm (Table 39).

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 foodstuffs¹⁸. They are present in trace amounts so there is a need for highly sensitive and selective analytical methods for these natural toxins¹⁹. Ochratoxin A (OTA) is one of the most frequent contaminants of several foods and feeds in Europe. It is nephrotoxic and carcinogenic and poses a serious threat to the health of both humans and animals. OTA is the most commonly occurring of a

³ <http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2006:364:0005:0024:EN:PDF>

⁴ <http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2007:255:0014:0017:EN:PDF>

⁵ <http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2010:035:0007:0008:EN:PDF>

⁶ <http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2010:052:0032:0043:EN:PDF>

⁷ <http://www.efsa.europa.eu/en/scdocs/doc/365.pdf>

⁸ <http://www.efsa.europa.eu/en/scdocs/doc/1626.pdf>

⁹ http://www.gipsa.usda.gov/GIPSA/documents/GIPSA_Documents/9181-2.pdf

¹⁰ <http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2006:162:0020:0077:EN:PDF>

¹¹ <http://www.cen.eu/CEN/Pages/default.aspx>

¹² <http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2002:221:0008:0036:EN:PDF>

¹³ http://ec.europa.eu/food/food/chemicalsafety/residues/Guideline_Validation_Screening_en.pdf

¹⁴ <http://www.labcompliance.de/documents/international/ich/h-307-ich-fda-methods-terminology-ichq2a.pdf>

¹⁵ <http://www.labcompliance.de/documents/international/ich/h-308-ich-fda-methods-methodology-ichq2b.pdf>

¹⁶ <http://www.labcompliance.de/documents/FDA/FDA-Others/Laboratory/f-507-bioanalytical-4252fnl.pdf>

¹⁷ <http://www.eurachem.org/guides/valid.pdf>

¹⁸ J. W. Bennett and M. Klich, *Clin. Microbiol. Rev.*, 2003, 16, 497.

¹⁹ I. Kralj Cigic and H. Prosen, *Int. J. Mol. Sci.*, 2009, 10, 62.



group of structurally related compounds (see Figure 2, Figure 3 and Table 2)²⁰. Ochratoxin A is classified as a group 2B potential human carcinogen by the International Agency for Research on Cancer²¹. Human exposure occurs mainly through consumption of improperly stored food products, particularly contaminated grain and pork products, as well as coffee, tea, wine grapes and dried grapes. The toxin has been found in the tissues and organs of animals, including human blood and breast milk. For these reasons, the regulatory legislation of the European Union enforcing food safety is more restrictive every time²². As ochratoxin A is moderately heat stable and will survive most physical food processing to some extent, it is important that levels of ochratoxin A are kept to a minimum in cereals that may be used to produce cereal based products, such as bread, biscuits and breakfast cereals. Instrumental methods for the analysis of OTA use mainly chromatographic based techniques²³, mostly based on high pressure liquid chromatography (HPLC) assisted with fluorescence detection (FLD)^{24,25}.

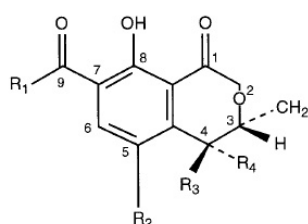


Figure 2: Different forms of ochratoxin and analogues

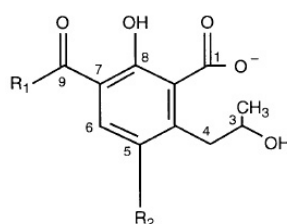


Figure 3: Open form of OTA

Table 2: Different structures of ochratoxine

NAME	ABBREVIATION	R1	R2	R3	R4
Ochratoxin A	OTA	Phenylalanine	Cl	H	H
Ochratoxin B	OTB	Phenylalanine	H	H	H
Ochratoxin C	OTC	Phenylalanine, ethylester	Cl	H	H
Ochratoxin α	OT α	OH	Cl	H	H
Ochratoxin β	OT β	OH	H	H	H
4R-HydroxyOchratoxin A	OTA-OH	Phenylalanine	Cl	H	OH
4S-HydroxyOchratoxin A	OTA-OH	Phenylalanine	Cl	OH	H
10-HydroxyOchratoxin A	OTA-OH	Phenylalanine	Cl	H	H

A number of methods have been validated by inter-laboratory collaborative studies, many of them under the auspices of international organisms such as AOAC International²⁶. Chromatographic methods generally require multiple sample preparation steps prior to detection, including extraction, sample clean up, concentration and sometimes derivatization of the analyte to improve the detection sensitivity²⁷. Particularly, sample concentration using solid phase extraction (SPE) and immunoaffinity clean-up (IAC) are very common in official methods of mycotoxin analysis using HPLC-IAC-FLD²⁸⁻³⁴, LC Coupled with electrospray ionization mass

²⁰ Weidenburner, M. (2001)- ENCYCLOPEDIA OF FOOD MYCOTOXINS. Springer-Berlin (DEU), 296p.

²¹ International Agency for Research on Cancer. Some Naturally Occurring Substances: Some Food Items and Constituents, Heterocyclic Aromatic Amines and Mycotoxins. In *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*; IARC: Lyon, France, 1993; Volume 56.

²² H. P. van Egmond, R. C. Schothorst and M. A. Jonker, *Anal. Bioanal. Chem.*, 2007, 389, 147

²³ N. W. Turner, S. Subrahmanyam and S. A. Piletsky, *Anal. Chim. Acta*, 2009, 632, 168

²⁴ E. Chiavaro, A. Lepiani, F. Colla, P. Bettoni, E. Pari and E. Spotti, *Food Addit. Contam.*, 2002, 19, 575

²⁵ G. S. Shephard, A. Fabiani, S. Stockenstrom, N. Mshicileli and V. Sewram, *J. Agric. Food. Chem.*, 2003, 51, 1102

²⁶ M. W. Trucksess, C. M. Weaver, C. J. Oles, F. S. Fry, G. O. Noonan, J. M. Betz and J. I. Pader, *J. AOAC Int.*, 2008, 91, 511

²⁷ W. C. Stephen and K. P. Kwong, *J. AOAC Int.*, 2007, 90, 773

²⁸ N. H. S. Ammida, L. Micheli, S. Piermarini, D. Moscone and G. Palleschi, *Anal. Lett.*, 2006, 39, 1559

²⁹ Visconti A., Pascale M., Centonze G., 1999. *J. Chromatogr. A*, 864, 89-101

³⁰ Leitner A., Zöllner P., Paolillo A., Stroka J., Papadopoulou-Bourauoi A., Jaborek S., Anklam E., Lindner W., 2002. Comparison of methods for the determination of ochratoxin A in wine. *Anal. Chim. Acta*, 453, 33-41.

³¹ González-Peñas E., Leache C., Viscarret M., Pérez de Obanos A., Araguás C., López de Cerain A., *J. Chromatogr. A*, 2004. 1025, 163-168

³² Sáez J.M., Medina A., Gimeno-Adelantado J.V., Mateo R., Jiménez M., *J. Chromatogr. A*, 2004. 1029, 125-133

³³ Hernández M.J., Valme M., Durán E., Guillén D., Barroso C., *Anal. Chim. Acta*, 2006, 566, 117-121.



spectrometry (MS) has detection limits comparable with those of LC–FLD and the selectivity of IAC can be achieved by tandem (MS/MS) or sequential (MSⁿ) detection. Sophisticated LC-MS/MS equipment does not require sample clean-up especially when isotope labeled internal standards are used. However, the conditions during sample preparation and chromatographic separation are often a compromise, and even LC-MS/MS is not completely devoid of matrix interferences in the form of ionization suppression. In spite of its immense potential, it will take some time for LC-MS/MS to be implemented in routine analysis because of the high investment costs. To overcome the above limitations, a variety of immunoassay methods have been developed for mycotoxins^{35 36 37 38 39} with monoclonal and polyclonal antibodies raised against the specific mycotoxins^{40 41 42}, particularly for OTA^{43 44 45}. The very selective immunological basis of such methods makes them highly specific and, therefore, less dependent on sample cleanup. So far, enzyme-linked immunosorbent assay (ELISA) is the most common immunoassay technique used in OTA analysis due to simplicity and capability for parallel analysis of multiple samples^{46 47}. They are marketed as useful tools for screening and quantification at low ng/g levels⁴⁸ and often may be used for *in situ* measurements of mycotoxin occurrences. Although beneficial in terms of speed and sensitivity, they have some drawbacks such as cross reactivity and matrix interference^{49 50 51}. These problems are compounded by the fact that a mycotoxin assay may be used with many different kinds of commodity extract, and controlling for matrix effect in one commodity by, for instance, using an antigen-free extract in the assay would only correct for the effects of that particular matrix and would likely be incorrect for others. While most grains and cereals, with a relatively moderate matrix effect, may be easily screened for ochratoxin content, commodities such as coffee, cocoa, spices, and grape derivatives such as red wine are notoriously difficult to test due to matrix interferences.

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 OTA 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 mycotoxins⁵². A number of commercial ELISA kits are available for aflatoxins, deoxynivalenol, fumonisins, ochratoxin, 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^{53 54 55}. ELISA is one of the more affordable methods for detecting mycotoxins, and has the advantage that a

³⁴ Association of Official Analytical Chemists (AOAC), 2002. Determination of ochratoxin A in wine and beer. Official Method 2001.01, AOAC International

³⁵ Desaegeer S, Sibanda L, Desmet A, Vanpeteghem C. *Int. J. Food Microbiol.* 2002; 75: 135–142.

³⁶ Bird CB, Malone B, Rice LG *et al.* *J. AOAC Int.* 2002; 85: 404–410

³⁷ Nilüfer D, Boyacıoğlu D. *J. Agric. Food Chem.* 2002; 50: 3375–3379

³⁸ Escobar A, Regueiro S. *J. Food Protect.* 2002; 65: 219–221.

³⁹ Rodríguez Velasco ML, Calonge Delso MM, Ordoñez Escudero D. *Food Addit. Contam.* 2003; 20: 276–280

⁴⁰ A. Logrieco, D. W. M. Arrigan, K. Brengel-Pesce, P. Siciliano and I. Tothill, *Food Addit. Contam.*, 2005, 22, 335

⁴¹ A. L. Sun, Q. A. Qi, Z. L. Dong and K. Z. Liang, *Sens. and Instrum. Food Qual.*, 2008, 2, 43.

⁴² I. Y. Goryacheva, S. De Saeger, S. A. Eremin and C. Van Peteghem, *Food Addit. Contam.*, 2007, 24, 1169

⁴³ A. Visconti and A. De Girolamo, *Food Addit. Contam.*, 2005, Supplement 1, 37

⁴⁴ F. Y. Yu, T. F. Chi, B. H. Liu and C. C. Su, *J. Agric. Food Chem.*, 2005, 53, 6947

⁴⁵ S. H. Alarcon, G. Pallechi, D. Compagnone, M. Pascale, A. Visconti and I. Barna-Vetró, *Talanta*, 2006, 4, 1031

⁴⁶ S. Fujii, E. Y. S. Ono, R. M. R. Ribeiro, F. Garcia Algarte, C. R. Takabayashi, T. C. R. M. Oliveira, E. N. Itano, Y. Ueno, O. Kawamura and E. Y. Hirooka, *Braz. Arch. Biol. Technol.*, 2007, 50, 349.

⁴⁷ L. Monaci and F. Palmisano, *Anal. Bioanal. Chem.*, 2004, 378, 96

⁴⁸ European Mycotoxins Awareness Network (<http://www.mycotoxins.org/>)

⁴⁹ I. Y. Goryacheva, S. De Saeger, I. S. Nesterenko, S. A. Eremin and C. Van Peteghem, *Talanta*, 2007, 72, 1230

⁵⁰ Trucksess MW, Koeltzow DE. Evaluation and application of immunochemical methods for mycotoxins in food. In: Nelson JO, Karu AE, Wong RB, eds. *Immunoanalysis of Agrochemicals in Emerging Technologies*, American Chemical Society, Washington, DC, 1995: 326–334.

⁵¹ Barna-Vetró, I., Soltó, L., Téren, J., Gyöngyösi, A., Szabo, E. and Wölfling A., Sensitive ELISA test for the determination of ochratoxin A., *J. Agric. Food Chem.*, 1996, 44, 4071–4074

⁵² Michael Z. Zheng, John L. Richard, Johann Binder, *Mycopathologia* 2006, 161: 261–273

⁵³ CAST. Management and detection of mycotoxins. In *Mycotoxins: Risks in Plant, Animal, and Human Systems*. Council for Agricultural Science and Technology: Council for Agricultural Science and Technology, Ames, IA, USA, 2003: 86–103.



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 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 results⁵⁶. 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^{57 58 59} 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 unbound and color the line red. If the sample was contaminated, the gold particles will be already bound 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 ZEA. 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.

Fluorescence polarization immunoassay (FPIA)

Most of the competitive ELISA assays are surface-based. That is, they require an antibody to be immobilized onto the surface of the wells. This is done to facilitate separation of the bound and unbound forms of the competing reagents. In typical ELISA formats the signal developed depends upon the presence of an enzymatic tracer. Generally the tracer is the mycotoxin that has been labeled with an enzyme (antibody is immobilized). Assays of this nature, which require separation of the ‘free’ and ‘bound’ tracer are termed heterogeneous and encompass the vast majority of mycotoxin immunoassays. The separation can be achieved in various ways, for instance chromatographically (as in lateral flow test strips) or washing (as in ELISAs). Fluorescence polarization immunoassay (FPIA) differs from ELISA in that it is a homogeneous assay conducted in solution phase⁶⁰. Unlike heterogeneous immunoassays, homogeneous assays do not require the separation of the free and bound tracer. It eliminates the need for additional manipulations, such as the washing steps of competitive ELISAs. When a fluorophore in solution is exposed to plane-polarized light at its excitation wavelength the resulting emission is depolarized. Because of this, the more rapid the motion of the fluorophore the more the emission is depolarized. As a relatively large molecule such as an intact IgG (MW approximately 150 kDa) binds to a small fluorophore (less than 1 kDa) the rate of the tumbling motion of the fluorophore is reduced, resulting in an increase in observed polarization. In order to make the assay specific for a mycotoxin, the mycotoxin can be covalently linked to the fluorophore to make a fluorescent

⁵⁴ Anklam E, Stroka J, Boenke A. *Food Control* 2002; 13: 173–183

⁵⁵ Seefelder W, Gossman M, Humpf HU. *J. Agric. Food Chem.* 2002; 50, 2778–2781.

⁵⁶ Steven J. Saul and , Mark Tess, Lateral Flow Quantitative Method for the Detection of Mycotoxins, Food Contaminants, Chapter 19, pp 314–319

⁵⁷ United States Patent 6.475.805

⁵⁸ United States Patent 7.097.983

⁵⁹ United States Patent 6.319.466

⁶⁰ Chris Maragos, *Toxins* 2009, 1, 196-207



tracer. In this case the tracer competes with mycotoxin (from the sample) for a limited amount of mycotoxin-specific antibody. In the absence of mycotoxin the antibody binds the tracer, restricting its motion and causing a high polarization. In the presence of mycotoxin less of the tracer is bound to the antibody and a greater fraction exists unbound in solution, where it has a lower polarization. With this format the polarization is inversely related to the mycotoxin concentration. The polarization fluorescence can be measured at equilibrium, even though it can also be measured during the reaction by monitoring the tracer-antibody system (kinetic FPIA, used by Aokin), in the latter a polarization-time kinetic curve is constructed. Kinetic FPIA offers several advantages: (a) by lowering the LOD and LOQ (b) raising the repeatability and reproducibility (c) reducing the dependence on the background signal (which does not change with time due to the dynamic character of the kinetic parameter increasing the accuracy (d) long incubation times are not necessary⁶¹.

⁶¹ Perez et al., *Journal of pharmaceutical and biomedical analysis*, 14, (1996), 917-930

Inventory



2.1 Inventory results of providers of OTA kits

Table 3 gives an overview of the kit manufactures that were contacted for the present evaluation.

Table 3: Overview of contacted manufacturers and their kits

Manufacturer	Address	Scientific contact	Web site	Kits	Range of quantification	Quan/Qual
AOKIN	Robert Rössle Str 10, Berlin D-13125 Germany,	Dr. Ursula Dahmen-Levison Chief Chemist	http://www.aokin.com/	Mycontrol OTA	0.1 – 100 ng/g	Quantitative
		Robert-Rössle-Str 10 13125 Berlin Germany				
CHARM	659 Andover Street, Lawrence, MA, USA 01743-1032	Mr. Wilbert Kokke Marketing Consultant EU	http://www.Charm.com/	ROSA Ochratoxin Grain	0 – 100 ng/g	Quantitative
		Logistiekweg 20-27 7007 CJ Doetinchem The Netherlands		ROSA Ochratoxin wine and grape juice	0 – 150 ng/g	Quantitative
DIAGNOSTIX (ThermoFisher Scientific)	2845 Argentia Road, Unit 5 Mississauga, Ontario Canada	Mr. Bob Robertson Vice President 2845 Argentia Road, Unit 5 Mississauga, Ontario Canada	http://www.diagnostix.ca/	EZ-Quant™ Ochratoxin*	Not specified	Quantitative
EUROPROXIMA	Beijenckweg 18, 6827 BN Arnhem, The Netherlands,	Dr. C.J.M. Arts Product Specialist Food Safety	http://www.europroxima.com	OCHRATOXIN-A ELISA	1.25 – 40 ng/g	Quantitative
		Beijenckweg 18 NL-6827 BN Arnhem The Netherlands		Ochratoxin A FTRT	LOD: 4 ng/g	Qualitative
NEOGEN	Cunningham Building Auchincruive, Ayr Scotland KA6 5HW United Kingdom	Rob Langley Distribution Manager	http://www.neogeneurope.com/home.html	Ochratoxin-A FTRT	Cut-off 4ng/g	Qualitative
		Cunningham Building Auchincruive, Ayr Scotland KA6 5HW United Kingdom		Veratox® For Ochratoxin	2-25 ng/g	Quantitative
LCTech	Bahnweg 41, 84405 Dorfen, Germany, wuppermann@LCTe ch.de	Mr. Hans Cornet Regional Sales Manager P.O. Box 1360 B-84403 Dorfen Germany	http://www.LCTech.de	otaREAD	0.6 (beer) – 20 (coffee) ng/g	Quantitative
R-BIOPHARM	D-64297 Darmstadt, Germany	Dr. Walter Lübbe Veterinarian An der neuen Bergstraße 17 D-64293 Darmstadt Germany	http://www.r-biopharm.com	R1311 Ridascreeen Ochratoxin A	1 - 36 ng/g	Quantitative
				R1301 Ridascreeen Ochratoxin A	1 - 50 ng/g	Quantitative
				R5402 Ridascreeen FAST Ochratoxin A	1-40 ng/g	Quantitative
				OCHRACARD	Various cut off points (matrix rdependant)	Qualitative
ROMERLABS	Technopark 1, 3430 Tulln, Austria	Mr. Gustav Kichler General Manager Technopark 1 3430 Tulln Austria	http://www.romerlabs.com/romer.htm	AgraQuant® Ochratoxin	2-40 ng/g	Quantitative
TECNALAB	Area research Park Loc. Padrciano, 99 34012 Trieste Italy	Dr. Federica Tamburlini Export Manager Area research Park Loc. Padrciano, 99 34012 Trieste Italy	http://www.tecnalab.it/itengli/sh/home	I'screen OCHRA	1-100 ng/g (cereals)	Quantitative.
TRANSIA	Dieselstrasse 9A 61239 Ober-Mörlen Germany	Mr. Klaus Jürgen Daniel General Manager	http://www.transia.de/	Ochratoxin A	Not specified	Quantitative
		Dieselstrasse 9A 61239 Ober-Mörlen Germany		Ochratoxin A membrane test	Cut-off 4ng/g	Qualitative
				Ochratoxin A of wine membrane test	Not specified	Quantitative

Of the 19 identified kits about $\frac{3}{4}$ are for quantitative determination, about $\frac{1}{4}$ or for qualitative identification.

Table 4 gives an overview of the kits for which we received an answer from the manufacturers.



Table 4: Overview of kits involved in the administrative evaluation

Kit name	Provider	ELISA	LFD	FPIA
Quantitative				
AgraQuant® Ochratoxin	Romerlabs	v	-	-
ROSA Ochratoxin Grain	Charm	-	v	-
RIDASCREEN® Ochratoxin A 30/15	R-Biopharm	v	-	-
Mycontrol OTA	Aokin	-	-	v (kinetic)
OCHRATOXIN-A Elisa	Europroxima	v	-	-
OtaREAD	LCTech GmbH	v	-	-
Qualitative				
Ochracard P48	R-Biopharm	-	v	-
Ochratoxin-A FTRT	Europroxima	-	v	-

As quantitative kits clearly dominate the market and are the most used in the field, it was decided to focus the experimental assessment on quantitative kits (Table 5).

Table 5: Overview of kits involved in the experimental evaluation

	Manufacturer	Format	Matrices	Practical use	Range
Rosa Ochratoxin Grain	Charm Sciences Europe	Lateral Flow Strips	Wheat, Barley, Malted Barley, Corn, Oats, Rice, Sorghum, Soybean, Wine	Field/lab	0-100 ng/g
OCHRATOXIN-A ELISA	EuroProxima BV	Competitive ELISA Microwells (Plates)	Cereals, feed, Coffee, wine	lab	1.25-40 ng/g
RIDASCREEN® Ochratoxin A 30/15	R-Biopharm AG	Competitive ELISA Microwells (Plates)	Cereals, feed, Beer, pig serum, coffee, wine, paprika, tissue (liver, kidney)	lab	1-36 ng/g
AgraQuant® Ochratoxin ELISA Test Kit	Romerlabs Division Holding GmbH	Competitive ELISA Microwells (Plates)	Corn, milo, barley, green coffee, wheat, soybeans, Cereal, raisin, cocoa, birdseeds, beer	lab	2-40 ng/g

Tecnalab and Transia preferred not to take part in the assessment. Diagnostix withdrew its OTA kit available on the market at that moment and planned to launch a new one the forthcoming year so they decided not to cooperate. It may be clear that the vast majority of the available kits are quantitative.

It should also be brought under the attention that Aokin will replace the kit for which a validation dossier was sent in, the data reported here are not applicable for their new kit that will be soon available.

Administrative evaluation



3.1 Quantitative kits

3.1.1 Overview of the kits

Table 6 gives an overview of the 6 quantitative kits involved in the administrative evaluation.

Table 6: Parameters for quantitative kits

Manufacturer	Kit	Formats	Matrices	Use	Range	Approval	Confidentiality	References
Aokin AG	Aokin mycontrol OTA	Kinetic FPIA	For common matrices	Lab	0.1-100 ng/g	ISO/IEC 9000/9001 (Tüf Reinland)	NS	NS
Charm Sciences Europe	ROSA OCHRATOXIN GRAIN	LFD	Wheat, Barley, Malted Barley, Corn, Oats, Rice, Sorghum, Soybean, Wine	Field/Lab	0-100 ng/g	Certificate of Conformance from USDA-GIPSA FGIS 2008-104	NS	US Patent 5.985.675 6.475.805B1
EuroProxima BV	OCHRATOXIN A ELISA	Competitive ELISA	Cereals, feed, Coffee, wine	Lab	1.25-40 ng/g	ISO/IEC 9000/9001 Bureau Veritas Certification BV	EuroProxima BV	NS
LCTech GmbH	OtaREAD	LFD	Coffee, wine	Field/Lab	0.6 ng/ml (beer) to 20 ng/g (coffee)	NS	LCTech GmbH	NS
R-Biopharm AG	RIDASCREE N® Ochratoxin A 30/15	Competitive ELISA	Cereals and feed	Lab	1-36 ng/g	ISO/IEC 9000/9001, (DQS GmbH, Frankfurt)	NS	NS
Romerlabs Division Holding GmbH	AgraQuant® Ochratoxin ELISA Test Kit	Competitive ELISA	Corn, milo, barley, green coffee, wheat, soybeans, Cereal, raisin, cocoa, birdseeds, beer	Lab	2-40 ng/g	NS	AgraQuant® Ochratoxin Test kit is proprietary of Romerlabs	Zheng et al. (2005) Mycopathologia. 159:2, 265-272

3.1.2 Quality assurance

Information on the quality control systems established by the manufacturers is presented in Table 7.

Table 7: Quality assurance

Manufacturer	Quality control	Intensity of response	Detectability	Specificity.	Sensibility	Homogeneity
Aokin AG	Internal	YES	YES	YES	YES	YES
Charm Sciences Europe	Internal	YES	YES	NO	NR	YES
EuroProxima BV	Internal/external (FAPAS)	YES	YES	YES	YES	YES
LCTech GmbH	Internal	YES	YES	NS	YES	YES
R-Biopharm AG	Internal	YES	YES	YES	YES	YES
Romerlabs Division	Internal/external (FAPAS)	YES	YES	YES	YES	NO



3.1.3 International approval frames

The compliancy to guidelines is summarised in Table 8.

Table 8: Guidelines used for performing the validation

	Aokin	Charm	EuroProxima	LCTech	R-Biopharm	Romerlabs
European Guidelines	NS	NO	YES	NS	NO	NO
AOAC Guidelines:	NS	NO	NO	NS	NO	NO
USDA/GIPSA/FGIS	NS	YES	NO	NS	NS	YES
Other Guidelines	NS	NO	NO	NS	YES (NS)	NO

3.1.4 Characterisation by appropriate analytical performance criteria

For a reliable screening analytical methods have to fulfil several critical steps of validation as presented in reference⁶², the ones focused on in this review are summarised in Table 9. A comparison of various international guidelines for analytical method validation can be found in ref⁶². Table 10 indicates how recovery, confidence intervals, standard combined uncertainty and expanded uncertainty are calculated.

Table 9: Brief definition of various validation parameters

Performance characteristics	Activity
Specificity	Ability to measure desired analyte in a complex matrix
Recovery	Amount actually extracted
Accuracy	Agreement between measured and real value
Precision	Agreement between a series of measurements
Linearity	Proportionality of measured value to concentration
Range	Interval where method is precise, accurate and linear
Detection limit (LOD)	Lowest amount of analyte that can be detected
Quantification limit (LOQ)	Lowest amount of analyte that can be quantified

Table 10: Calculation of parameters

Parameter	Equation
Recovery	Equation 1 $\text{Rec}\% = (x_c/x_s) \cdot 100$
95% confidence interval	Equation 2 $U_{(95\%)} = t_{0,95 (N-1)} \cdot \text{RSD} / \sqrt{N}$
Standard combined uncertainty	Equation 3 $u_{(\text{comb})} = \sqrt{(\text{CV}^2 + u_{(\text{bias})}^2)}$
Expanded uncertainty	Equation 4 $U_{(\text{comb})} = k \cdot u_{(\text{comb})}$
Squared root mean CV	Equation 5 $\text{SQR CV} = \sqrt{(\text{CV}_1^2 + \text{CV}_2^2)}$

⁶² Chandran S, Singh RS., Pharmazie. 2007 Jan;62(1):4-14.



To verify the agreement between the immunoassays and the reference values linear regression was used with Lin's concordance coefficient as criterion. Table 11 gives an overview of the scales used for values of the coefficient.

Table 11: Extent of agreement based on Lin's concordance coefficient⁶³

Agreement	ρ_c
Almost perfect	>0.99
Substantial	0.95-0.99
Moderate	0.90-0.95
Poor	<0.90

3.1.4.1 Calibration requirements

AOAC demands at least 5 calibration points, the blank not included. Eurachem further asks for a range of 0-150% of the intended target concentration. From Table 12 it can be seen that only Charm sciences calibration curve extends above the target sample range. Note also that we used the AOAC and Eurachem guidelines are not legal requirements to be fulfilled. It is also clear that the calibration range of all manufacturers covers the legal limits set for OTA.

Table 12: Evaluation of the calibration curves

	Aokin AG	Charm	EuroProxima	LCTech	R-Biopharm	Romerlabs
Calibration range (ng/g)	0.5-25	0- 150	1.25-40	0- 1	0-36	2-40
Range in target matrix (ng/g)	0.1-100	0-100	1.25-40	0.6-20	1-36	2-40
Number of standards	7	7	7	7	6	5
Points (ng/g)	0, 0.5, 1, 3, 6, 12, 25*	0, 1, 3, 5, 9, 20, 100	0, 0.25, 0.5, 1, 2, 4, 8*	0, 0.1, 0.2, 0.4, 0.6, 0.8, 1*	0, 0.05, 0.1, 0.3, 0.9, 1.8*	0, 2, 5, 20, 40
Calibration curves are not required to be linear	Curvilinear	Internal Reader	Linear	Cubic Spline	Cubic spline	Linear

*)have to be multiplied with appropriate dilution factor to obtain the concentration in the matrix (ng/g)

3.1.4.2 Matrix applicability

In Table 13 the matrices that the kit manufacturers indicated as the reference matrices are summarised.

As OTA maximum admissible limits are set by the EU for cereals and derivatives kits should preferably be able to handle these matrices (Table 14).

Table 13: Reference Matrices

	Aokin AG	Charm	EuroProxima	LCTech	R-Biopharm	Romerlabs
Target Matrix	Common matrices	Wheat	Cereals/feed	0.6 ng/ml (beer) to 20 ng/g (coffee)	Cereals and feed	Corn, milo, barley, green coffee, wheat, soybeans

⁶³ National Institute of Water and Atmospheric Research (<http://www.niwa.co.nz/>)



Table 14: Applicable matrices

	Aokin	Charm	EuroProxima	LCTech	R-Biopharm	Romerlabs
Wheat	✓	✓				✓
Barley		✓		✓		✓
Malted Barley		✓				
Corn		✓		✓		✓
Oats		✓				
Rice		✓				
Sorghum		✓				
Soybean		✓				✓
birdseed						✓
Cereals			✓	✓	✓	✓
Feed				✓	✓	
Milo				✓		✓
Wine		✓	✓	✓	✓	
Beer				✓	✓	✓
Roasted Coffee	✓		✓	✓	✓	
Instant Coffee				✓		
Green coffee			✓	✓		✓
Chocolate/cacao			✓			✓
Almonds				✓		
Pistachios				✓		
Pepper	✓					
Paprika					✓	
Grapes	✓					
Dried fruits				✓		
Raisin						✓
Pig serum					✓	
Tissue					✓	

3.1.4.3 Limit of detection & quantification

Table 16 summarised the calculation methods used by the kit manufacturer. The AOAC recommends using the following formulas for the calculation of the LOD and LOQ:

Equation 6 $LOD = \text{Mean value of blank matrix} + 3 SD$

Equation 7 $LOQ = \text{Mean value of blank matrix} + 10 SD$

Table 15: LOD and LOQ for different matrices

Manufacturer	Matrix	LOD (ng/g)	LOQ (ng/g)
Aokin	cereals	0.4	0.4
	coffee	0.5	0.7
	grapes	0.5	0.7
	pepper	0.5	0.7
CHARM	wheat	< 1	4.1
	barley	1	4.2
Europroxima	cereal	0.4	0.55
	wine/beer	0.6	1
	chocolate/cacao	0.8	1.1
	green coffee	1	1.2
LCTech GmbH	coffee	NS	2
	wine	NS	0.8
R-Biopharm	cereals	2.5	NS
	paprika	1.2	2
Romerlabs	cereals	< 1	< 1
	milo	3.8	4
	wheat	3.5	4.3
	barley	2.8	3.1
	soybeans	2.5	3
green coffe	3.3	3.4	



Table 16: Calculation of LOD and LOQ

		Aokin AG	Charm	EuroProxima	LCTech	R-Biopharm	Romerlabs
LOD	AOAC	v		v		v	
	Other		Matrix + 2SD				Matrix + 2SD
LOQ	AOAC	v		v		v	
	Other		Matrix + 10SD				Matrix + 3SD

Unlike for DON⁶⁴, no explicit LOD requirements are set for OTA. Evidently the LOD, and preferably also the LOQ, should be lower than the legal limits set for a certain matrix if applicable. For wheat in the EU, the limit is set at 5 ng/g for unprocessed wheat and 3 ng/g³ for flour. From Table 15 it can be seen that all kits are compliant.

3.1.4.4 Recovery

EU directives¹⁰ state that recovery should be between 70 and 110% for the concentration range 1-10 ng/g. Most kits are compliant with the limits set for OTA recovery. The error range is in general higher for lower concentrations, which is to be expected^{65 66}.

Table 17: Recovery

		Aokin					
Matrix	ng/g	Rec%	N	CV%	U _{comb k=2} (%)		
Common Matrix	2	85	3	8.2	34.2		
	5	89	3	11.2	31.4		
	10	79	3	11.4	47.8		
		Charm Sciences					
Matrix	ng/g	Rec%	N	CV%	U _{comb k=2} (%)		
Wheat	1	80	21	50	107.7		
	5	120	21	13.3	48.0		
	10	116	21	6	34.2		
		EuroProxima					
Matrix	ng/g	Rec%	N	CV%	U _{comb k=2} (%)		
Cereal	2.5	95	6	6.7	16.7		
	5	90	6	8.3	26.0		
	7	73	6	4.7	54.8		
Green Coffee	2.5	74	6	4	52.6		
	5	97	6	3	8.5		
	7	114	6	4	29.1		
Beer	2.5	79	20	5	43.2		
	5	86	6	4	29.1		
	7	93	6	3	15.2		
Chocolat	2.5	115	8	5	31.6		
	5	82	6	5	37.4		
	7	101	6	3	6.3		

⁶⁴ <http://www.gipsa.usda.gov/GIPSA/webapp?area=home&subject=lr&topic=hb-don>

⁶⁵ W. Horwitz, L.R. Kamps and K.W. Boyer, *J. Assoc. Off. Anal. Chem.*, **63**, 1344 (1980)

⁶⁶ W. Horwitz, *J. Assoc. Off. Anal. Chem.*, **86**, 109 (2003)



Table 17: Recovery

LCTech						
Matrix	ng/g	Rec%	N	CV%	U _{comb k=2} (%)	
Roasted Coffee	5	102.2	2	6.5	13.7	
	10	99.5	2	16.8	33.6	
Rice	5	119	4	12.7	45.7	
	10	108	4	17.3	38.1	
Beer	5	119.2	2	4.8	39.6	
	10	124.8	2	13.2	56.2	
Almonds	5	96.9	2	11.4	23.6	
	10	104.7	2	8.7	19.8	
Pistachio	5	131.9	2	12.8	68.7	
	10	107.5	2	0.7	15.1	
R-Biopharm						
Matrix	ng/g	Rec%	N	CV%	U _{comb k=2} (%)	
Cereal	3	105	6	13	27.9	
	5	109	6	6	21.6	
	15	100	6	7.5	15.0	
Paprika	5	86	7	15	41.0	
	10	81	7	6.6	40.2	
	20	87	7	9.5	32.2	
Romerlabs						
Matrix	ng/g	Rec%	N	CV%	U _{comb k=2} (%)	
Wheat	5	91.4	36	29.2	60.9	
	20	64.8	36	10.7	73.6	
Corn	5	91.8	90	12.8	30.4	
	10	85.3	90	8.2	33.7	
	20	85.1	90	9	34.8	
	30	86.6	90	10.3	33.8	
Milo	5	117.2	36	24	59.1	
	20	98.7	36	12.1	24.3	
Barley	5	85	36	8.5	34.5	
	20	65.1	36	5.5	70.7	
Soybeans	5	70.8	36	13.5	64.3	
	20	60.8	36	4.1	78.8	
Green Coffee	5	123.8	36	17.1	58.6	
	20	94.1	36	6.9	18.2	

3.1.4.5 Accuracy

EU directive 657/2002 demands that the accuracy should lie between -30% and +10% for a concentration range of 1-10 ng/g when analysing CRMs or spikes.



3.1.4.5.1 Accuracy and measurement uncertainties

Table 18: Accuracy and measurement uncertainties

Charm Sciences						
Matrix	True (ng/g)	Measured (ng/g)	Rec%	N	CV%	U _{comb k=2} (%)
Wheat	0	0.1	/	21	40	
	1	0.8	80.0	21	50	107.7
	5	6	120.0	21	13.33	48.1
Barley	0	0.1	/	21	20	
	3.5	4.9	140.0	21	10.2	82.6
	20	21.4	107.0	21	10.28	24.9
EuroProxima BV						
Matrix	True (ng/g)	Measured (ng/g)	Rec%	N	CV%	U _{comb k=2} (%)
Green Coffee (FAPAS)	7.53	7.7	102.3	8	2.3	6.4
	7.67	8.4	109.5	8	9.5	26.9
	6.24	7	112.2	8	12.2	34.5
Barley (FAPAS)	3.52	3.6	102.3	8	2.3	6.5
Wine (FAPAS)	1.02	1	98.0	8	2	5.6
	1.63	1.24	76.1	8	24	67.8
LCTech GmbH						
Matrix	True (ng/g)	Measured (ng/g)	Rec%	N	CV%	U _{comb k=2} (%)
Roasted Coffee (ERM)	6.1	5.5	90.2	4	6	23.0
Green Coffee (FAPAS)	9.17	7.4	80.7	4	11.4	44.8
Instant Coffee (FAPAS)	14.9	15.3	102.7	4	10.2	21.1
Coffee	15.2	18.6	122.4	3	21.6	62.2
R-Biopharm						
Matrix	True (ng/g)	Measured (ng/g)	Rec%	N	CV%	U _{comb k=2} (%)
Wheat	2	2.5	125.0	3	7	51.9
	5	5.2	104.0	3	13.9	28.9
	7.4	6.7	90.5	3	10.1	27.7
Romerlabs						
Matrix	True (ng/g)	Measured (ng/g)	Rec%	N	CV%	U _{comb k=2} (%)
Corn	5	4.59	91.8	90	12.8	30.4
	10	8.53	85.3	90	8.2	33.7
	20	17.02	85.1	90	9	34.8
	30	25.97	86.6	90	10.3	33.9

Accuracies are well within the mandatory range, only Charm for barley and LCTech for coffee bias somewhat. Expanded measurement uncertainty is in general lower than 50%, Charm is somewhat higher at 1 ng/g.

3.1.4.5.2 Confirmation (ELISA/LFD versus reference values)

For the two kit producers that provided extensive information on accuracy for cereals, the data were assessed using the following methodology;

- Descriptive comparison of data obtained via ELISA/LFD
- Ordinary linear regression with Lin's concordance coefficient



3.1.4.5.2.1 Charm Science

3.1.4.5.2.1.1 [Descriptive comparison](#)

Figure 4 summarizes the difference in relative values. When looking at the results it seems that at the legal limit samples tend to bias a little high, at high concentrations they bias a little bit low.

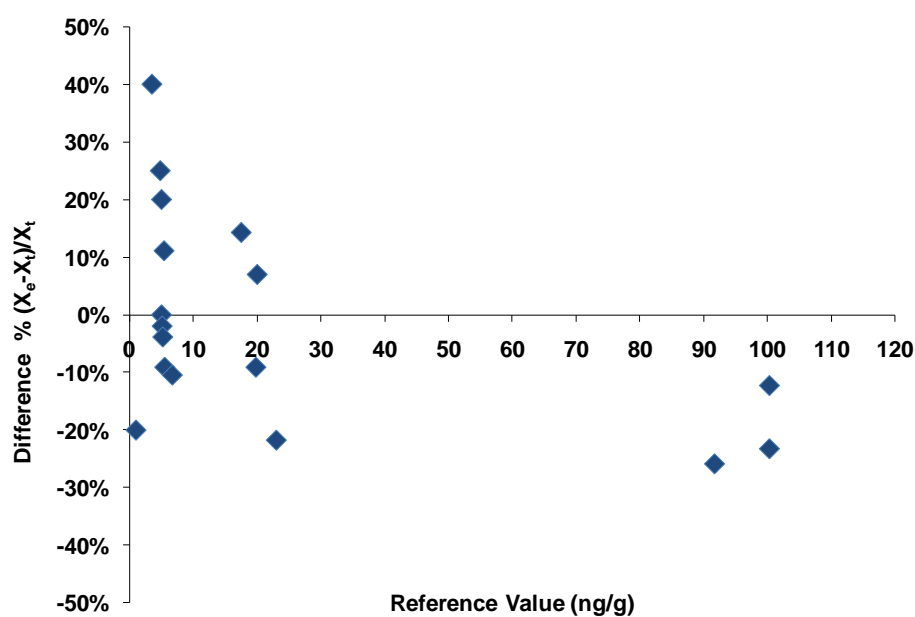


Figure 4: Descriptive comparison of data in relative values obtained via Charm

3.1.4.5.2.1.2 [Ordinary linear regression](#)

Concordance between the LFD and the reference values was checked by linear regression, results are summarised in Figure 5 and Table 19. ρ_c indicates overall good agreement.

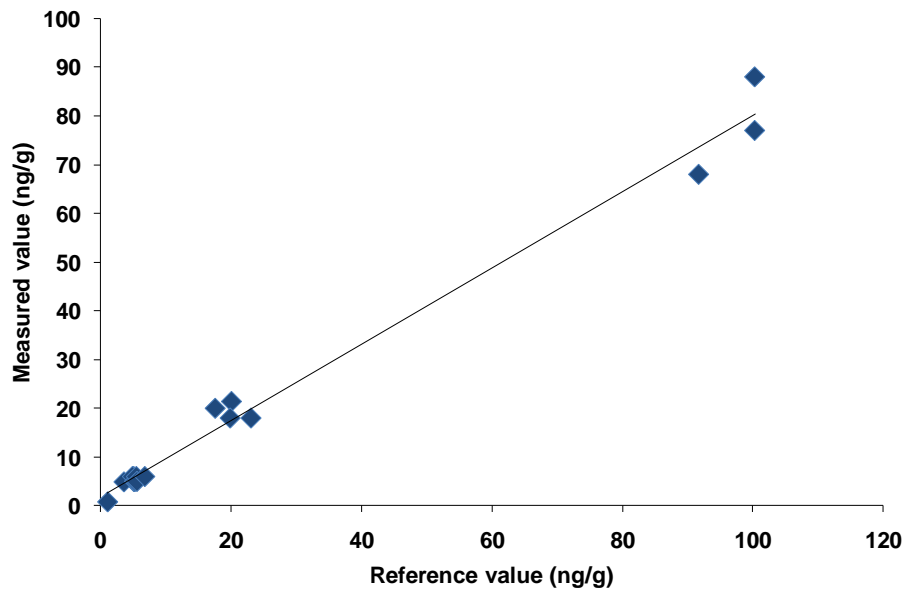


Figure 5: Linear fit of measured OTA concentration versus reference value for Charm

Table 19: Summary of the ordinary linear regression for Charm

Coefficients	
Slope	0.783
Intercept	1.837
R ²	0.988
ρ _c	0.960
T-test	0.750

3.1.4.5.2.1.3 [Conclusion](#)

An overall good agreement between the reference values is obtained, only at low or high concentrations there is some deviation.

3.1.4.5.2.2 [R-Biopharm](#)

3.1.4.5.2.2.1 [Descriptive comparison](#)

The results (Figure 6) shows random scattering around the ordinate.

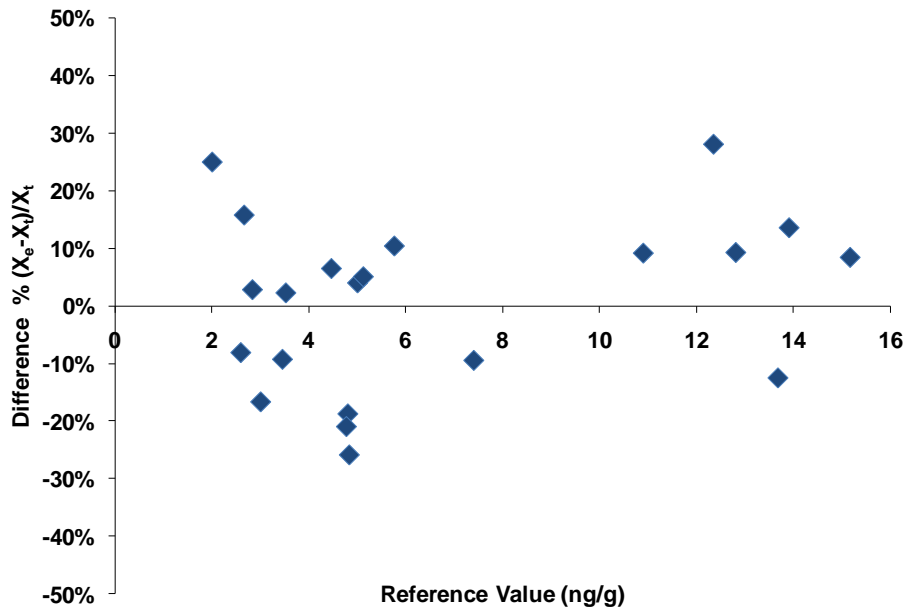


Figure 6: Descriptive comparison of data in relative values obtained via R-Biopharm

3.1.4.5.2.2.2 [Ordinary linear regression](#)

The results of the linear regression are summarised in Figure 7 and Table 20. ρ_c indicates excellent agreement.

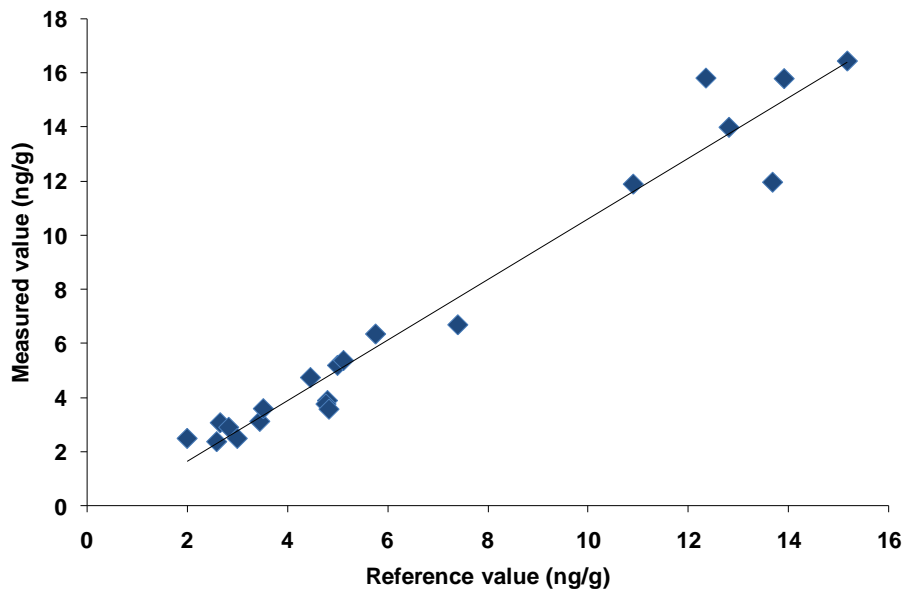


Figure 7: Linear fit of measured OTA concentration versus reference value for R-Biopharm



Table 20: Summary of the ordinary linear regression for R-Biopharm

Coefficients	
Slope	1.119
Intercept	-0.580
R ²	0.958
q _c	0.969
T-Test	0.880

3.1.4.5.2.2.3 Conclusion

An overall good agreement is seen between results obtained by R-Biopharm and the reference value. $OTA_{\text{immunoassa}}$ - $OTA_{\text{reference}}$ is not statistically dependant on OTA levels.

3.1.4.6 *Intra-run repeatability, inter-run repeatability and reproducibility*

According to directive 401/2006^{ref10} the relative standard deviation, calculated from results generated under repeatability conditions should be $\leq 20\%$ (1-10 ng/g), relative standard deviation calculated from results generated under reproducibility conditions should be $\leq 30\%$ (1-10 ng/g).

3.1.4.6.1 Intra-run repeatability (same batch/operator)

The extended measurement uncertainty is in general well below 40%, (Table 21) indicating results are reproducible. For the Charm kit it can be seen that results vary significantly at low concentration, at high concentration the kit generates very reproducible results.



Table 21: Intra-run repeatability

Charm Sciences							
Conc (ng/g)	Run	N	Rec%	Mean Rec%	CV%	SQR CV%	U _{comb k=2} (%)
1	1	21	65	88	47.5	41.7	87.1
	2	21	110		35.0		
5	1	21	100	100	11.7	12.5	25.1
	2	21	100		13.3		
10	1	21	95	89	8.0	8.9	28.0
	2	21	84		9.8		
Europroxima							
Conc (ng/g)	Run	N	Rec%	Mean Rec%	CV%	SQR CV%	U _{comb k=2} (%)
2	1	6	95	/	/	/	/
10	1	6	91	/	/	/	/
50	1	6	73	/	/	/	/
LCTech							
Conc (ng/g)	Run	N	Rec%	Mean Rec%	CV%	SQR CV%	U _{comb k=2} (%)
1.23	1	8	253	/	/	/	/
	2	8	214	/	/	/	/
5.31	1	8	137	/	/	/	/
	2	8	131	/	/	/	/
10.4	1	8	120	/	/	/	/
	2	8	125	/	/	/	/
R-Biopharm							
Conc (ng/g)	Run	N	Rec%	Mean Rec%	CV%	SQR CV%	U _{comb k=2} (%)
3	1	6	92	99	7.9	10.8	21.7
	2	6	105		13.0		
5	1	6	83	96	9.0	7.6	17.3
	2	6	109		6.0		
15	1	6	80	89	2.3	5.3	24.4
	2	6	98		7.1		
Romerlabs							
Conc (ng/g)	Run	N	Rec%	Mean Rec%	CV%	SQR CV%	U _{comb k=2} (%)
10	1	3	94	97	3.0	2.7	8.1
	2	3	101		3.0		
	3	3	96		2.0		
30	1	3	106	107	3.0	2.5	14.3
	2	3	109		1.0		
	3	3	105		3.0		

3.1.4.6.2 Inter-run repeatability (same batch/different operator)

In general the extended measurement uncertainty is below 40% (Table 22). If the measurement uncertainty is compared (Table 21 versus Table 22), the variability increases if different operators perform the test.



Table 22: Inter-run repeatability

Charm Sciences							
Conc (ng/g)	Operator	N	Rec%	Mean Rec%	CV%	SQR CV%	$U_{\text{comb } k=2} (\%)$
1	1	21	100	100	40.0	43.6	87.2
	2	21	80		50.0		
	3	21	120		40.0		
5	1	21	118	120	10.2	12.9	47.6
	2	21	120		13.3		
	3	21	122		14.8		
10	1	21	107	114	11.2	9.1	32.9
	2	21	116		6.0		
	3	21	118		9.4		
Europroxima							
Conc (ng/g)	Operator	N	Rec%	Mean Rec%	CV%	SQR CV%	$U_{\text{comb } k=2} (\%)$
2	1	6	95	92	6.7	8.5	23.4
	2	6	89		10.0		
10	1	6	91	92	3.8	5.7	19.6
	2	6	93		7.1		
50	1	6	73	79	4.7	7.3	45.4
	2	6	84		9.2		
Romerlabs							
Conc (ng/g)	Operator	N	Rec%	Mean Rec%	CV%	SQR CV%	$U_{\text{comb } k=2} (\%)$
5	1	5	91	92	21.9	19.7	42.6
	2	5	90		22.9		
	3	5	94		12.5		
20	1	5	88	85	8.7	9.3	35.1
	2	5	86		9.5		
	3	5	82		9.8		
40	1	5	87	85	7.7	10.0	36.8
	2	5	86		10.2		
	3	5	80		11.7		

3.1.4.6.3 Reproducibility (different batch/different operator)

If Table 21, Table 22 and Table 23 are compared the measurement uncertainty increases from intra-run repeatability to inter-repeatability but not significantly when inter-repeatability and reproducibility are compared. So operator variability has a relatively large effect.



Table 23: Reproducibility

Charm Sciences							
Conc (ng/g)	Batch	N	Rec%	Mean Rec%	CV%	SQR CV%	U _{comb k=2} (%)
5	1	21	100	101	16.0	14.1	28.3
	2	21	114		14.0		
	3	21	90		12.0		
Europroxima							
Conc (ng/g)	Batch	N	Rec%	Mean Rec%	CV%	SQR CV%	U _{comb k=2} (%)
2	1	8	95	95	6.9	10.1	22.5
	2	8	95		12.5		
10	1	8	91	91	3.8	4.1	19.8
	2	8	91		4.4		
50	1	8	73	73	9.4	11.3	58.6
	2	8	73		13.0		
LCTech							
Conc (ng/g)	Batch	N	Rec%	Mean Rec%	CV%	SQR CV%	U _{comb k=2} (%)
4	1	8	90	105	0.8	1.1	10.3
	2	8	110		0.8		
	3	8	115		1.6		
10	1	8	98	96	0.6	0.5	8.1
	2	8	98		0.4		
	3	8	92		0.6		
16	1	8	96	96	0.5	0.4	7.5
	2	8	91		0.4		
	3	8	101		0.2		
R-Biopharm							
Conc (ng/g)	Batch	N	Rec%	Mean Rec%	CV%	SQR CV%	U _{comb k=2} (%)
2	1	3	130	125	9	12	28
	2	4	120		8		
5	1	3	92	105	9	31	32
	2	5	112		11		
	3	4	112		26		
	4	4	104		10		
7.4	1	3	92	94	4	20	21
	2	5	93		12		
	3	4	101		13		
	4	4	91		9		
Romerlabs							
Conc (ng/g)	Batch	N	Rec%	Mean Rec%	CV%	SQR CV%	U _{comb k=2} (%)
5	1	5	91	93	21.9	17.8	38.7
	2	5	94		12.5		
10	1	5	88	85	8.7	9.3	35.5
	2	5	82		9.8		
30	1	5	80	84	7.7	9.9	38.0
	2	5	87		11.7		



3.1.4.7 Cross-reactivity

Indicated cross-reactivity for non-targeted components is summarised in Table 24. Only Charm and Romerlabs report significant false positive results for OTB. Interestingly, cross-reactivity has not been checked for other mycotoxins or non-targets.

Table 24: Cross-reactivity in%

Manufacturer	OTA	OTB	OTC	Ot α	Cou	4-HC	CIT	Phe	ZEA	AFLA B1
Aokin	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Charm	100	200	NS	NS	NS	NS	NS	NS	NS	NS
EuroProxima	100	9.3	NS	<0.1	<0.1	<0.1	NS	NS	<0.1	<0.1
LCTech	YES	YES	NS	NO	NS	NS	NO	NO	NO	NO
R-Biopharm	100	14	44	<0.1	NS	NS	NS	NS	NS	NS
Romerlabs	100	108	NS	NS	NS	NS	NS	NS	NS	NS

3.1.5 Materials, time requirements and estimated costs

Besides the evident material to perform a test (the kit itself, extraction solvent, mixer, centrifuge (or filtration paper) pipettes and a reader/incubator) no specialized equipment is required for using immunoassays. With a fast process time (< 2 hours) labor costs should be controllable with the fast kits. Only Charm communicated the indicative retail price for their kit in the Benelux but the other kits will fall in the same range.

Table 25: Materials, time requirements and estimated costs

	Charm	Europroxima	LCTech	R-Biopharm	Romerlabs
Time incubation (min)	10	45	130	45	15
Time entire test (min)	13.5	45	150	120	25
Storage T (°C)	4	2-8	4-8	4-8	2-8
Shelf life (months)	6	12	12	12	12
Sample (g)	50	2.5	20 (solid matrix) 10 ml (beer/wine)	5	20
Extractant	70% methanol	Dichloromethane	70% methanol	0.13 M NaHCO ₃	70% methanol
Volume of extractant (ml)	100	10	25-100	100	100
Other chemicals	No	n-Hexane, HCL, NaHCO ₃	Sodium chloride (special matrices), n-hexane	NS	NR
Clean-up	Filtration	Filtration Defatting hexane	Filtration	Filtration	Filtration
Incubator	Proprietary Charm	37°C	NO	No	No
Reader	Proprietary Charm	Microtiter plate reader	Microtiter plate reader	Microtiter plate reader	Microtiter plate reader
Number of wells/LFD	100	96	96	96	96
Calibrations/verifications per run	1	12	NS	12	5
Samples per run (except calibration)	One	40	NS	42	43
Costs per kit (euros)	€ 220/20 strips	NS	NS	NS	NS



3.2 Qualitative kits

3.2.1 Overview of the kits

Table 26 gives an overview of the 2 qualitative kits involved in the administrative evaluation, references are summarised in Table 27.

Table 26: Parameters for quantitative kits

Manufacturer	Kit	Contact	Approval	Format	Matrices	Use	Purpose	Cut-off
Europroxima	Ochratoxin-A FTRT	EuroProxima BV, Beijerinckweg 18, 6827 BN Arnhem, The Netherlands.	ISO9001 2000 Delivered by Bureau Veritas Certification BV Amersfoort	Flow Through	Cereals, green coffee, feed	Field & Lab	Screening	4 (ng/g)
R-Biopharm	OCHRACA RD	R-BIOPHARM AG An der neuen Bergstrass 17 64297 Darmstadt Germany	DQS GmbH	CARD	Cereal, coffee, wine, dried fruit, spices, Animal feed, cocoa, tobacco	Lab	Screening	Various cut off points depending on matrix tested

Table 27: References

Europroxima	R-Biopharm
Sibanda et al. J.Agric.Food Chem (2002), 50, 6964-6967-1602	Assessment by Nestle UK of OCHRASCAN, a Rapid Screening Test for Ochratoxin A in Coffee (External Poster)
Sibanda et al. J.Food Protection (2001), 64, 1597-1602	Development of OCHRACARD for the Determination of Ochratoxin A in Cereals, Green Coffee and Dried Fruit (Poster)
	Membrane Cards for Screening Aflatoxins & Ochratoxin A in a Range of Materials (Poster)

3.2.2 Quality assurance

Both Europroxima and R-Biopharm use an internal quality control on their kit (Table 28).

Table 28: Standards provided for verifying the test

	Internal	External	Not Specified
Europroxima	√		
R-Biopharm	√		



3.2.3 International approval frames

The compliancy to guidelines is summarised in Table 29.

Table 29: Guidelines used for performing the validation

	Europroxima	R-Biopharm
2002/657/EC	Yes	No
AOAC Guideline	Yes	No
USDA/GIPSA/FGIS	No	No
Other Guidelines	No	No

3.2.4 Characterisation by appropriate analytical performance criteria

For a reliable screening analytical methods have to fulfil several critical steps of validation as presented in reference², the ones focused on in this review are summarised in Table 9.

Precision: expressed as true and false positive (and negative) rates and it has to be taken into account that these rates are related to sensitivity and specificity.

Sensitivity: the ability of a method to detect truly positive samples as positive, so the sensitivity rate is the probability, for a given concentration, that the method will classify the test sample as positive, given that the test sample is a known positive.

Specificity: the ability of a method to detect truly negative samples as negative. In the same way, the specificity rate is the probability, for a given concentration, that the method will classify the test sample as negative, given that the test sample is a known negative.

False positive rate: the probability that a test sample is a known negative, given that the test sample has been classified as positive by the method.

False negative rate: the probability that a test sample is a known positive, given that the test sample has been classified as negative by the method.

Unreliability region: In quantitative analysis, the uncertainty is the numerical value related to the interval in which the component may be found with a given probability. However, for qualitative methods, having binary responses of the YES/NO type, there is no meaning for a number associated with the result and expressed as a semi-interval that is attached to it, so uncertainty is not expressed as a numerical value but as a region of probabilities of committing error. Moreover, following the nomenclature used until now, it corresponds to the region in which false responses are obtained (either false positive or negative). As we are dealing with a region where there are certain error probabilities, some authors prefer to call it an unreliability region rather than an uncertainty region. This region is defined by an upper and a lower concentration limit, between which the qualitative method can provide false responses. As these false responses can be either positive or negative, the upper and lower limits that define this unreliability region depend on the probability of obtaining these false responses, which is fixed by the analyst.

Cut-off value: a limiting value in the response domain, at which the decision about whether the analyte is above or below the specific concentration level must be taken. Regarding the qualitative methods with sensorial responses, this value means the concentration level where the qualitative method differentiates the samples with a certain



probability of error, usually of 5%. In the particular case of problems related to the maximum permitted level, the cut-off value is related to the sensitivity, as it corresponds to the concentration level at which the sensitivity rate is 95%, when the β -type error probability has been set at 5%.

3.2.4.1 Calibration requirements

Europroxima doesn't include a solution to verify the performance of the kit (Table 30).

Table 30: Standards

	Europroxima	R-Biopharm
Negative Control	No	No
Cut-off control	No	Yes
Multi Cut-off control	No	No

3.2.4.2 Determination of unreliability region

Table 31: Unreliability region

	Matrix	Unreliability region
Europroxima	Coffee (roasted, ground and green)	3.5-4 ng/g
R-Biopharm	Green coffee, Cereals	NS

The unreliability region for the Europroxima kit lies below the legal limit imposed for coffee (5 ng/g).

3.2.4.3 Precision, sensitivity and specificity

Table 32: False positives/negatives & true positives/negatives rates

Performance parameters	Formulas	Europroxima	R-Biopharm
Number samples	/	126	NS
False positive rate	$FP/(FP + TN)$	1.6%	NS
False negative rate	$FN/(TP + FN)$	0%	NS
Sensitivity rate	$TP/(TP + FN)$	100%	NS
Specificity rate	$TN/(TP + FP)$	97%	NS
Positive predictive value	$TP/(TP + FP)$	98%	NS
Negative predictive value	$TN/(TN + FN)$	100%	NS



Table 33: Calculation of false positives/negatives & true positives/negatives

	Samples spiked above the cut-off value	Samples spiked below the cut-off value	Total number
Positive	TP (True positive)	FP (False positive)	TP +FP
Negative	FN (False negative)	TN (True Negative)	FN + TN
Total number	TP + FN	FP + TN	N =TP + FN +FP +TN

Screening methods for OTA should be compliant to the following criteria⁶⁷:

- Sensitivity should be 100% when exceeding the action limit
- Specificity should be > 95% when exceeding the action limit

The Europroxima kit is compliant to these requirements (Table 32).

3.2.4.4 Relative accuracy after confirmation of screening materials against reference method

The Calculation of Relative Accuracy, Relative Sensitivity and Relative Specificity starting from a contingency table is summarized in Table 34 and

Table 35.

Table 34: Contingency table

	Reference method positive	Reference method negative	Total number of samples
Positive	PA (Positive agreement)	PD (Positive deviation)	PA+PD
Negative	ND (Negative deviation)	NA (Negative agreement)	ND+NA
Total number of samples	PA+ND	PD+NA	PA+NA+PD+ND

Table 35: Calculation of Relative Accuracy, Relative Sensitivity and Relative Specificity

Relative Accuracy RA (%)	Relative Sensitivity SE (%)	Relative Specificity SP (%)
$(PA+NA)/(PA+NA+PD+ND)$	$PA/(PA+ND)$	$NA/(PD+NA)$

Table 36: Relative accuracy, relative sensitivity and relative specificity

	Europroxima			R-Biopharm		
	Relative Accuracy RA (%)	Relative Sensitivity SE (%)	Relative Specificity SP (%)	Relative Accuracy RA (%)	Relative Sensitivity SE (%)	Relative Specificity SP (%)
Rye	100%	100%	100%	NS	NS	NS
Barley	100%	100%	100%	NS	NS	NS
Coffee	100%	100%	100%	NS	NS	NS
Wine	91%	100%	100%	NS	NS	NS

⁶⁷ Guidelines and acceptance criteria for analysis methods mycotoxins (DON, ZEN and OTA) in raw materials for animal feed, Productschap diervoeder (PDV), Quality series n° 96, 2004



Specific criteria for relative accuracy, relative sensitivity and relative specificity are not set by regulatory organs, but the data for the Europroxima kit (Table 36) indicate no specific problems.

3.2.4.5 Cross-reactivity

Table 37: Cross-reactivity in%

Manufacturer	OTA	OTB	OTC	Ota	Cou	4-HC	CIT	PhA	ZEA	AFLA B1
EuroProxima BV	100	9	NS	NS	NS	NS	NS	NS	NS	NS
R-Biopharm AG	100	NS	NS	<0.1	NS	NS	NS	NS	NS	NS

At $\pm 9\%$ Europroxima reports acceptable cross-reactivity for OTB.

3.2.5 Materials, time requirements and estimated costs

A summary of the required materials and reagents is given in Table 38.

Table 38: Materials, time requirements and estimated costs

	Europroxima	R-Biopharm
Time incubation (min)	5	5
Time entire test (min)	< 15	10
Storage temperature (°C)	2-8°C	2-8°C
Shelf life (months)	10	12
Sample (g)	5-50	50
Extractant	Methanol	Methanol
Volume of extractant (ml)	15	100
Other consumables	No	NS
Clean-up	No	Immunoaffinity, columns provided in kit
Incubator	Room Temperature	NS
Reader	Visual	Visual
Number of wells/LFD/FTRT	10	20
Number of runs per kit	10	20
Number of calibrations per run	NR	NR
Indicative price of incubator (euro)	NS	NS
Indicative price of reader (euro)	NS	NS
Price per kit (euro)	150-200	400



3.3 Conclusions of the administrative evaluation

The performance of ELISA and LFD kits for OTA was compared for the following parameters: concentration range, applicable matrices, limit of detection, limit of quantification, recovery, accuracy, precision, repeatability, reproducibility, measurement uncertainty and cross-reactivity. For qualitative kits, false negative/false positive rates, accuracy and cross-reactivity were compared.

From this exercise it is clear that the kits fulfil the requirements to be used as fast screening tools. In terms of purchasing cost, material and consumable requirements and labour cost they are competitive compared to the reference methods. Results are in general biased slightly positively leading to an increase in false positive rate. As samples labelled as positive are to be screened by a significantly more expensive reference method, a too high false positive result rate will compromise the cost efficiency.

Experimental evaluation



4.1 Materials and methods

4.1.1 Kits used

The following kits were assessed: AgraQuant® Ochratoxin from Romerlabs, ROSA OCHRATOXIN GRAIN by Charm, OCHRATOXIN-A ELISA by Europroxima and RIDASCREEN® Ochratoxin A 30/15 by R-Biopharm. The kit of Aokin arrived too late to be included in this assessment (Table 39).

Table 39: Summary of kits

Provider	Concerned kit	Batch	Format	Expire date
Romerlabs	AgraQuant® Ochratoxin	200831-09-04	96 wells	August, 31st 2010
Charm	ROSA OCHRATOXIN GRAIN	010C	100 strips	May 2010
Europroxima	OCHRATOXIN-A ELISA	KN6733	96 wells	February 2011
R-Biopharm	RIDASCREEN® Ochratoxin A 30/15	4209	96 wells	Octobre 2010

4.1.2 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 40) of OTA, OTB, OT α , Citrinin, Zearalenone and AFLA B1 in acetonitril were purchased from Biopure (Tulln, Austria). Unfortunately, no commercial supplier of OTC could be found. Pure crystalline forms of Coumarin, 4-HydroxyCoumarin and L-phenylalanine were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Table 40: Standard solutions

	OTA	OTB	OT α	CIT	ZEA	AFLA B1	Coumar	4-hydrCou	PhA
Provider	Biopure	Biopure	Biopure	Biopure	Biopure	Biopure	Sigma	Sigma	Sigma
$\mu\text{g/ml}$	10.01	10.3	11.9	100.3	10	2.02	/	/	/
Quant	1 ml	1 ml	1 ml	1 ml	1 ml	1 ml	/	/	/
Solvent	ACN	ACN	ACN	ACN	ACN	ACN	MeOH	MeOH	H ₂ O

Stock solutions of 800 ng/ml of OTA, OTB, OT α , Citrinin, Zearalenone and AFLA B1 were prepared in pure acetonitril and stored at 4°C. Coumarin and 4-HydroxyCoumarin were dissolved in methanol whilst L-phenylalanine was dissolved in water. The former were stored at 4°C, L-phenylalanine was aliquoted to 2 ml vials and stored at -20°C. Daily working dilutions were prepared by diluting the stock solutions in the appropriate extraction solvent of the kit to a concentration of 8 ng/ml. Successive dilutions for direct application on the kit were made in the appropriate extraction solvent.



4.1.3 Equipment and materials

4.1.3.1 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 Charms LFD was done with the proprietary LFD reader of Charm.

4.1.3.2 Matrix effect

Matrix interferences were checked with 3 blank samples of wheat, winter barley and oats (OTA content < 0.6 ppb⁶⁸ OTA as checked by HPLC-IAC-FLD). Variability in the matrix (solid matrices can be very inhomogeneous) was minimized by pooling three different extracts.

4.1.3.3 Accuracy

Contaminated matrices, all barley, at levels of 4.13 ng/g, 13.25 ng/g and 35.40 ng/g (as by HPLC-IAC-FLD) were used to assess the accuracy.

We used samples that were characterised in our laboratory using our HPLC-IAC-FLD. Unfortunately there was not enough of each sample to do all kits on the same one. So different kits might be characterised using different CRMs.

4.1.3.4 Recovery

To assess the recovery a certified reference solution at approximately 10 mg/ml OTA in acetonitril from Biopure was diluted with 50% MeOH/H₂O to obtain a working solution of \pm 500 ng/ml that was stored until use at 4°C. Whole wheat flour (used in the recovery experiments for our accredited HPLC-IAC method and having a OTA content of about 0.8 ng/ml) is spiked with an appropriate volume of working solution to obtain the desired spiking level in ng OTA per g flour. The wheat is left overnight in an open container in the dark at room temperature to allow the solution to evaporate.

4.1.3.5 Standard solutions

Verification of standard concentration was performed on a UPLCTM linked to a Quattro Premier XETM system, both by Waters (Milford, MA, USA).

4.1.4 Work packages

4.1.4.1 Work package I: Concentration accuracy of standards

4.1.4.1.1 Study design

A calibration curve with at least 6 points of OTA in 50% MeOH/water prepared from a commercially purchased reference standard (BIOPURE) will be measured on the UPLC-MS. From each kit all the calibrator solutions will be measured to determine the accuracy in OTA content of the calibrators.

⁶⁸ G. M. Wood, S. Patel b, A. C. Entwisle; A. C. Williams; A. Boenke; P. J. Farnel, Food Additives & Contaminants: Part A, Volume 14, Issue 3 April 1997, pages 237-248



4.1.4.1.2 Data handling and statistical analyses

The concentration of OTA in the internal standard included in the kit is calculated by linear regression of the area response versus concentration of the OTA standard. A t-test will be used to determine whether the concentration of the standards deviates significantly from those of the certified reference standard.

4.1.4.2 Work package II: Cross reactivity

4.1.4.2.1 Cross reactivity in solvent

4.1.4.2.1.1 Study design

The stock solutions of OTA, OTB, OT α , Citrinin, Zearalenone and AFLA B1, Coumarin, 4-HydroxyCoumarin and L-phenylalanine were diluted using the appropriate extraction solvent of the kit to obtain a calibration curve. This calibration curve has an equal number of points with comparable concentration as the calibrants for the kit. The assay procedure as described by the manufacturer was further followed.

Concentrations on the abscissa are given in ng/g concentration of OTA in the cereal, not the concentration in the solvent. This makes a comparison between different kits more easy as they do not always use the same standard concentration.

4.1.4.2.1.2 Data handling and statistical analyses

4.1.4.2.1.2.1 [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% extinction occurs is calculated. In a typical log versus logit curve of an ELISA kit this corresponds to the intercept. The relative cross-reactivity in % can be calculated as follows:

$$\text{Equation 8} \quad \text{CR} = C_{50\%[\text{OTA}]} / C_{50\%[\text{COMPONENT}]} * 100$$

With:

$C_{50\%[\text{OTA}]}$: concentration of OTA required for a 50% signal reduction of the zero standard

$C_{50\%[\text{COMPONENT}]}$: concentration of specified component required for a 50% signal reduction of the zero standard

4.1.4.2.1.2.2 [LFD](#)

As the LFD reader of Charm 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.

$$\text{Equation 9} \quad \text{CR} = \text{Slope}_{[\text{COMPONENT}]} / \text{Slope}_{[\text{OTA}]} * 100$$

4.1.4.2.2 Cross reactivity in matrix

4.1.4.2.2.1 Study design

For the components that generated a significant cross-reactivity (>15%) the exercise was repeated in matrix instead of pure extraction solvent. 3 different matrices (wheat, winter barley and oats) blank (<0.6 ng/g) for OTA as determined by HPLC were extracted according to the manufactures' guidelines (see Table 43). The stock solution of 800 ng/ml was diluted to the working solution (8 ng/ml) with the extract. Calibration curves of the components of



interest were subsequently constructed in the extracts. Besides these modifications the same protocol was used as in 4.1.4.2.1.

4.1.4.2.2.2 Data handling and statistical analyses

The same protocol was used as described in 4.1.4.2.1.2.

4.1.4.3 Work package III: Matrix effect

4.1.4.3.1 Study design

To assess the matrix effect a calibration curve of OTA is constructed in pure extraction solvent, in extract of a matrix (wheat, winter barley and oats) and in extract diluted 5 times with pure extraction solvent (see Table 41). For all kits, the same sample of wheat, winter barley and oats was used. The extracts were pooled to minimize matrix inhomogeneity.

Table 41: Example of dilution scheme for matrix affect (calibration curve with 4 points)

		Concentration 1	Concentration 2	Concentration 3	Concentration 4
Control	Water+std (9:1)				
Wheat	Undiluted Extract+Std (9:1)				
	Diluted Extract +std (9:1)				
Winter barley	Undiluted Extract+Std (9:1)				
	Diluted Extract +std (9:1)				
Oats	Undiluted Extract+Std (9:1)				
	Diluted Extract +std (9:1)				

It is very well known in the art that mycotoxin identification and quantification can be hampered by matrix effects. Immunoassays are no exception to this. One common way to overcome the detrimental effects of matrix compounds is to dilute the sample, implying the assumption that less matrix means less matrix effect. Diluting has some drawbacks: it might increase significantly the LOD and LOQ as the component of interest is also diluted. Moreover it introduces an extra manipulation step (pipetting) whose error is afterwards multiplied by the dilution factor. Matrix effects are considered to be significant when higher than 10% compared to the solvent⁶⁹.

Matrix effect may be defined as the sum of the effects of all the components in a system with the exception of the analyte to be measured⁷⁰ (see also Equation 11).

4.1.4.3.2 Data handling and statistical analyses

4.1.4.3.2.1.1 Elisa kits

Dose-response curves were constructed by plotting the theoretical concentration against the relative absorbance (B/Bo).

Two methods can be used to assess the matrix effect.

⁶⁹ Lee NA, Wang S, Allan RD, Kennedy IR. J Agric Food Chem. 2004 May 19; 52(10):2746-55.

⁷⁰ Wood WG, Matrix effects in immunoassays, Scand. J. Clin. Lab. Invest. Suppl., 1991, 205, 105



- **C50% reduction**

From this calibration curve the concentration at which 50% extinction occurs is calculated. Using the concentrations of 50% reduction of absorbance, the matrix effect in % can be calculated as follows:

Equation 10
$$CR = IC_{50[\text{Diluted matrix}]} / IC_{50[\text{Undiluted matrix}]} * 100$$

With:

$C_{50\%[\text{Undiluted matrix}]}$: concentration of OTA in undiluted matrix

$C_{50\%[\text{Diluted matrix}]}$: concentration of OTA in matrix 5 times diluted with extraction solvent

Percentage matrix effect can be calculated as:

Equation 11
$$\frac{\text{OTA in (undiluted) matrix required to induce 50\% signal reduction}}{\text{OTA in solvent (diluted matrix) required to induce 50\% signal reduction}} * 100$$

- **% Control Color Reduction**

Using the absorbance values the color reduction was calculated as follows⁷¹.

Table 42: Absorbance of the calibration curve in extraction solvent (A), undiluted matrix (B) and diluted matrix (C)

Standards						
Control	A ₀	A ₁	A ₂	A ₃	A ₄	A ₅
Undiluted	B ₀	B ₁	B ₂	B ₃	B ₄	B ₅
Diluted	C ₀	C ₁	C ₂	C ₃	C ₄	C ₅

- ❖ For undiluted extracts:

Equation 12
$$\%CR_{\text{matrix assisted}} = [(1-B_0/A_0) + (1-B_1/A_1) + (1-B_2/A_2) + (1-B_3/A_3) + (1-B_4/A_4) + (1-B_5/A_5)] * 100/6$$

- ❖ For diluted extracts:

Equation 13
$$\%CR_{\text{matrix assisted}} = [(1-C_0/A_0) + (1-C_1/A_1) + (1-C_2/A_2) + (1-C_3/A_3) + (1-C_4/A_4) + (1-C_5/A_5)] * 100/6$$

- ❖ Difference:

Equation 14
$$\text{Diff} = [(B_0/A_0 - C_0/A_0) + (B_1/A_1 - C_1/A_1) + (B_2/A_2 - C_2/A_2) + (B_3/A_3 - C_3/A_3) + (B_4/A_4 - C_4/A_4) + (B_5/A_5 - C_5/A_5)] * 100/6$$

- ❖ % Control Color Reduction:

Equation 15
$$\% \text{Control Color Reduction} = (A_{\text{control}} - \text{Diff}) / (A_{\text{control}}/100)$$

⁷¹ Sabina Cairoli, Anna Arnoldi, and Silvia Pagani, *J. Agric. Food Chem.*, 1996, 44 (12), pp 3849–3854



4.1.4.3.2.1.2 LFD

As the LFD reader of Charm generates directly readings in concentration (ng/g), a different approach was used. Calibration curves using linear regression were constructed, the matrix effect was calculated by comparing the slopes⁷².

$$\text{Equation 16} \quad \text{CR} = \text{Slope}_{[\text{Diluted matrix}]} / \text{Slope}_{[\text{Undiluted matrix}]} * 100$$

4.1.4.4 *Work package IV: Accuracy*

4.1.4.4.1 Study design

4.1.4.4.2 Data handling and statistical analyses

Samples are firstly analysed using the manufacturers protocol.

The following protocol was used⁷³.

$$\text{Equation 17} \quad x_e - x_t = (t * s_d) / \sqrt{N}$$

With:

x_e : experimental mean (ng/g)

x_t : true value (ng/g)

t: statistic value at 95% confidence level for levels of probability

N: Number of replicate measurements

4.1.4.5 *Work package V: Blank sample analysis*

4.1.4.5.1 Study design

3 extracts of blank matrices (wheat, oats, winter barley) are prepared according to the manufacturers guidelines. On three different days each extract was determined.

4.1.4.5.2 Data handling and statistical analyses

Samples are firstly analysed using the manufacturers protocol preferably at least three analyses per sample. Using a protocol described by reference⁷³ for estimation of detection limit.

⁷² Matuszewski BK. J Chromatogr B Analyt Technol Biomed Life Sci. 2006 Jan 18;830(2):293-300

⁷³ Skoog, West & Holler, Fundamentals of Analytical chemistry, 7th Edition



Equation 18
$$\Delta X_{\min} = x_e - x_t > (t * sd) * \sqrt{(N_1 + N_2) / (N_1 * N_2)}$$

With:

x_e : experimental mean (ng/g)

x_t : true value (ng/g)

sd: standard deviation (ng/g)

t: statistic value at 95% confidence level for levels of probability

N: Number of replicate measurements

4.1.5 Extraction protocols

In Table 43, the extraction protocols of the four kits are schematised.

Table 43: Extraction protocols

	Charm Sciences	Europroxima	R-Biopharm	Romerlabs
Sample	10 g ≤ sample ≤ 50 g	2.5 g	5 g	20 g
Solvent	70 % MeOH 2 (vol) on 1 (mass)	1. H ₃ P0 ₄ :10 ml + CH ₂ Cl ₂ : 10 ml 2. Evaporation 3. Reconstitution proprietary buffer 4. Defatting hexane	0.13 M NaHCO ₃ 100 ml	70 % MeOH 100 ml
Extraction	Shaking	Shaking:	Shaking	Shaking/blending
Extraction Time	2-3 min	20 min	15 min	3 min



4.2 ROSA OCHRATOXIN GRAIN by CHARM

4.2.1 Concentration accuracy of standards

Unlike the well-type ELISA kits further discussed the LFD doesn't rely on an end users defined calibration curve. The reader uses a proprietary internal calibration.

4.2.2 Cross-reactivity

4.2.2.1 Solvent

The external OTA concentration in the extraction solvent (equivalent from 0 to 8 ng/g in cereal) was used as reference value for the other compounds. Standard concentrations used are equidistant, this might seem unusual for an immunoassay but Charm's kit doesn't generate results as a logistic regression dose-response curve. Using Equation 8, the relative cross-reactivity was calculated (Table 44). The calibration curve (Figure 8) clearly shows that the kit is not able to differentiate between OTA and OTB (the dechloro analog of OTA, Table 2). The CR% based on the slope difference is marginal and Lin's concordance coefficient (ρ_c) clearly indicates that there is no statistically significant difference in reaction of OTB and OTA.

OTB can be produced by the same fungi as OTA. If it would interact with the anti-bodies in the same manner as OTA it could increase the signal (a translational bias indicated on recovery curves by an intercept statistically different from 0, non-proportional recovery will be manifested as a slope statistically different from zero) leading to an increased false positive rate. However, OTB is usually far less abundantly present in cereals than OTA⁷⁴. As quick tests are intended for fast screening to detect possible screen positive samples a (slightly!) increased response should be a manageable problem.

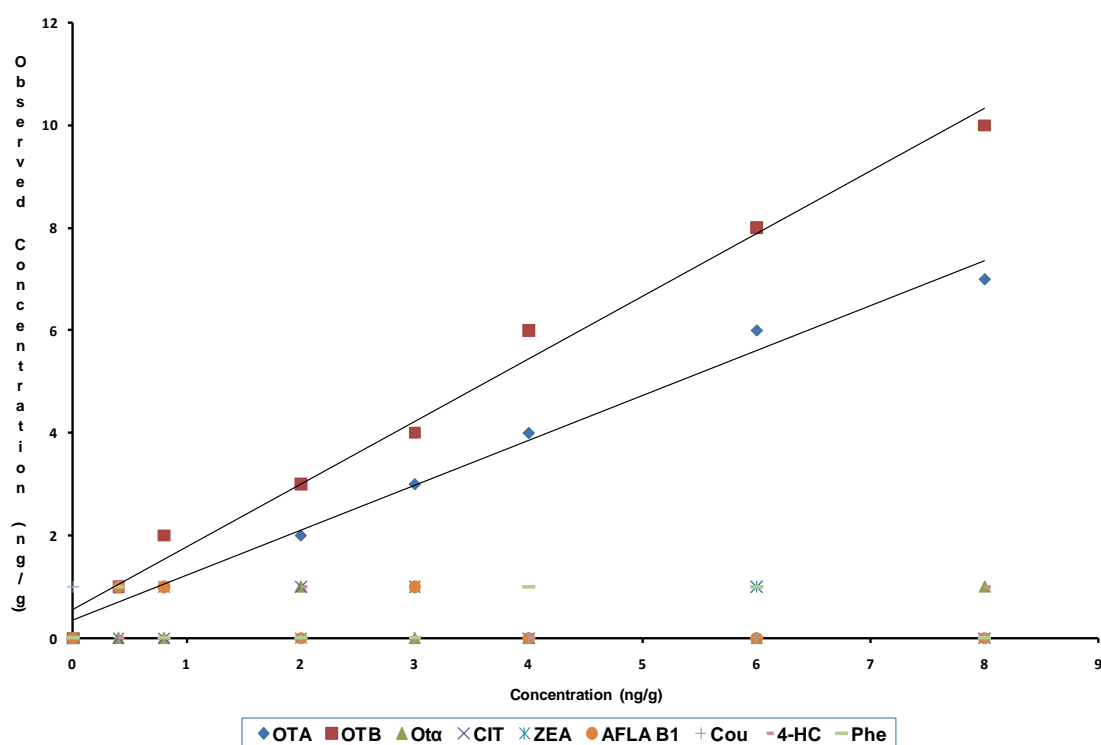


Figure 8: Standard curve of OTA, OTB, OT α , Citrinin, Zearalenone, AFLA B1, Coumarin, 4-HydroxyCoumarin and L-phenylalanine (n=1)

⁷⁴ A, Visconti and A. Bottalico, J. Agric. Food Chem, 31, 1122-1123 (1983)



Table 44: Cross-reactivity in solvent

	OTA	OTB	OT α	CIT	ZEA	AFLA B1	Cou	4-HC	Phe
SLOPE	0.876	1.222	0.070	0.034	-0.034	-0.086	-0.053	0.122	0.023
INTERCEPT	0.349	0.554	0.039	0.272	0.602	0.636	0.287	0.005	0.304
SE Intercept	0.159	0.220	NR	NR	NR	NR	NR	NR	NR
SE Slope	0.040	0.055	NR	NR	NR	NR	NR	NR	NR
R ²	0.988	0.988	0.184	0.035	0.032	0.224	0.185	0.452	0.017
CR (%)	100.00%	139%	8%	4%	-4%	-10%	-6%	14%	3%
q _c	1	0.859	0.057	0.040	0.020	0.079	0.054	0.119	0.120

Charm reported a response roughly 2 times higher for OTB than for OTA in their validation dossier, but no cross-reactivity for the other components which concurs to our findings.

4.2.2.2 Matrix

Significant cross reactivity was observed for OTB compared to OTA. The exercise was repeated for these two mycotoxins by constructing calibration curves in extracts derived from wheat, winter barley and oat. The results are summarised in Table 45.

Table 45: Cross-reactivity in matrix

	OTA				OTB			
	Solvent	Wheat	Barley	Oats	Solvent	Wheat	Barley	Oats
SLOPE	0.876	1.100	1.009	1.080	1.222	1.149	1.203	1.166
INTERCEPT	0.349	-0.577	0.322	-0.018	0.554	0.299	0.860	0.472
SE Intercept	0.159	0.304	0.325	0.261	0.220	0.317	0.538	0.657
SE Slope	0.040	0.075	0.081	0.065	0.055	0.079	0.134	0.163
R ²	0.988	0.958	0.963	0.979	0.988	0.967	0.931	0.895
CR (%)	100	126	115	123	100	94	98	95
T-TEST	1.00	0.86	0.79	0.86	1.00	0.83	0.89	0.89
LOD (ng/g)	0.60	1.14	1.06	0.80	0.59	0.97	1.48	1.86
LOQ (ng/g)	1.82	3.46	3.22	2.42	1.80	2.93	4.47	5.63

When the calibration curves in the three matrices are compared to the calibration curve for OTA or OTB there is no statistical relevant effect noticeable based on the t-test. This indicates that there is no apparent matrix effect. Note also that the cross-reactivity for OTB does not disappear when a “real-life” extract is used.

The LOD and LOQ were determined using a protocol described by the ICH using the standard deviation of the intercept of the calibration curve^{14,15}.

Equation 19 $LOD = 3.3 * (SE \text{ intercept} / \text{slope})$

Equation 20 $LOQ = 10 * (SE \text{ intercept} / \text{slope})$

For ochratoxin A they lie around 1 ng/g.



4.2.3 Matrix effect

Extracts of wheat, winter barley and oats were prepared according to the recommendations of Charm. Two calibration curves were constructed: one in the undiluted matrix and one in the 5 times diluted matrix using Charm proprietary dilution buffer. When the calibration curves are examined visually (Figure 9, Figure 10, and Figure 11) it's immediately clear there is no evident influence of the matrix.

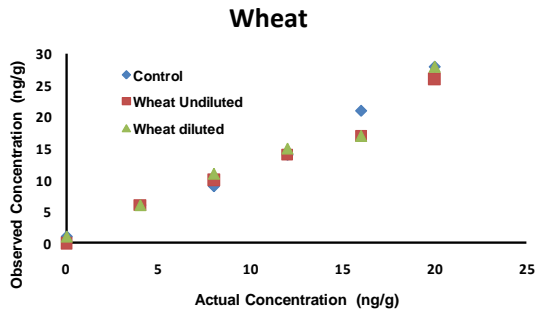


Figure 9: Standard curves of ochratoxin A (ng/g) in wheat versus control (proprietary buffer)

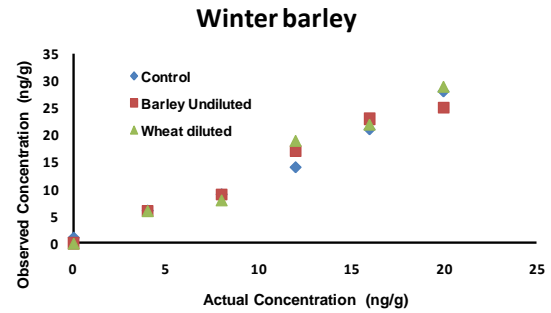


Figure 10: Standard curves of ochratoxin A (ng/g) in winter barley versus control (proprietary buffer)

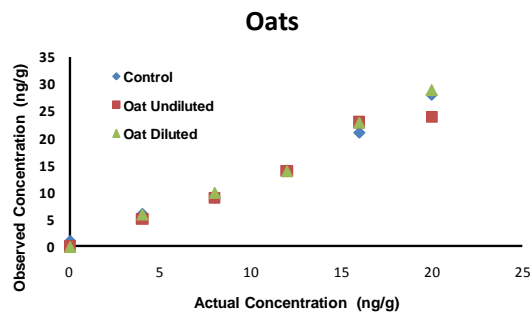


Figure 11: Standard curves of ochratoxin A (ng/g) in oats versus control (proprietary buffer)

The results in Table 46 confirm this: Lin's concordance coefficient (ρ_c) indicates there is no statistical difference between any of the calibration curves in the matrix (diluted or undiluted). Comparing the LOD/LOQ reveals no significant increase in variability.

Table 46: Matrix effect

	Contol	Wheat Undiluted	Wheat Diluted	Barley Undiluted	Barley Diluted	Oats Undiluted	Oats Diluted
SLOPE	0.66	0.60	0.61	0.66	0.73	0.64	0.71
INTERCEPT	-0.02	0.12	0.36	0.10	-0.29	-0.14	-0.31
R ²	0.98	0.97	0.96	0.98	0.97	0.97	0.98
CR%	100.0	90.27	92.97	99.46	110.27	96.76	108.11
ρ_c	1.00	0.98	0.98	0.98	0.97	0.98	0.99
LOD (ng/g)	1.42	1.62	2.14	1.41	1.61	1.60	1.32
LOQ (ng/g)	4.31	4.90	6.48	4.28	4.89	4.86	3.99



4.2.4 Accuracy

To assess the accuracy of the kit two reference materials were analyzed. The results, calculated according to the method described in ref²³, are summarized in Table 47.

Table 47: Accuracy on two reference materials

	FAPAS	BIPEA
TRUE VAL (ng/g)	4.13	13.25
N	5.00	9.00
DF	4.00	8.00
t (95)	2.78	2.31
SD (ng/g)	0.55	2.28
Q1 (ng/g)	3.00	9.00
Q3 (ng/g)	4.00	12.00
Mean (ng/g)	3.40	10.56
$x_c - x_i$ (ng/g)	-0.73	-2.69
Criterion (ng/g)	0.68	1.76

From these results it is clear that both materials are biased slightly negatively in a statistical significant manner.

4.2.5 Blank sample analysis

To assess the LOD of the kit another method using the analysis of blank samples (wheat, winter barley and oats) was used.

Blank matrices were extracted 3 times on 3 different days. These data were pooled and used to calculate the LOD according to the protocol described in 4.1.4.5.2. and are summarized in Table 48.

Table 48: Minimum detectable limit

	Wheat	Winter barley	Oats
Mean (ng/g)	0.11	0.67	0.33
N	9.00	9.00	9.00
Degrees freedom	8.00	8.00	8.00
t (95%)	2.31	2.31	2.31
SD (ng/g)	0.33	0.50	0.73
Q1 (ng/g)	0.00	0.00	0.00
Q3 (ng/g)	0.00	1.00	0.00
Δx_{min} (ng/g)	0.81	1.22	1.77
Detection limit (ng/g)	0.92	1.88	2.21

These LOD's agree well to the data obtained in 4.2.2 or those reported by Charm (LOD \approx 1 ng/g).



4.2.6 Conclusions

Cross-reactivity with OTB was observed but this should not be a problem, OTB is far less present in real-life samples and always together with OTA. Matrix effects were practically unobservable. As can be expected for an LFD, the kit was very easy to use and requires almost no experience. All together the kit is very well suited for fast and reliable screening of samples.



4.3 RIDASCREEN® Ochratoxin A 30/15 by R-Biopharm

4.3.1 Concentration accuracy of standards

The kit of R-Biopharm is supplied with 6 ready to use standard solutions with a concentration ranging from 0 (blank) to 1.8 ng/ml OTA, corresponding to a concentration of 0 to 36 ng OTA per gram cereal. The results, summarized in Table 49, don't highlight any anomalies in the standards.

Table 49: Accuracy of calibrants

Calibrant (ng/g)	LC-MS/MS (ng/g)	% Dev	T-Test
0	0.000	/	0.89226
0.05	0.069	38	
0.1	0.126	26	
0.3	0.383	28	
0.9	1.073	19	
1.8	1.846	3	

4.3.2 Cross-reactivity

4.3.2.1 Solvent

The cross-reactivity was checked on five different concentrations, corresponding to the concentration of the standards. 0.13 M NaHCO₃ in deionised water was used as extraction solvent. From Table 50 and Figure 12 it is clear that no component (other than OTA) shows significant cross-reactivity. Only for OTB and OT α there was some slight cross-reactivity, only visible at very high concentration. For the other components the IC₅₀ was virtually non-existing.

Table 50: Cross-reactivity in solvent

	OTA	OTB	OT α	CIT	ZEA	AFLA B1	COU	4-HC	Phe
Slope	-1.0454	-0.4909	-0.5535	0.2177	-0.9677	0.2592	0.1230	0.1448	-0.1356
Intercept	1.1448	1.3183	1.2364	1.2022	2.8292	1.3339	1.0879	0.9117	1.1039
R ²	0.9976	0.8922	0.8152	NR	NR	NR	NR	NR	NR
SE Slope	0.0359	0.0985	0.1522	NR	NR	NR	NR	NR	NR
SE Intercept	0.0389	0.0954	0.1474	NR	NR	NR	NR	NR	NR
IC ₅₀ (ng/g)	12.45	484.63	171.28	3,E+05	8,E+02	1,E+05	7,E+08	2,E+06	1,E+08
Relative Crossreactivity	100.0%	2.6%	7.3%	0.0%	1.5%	0.0%	0.0%	0.0%	0.0%

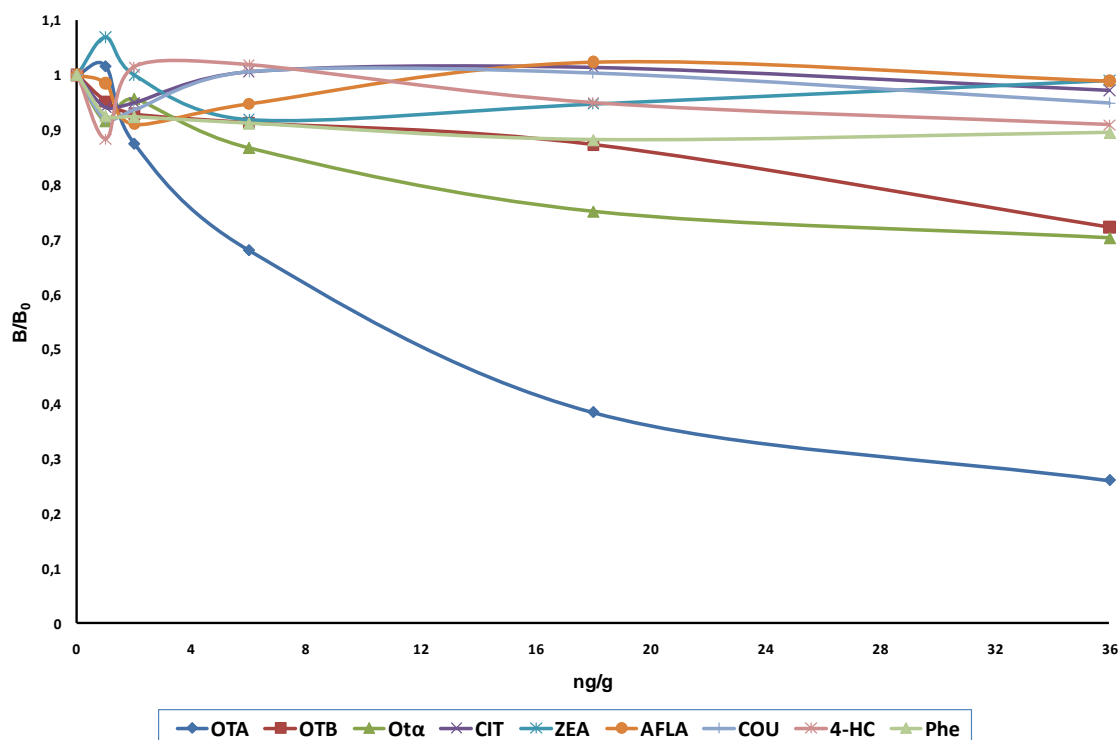


Figure 12: Standard curve of OTA, OTB, OT α , Citrinin, Zearalenone, AFLA B1, Coumarin, 4-HydroxyCoumarin and L-phenylalanine (n=2)

4.3.2.2 Matrix

For the compounds that the kit showed clear cross-reactivity in solvent, OTA, OTB and OT α , the exercise was repeated by constructing calibration curves in extracts of wheat, winter barley and oats. The results are summarised in Table 51.

Table 51: Cross-reactivity in matrix

	OTA			OTB			Ot α		
	Wheat	Barley	Oats	Wheat	Barley	Oats	Wheat	Barley	Oats
Slope	-1.242	-1.036	-0.836	-0.779	-0.883	-0.564	-0.579	-0.651	-0.505
Intercept	0.967	0.712	0.477	1.552	1.632	1.238	1.095	1.146	0.968
R ²	0.941	0.992	0.776	0.821	0.938	0.937	0.911	0.975	0.967
SE Intercept	0.077	0.053	0.252	0.203	0.127	0.081	0.101	0.059	0.052
SE Slope	0.079	0.054	0.260	0.210	0.131	0.084	0.105	0.061	0.054
IC ₅₀ (ng/g)	6.011	4.868	3.715	98.298	70.422	157.419	77.891	57.469	82.434

When examining these results it can be seen that for OTA the concentration needed for 50% extinction of the signal in the matrix decreased significantly compared to the concentration needed when solvent was used. The IC₅₀ also decreased in the following order: wheat > winter barley > oats. This is clearly indicative of a positive matrix effect, possibly leading to false positives. A similar trend can be observed for OTB and OT α . But as the reaction with OTB and OT α is much weaker, it's possible that the results are not that reproducible.



4.3.3 Matrix effect

To assess the matrix effect calibration curves were constructed for OTA in solvent and in extract for three matrices (wheat, winter barley and oats), for each matrix one 5 times diluted and one undiluted. The results of this exercise are summarized in Table 52 and Table 53. From these results it can be clearly seen that the amount of OTA necessary to obtain a 50% signal reduction is lower in the matrix than in the solvent, suggesting a matrix effect possibly leading to false positive results. When the matrix is diluted the IC₅₀ increases towards the value obtained for the solvent, suggesting the matrix effect is suppressed by diluting, not uncommonly observed in ELISA⁷⁵. The R² is somewhat higher and the standard deviations on the slope and intercept somewhat lower for the solvent, likely due to some degree of variability introduced by the matrix.

Table 52: Matrix-effect calculated as relative (IC₅₀ ng/g) extinction

	Control OTA	Wheat Undiluted	Wheat Diluted	Winter Barley Undiluted	Winter Barley Diluted	Oats Undiluted	Oats Diluted
Slope	-0.784	-0.708	-0.756	-1.020	-0.970	-0.944	-0.967
Intercept	1.320	1.106	1.212	1.542	1.637	1.457	1.545
R ²	0.975	0.918	0.820	0.880	0.960	0.967	0.969
SE Intercept	0.070	0.118	0.198	0.211	0.110	0.097	0.097
SE Slope	0.072	0.122	0.205	0.218	0.114	0.101	0.100
IC ₅₀ (ng/g)	48.311	36.570	40.101	32.452	48.819	34.963	39.645
CR	100%	76%	83%	67%	101%	72%	82%

Table 53: Matrix-effect calculated as percentage color reduction to the control (%CCR)

	Control OTA	Wheat Undiluted	Wheat Diluted	Winter Barley Undiluted	Winter Barley Diluted	Oats Undiluted	Oats Diluted
% Control Color Reduction	100%	77%	82%	77%	92%	60%	85%
CV	/	7%	8%	8%	2%	6%	2%

4.3.4 Accuracy

Accuracy was assessed on two reference materials, one from FAPAS (barley, 4.13 ng/g OTA) and one from BIPEA (barley, 35.4 ng/g OTA) The results in Table 54 indicate a very slight underestimation of the true value.

Table 54: Accuracy on two reference materials

	FAPAS	BIPEA
TRUE VAL (ng/g)	4.13	35.4
N	13	12
Degrees of freedom	12	11
t (95)	2.18	2.2
SD (ng/g)	0.60	6.42
Q1 (ng/g)	2.29	28.31
Q3 (ng/g)	3.55	35.39
Mean (ng/g)	2.95	31.21
x _e -x _t (ng/g)	-1.18	-4.19
Criterion (ng/g)	0.36	4.08

⁷⁵ Simone Fujii et al, Brazilian archives of biology and technology, Vol.50, n. 2 : pp.349-359, March 2007



4.3.5 Blank sample analysis

The results of three blank materials are summarized in (Table 55). The kit gives good results for wheat and winter barley but it generates an elevated signal for oats.

Table 55: Detection limit for three matrices

	Wheat	Winter Barley	Oats
N	9	9	9
DF	8	8	8
t (95%)	2.31	2.31	2.31
SD (ng/g)	0.16	0.09	0.33
Q1 (ng/g)	0.20	0.19	1.90
Q3 (ng/g)	0.47	0.31	2.20
Mean (ng/g)	0.31	0.25	2.08
Δx_{\min} (ng/g)	0.12	0.07	0.25
Detection limit (ng/g)	0.43	0.32	2.33

R-Biopharm remarked correctly that the matrix effect for oat is somewhat higher compared to the wheat and winter barley. This is in contrast to Table 52 where the difference was not evident. We suspect two reasons could be responsible for this. Variability in the matrix (solid matrices can be very inhomogeneous) was minimized by pooling three different extracts but it's possible that even this didn't cancel out the variability of the matrix. The experimental set-up of the cross reactivity: used relatively low concentrations of OTA. This could increase the variability of the response.

4.3.6 Conclusions

Cross-reactivity with OTB was not observed with this kit (making it the only one in this review). There was indication for a slight matrix effect. The kit was easy to use with a very straightforward and cheap extraction protocol.



4.4 AgraQuant® Ochratoxin by Romerlabs

4.4.1 Concentration accuracy of standards

The kit of Romerlabs is supplied with 5 ready to use standard solutions with a concentration ranging from 0 (blank) to 8 ng/ml OTA, corresponding to a concentration of 0 to 40 ng OTA per gram cereal. The results, summarized in Table 56, don't highlight any anomalies in the standards.

Table 56: Accuracy of calibrants

Calibrant (ng/g)	LC-MS/MS (ng/g)	% Dev	T-Test
0.00	0.00	/	0.96
0.40	0.53	33	
1.00	1.07	7	
4.00	4.52	13	
8.00	7.88	-2	

4.4.2 Cross-reactivity

4.4.2.1 Solvent

Results of the cross-reactivity are summarized in Table 57 and Figure 13. Clearly the kit doesn't differentiate between OTA and its dechloro form OTB.

Table 57: Cross-reactivity in solvent

	OTA	OTB	Otx	CIT	ZEA	AFLA B1	COU	4-HC	Phe
Slope	-1.73	-1.20	0.62	-0.83	-0.24	-0.48	0.00	-0.16	0.27
Intercept	2.05	1.08	3.03	3.55	1.83	2.00	1.06	1.09	0.96
R ²	0.97	0.98	0.02	NR	NR	NR	NR	NR	NR
SE Intercept	0.26	0.13	3.64	NR	NR	NR	NR	NR	NR
SE Slope	0.23	0.12	3.31	NR	NR	NR	NR	NR	NR
IC ₅₀ (ng/g)	15.30	8.00	76966	18848	5E+7	14789	9E+32	4E+6	3169
Relative Crossreactivity	100%	191%	0%	0%	0%	0%	0%	0%	0%

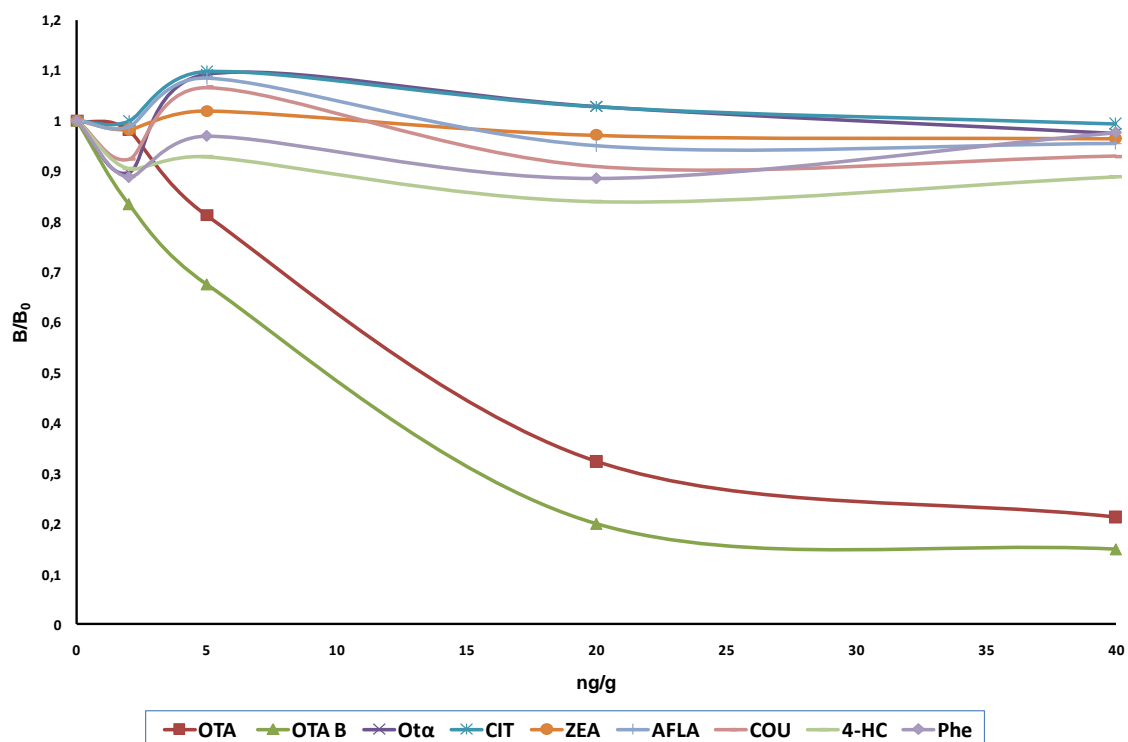


Figure 13: Standard curve of OTA, OTB, OT α , Citrinin, Zearalenone, AFLA B1, Coumarin, 4-HydroxyCoumarin and L-phenylalanine (n=2)

4.4.2.2 Matrix

For the compounds the kit showed clear cross-reactivity, OTA, and OTB, the exercise was repeated by constructing calibration curves in extracts of wheat, winter barley and oats. The results are summarised in Table 58. When examining these results it can be seen that the concentration needed for 50% reduction of the signal increased slightly (except for OTB in wheat) in the matrix. Cross-reactivity for OTB is still present in the matrix.

Table 58: Cross-reactivity in matrix

	OTA			OTB		
	Wheat	Barley	Oats	Wheat	Barley	Oats
Slope	-1.038	-1.559	-1.437	-1.158	-0.818	-1.323
Intercept	1.264	1.826	1.786	0.970	0.898	1.332
R ²	0.995	0.994	0.990	0.993	0.991	0.998
SE Intercept	0.057	0.092	0.180	0.077	0.062	0.048
SE Slope	0.052	0.084	0.143	0.070	0.056	0.043
IC ₅₀ (ng/g)	16.518	14.838	17.480	6.882	12.535	10.156



4.4.3 Matrix effect

To assess the matrix effect calibration curves were constructed for OTA in solvent and in extract for three matrices (wheat, winter barley and oats), for each matrix one 5 times diluted and one undiluted. The results of this exercise are summarized in Table 59 and Table 60. From these results it can be clearly seen that the amount of OTA necessary to obtain a 50% signal reduction is higher in the matrix than in the solvent, the result of a matrix suppression effect.

Table 59: Matrix-effect calculated as relative (IC_{50}) extinction

	Control OTA	Wheat Undiluted	Wheat Diluted	Winter Barley Undiluted	Winter Barley Diluted	Oats Undiluted	Oats Diluted
Slope	-0.928	-0.764	-1.265	-0.545	-0.665	-0.660	-0.586
Intercept	1.539	1.485	2.234	1.228	1.386	1.494	1.011
R ²	0.919	0.968	0.934	0.881	0.978	0.962	0.921
SE Intercept	0.215	0.108	0.262	0.156	0.077	0.102	0.133
SE Slope	0.195	0.098	0.238	0.142	0.070	0.092	0.121
IC ₅₀ (ng/g)	45.545	87.590	58.347	179.355	121.502	184.277	53.175
Relative Crossreactivity	100%	192%	128%	394%	267%	405%	117%

Table 60: Matrix-effect calculated as percentage color reduction to the control (%CCR)

	Control OTA	Wheat Undiluted	Wheat Diluted	Winter Barley Undiluted	Winter Barley Diluted	Oats Undiluted	Oats Diluted
% Control Color Reduction	100%	101%	108%	85%	93%	100%	81%
CV	/	13%	9%	12%	8%	11%	12%

4.4.4 Accuracy

Accuracy was assessed on two reference materials, one from FAPAS (barley, 4.13 ng/g OTA) and one from BIPEA (also barley, 13.25 ng/g OTA) The results in Table 61 indicate a slight underestimation of the true value.

Table 61: Accuracy on two reference materials

	FAPAS	BIPEA
TRUE VAL (ng/g)	4.13	13.25
N	9	9
Degrees of freedom	8	8
t (95)	2.31	2.31
SD (ng/g)	1.60	3.03
Q1 (ng/g)	1.53	4.90
Q3 (ng/g)	2.73	8.76
Mean (ppb)	2.18	7.01
$\bar{x} - x_i$ (ng/g)	-1.95	-4.19
Criterium (ng/g)	1.23	2.33



4.4.5 Blank sample analysis

The results of three blank materials are summarized in (Table 62). The kit gives good results for wheat and to a lesser extent for oats, but struggles with winter barley.

Table 62: Detection limit for three matrices

	Wheat	Winter Barley	Oats
N	9	9	9
Degrees freedom	8	8	8
t (95%)	2.310	2.310	2.310
SD (ng/g)	0.197	0.265	0.242
Q1 (ng/g)	0.197	0.265	0.242
Q3 (ng/g)	0.239	1.665	1.153
Mean (ng/g)	0.714	1.524	1.030
Δx_{min} (ng/g)	0.480	0.645	0.590
Detection limit (ng/g)	1.194	2.169	1.620

4.4.6 Conclusions

Cross-reactivity with OTB was observed with this kit, it is incapable of differentiating between OTA and OTB. Matrix effects leading to possible false negatives were observed.



4.5 OCHRATOXIN-A ELISA by Europroxima

4.5.1 Concentration accuracy of standards

The kit of Europroxima is supplied with 7 ready to use standard solutions with a concentration ranging from 0 (blank) to 8 ng/ml OTA, corresponding to a concentration of 0 to 40 ng OTA per gram cereal. The results, summarized in Table 63, don't highlight any anomalies in the standards.

Table 63: Accuracy of calibrants

Calibrant (ng/g)	LC-MS/MS (ng/g)	% Dev	T-Test
0.00	0.00	/	0.87
0.25	0.29	16	
0.50	0.61	23	
1.00	1.18	18	
2.00	2.34	17	
4.00	4.50	12	
8.00	8.80	10	

4.5.2 Cross-reactivity

4.5.2.1 Solvent

The cross-reactivity was checked on 7 different concentrations, corresponding to the OTA concentration Europroxima uses in its kit for 8 components and compared to the extinction of OTA. The solvent used was the proprietary dilution buffer supplied with the kit. From Table 64 and Figure 14 it is clear that OTB interacts strongly with the anti-bodies. For the other components the IC₅₀ was virtually non-existing.

Table 64: Cross-reactivity in solvent

	OTA	OTB	Otz	CIT	ZEA	AFLA B1	COU	4-HC	Phe
Slope	-0.541	-1.046	-0.281	0.266	0.056	-0.251	0.035	-0.186	-0.121
Intercept	0.556	0.080	1.105	0.761	0.648	1.026	0.468	0.735	0.606
R ²	0.965	0.988	0.548	0.189	0.200	0.830	0.068	0.538	0.241
SE Intercept	0.051	0.057	NR	NR	NR	NR	NR	NR	NR
SE Slope	0.051	0.057	NR	NR	NR	NR	NR	NR	NR
IC ₅₀ (ng/g)	10.677	1.193	8608.887	732.598	3.1E+11	1.2E+04	1.8E+13	8.9E+03	9.9E+04
Relative Crossreactivity	100%	895%	0%	1%	0%	0%	0%	0%	0%

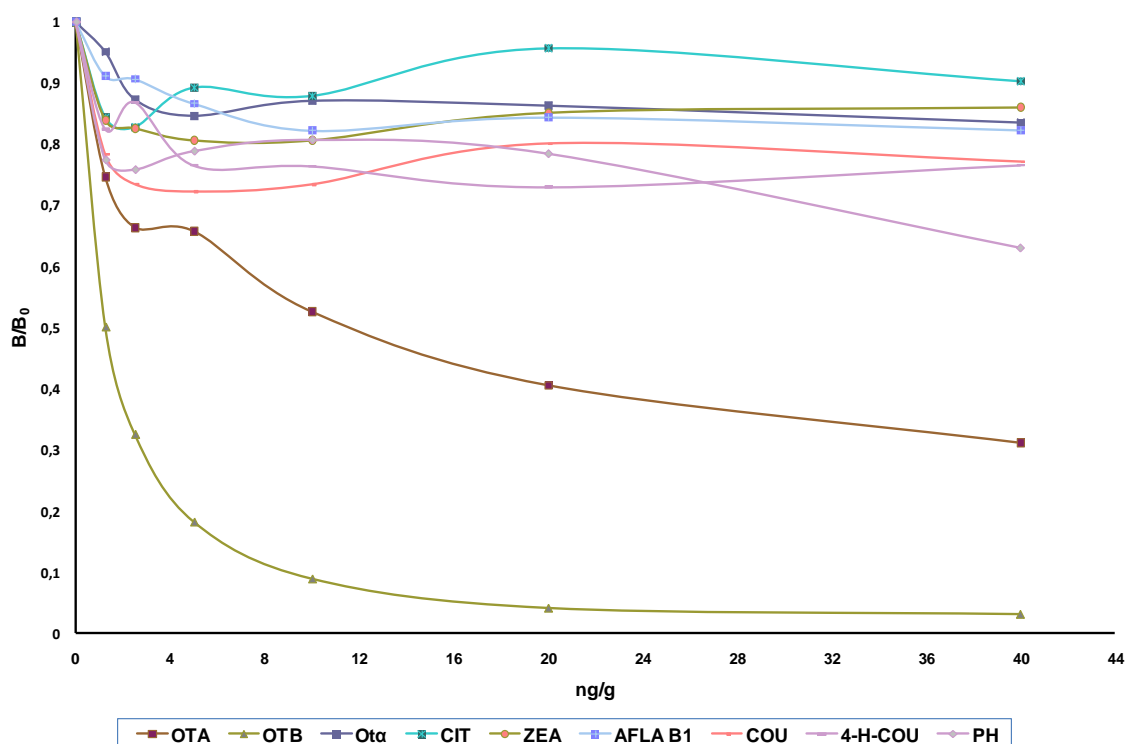


Figure 14: Standard curve of OTA, OTB, OT α , Citrinin, Zearalenone, AFLA B1, Coumarin, 4-HydroxyCoumarin and L-phenylalanine (n=2)

4.5.2.2 Matrix

In Table 65 it can be seen that the IC₅₀ is strongly dependent on the matrix.

Table 65: Cross-reactivity in matrix

	OTA			OTB		
	Wheat	Barley	Oats	Wheat	Barley	Oats
Slope	-1.010	-1.205	-0.824	-0.953	-1.096	-0.909
Intercept	1.634	2.248	1.173	0.306	0.699	0.281
R ²	0.910	0.888	0.931	0.967	0.960	0.943
SE Intercept	0.158	0.212	0.111	0.087	0.111	0.111
SE Slope	0.159	0.214	0.112	0.088	0.112	0.112
IC ₅₀ (ng/g)	41.473	73.298	26.509	2.095	4.344	2.036

4.5.3 Matrix effect

The results of this exercise are summarized in Table 66 and Table 67. The extraction protocol of the Ochratoxin-A-ELISA asks for a rather complex extraction procedure (Table 43). First 5 ml of 0.5 ml H₃PO₄ has to be added to 2.5 grams of sample and shaken, subsequently 10 ml of dichloromethane is added and shaken again for 10 minutes.



After centrifugation and removal of the upper-layer (0.5 ml H₃PO₄), the mixture is vortexed, recentrifuged and evaporated. The residue is then reconstituted in the proprietary extraction buffer. After a final defatting step with hexane the extract is ready to be pipetted on the plate. The more complex extraction procedure with dichloromethane and defatting with hexane should result in a cleaner extract and thus less matrix interference. From the results it can be clearly seen that the amount of OTA necessary to obtain a 50% signal reduction is highly dependent of the matrix and decreases significantly in matrix, suggesting a matrix effect. When the matrix is diluted the IC₅₀ increases to the value obtained for the solvent (but not for oats), suggesting the matrix effect becomes suppressed by diluting.

Table 66: Matrix-effect calculated as relative (IC₅₀) extinction

	Control OTA	Wheat Undiluted	Wheat Diluted	Winter Barley Undiluted	Winter Barley Diluted	Oats Undiluted	Oats Diluted
Slope	-1.140	-1.905	-1.178	-1.608	-0.944	-0.195	-0.851
Intercept	1.798	2.814	1.813	2.221	1.488	0.205	1.157
R ²	0.834	0.921	0.986	0.856	0.935	0.876	0.985
SE Intercept	0.319	0.350	0.089	0.413	0.156	0.046	0.067
SE Slope	0.293	0.322	0.082	0.380	0.144	0.042	0.062
IC ₅₀ (ng/g)	37.760	29.992	34.556	24.067	37.666	11.203	22.893
Relative Crossreactivity	100%	79%	92%	64%	100%	30%	61%

Table 67: Matrix-effect calculated as percentage color reduction to the control (%CCR)

	Control OTA	Wheat Undiluted	Wheat Diluted	Winter Barley Undiluted	Winter Barley Diluted	Oats Undiluted	Oats Diluted
% Control Color Reduction	100%	81%	95%	94%	104%	69%	88%
CV	/	16%	27%	17%	4%	16%	16%

4.5.4 Accuracy

From Table 68 it can be seen that the statistical criterion outranges the actual value of the reference material. The large SD and interquartile distance clearly indicate a large variability in the results. During the work in the lab we had also some difficulties of obtaining good reproducible results for the calibration curve. The kit didn't generate any false negative results however.

Table 68: Accuracy on two reference materials

	FAPAS	BIPEA
TRUE VAL (ng/g)	4,130	13,250
N	3	3
Degrees of freedom	2	2
t (95)	4,130	4,130
SD (ng/g)	1,874	10,765
Q1 (ng/g)	2,393	15,924
Q3 (ng/g)	3,730	29,345
Mean (ppb)	3,138	22,069
xe-x _i (ng/g)	0,992	-8,819
Criterion (ng/g)	4,469	25,670



4.5.5 Blank sample analysis

Table 69: Detection limit for three matrices

	Wheat	Winter Barley	Oats
N	3	3	3
Degrees freedom	2	2	2
t (95%)	4.13	4.13	4.13
SD	0.503	0.095	2.495
Q1	1.199	0.157	8.643
Q3	1.284	0.200	11.376
Mean (ng/g)	1.472	0.185	9.662
Δx_{\min} (ng/g)	2.546	0.481	12.620
Detection limit (ng/g)	4.019	0.666	22.282

The results of three blank materials are summarized in (Table 69). The kit gives good results for winter barley, for wheat the detection limit is close to the legal limit leading to a risk of false positives. The kit is clearly unable to cope with oats as a matrix confirming our findings in 0.

4.5.6 Conclusions

Cross reactivity with OTB was relatively high. The kit seems unable to cope with the oats we used. Also for one reference material it generated a very elevated response. The use of a phosphoric acid/dichloromethane extraction yielded the cleanest (based on visual observation) but was also the most time-consuming. With one sample we also observed the formation of an emulsion.



4.6 Conclusions of the experimental evaluation

The kit of R-Biopharm showed no cross-reactivity towards OTB. The other kits reviewed here cross-reacted with OTB. Whether the observed cross-reactivity poses a problem (or the lack of it is an important advantage) will depend on the end-users. At the present moment only OTA is regulated in the EU, there are no regulations for OTB. So cross-reaction for OTB poses a problem as it might give rise to a false non-compliant result forcing the end-user to verify the result by a (more expensive) reference method and decreasing the cost efficiency of the kit as a screening tool. On the other hand, OTB is usually far less abundantly present in cereals than OTA and as quick tests are intended for fast screening to detect possible screen positive samples a (slightly!) increased response should be a manageable problem.

Matrix effects were present (except the kit of Charm Sciences) but mostly biased to false positives, not false negatives. We observed problems with one kit however. The kit of Europroxima seems unable to cope with the oats we used.

The analysis of the standard solutions revealed no problems in terms of accuracy. All of the kits we received came each time from the same batch; it would be more interesting to monitor the interbatch variability.

Conclusion and future prospects



False positives due to cross-reactivity and/or matrix effect were last year during the evaluation of DON kits identified as a major problem.

The same is true for OTA kits. As mentioned in the conclusion of the administrative evaluation, they are competitive compared to reference methods in terms of cost efficiency given their false positive rate is well controlled. As positive labeled samples have to be double checked using a reference method, a too high false positive rate would compromise their cost effectiveness. Their strong point lies clearly in their ability to screen samples rapidly in-situ.

We also found that most kits biased somewhat to the low side during the assessment of accuracy.

The Rosa kit of Charm was the most robust test in terms of matrix effect and limit of detection. There is no need for a calibration curve and the number of manipulation steps is very limited. This gives it not only the edge in terms of labor cost but it is the only one suitable for use in the field.

The kit of R-Biopharm scored very well in terms of cross-reactivity, matrix effect and LOD. Of the ELISA kits it was the easiest and fastest to use. Romerlabs: biases somewhat to the low side. It also needs more manipulation steps than the kit of R-Biopharm. The Europroxima kit suffered somewhat from cross-reactivity for OTB. The kit had some troubles to deal with oats. The main drawback though was the complicated extraction procedure. Although it might lead to a cleaner extract the large number of clean-up steps makes it more labor intensive.

This exercise will be repeated the following next year for Aflatoxin kits. Focus will lie on total aflatoxin in cereals.

For the cross-reactivity it would be more meaningful to extract matrices spiked with the possible cross-reactants. Measuring cross-reactivity on spiked solvent or spiked matrix extracts might be interesting from a scientific point of view; they are irrelevant if the possible cross-reactant is not extracted by the solvent. Assessing the accuracy of the standards will not be repeated, this would only make sense if the kits came from different lot numbers which was not the case.

Recovery should be interesting to evaluate; not only are explicit legal limits set for mycotoxin recovery, this could also explain why accuracy biases somewhat negatively.

