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Evaluation of rapid test kits as potential screening tools for Ochratoxin A (OA) determination in wheat and barley

by

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Contents

Abstract

Existing validated laboratory test procedures for Ochratoxin A (OA) analysis are accurate and sensitive but not ideally suited to routine screening due to their time consuming nature and the necessary investment required in specialist personnel and sophisticated laboratory instrumentation. The aim of this project was to survey the scientific literature to identify rapid test approaches and to evaluate tests having the potential to screen wheat and barley samples for OA contamination.

A literature search identified that rapid test kits based on antibody capture of OA molecules and colorimetric or UV detection were the only commercially available rapid test kits suitable for purpose. Kits based on lateral flow devices (LFDs), that behave in a similar manner to pregnancy test kits, proved to be the simplest to use, exhibited acceptable levels of repeatability and reproducibility and provided consistent analytical data for screening at a defined threshold concentration of 3 or 4 parts per billion (ppb) (Ochratoxin A, BioControl Systems Diagnostics Ltd as an example). One fully quantitative LFD-style test kit (ROSA® Ochratoxin (Quantitative) Test, Charm Sciences Inc.) was also tested and shown to be fit for purpose. The microtiter plate-based assays (Veratox for Ochratoxin, Neogen Europe and Ridascreen® Ochratoxin A 30/15, r-Biopharm-rhône) provided the facility to analyse samples using calibrations applicable to screening at different legislative limits but the analysis of batched samples took several hours to produce a set of results and it might be argued that these approaches do not strictly conform to the definition of rapid tests. Test kits based on the use of immuno-affinity (IMA) columns and UV detection (Ochracard P48, r-Biopharm-rhône and Ochrascan, r-Biopharm-rhône) were also evaluated. In general, these kits required more time-consuming or complex approaches for sample preparation and were not found to provide compensating advantages.

The overall conclusion from the evaluation was that all test kits selected were capable of detecting OA in ground wheat and barley samples, conforming to manufacturers' stated claims and fulfilling the requirements of being "fit for purpose." However, repeatability and reproducibility of data remain a major challenge to the analysis of OA because the detection of OA contamination in wheat and barley has a much greater dependence on the sampling regime than is the case for other cereal mycotoxins, e.g. deoxynivalenol (DON).

Introduction

Fungi belonging to *Penicillium* and *Aspergillus* species are important cereal crop pathogens. The potential to generate ochratoxins (secondary metabolites of the initial infection) is a major food safety concern for cereal growers and processors.

A risk assessment by the joint FAO/WHO Experts Committee on Food Additives in 2001 concluded that over 50% of human exposure to ochratoxins was attributable to cereals and cereal-based products (JECFA, 2001). The reduction of these cereal contaminants is therefore considered desirable.

The scientific literature identifies Ochratoxin A (OA) as the most important Ochratoxin. OA can be isolated from cereals (barley, wheat, maize and oat), green coffee beans, malt containing beverages, peanuts, sorghum, olives, beer, pork and poultry, cheese, fruits and wine infected with *Aspergillus* species or *Penicillium verrucosum* (Pitt, 2000; Bennett and Klich, 2003; Pardo *et al*., 2006a).

The presence of OA in a variety of raw material commodities presents a significant threat to health for both humans and monogastric livestock. Acute contamination (Ochratoxicosis) is characterised by nephropathy (a reduction in kidney function), enteritis (fatty liver), necrosis of the lymph nodes and suppression of the immune system (Krogh, 1974). Accumulation of OA in the tissues of livestock has major human health implications. Epidemiological studies support the hypothesis that OA is a causative agent of nephropathy in humans (Krogh, 1976).

As such concerns in relation to human health are well-known, the UK has undertaken a programme of annual surveys of grain for a number of years, which has demonstrated that the incidence of OA levels in grain which exceed legislative limits is very low. Nevertheless, the availability of rapid methods of analysis for OA will aid the grain chain in its ongoing management of the risk from mycotoxins. To facilitate this, evaluation of the performance of commercially available kits was required.

OA is synthesised post-harvest during storage. The occurrence and formation of OA varies from year to year and is dependent on level of infection, microbial species present, grain moisture, temperature of storage and time of storage (Scudamore *et al*., 1999; Bennett and Klich, 2003). Damaged grains are more susceptible to the disease than intact grains; therefore, conditions such as frost damage, drought stress

and higher than average rainfall may all elevate levels of the disease (Scudamore *et al*., 1999).

A number of factors can affect growth and toxin production. Specifically a water activity (a_w), defined as the vapour pressure of water divided by that of pure water, of 0.8 (approximately 14%) is required for the growth of OA producing fungi on barley (Pardo *et al*., 2006a and b). Growth has not been observed at water activity levels <0.8. Optimal growth and/or toxin production is significantly affected by different substrates and temperature and this is species dependent (Pardo *et al*., 2006b). Typically growth is inhibited at temperatures less than 10°C (Pardo *et al*., 2006b). However, spore germination occurs in a wider range of a_w and temperature than mycelial growth (Pardo *et al*., 2006a). Growth on synthetic media occurs at more extreme factors than growth on a naturally contaminated material (Pardo *et al*., 2006a). Nutrititional status, surface effects, moisture level, temperature and the presence of competitive flora will all affect OA production (Bennett and Klich, 2003). For example, spore germination of *P. verrucosum* on barley meal extract agar has been shown to be maximal at 0.99 a_w and at 20-30°C (Pardo *et al*., 2006a). Decreasing the temperature from 20 to 10°C increased the lag time. The a_w for growth was 0.85 at 30°C and 0.9 at 10-20°C (Pardo *et al*., 2006a). Ribeiro *et al*. (2006) showed that optimal OA production by *Aspergillus ochraceus* on barley rootlets occurred at 0.82 a_w at 25 and 30°C and that length of incubation, water activity and temperature had a synergistic effect on OA production.

It can be concluded that the growth of OA fungal species *in vitro* is dependent on the interplay of a number of factors including fungal strain, water activity, temperature, surface effects and nutritional status. Spore germination and growth *in vitro* tend to be at the extremes and are not necessarily a reflection of growth *in vivo*, where other factors such as the presence of competitive flora can be of influence.

The introduction of EU legislation in 2006 (EC 1881/2006 and 2006/576/EC) established targets for grain traders and cereal processors to meet in order to prevent samples heavily contaminated with OA from entering the food and feed supply. Table 1 relates to foodstuffs for human consumption. Table 2 relates to the maximum permitted OA level in animal feed.

Classical analysis techniques for OA determination involve chromatographic separations followed by fluorescence detection. These methods are time consuming and require considerable investment in sophisticated laboratory equipment as well as

highly skilled staff to attain consistent results. For routine situations a different approach is required. Rapid test kits based on antibody-antigen reactions have potential for use within the grain chain to assure safety of supply into the human and animal feed chain. Testing must provide the ability to screen raw materials and processed products to the legislative requirements and provide documentation in support of traceability.

Table 1: EU legislative limits for Ochratoxin A in foods (data taken from Commission Regulation EC No 1881/2006)

Foodstuff	Maximum level (µg/kg)
Unprocessed cereals	5.0
All products derived from unprocessed cereals, including processed cereal products and cereals intended for direct consumption.	3.0
Processed cereal-based foods and baby foods for infants and young children.	0.5
Dietary foods for special medical purposes intended specifically for infants.	0.5

Table 2: EU legislative limits for Ochratoxin A in animal feeds (data taken from Commission Recommendation 2006/576/EC - figures based on 12% moisture)

Foodstuff	Maximum level (µg/kg)
Feed materials	250
Complementary and complete feedingstuffs for pigs	50
Complementary and complete feedingstuffs for poultry	100

Project Objectives

The overall aim of the project was to evaluate the suitability of commercially available test kits to screen for samples with an OA level above the legislative limits with rapid turnaround of results.

The objectives were:

- to provide an independent evaluation of commercially available test kits for OA estimation.
- to assist cereal processors in demonstrating due diligence in meeting legislative requirements.

Materials and Methods

Test kits

The evaluation was completed in a single phase by analysis of all samples using a short-list of test kits selected from a larger available range (Tables 3-5).

Table 3: Commercially available test kits for OA based on lateral flow devices (LFD)

Supplier	Web address	Test kit/No Tests	LOD (ppb)	Screening Range (ppb)
Charm Sciences Inc	www.charm.com	ROSA Ochratoxin (Quantitative) Test[a] 20/100/500 LFD Tests	<1.0	0 - 12
BioControl Systems Diagnostics Ltd	www.biocontrolsys.com	Ochratoxin-A Flow-Through Rapid Test 10 LFD Tests	N/A[b]	4[c]

[a]GIPSA approved (Certificate No. FGIS 2008-104)
[b]Screening test
[c]Screening level

Table 4: Commercially available test kits for OA based immunoaffinity (IMA) columns

Supplier	Web address	Test kit/No Tests	LOD (ppb)	Screening Range (ppb)
r-Biopharm-rhône	www.r-biopharmrhone.com	Ochracard P48 20 Tests	N/A[a]	3 - 10
		Ochrascan 25 Tests	2	0 - 20[b]

[a]Screening test
[b]Dependent on extract volume analysed

Table 5: Commercially available microtiter-plate based ELISA test kits for OA

Supplier	Web address	Test Kit/ No tests	LOD (ppb)	Range (ppb)	Cross-reactivity (%)
BioControl Systems Diagnostics Ltd	www.biocontrolsys.com (previously found at www.raisio.com)	Ochratoxin-A EIA (96 wells) (16 standards/controls plus 40 samples)[a]	1.0[b]	0.25 – 5.0	100 Ochratoxin A 9.3 Ochratoxin B <0.1 Ochratoxin α[f] <0.1 Coumarin <0.1 4-Hydroxy-coumarin
Neogen Europe	www.neogeneurope.com	Veratox for Ochratoxin (48 wells) (10 standards/controls plus 19 samples)	1.0	2.0 –25.0	100 Ochratoxin A 18 Ochratoxin B
r-Biopharm-rhône	www.r-biopharmrhone.com	Ridascreen Ochratoxin A (96 wells) (10 standards/controls plus 19 samples)	0.63	0 - 2.03[d] 0-50[e]	100 Ochratoxin A 14 Ochratoxin B 44 Ochratoxin C[g] <0.1 Ochratoxin α
		Ridascreen Ochratoxin A 30/15 (96 wells) 12 standards/controls plus 36 samples)	1.25[c]	0 – 1.8[d] 0-36[e]	
Romer Labs	www.romerlabs.com	AgraQuant Ochratoxin Assay 2/40 48 and 96 wells (10 standards/controls plus 19/38 samples)	1.9	2.0 – 40.0	100 Ochratoxin A 108 Ochratoxin B

[a]Based on duplicates
[b]Based on ±3SD
[c]2.5µgKg^{-1} for rapid screening
[d]Stated
[e]Accounting for dilution
[f]Metabolite of Ochratoxin A
[g]Metabolite of Ochratoxin A

A final selection of 6 test kits (Table 6) was made from those represented in Tables 3-5.

Table 6: Test kits evaluated

Test kit	Assay type	Manufacturer	LOD (ppb)	OA Range (ppb)
Ridascreen Ochratoxin A 30/15	ELISA	r-Biopharm-rhône	1.25	0 - 36
Veratox for Ochratoxin	ELISA	Neogen Europe	1.0	2.0 - 25.0
ROSA Ochratoxin (Quantitative)	LFD	Charm Sciences Inc.	<1.0	0.0 - 12.0
Ochratoxin-A	LFD	BioControl Systems Diagnostics Ltd	N/A	4.0
Ochracard P48	LFD	r-Biopharm-rhône	N/A	3.0 - 10.0
Ochrascan	IMA	r-Biopharm-rhône	2.0	0 - 20

Kits were selected on the basis of:

- Suitability to detect OA in cereals.
- Type i.e. ELISA/LFD/IMA.
- Range of measurement covered by the calibration e.g. 0-2ppb, 0-20ppb.
- Availability.

The criteria for the evaluation were produced by CCFRA and BRI in consultation with the UK cereals industry and included:

- Ease of use.
- Analysis turnaround time <30 minutes.
- Reduced capital outlay on ancillary equipment.
- Sensitivity of detection at legislative levels.
- Reliability of results.
- Specificity i.e. no significant cross-reactivity.
- Cost per test.

Thus, suitability and potential use at different points in the cereal grain supply chain were evaluated rather than a full statistical treatment of data outcomes. The criteria

were supplied to each test kit manufacturer and an invitation to join the evaluation was offered. Acceptance of the project conditions was obtained from each manufacturer/supplier prior to the evaluation of each test kit. For 2 of the test kits (Veratox for Ochratoxin and ROSA Ochratoxin (Quantitative)), ancillary equipment was provided on loan. This will be described under the relevant section.

Wheat and barley samples

The sample set consisted of 16 wheat, 9 barley and 7 barley-malt samples. All samples were naturally contaminated with OA in the range <1ppb to 17.8ppb. Levels of OA (Table 7) were confirmed by analysis by high-performance liquid chromatography (HPLC) with fluorescence detection based on BS EN 14132:2003 with a stated LOQ of 0.1ppb.

Table 7: Results of confirmatory test analysis for wheat, barley and malt samples by HPLC

Sample ID	HPLC	Sample ID	HPLC
Wheat		Barley[b]	
103379/X	ppb	/Malt[c]	ppb
1	<0.10	17B	<0.10
2	<0.10	18M	<0.10
3	0.43	19M	0.80
4	1.34	20B	17.80
5	<0.10	21B	1.00
6	1.90	22B	9.50
7	<0.10	23M	<0.10
8	<0.10	24B	<0.10
9	<0.10	25M	0.20
10	<0.10	26M	<0.10
11	1.28	27M	<0.10
12	0.64	28B	0.20
13	<0.10	29B	<0.10
14	0.59	30B	<0.10
15	10.53	31B	<0.10
16	4.39	32M	<0.10

[b]B=Barley; [c]M=Malt

Sampling

The objective of sample preparation is to produce an isolate of the target analyte for analysis. Ideally, the test portion should contain proportionally the same concentration of OA as the larger sample from which the test portion is taken. All test kit manufacturers recommend that a clearly defined sample protocol is followed to ensure that the sub-sample tested is representative of the bulk from which it is taken. The sample should be ground to a consistent particle size and mixed prior to analysis.

For the current study, all samples were prepared at CCFRA according to:

FTWG Procedure 04p Grinding of Whole Grain to Produce a Fine Wholemeal (CCFRA, 2002).

500g of each sample was ground using an LM 3100 mill (Perten Instruments, Sweden), fitted with an 800µm screen. Following grinding, all samples were thoroughly homogenised using a rotary mixer (Chopin Instruments, France) for 20 minutes.

Additional equipment

The following lists additional equipment used in this study. Not all of the additional items were required for each kit and more detail on the specific requirements is included in the following sections.

- Analytical balance capable of weighing up to 50.0g ± 0.1g.
- Laboratory glassware e.g. beakers, volumetric flasks, measuring cylinders and test tubes.
- Vortex mixer or shaking device.
- Micropipettes (for the microtiter-plate based ELISA assays, a multichannel pipette is required).
- Spectrophotometer or Microwell reader.
- Methanol (Analytical grade).
- Chloroform (Analytical grade).
- Sodium Hydrogen Carbonate (Analytical Grade).
- 5% Sodium hypochlorite solution, to clean reusable glassware.

Results and Discussion

Test kits were evaluated in no specific order. Wheat analysis was conducted at CCFRA and the barley and malt analysis was conducted at BRI.

Quantitative Enzyme-linked Immunosorbent Assay (ELISA)

Veratox ® for Ochratoxin (Neogen Europe)

Veratox for Ochratoxin is a fully quantitative and competitive direct enzyme-linked immunosorbent assay (CD-ELISA). The kit is available in 48-microtiter well format. For quantification, a spectrophotometer (fitted with a 650nm filter) capable of reading microtiter plates is required. Alternatively, the StatFax microwell reader (Neogen Corp.) provides a lower cost option (Figure 1).

Figure 1: Neogen Microwell (StatFax) Strip Reader

All Neogen Veratox® format tests are pre-programmed in the StatFax instrument. The OA calibration is also contained within the instrument. When using the StatFax instrument, the manufacturer's recommendations regarding the layout of standards and samples should be followed. A series of OA standards (0ppb, 2ppb, 5ppb, 10ppb and 25ppb) is provided with the test kit. There are two incubation stages totalling 10 minutes. The second incubation reaction (between chromogen and substrate) is light sensitive, so measurements must be made within 20 minutes of the end of the incubation period. The colour change is from pink to blue. Limit of detection (LOD) is 1ppb. Due to the format of the test kit and the requirement to run a standard curve (with each analysis) the assay is only cost effective for multiple or batch analysis. The Veratox® assay covers the widest quantitative range of the test kits evaluated in this study.

Analytical data are presented in Table 8.

Table 8: Analysis of wheat, barley and malt using Veratox® Ochratoxin A

Sample ID	HPLC	Veratox			Sample ID	HPLC	Veratox		
Wheat		Lot: 21580	Lot: 21580	Lot: 21581	Barley		Lot: 21580	Lot: 21580	Lot: 21581
103379/X	µg/kg	µg/kg	µg/kg	µg/kg	/Malt	µg/kg	µg/kg	µg/kg	µg/kg
1	<0.10	<2.0	<2.0	<2.0	17B	<0.10	nr	nr	<2.0
2	<0.10	2.3	2.0	2.6	18M	<0.10	nr	nr	<2.0
3	0.43	<2.0	<2.0	<2.0	19M	0.80	nr	nr	<2.0
4	1.34	2.1	2.5	2.5	20B	17.80	nr	nr	4.9
5	<0.10	<2.0	<2.0	<2.0	21B	1.00	nr	nr	<2.0
6	1.90	3.8	4.2	3.3	22B	9.50	nr	nr	4.7
7	<0.10	<2.0	<2.0	<2.0	23M	<0.10	nr	nr	<2.0
8	<0.10	<2.0	<2.0	<2.0	24B	<0.10	nr	nr	<2.0
9	<0.10	<2.0	<2.0	<2.0	25M	0.20	nr	nr	<2.0
10	<0.10	<2.0	<2.0	<2.0	26M	<0.10	nr	nr	<2.0
11	1.28	<2.0	2.0	<2.0	27M	<0.10	nr	nr	<2.0
12	0.64	<2.0	<2.0	<2.0	28B	0.20	nr	nr	<2.0
13	<0.10	<2.0	<2.0	<2.0	29B	<0.10	nr	nr	<2.0
14	0.59	<2.0	<2.0	<2.0	30B	<0.10	nr	nr	<2.0
15	10.53	8.7	9.6	10.8	31B	<0.10	nr	nr	<2.0
16	4.39	4.2	4.6	5.4	32M	<0.10	nr	nr	<2.0

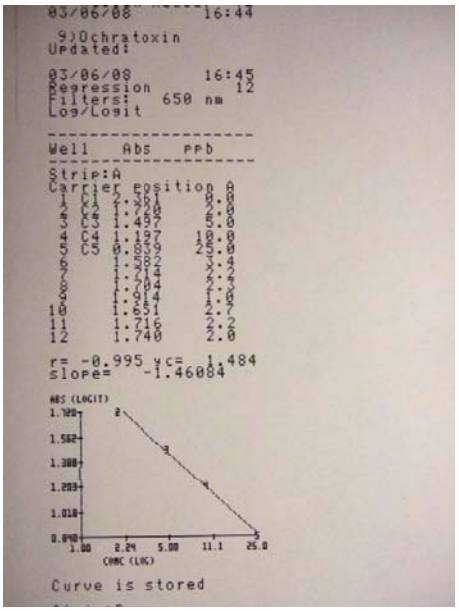
nr=no result

Key analytical outcomes are based on:

1. The ability of the test kit to detect OA contamination in wheat, barley and malt samples.
2. Assay performance.

In relation to point 1, the Veratox® Ochratoxin A test was sufficiently sensitive to detect contamination in the test samples. However, calibration issues experienced with the barley and malt analysis at BRI meant that no analytical data were returned for kit lot no 21580, the reason being that quantification of data relies on a specific calibration curve-fit (log/logit). The barley and malt data were measured on a conventional microtiter-plate reader controlled by software which automatically defaults to the "best" calibration curve-fit. If the calibration returned is not that specified by the manufacturer's instructions (stated in the kit protocol), results obtained will be inaccurate. No such issue was experienced for the analysis of the wheat samples. The StatFax reader is pre-programmed with the log/logit calibration (Figure 2).

Figure 2: Calibration data output from the StatFax (Neogen Corp.)



Comparison with the confirmatory test was used as a measure of assay performance. To ensure consistency of approach for all such kits, values exceeding limits (±35.6% of the confirmatory test result) based on current uncertainty estimates for OA analysis (Biselli, 2007) have been highlighted (Table 9). The underlying basis for such an assessment is that the difference between fully quantitative confirmatory and test kit data should not be greater than two times the standard deviation for each method - the uncertainty of analysis.

Table 9: Comparison of confirmatory and kit data[a]

Sample ID	HPLC	Veratox			Sample ID	HPLC	Veratox		
Wheat		Lot: 21580	Lot: 21580	Lot: 21581	Barley		Lot: 21580	Lot: 21580	Lot: 21581
103379/X	µg/kg	µg/kg	µg/kg	µg/kg	/Malt	µg/kg	µg/kg	µg/kg	µg/kg
3	0.43	<2.0	<2.0	<2.0	19M	0.80	nr	nr	<2.0
4	1.34	2.1	2.5	2.5	20B	17.80	nr	nr	4.9
6	1.90	3.8	4.2	3.3	21B	1.00	nr	nr	<2.0
11	1.28	<2.0	2.0	<2.0	22B	9.50	nr	nr	4.7
12	0.64	<2.0	<2.0	<2.0	25M	0.20	nr	nr	<2.0
14	0.59	<2.0	<2.0	<2.0	28B	0.20	nr	nr	<2.0
15	10.53	8.7	9.6	10.8					
16	4.39	4.2	4.6	5.4					

[a]Highlighted cells represent results of the kit analysis which were outside ±35.6% of the value obtained using the confirmatory test

nr=no result

Due to analytical issues related to the barley analysis, statements regarding assay precision will be limited to wheat. Repeatability of the assay for wheat was 10.9% at 9.7ppb (average of 6 samples). Using the underlying data generated by the kit, correlation with the confirmatory test was acceptable (R^2=0.91).

It was encouraging that the test kit positively identified the most contaminated samples. In terms of assay performance compared with the confirmatory test, in all cases for wheat where the test results were outside the uncertainty limits on the confirmatory test, no samples would have been erroneously rejected at grain intake and none would have caused subsequent problems if processed. However, persistent problems occurred with the barley analysis, which were not resolved within the project. Values were only obtained for one kit lot for barley and final conclusions on performance for this cereal species may not be drawn as a result. Subsequent discussion with the kit manufacturer has indicated that the extraction protocol for barley has been modified from 50% to 70% methanol which, in their view, would resolve the issues encountered with barley during the kit evaluation.

Ridascreen® Ochratoxin A 30/15 (r-Biopharm-rhône)

The assay is described as a competitive enzyme immunoassay for the quantitative analysis of OA in cereals, feed, beer and pig serum. The assay is available in 48 and 96 microtiter well format. For quantification, a spectrophotometer fitted with a 450nm filter is required to detect a colour change from blue to yellow (Figure 3).

Figure 3: The Thermoskan Ascent microtiter plate reader

The assay is capable of measuring at sub parts per billion (parts per trillion, ppt) levels. Six standards are supplied with the kit (0ppb, 0.05ppb,0.1ppb,0.3ppb, 0.9ppb

and 1.8ppb), which must be run with each assay in order to generate a calibration curve against which unknowns are quantified. After allowing for dilution, the calibration levels equate to a range of 0ppb - 36ppb in cereal samples. Extraction protocols are described which vary depending on the matrix from which OA is to be extracted and the screening level selected. There are two incubation steps, totalling 45 minutes. Incubation step two (reaction between chromogen and substrate) is light sensitive, so measurements must be made within 30 minutes of the end of the incubation period. The colour change is from blue to yellow. The quoted limit of detection (LOD) is 1.25ppb. Due to the format of the test kit and the requirement to run a standard curve (with each analysis) the assay is only cost effective for multiple or batch analysis. The Ridascreen® Ochratoxin A 30/15 assay covers a quantitative range of 0ppb-36ppb and the sensitivity of the assay has potential use for screening at <1.0ppb levels.

Results are shown in Table 10.

Table 10: Analysis of wheat, barley and malt using Ridascreen® Ochratoxin A 30/15

Sample ID	HPLC	r-Bio Ridascreen OA 30/15		Sample ID	HPLC	r-Bio Ridascreen OA 30/15	
Wheat		Lot:05477	Lot: 05477	Barley		Lot:05477	Lot: 05477
103379/X	µg/kg	µg/kg	µg/kg	/Malt	µg/kg	µg/kg	µg/kg
1	<0.10	<0.5	<0.5	17B	<0.10	<0.5	<0.5
2	<0.10	<0.5	<0.5	18M	<0.10	<0.5	<0.5
3	0.43	<0.5	<0.5	19M	0.80	<0.5	<0.5
4	1.34	1.56	1.29	20B	17.80	16.9	16.9
5	<0.10	<0.5	<0.5	21B	1.00	0.75	0.75
6	1.90	2.27	2.11	22B	9.50	10.0	10.0
7	<0.10	<0.5	<0.5	23M	<0.10	<0.5	<0.5
8	<0.10	<0.5	<0.5	24B	<0.10	<0.5	<0.5
9	<0.10	<0.5	<0.5	25M	0.20	<0.5	<0.5
10	<0.10	<0.5	<0.5	26M	<0.10	<0.5	<0.5
11	1.28	0.98	1.0	27M	<0.10	<0.5	<0.5
12	0.64	0.88	0.81	28B	0.20	<0.5	<0.5
13	<0.10	<0.5	<0.5	29B	<0.10	<0.5	<0.5
14	0.59	<0.5	<0.5	30B	<0.10	<0.5	<0.5
15	10.53	12.2	11.12	31B	<0.10	<0.5	<0.5
16	4.39	4.86	4.45	32M	<0.10	<0.5	<0.5

The Ridascreen® Ochratoxin A 30/15 test kit was selected on the basis that a high percentage of the test samples used in the study (75%) had low levels of OA

(<1.0ppb). Due to systems of storage control implemented in the UK, the sample set provides a "snap-shot" of the actual situation faced by grain handlers and cereal processors. Cereal-based products intended for infants and for medical use require analysis at <1.0ppb levels. The use of the Ridascreen® Ochratoxin A 30/15 test kit provides potential for screening at low levels of OA contamination.

Results obtained from Table 10 provide evidence that the Ridascreen® Ochratoxin A 30/15 test kit is capable of OA detection at levels of <1.0ppb, in wheat, barley and malt samples. Heavily contaminated samples, i.e. beyond the highest concentration standard level of 1.8ppb, required re-analysis, with sample dilution to allow results to fall within the calibration range. The data show comparable results to analysis using the confirmatory procedure.

As stated above, comparison with the confirmatory test was used as a measure of assay performance. Limits (±35.6% of the confirmatory test result) based on current uncertainty estimates for OA analysis (Biselli, 2007) were used for this assessment. For this kit, all of the results fell within these limits, which was an encouraging outcome of the analysis (Table 11).

Table 11: Comparison of confirmatory and kit data

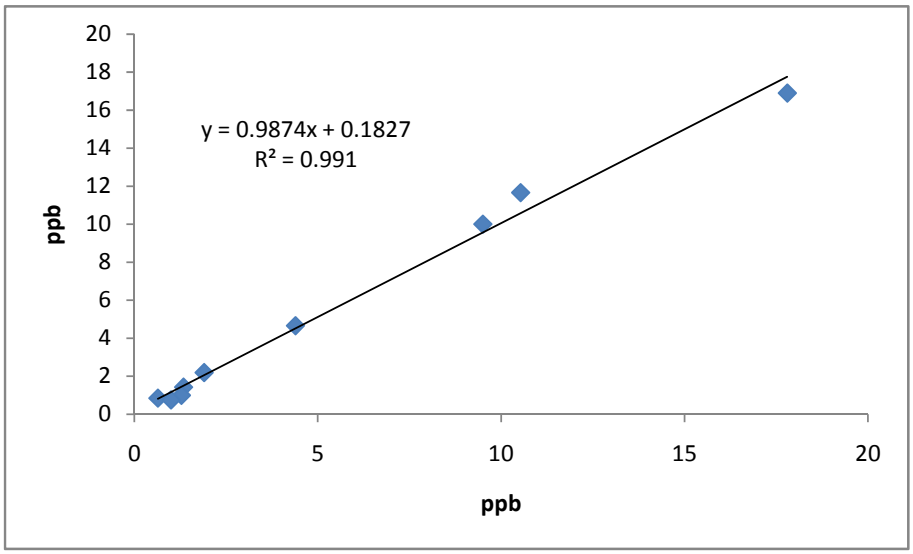
Sample ID	HPLC	r-Bio Ridascreen OA 30/15		Sample ID	HPLC	r-Bio Ridascreen OA 30/15	
Wheat		Lot:05477	Lot: 05477	Barley		Lot:05477	Lot: 05477
103379/X	µg/kg	µg/kg	µg/kg	/Malt	µg/kg	µg/kg	µg/kg
4	1.34	1.56	1.29	20B	17.80	16.9	16.9
6	1.90	2.27	2.11	21B	1.00	0.75	0.75
11	1.28	0.98	1.0	22B	9.50	10	10
12	0.64	0.88	0.81				
15	10.53	12.2	11.12				
16	4.39	4.86	4.45				

Data provided using the Ridascreen® Ochratoxin A 30/15 test kit highlighted the potential to identify OA samples both within and outside legislative limits for grain intake, processed cereals and processed cereal products intended for infants or medical applications. Study samples were characterised correctly (in relation to the confirmatory test).

Assay performance was limited to within batch precision (test kits were available in production runs and only one batch was available for the evaluation). Repeatability of the assay was very good, achieving an average of 4.3% over the range 0.75ppb to

16.9ppb (average of 4 analyses for each sample). Correlation with the confirmatory test was also good, R^2=0.99 (Figure 4).

Figure 4: Comparison of HPLC OA data vs. Ridascreen® Ochratoxin A 30/15 test kit for selected wheat, barley and malt samples



All samples were correctly categorised and at different legislative levels, satisfying the analytical requirements for the test kit. However, there were concerns over the limited range of calibration (a function of the operating range for non-diluted extracts) compared with EU legislative limits as well as the duration of the test. For the latter, one of the pre-requisites of the evaluation was that turnaround of results should be no longer than 30 minutes. The extraction process (for both wheat and barley) was found to be laborious and the total time to produce a set of 16 results was 6-7 hours. As a result, it is concluded that the test is ideally suited to a central laboratory function and not to processing situations.

Quantitative assay based on Lateral Flow Devices (LFD)

ROSA® Ochratoxin (Quantitative) Test (Charm Sciences Inc.)

The ROSA® Ochratoxin (Quantitative) test is an immunoassay in lateral flow format. OA is extracted from ground grain using a methanol water solution. Extracted OA interacts with coloured beads on an inert strip and, following a short incubation step, colour development in the test zone is read (using the ROSA®-M-Reader) as a ppb concentration figure.

To operate this test kit, the portable ROSA®-M-Reader and incubator (2 or 4-channel) must be purchased separately. The ROSA®-M-Reader carries a pre-programmed

calibration for OA measurement and may also be used for ROSA kits for other mycotoxins. The ROSA®-M-Reader is supplied with calibration "sticks" to monitor performance of the instrument over time. In keeping with good laboratory practice, it is advised that the calibration is verified prior to each batch of analysis. Test kits are available in sets of 20, 100 and 500 test sticks and instructions for preparing a negative (0ppb) control and for reconstitution of the positive (3.5ppb) control (supplied with the kit) are included in the test kit instructions. There is a single incubation step (10 minutes) for which there is a timing device built into the portable incubator (controlled at 45±1ºC). At the end of the incubation period, an alarm sounds. The test strip must be read within two minutes of the first alarm for accurate estimations of OA. The LOD is <1.0ppb, though the reader also returns values below 1.0ppb, and the analytical range is from 0ppb-12ppb. Samples above this concentration should be diluted and re-analysed according to the manufacturer's instructions.

Results are shown in Table 12.

Table 12: Analysis of wheat, barley and malt using ROSA® Ochratoxin (Quantitative) Test

Sample ID	HPLC	ROSA OA (ppb)			Sample ID	HPLC	ROSA OA (ppb)		
Wheat		Lot:007	Lot: 007	Lot:006	Barley		Lot:007	Lot: 007	Lot:006
103379/X	µg/kg	µg/kg	µg/kg	µg/kg	/Malt	µg/kg	µg/kg	µg/kg	µg/kg
1	<0.10	0.3	0.1	0.3	17B	<0.10	0.2	0.2	1.3
2	<0.10	0.2	0.4	0.1	18M	<0.10	0.5	0.5	1.0
3	0.43	0.6	0.5	0.6	19M	0.80	0.6	0.6	1.9
4	1.34	1.5	1.2	1.7	20B	17.80	>12	>12	9.2
5	<0.10	0	0.1	0.1	21B	1.00	1.6	1.6	1.1
6	1.90	2.2	2.1	1.8	22B	9.50	10.7	10.7	7.0
7	<0.10	0.2	0.3	0.2	23M	<0.10	0.4	0.4	0.9
8	<0.10	0.1	0.2	0	24B	<0.10	0.3	0.3	0.6
9	<0.10	0.1	0.1	0	25M	0.20	0.5	0.5	0.6
10	<0.10	0	0.1	0.1	26M	<0.10	0.2	0.2	1.0
11	1.28	1.4	1.3	1.0	27M	<0.10	0.7	0.7	1.5
12	0.64	0.9	0.5	0.6	28B	0.20	0.9	0.9	0.9
13	<0.10	0.2	0	0	29B	<0.10	0.3	0.3	1.5
14	0.59	0.8	1.2	0.9	30B	<0.10	0.3	0.3	0.8
15	10.53	10.8	11.3	9.8	31B	<0.10	0	0	0.9
16	4.39	4.1	3.9	4.4	32M	<0.10	1.1	1.1	0.7

The ROSA® Ochratoxin (Quantitative) test is an example of an LFD. The flexibility of being able to conduct analysis on single or multiple (four maximum) samples offers

potential use in cereal production environments. Additionally, ease-of-use and a simple analysis protocol allow users without extensive laboratory training to use test kits based on LFDs. The ROSA® Ochratoxin (Quantitative) test has the advantage that the data outputs are fully quantitative and comparable to laboratory-based analytical procedures.

Results obtained from Table 12 indicate the ability of the ROSA® Ochratoxin (Quantitative) test in the detection of OA in wheat, barley and malt at levels of <1.0ppb to the most heavily contaminated sample at 17.8ppb (following re-analysis according to the manufacturer's instructions).

Table 13: Comparison of confirmatory and kit data[a]

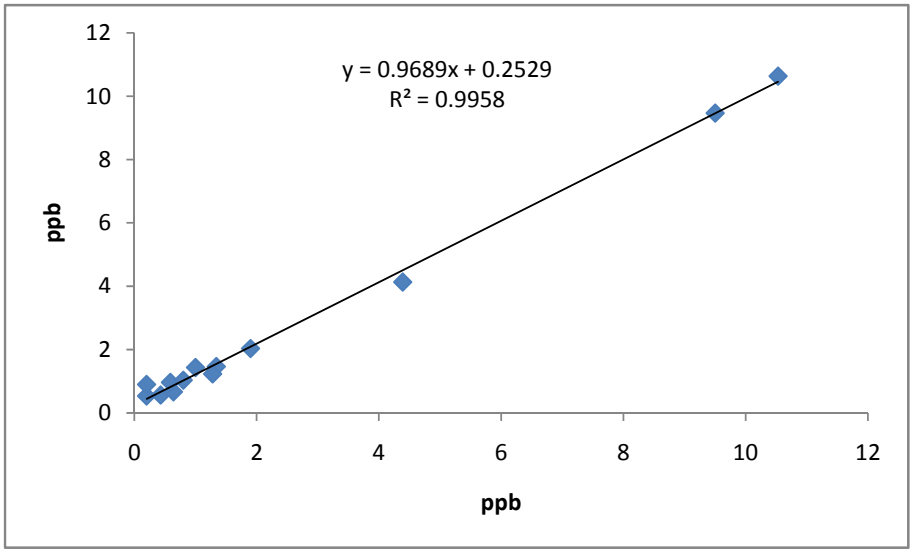
Sample ID	HPLC	ROSA OA (ppb)			Sample ID	HPLC	ROSA OA (ppb)		
Wheat		Lot:007	Lot: 007	Lot:006	Barley		Lot:007	Lot: 007	Lot:006
103379/X	µg/kg	µg/kg	µg/kg	µg/kg	/Malt	µg/kg	µg/kg	µg/kg	µg/kg
3	0.43	0.6	0.5	0.6	19M	0.80	0.6	0.6	1.9
4	1.34	1.5	1.2	1.7	20B	17.80	>12	>12	9.2
6	1.90	2.2	2.1	1.8	21B	1.00	1.6	1.6	1.1
11	1.28	1.4	1.3	1	22B	9.50	10.7	10.7	7.0
12	0.64	0.9	0.5	0.6	25M	0.20	0.5	0.5	0.6
14	0.59	0.8	1.2	0.9	28B	0.20	0.9	0.9	0.9
15	10.53	10.8	11.3	9.8					
16	4.39	4.1	3.9	4.4					

[a]Highlighted cells represent results of the kit analysis which were outside ±35.6% of the value obtained using the confirmatory test

The ROSA® Ochratoxin (Quantitative) test correctly identified the most contaminated samples. Comparison with the confirmatory test was used as a measure of assay performance. To ensure consistency of approach for all such kits, values exceeding limits (±35.6% of the confirmatory test result) based on current uncertainty estimates for OA analysis (Biselli, 2007) have been highlighted (Table 13). In terms of assay performance compared with the confirmatory test, in all cases for wheat where the test results were outside the uncertainty limits on the confirmatory test, no samples would have been erroneously rejected at grain intake and none would have caused subsequent problems if processed. This is further demonstrated by the result for sample 20B where, although one of the individual replicates showed deviations below the lower uncertainty limit on the confirmatory test, the result would still have led to further investigation of these samples, i.e. they were correctly categorised.

Repeatability of the assay ranged from CV%=10.8% at 0.5ppb (average of 6 samples) to 7.2% at 10.6ppb (average of 6 samples). Correlation with the confirmatory test was good, R^2=0.99 (Figure 5).

Figure 5: Comparison of HPLC OA data vs. ROSA® Ochratoxin (Quantitative) test kit for selected wheat, barley and malt samples



Threshold Tests

Ochratoxin A Flow-Through Assay (BioControl Systems Diagnostics Ltd)

This test kit contains 10 test membranes, each coated with antibodies. Following the addition of extract to the membrane, reagents are applied in a specific order to the membrane surface. Each reagent must pass or "flow-through" the membrane before the addition of the next reagent in the sequence. A chromogen is added to the membrane with the development of a blue coloured product. The results are interpreted visually through a window on the test membrane. The depth of the blue coloured test line is inversely proportional to OA in the sample extract. The absence of a coloured line identifies OA contamination of >4ppb.

Test data are shown in Table 14.

Table 14: Analysis of wheat, barley and malt using Ochratoxin A Flow-Through Assay (BioControl Systems)

		BioControl(ppb)					BioControl(ppb)		
Sample ID	HPLC	Lot: IN6706	Lot: IN6706	Lot: JN5012	Sample ID	HPLC	Lot: IN6706	Lot: IN6706	Lot: JN5012
Wheat		08-Aug	08-Aug	09-Aug	Barley		08-Aug	08-Aug	09-Aug
103379/X	µg/kg	µg/kg	µg/kg	µg/kg	/Malt	µg/kg	µg/kg	µg/kg	µg/kg
1	<0.10	<4.0	<4.0	<4.0	17B	<0.10	<4.0	<4.0	<4.0
2	<0.10	<4.0	<4.0	<4.0	18M	<0.10	<4.0	<4.0	<4.0
3	0.43	<4.0	<4.0	<4.0	19M	0.80	<4.0	<4.0	<4.0
4	1.34	<4.0	<4.0	<4.0	20B	17.80	>4.0	>4.0	>4.0
5	<0.10	<4.0	<4.0	<4.0	21B	1.00	<4.0	<4.0	<4.0
6	1.90	<4.0	<4.0	<4.0	22B	9.50	>4.0	>4.0	>4.0
7	<0.10	<4.0	<4.0	<4.0	23M	<0.10	<4.0	<4.0	<4.0
8	<0.10	<4.0	<4.0	<4.0	24B	<0.10	<4.0	<4.0	<4.0
9	<0.10	<4.0	<4.0	<4.0	25M	0.20	<4.0	<4.0	<4.0
10	<0.10	<4.0	<4.0	<4.0	26M	<0.10	<4.0	<4.0	<4.0
11	1.28	<4.0	<4.0	<4.0	27M	<0.10	<4.0	<4.0	<4.0
12	0.64	<4.0	<4.0	<4.0	28B	0.20	<4.0	<4.0	<4.0
13	<0.10	<4.0	<4.0	<4.0	29B	<0.10	<4.0	<4.0	<4.0
14	0.59	<4.0	<4.0	<4.0	30B	<0.10	<4.0	<4.0	<4.0
15	10.53	>4.0	>4.0	>4.0	31B	<0.10	<4.0	<4.0	<4.0
16	4.39	>4.0	>4.0	>4.0	32M	<0.10	<4.0	<4.0	<4.0

The Ochratoxin A Flow-Through Assay is an example of a limit or threshold test. Interpretation of results is at a pre-defined value (in this case 4ppb). Evaluation of threshold test kits requires a different rationale than that applied to quantitative tests. The criteria applied relate to:

1. The ability of the kit to identify OA contamination at the defined threshold value.
2. The presence or absence of "false positives" and "false negatives".

From a user perspective, the Ochratoxin A Flow-Through Assay was the easiest test kit to use out of all those evaluated. The instructions supplied were clear and concise. The time from production of a ground sample to interpretation of the test data was under 30 minutes, conforming to the definition of a rapid test. The most contaminated samples were correctly identified in wheat, barley and malt samples, providing evidence of the kit being fit-for-purpose.

When comparing test kit data to the confirmatory test, an absence of "false positives" and "false negatives" was recorded. In all cases, analytical data were verified and

correctly categorised. The only concern with using this test kit is the amount of "background interference" observed on the membrane, which has the potential to delay interpretation. However, this is a relatively minor issue. As a surveillance or monitoring tool, Ochratoxin A Flow-Through Assay would be suitable for use at grain intake.

Threshold tests involving the use of immunoaffinity (IMA) columns

Ochracard P48 (r-Biopharm-rhône)

The Ochracard P48 test kit contains comprehensive instructions for the analysis of OA in a variety of foods. In relation to cereals, a water-based extraction method is followed by application to an IMA column. The desired screening level determines the volume of (eluting) solvent passed through the IMA column (Table 15).

Table 15: Solvent requirements for eluting OA from the IMA column using the Ochracard P48 test kit

	Screening level (ppb)			
	3	4	5	6
Volume (ml)	25	20	15	7.5

The routine for removal of OA from the column (desorption) is described in the test kit instructions. Each test card must be equilibrated at room temperature 30 minutes prior to measurement. Having determined the screening level required, a 500µL aliquot (of the total eluent volume) is applied to one port of the Ochracard device (two tests can be conducted using one Ochracard). A series of colour-coded reagents are added in sequence to the Ochracard. A 5 minute incubation precedes the application of the stop solution. The Ochracard is visually interpreted.

Interpretation of the result relies on the presence or absence of a purple spot in the viewing window. The presence of a purple spot indicates OA contamination below the chosen screening threshold and the absence of a spot indicates OA contamination above the screening threshold.

Data are shown in Table 16 (screening level 3ppb).

Table 16: Analysis of wheat, barley and malt using Ochracard P48 (r-Biopharm-rhône)

Sample ID	HPLC	Ochracard P48 OA (ppb)			Sample ID	HPLC	Ochracard P48 OA (ppb)		
Wheat		Lot: UH389/ A	Lot: UH389/ A	Lot: UH389/ A	Barley		Lot: UH389/ A	Lot: UH389/ A	Lot: UH389/ A
103379/ X	µg/kg	05-Aug	05-Aug	08-Aug	/Malt	µg/kg	05-Aug	05-Aug	08-Aug
1	<0.10	<3.0	<3.0	<3.0	17B	<0.10	<3.0	<3.0	<3.0
2	<0.10	<3.0	<3.0	<3.0	18M	<0.10	<3.0	<3.0	<3.0
3	0.43	<3.0	<3.0	<3.0	19M	0.80	<3.0	<3.0	<3.0
4	1.34	<3.0	<3.0	<3.0	20B	17.80	>3.0	>3.0	>3.0
5	<0.10	<3.0	<3.0	<3.0	21B	1.00	<3.0	<3.0	<3.0
6	1.90	<3.0	<3.0	<3.0	22B	9.50	>3.0	>3.0	>3.0
7	<0.10	<3.0	<3.0	<3.0	23M	<0.10	<3.0	<3.0	<3.0
8	<0.10	<3.0	<3.0	<3.0	24B	<0.10	<3.0	<3.0	<3.0
9	<0.10	<3.0	<3.0	<3.0	25M	0.20	<3.0	<3.0	<3.0
10	<0.10	<3.0	<3.0	<3.0	26M	<0.10	<3.0	<3.0	<3.0
11	1.28	<3.0	<3.0	<3.0	27M	<0.10	<3.0	<3.0	<3.0
12	0.64	<3.0	<3.0	<3.0	28B	0.20	<3.0	<3.0	<3.0
13	<0.10	<3.0	<3.0	<3.0	29B	<0.10	<3.0	<3.0	<3.0
14	0.59	<3.0	<3.0	<3.0	30B	<0.10	<3.0	<3.0	<3.0
15	10.53	>3.0	>3.0	>3.0	31B	<0.10	<3.0	<3.0	<3.0
16	4.39	>3.0	>3.0	>3.0	32M	<0.10	<3.0	<3.0	<3.0

The use of IMA columns in the analytical process represents a slight departure from methods already described. Antibody coated columns specific to the OA antigen provide a means to isolate the contaminant at the expense of possible interferences. The screening level selected for analysis was 3ppb.

Data from Table 16 highlight the ability to detect OA contamination in the study samples using the Ochracard P48 test kit. All samples contaminated above the screening level (3ppb) were correctly identified, although, in total, only 4 samples exceeded this threshold. The method was easy to use and the instructions supplied were comprehensive.

There was an absence of "False positives" and "False negatives", when comparing the Ochracard P48 test data to the results of the confirmatory analysis, which is an adequate indicator of assay performance for a threshold test kit.

However, the authors agree that the extraction process is time consuming and labour intensive. One batch of 8 duplicate samples would take 5-6 hours. This is considered a disadvantage in relation to some of the other test kits already described.

Ochrascan (r-Biopharm-rhône)

The Ochrascan (r-Biopharm-rhône) test kit describes a simple OA extraction procedure followed by application of the extract to an IMA column containing a monoclonal antibody which selectively retains the OA antigen. An organic solvent (chloroform) is then applied to the column desorbing the OA antigen which passes through the column and into a florisil tip. The tip is then read at 366nm. The natural fluorescence of OA is compared to a comparator card (supplied with the test kit), with assigned OA values. Two series of values are available representing two screening levels, the upper series relating to screening at 10ppb and the lower series representing screening at 4ppb.

Data are shown in Table 17.

The data shown in Table 17 represent the ability of the Ochrascan (r-Biopharm-rhône) test to detect OA contamination in wheat, barley and malt samples at the 4ppb screening level. The data positively identify the most contaminated samples, but reveal an overestimation of OA levels in selected cases in the study sample set. Indeed comparison of data generated by the confirmatory test with that obtained using the Ochrascan (r-Biopharm-rhône) test kit revealed a number of "False positives" (Table 18).

In all cases the samples identified were lower than the legislative limit for grain intake (using confirmatory test data and data obtained from other rapid tests) and could adversely affect acceptance of the test kit as a screening tool for OA analysis. Additionally, the authors had concerns regarding the complex sample preparation procedure (which is more complex than the confirmatory test). There were also concerns regarding the use of chloroform in the test procedure.

Table 17: Analysis of wheat, barley and malt using Ochrascan (r-Biopharm-rhône)

Sample ID	HPLC	Ocrascan OA (ppb)			Sample ID	HPLC	Ocrascan OA (ppb)		
Wheat		Lot:VA480	Lot:VA480	Lot:CE329	Barley		Lot:VA480	Lot:VA480	Lot:CE329
103379/X	µg/kg	µg/kg	µg/kg	µg/kg	/Malt	µg/kg	µg/kg	µg/kg	µg/kg
1	<0.10	nd	nd	nd	17B	<0.10	nd	nd	nd
2	<0.10	nd	nd	nd	18M	<0.10	10	10	10
3	0.43	nd	nd	nd	19M	0.80	nd	nd	nd
4	1.34	nd	nd	nd	20B	17.80	20	20	20
5	<0.10	nd	nd	nd	21B	1.00	nd	nd	nd
6	1.90	>4<10	>4<10	>4<10	22B	9.50	>4<10	>4<10	>4<10
7	<0.10	nd	nd	nd	23M	<0.10	nd	nd	nd
8	<0.10	nd	nd	nd	24B	<0.10	nd	nd	nd
9	<0.10	nd	nd	nd	25M	0.20	nd	nd	nd
10	<0.10	nd	nd	nd	26M	<0.10	nd	nd	nd
11	1.28	>4<10	>4<10	>4<10	27M	<0.10	>4<10	>4<10	>4<10
12	0.64	nd	nd	nd	28B	0.20	nd	nd	nd
13	<0.10	nd	nd	nd	29B	<0.10	nd	nd	nd
14	0.59	nd	nd	nd	30B	<0.10	nd	nd	nd
15	10.53	10	10	10	31B	<0.10	nd	nd	nd
16	4.39	>4<10	>4<10	>4<10	32M	<0.10	10	10	10

nd= not detected

Table 18: False positive identification using Ochrascan (r-Biopharm-rhône)

Sample ID	HPLC	Ochrascan OA (ppb)			Sample ID	HPLC	Ochrascan OA (ppb)		
Wheat		Lot:VA480	Lot:VA480	Lot:CE329	Barley		Lot:VA480	Lot:VA480	Lot:CE329
103379/X	µg/kg	µg/kg	µg/kg	µg/kg	/Malt	µg/kg	µg/kg	µg/kg	µg/kg
6	1.90	FP (>4<10)	FP (>4<10)	FP (>4<10)	18M	<0.10	FP (10)	FP (10)	FP (10)
11	1.28	FP (>4<10)	FP (>4<10)	FP (>4<10)	27M	<0.10	FP (>4<10)	FP (>4<10)	FP (>4<10)
					32M	<0.10	FP (10)	FP (10)	FP (10)

Summary of results and conclusions

1. Six test kits were evaluated in this study, using sample sets of wheat, barley and malt.
2. All test kits were capable of OA detection and conform to the manufacturers' description of being "fit-for-purpose".
3. Considering the diffuse distribution of OA in foods, the performance of test kits compared favourably with data obtained using the confirmatory test.
4. Repeatability and reproducibility of analysis was acceptable.
5. False positives were an issue with one of the threshold kits.
6. Test kits based on microtiter plate (ELISA) provided fully quantitative data comparable to confirmatory tests.
7. LFD kits are available which provide fully quantitative data comparable to the confirmatory procedure.
8. Threshold tests based on LFD technology were simple to use and are effective screening tools for use at grain intake.
9. Where it is likely that screening is required at more than one legislative limit (such as for barley and malt), care should be taken that the kit selected is capable of screening at the limits required.
10. Threshold test kits based on IMA columns generally provided adequate data but are complicated, time consuming and did not provide any advantages over the other approaches evaluated.
11. The use of 'real world' samples allowed kits to be evaluated in this project but a general lack of standard materials (ideally naturally contaminated OA wheat/barley) makes routine assessment of assay and operator performance difficult.

Acknowledgements

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Appendix - initial literature review

Analytical approaches to the determination of Ochratoxin A (OA) in foods, with specific reference to cereals (including wheat and barley) and products based on processed barley - a literature review of methods

Table of contents for literature review

Abbreviations used

A.	-*Aspergillus*
a_w	-water activity
b.w.	-body weight
CZE-LIF	-Capillary Zone Electrophoresis-Laser Induced Fluorescence
ELISA	-Enzyme-Linked Immunosorbent Assay
EU	-European Union
FAO/WHO	-Food and Agriculture Organization of the United Nations/ World Health Organisation
FAPAS	-Food Analysis Performance Assessment Scheme
HACCP	-Hazard Analysis and Critical Control Point
IMA	-Immunoaffinity
JECFA	-Joint Expert Committee on Food Additives
LC	-Liquid Chomatography
LC-MS	-Liquid Chromatography-Mass Spectrometry
LFD	-Lateral Flow Device
LOD	-Limit of Detection
MIP	-Molecular Imprinted Polymer
μgkg^{-1}	-microgram per kilogram (equivalent to ppb)
μgl^{-1}	-microgram per litre (equivalent to ppb)
$ngkg^{-1}$	-nanogram per kilogram (equivalent to ppt)
OA	-Ochratoxin A
OB	-Ochratoxin B
P.	-*Penicillium*
PTWI	-Provisional Tolerable Weekly Intake
RSD_r	-Relative standard deviation for repeatability
RSD_R	-Relative standard deviation for reproducibility
SCF	-Scientific Committee for Food
SPE	-Solid-Phase Extraction
SRM	-Standard Reference Material
TDI	-Tolerable Daily Intake Level

Summary of review

Ochratoxins are secondary metabolites produced by certain species of mycopathogenic fungi. The genera identified are *Penicillium* and *Aspergillus*.

The scientific literature identifies Ochratoxin A (OA) as the most important Ochratoxin. Its abundance in a variety of raw material commodities presents a significant threat to health of both humans and monogastric livestock. Acute contamination (Ochratoxicosis) is characterised by nephropathy (a reduction in kidney function), enteritis (fatty liver) and necrosis of the lymph nodes and suppression of the immune system (Krogh, 1974). Accumulation of OA in the tissues of livestock has major human health implications. Epidemiological studies support the hypothesis that OA is a causative agent of nephropathy in humans (Krogh, 1976).

A risk assessment by the joint FAO/WHO Experts Committee on Food Additives (JECFA, 2001) concluded that over 50% of human exposure to OA was attributable to cereals and cereal-based products, and that reduction of OA is considered desirable.

The introduction of Commission Regulation EC No 1881/2006 – setting maximum levels for certain contaminants in foodstuffs, and Commission Recommendation 2006/576/EC – on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 and fumonisins in products intended for animal feeding, establishes targets for grain traders and cereal processors to meet in order to prevent heavily contaminated material entering the food and feed supply. This implies that a regime of routine analysis for surveillance and control purposes will be required in order to meet legislative limits.

This review summarises published analytical approaches which have been used to estimate OA in raw and processed cereals. It documents examples of the fully validated confirmatory procedures based on chromatographic separations to the innovation of rapid test technology.

The rapid test methods described are based on: immunochemistry, biosensor technology, fluorescence polarisation, electrophoresis, molecular imprinted polymers and chromatography-mass spectrometry.

Of the methods considered, immunochemistry provides the most realistic approach, both in terms of commercially available technology (in a variety of test kit formats) and ease of use.

Introduction

Ochratoxins are secondary metabolites produced by certain species of fungi (a single *Penicillium* species: *P.verrucosum* and several species of *Aspergillus*: *A. ochraceus, A.carbonarius* and *A.niger*).

OA has been isolated from cereals (barley, wheat, maize and oat), green coffee beans, malt containing beverages, peanuts, sorghum, olives, beer, pork and poultry, cheese, fruits and wine infected with *Aspergillus* species or *P.verrucosum* (Pitt, 2000; Bennett and Klich, 2003; Pardo *et al.*, 2006). These are called storage fungi that synthesise the OA post-harvest during drying or storage. The occurrence and formation of the fungi might vary from year-to-year and is dependent on level of infection, microbial species present, grain moisture, temperature of storage and time of storage (Scudamore *et al.*, 1999; Bennett and Klich, 2003). Damaged grains are more susceptible to the disease than intact grains; therefore, conditions such as frost damage, drought stress and higher than average rainfall may all elevate levels of the disease (Scudamore *et al.*, 1999).

The scientific literature identifies Ochratoxin A (OA) as the most important ochratoxin. Its abundance in a variety of raw material commodities presents a significant threat to health of both humans and monogastric livestock. OA present in feed materials contaminates most of the edible tissues resulting in kidney disorders which may render the carcass unfit for human consumption. Acute contamination (Ochratoxicosis) is characterised by nephropathy (a reduction in kidney function), enteritis (fatty liver) and necrosis of the lymph nodes and suppression of the immune system. Accumulation of OA in the tissues of livestock has major human health implications. Epidemiological studies support the hypothesis that OA is a causative agent of nephropathy in humans (Krogh, 1976).

A risk assessment by the joint FAO/WHO Experts Committee on Food Additives (JECFA, 2001) concluded that over 50% of human exposure to OA was attributable to cereals and cereal-based products, and that reduction of OA is considered desirable. The Committee upheld previous provisional tolerable weekly intake (PTWI) levels of 100 $ngkg^{-1}$ body weight (b.w.)/week, corresponding to 14 $ngkg^{-1}$ b.w./day. The European Scientific Committee for Food (SCF) advised food processors to reduce OA to levels closer to the lower Tolerable Daily Intake level (TDI), i.e. below 5 $ngkg^{-1}$ b.w./day. Legislators have endorsed this viewpoint by introducing maximum

permissible limits for OA in cereal-based products. To harmonize different legislation in EU member states, Commission Regulation (EC) No 1881/2006 established maximum Community limits for contaminants. The maximum permissible level for OA in raw, unprocessed cereal grains was set at 5.0 μgkg^{-1}. For cereal-based foods destined for human consumption, the maximum permissible limit is 3.0 μgkg^{-1} while for cereal-based foods destined for consumption by infants and young children as well as dietary foods for special medical purposes intended specifically for infants the limit is 0.5 μgkg^{-1}.

Both *Aspergillus* and *P. verrucosum* can produce OA in cereals; however, *P. verrucosum* tends to be the predominant OA-producing species on barley in northern Europe. Research shows that contaminating fungi tend to be distributed heterogeneously throughout barley lots (Lund and Frisvad, 2003). It is thought that if greater than 7% of the grains were contaminated with fungi then OA is likely to be present in the sample. Levels of OA are typically higher in barley than in wheat or oats (Scudamore *et al.*, 1999). Regional differences in OA concentrations have also been demonstrated, with samples from the north and west of the UK being more frequently contaminated (Scudamore *et al.*, 1999). These authors also showed that OA levels increased with storage time and with increasing moisture content. The maximum frequency was detected when the stores were being emptied prior to the next harvest intake.

Prevention of OA formation and accumulation in cereals relies on efficient and effective storage practices. There are two governing principles.

- To prevent or reduce infection by OA-toxigenic moulds.
- To avoid conditions conducive to mould growth.

Implementing a management system based on Hazard Analysis and Critical Control Point (HACCP) is the only effective means to manage the risk of contamination of stored food commodities. Control of the environmental and storage conditions conducive to mould growth would effectively reduce the development and accumulation of OA. Analysis for residual contamination is conducted in order to:

1. Validate installed management and control strategies.

2. Verify the efficiency and effectiveness of installed management and control procedures.

The robustness of analytical results is paramount when used as a surveillance or control measure for OA estimates in relation to: monitoring standards for trading, grain storage and processing; and studies involving the intake and OA turnover in toxicological studies. It is unlikely, therefore, that a single analytical approach will be suitable or applicable to all situations. The aim of this review is to provide a simple treatment of available and published methodologies related to OA measurement in foodstuffs, emphasising analytical approaches for determination in cereal-based foods, and the suitability of their application as surveillance tools in a variety of grain handling and processing situations. Validation and accreditation of methods and use of standard reference materials will also be investigated as part of the overall project with a view to providing the potential end-user with additional confidence in the analytical approach.

Sample considerations

Sampling

Sampling is arguably the most important facet of any analysis (Miraglia *et al.*, 2005), and is particularly relevant in the case of OA (as it is in all mycotoxin analysis). It is recognized that the distribution of OA is less homogeneous than aflatoxins, although specific studies focussed on the distribution of OA in contaminated cereal parcels are not widely published.

A recent study by Biselli (2007) concluded that sampling has a profound effect on the consistency of analytical results. The reasons are two-fold:

1. Heterogeneity with respect to OA distribution.
2. OA is present in low concentrations.

Using the EU standard protocol introduced in 2005 (Commission Directive 2005/EC 2005), the impact of sampling at various stages of the analysis was assessed. Addition of the sampling components provides an estimate of the total measurement Uncertainty (U_{TOTAL}):

$U_{TOTAL} = U_{SAMPLING} + U_{SAMPLE\ PREPARATION} + U_{SAMPLE\ ANALYSIS}$

With regard to OA measurement, U_{TOTAL} was estimated at 35.6%. (A factor of 2 is usually applied to obtain an extended Uncertainty U_{TE} = 71.3%). The estimation of the contribution made by the initial sampling component ($U_{SAMPLING}$) was by far the greatest (Biselli, 2007).

For situations other than those for official control, implementing a sample protocol according to Commission Directive 2005/EC 2005 is not practicable.
In all cases, the sampling protocol which forms the basis on which analysis is conducted should be clearly stated.

Sample preparation

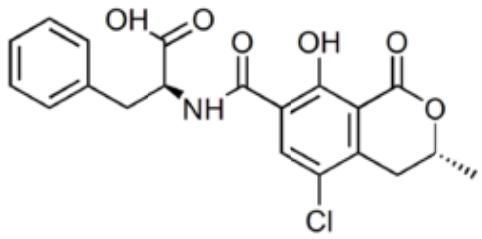
The objective of sample preparation is to produce an isolate of the target analyte, which can be taken into the analysis. Ideally, the test portion should contain proportionally the same concentration of OA as the larger sample from which the test portion is taken.

Sample preparation consists of two steps: grinding the test portion to a uniform particle-size distribution and isolation of the target analyte (in this case OA).

Analytical methods

Chemistry

Chemically, ochratoxins are complex structures. They are classified as pentaketides, the result of coupling dihydro-isocumarin with ß-phenylalanine (Pohland *et al.*, 1992). The chemical structure of OA is shown below:



OA and the less abundant dechloro analogue Ochratoxin B (OB) have been detected in a variety of food raw materials, e.g. cereals, dried vine fruits and coffee. Additionally, minute amounts of the methyl-, methyl ester and 4-hydroxy-derivatives have been detected.

OA has been detected in a variety of food raw material commodities. The purpose of this review is to concentrate on analytical methods suitable for detection of OA in cereal commodities and processed cereal products (specifically related to wheat and barley).

Methods based on liquid chromatography (LC)

The classical analytical approach is based on liquid chromatography (LC) with fluorescence detection. Isolation of the OA target molecule often includes a solid-phase extraction (SPE) concentration (clean-up) followed by elution using a reversed-phase C_{18} , silica gel SI-60 or immunoaffinity (IMA) columns.

The first collaborative study to validate a method suitable for the detection of OA in maize and barley was conducted by Nesheim *et al.* (1992). Extraction of the OA target analyte was effected by chloroform and aqueous phosphoric acid phases, clean-up using a reversed-phase C_{18} SPE cartridge, followed by liquid-liquid partitioning into an aqueous phase containing bicarbonate ions. The extract was analysed using a reversed-phase LC elution program and fluorescence detection. Accuracy was determined using the analyte recovery (spiking) method, using solutions of known concentration in the range 10-50 $\mu g kg^{-1}$. OA was confirmed by subsequent analysis. A collaborative ring trial involving 16 laboratories provided the data in Table 1.

The method is quantitative at ≥ 10 $\mu g kg^{-1}$ in maize and barley and was incorporated as a final-action AOAC International Official Method 991.44.

Larsson and Moller (1996) validated AOAC 991.44 in naturally contaminated wheat bran, barley and rye. Pure OA spikes were introduced in the range 2-9 μgkg^{-1} . The method was performance tested by 12 laboratories (Table 2).

Table 1: Results of the first collaborative ring trial for OA

Matrix	OA (μgkg^{-1})	Number of acceptable results	Mean recovery (%)	RSD_r (%)	RSD_R (%)
Maize	0.8	15	No Data	No Data	No Data
	8.2	15	82	No Data	21
	16	15	82	20	28
	40	14	77	No Data	32
Barley	0.8	15	No Data	No Data	No Data
	3	15	74	No Data	28
	7.4	15	74	No Data	27
	14	14	72	7.9	26

Table 2: Results for a collaborative ring trial to validate AOAC 991.44 in naturally contaminated wheat bran, barley and rye

Matrix	OA (μgkg^{-1})	Number of acceptable results	Mean recovery (%)	RSD_r (%)	RSD_R (%)
Wheat Bran	3.8	12	70	21	24
	4.5	12	68	17	26
Barley	2.9[a]	12	No Data	17	22
	3.0	12	No Data	15	23
Rye	2.8	12	64	22	29
	4.8	12	65	16	23

[a]Represents natural contamination

The European Committee for Standardisation (CEN) has adopted this method (EN ISO 15141-2) for OA determination in wheat bran, barley and maize.

A more sensitive method for OA determination in wheat was proposed by Majerus *et al.* (1994). Sample pre-treatment with hydrochloric acid plus magnesium chloride was followed by extraction with toluene. The mixture was allowed to separate into solid and liquid phases, filtered and applied to a mini-column of silica gel. The OA containing extract was analysed by reversed-phase LC and detected by fluorescence. The method was performance tested (in the range 0.4-1.2 μgkg^{-1}) by 13 laboratories (Table 3).

Table 3: Results of a collaborative ring-trial for OA involving 13 laboratories

Matrix	OA (μgkg^{-1})	Number of acceptable results	Mean recovery (%)	RSD_r (%)	RSD_R (%)
Wheat	0.4	13	80	15	26
Wholemeal	1.2	13	80	20	32

The European Committee for Standardisation (CEN) has adopted this method (EN ISO 15141-1) for OA determination in cereal and cereal products.

Use of immunoaffinity (IMA) columns

Isolation of OA during the extraction procedure has been enhanced by the advent of antibody-based immunoaffinity (IMA) columns. Entwisle *et al.* (2000) applied the technology to the determination of OA in barley. A crude OA extract was produced using an acetonitrile/water solution. The supernatant was filtered and diluted with a phosphate-buffered saline solution, which had been passed through the IMA column. As before, the eluent was analysed by LC and detected by fluorescence. The method was performance tested by 15 laboratories using naturally contaminated and spiked samples in the range 1.2.-4.5 μgkg^{-1} (Table 4).

The European Committee for Standardisation (CEN) has adopted this method (EN ISO 14132:2003) for OA determination in barley (and roasted coffee). Additionally, the method was adopted as First-Action Method AOAC 2000.03.

Table 4: Results of a 15 laboratory collaborative study to measure OA by HPLC-Fluorescence following IMA clean-up

Matrix	OA (μgkg^{-1})	Number of acceptable results	Mean recovery (%)	RSD_r (%)	RSD_R (%)
Barley	0.1	14	No Data	26	72
	1.3[a]	15	No Data	24	33
	3[a]	14	No Data	12	17
	3.7	12	93[b]	4	12
	4.5[a]	12	No Data	14	15

[a]Naturally contaminated samples

[b]Mean recovery for an average of 12 samples at 3.7 μgkg^{-1}

IMA protocols have been extended for the analysis of OA in wine and beer. Visconti *et al.* (2001) diluted the sample with water containing polyethylene glycol (PEG) and sodium bicarbonate and then applied it to an IMA column. The OA-containing eluent was analysed by reversed-phase LC and detected by fluorescence. The method was validated in a collaborative trial by 15 laboratories at concentrations of: 0.01 – 1.8 μgkg^{-1} for white wine, 0.01 – 2.5 μgkg^{-1} for red wine and 0.01 – 1.4 μgkg^{-1} for beer (Table 5).

The European Committee for Standardisation (CEN) has adopted this method (EN ISO 14133:2003) for OA determination in wine and beer. The method has also been adopted as First-Action Method AOAC 2001.01.

Table 5: Results of a collaborative study involving 15 laboratories to measure OA in wine and beer using HPLC-Fluorescence, following IMA clean-up

Matrix	OA (μgkg^{-1})	Number of acceptable results	Mean recovery (%)	RSD_r (%)	RSD_R (%)
White wine	< 0.01	No Data	No Data	No Data	No Data
	0.1	13	100	10	14
	0.28	15	No Data	11	15
	1	14	91	6.6	14
	1.8	14	88	8.5	13
Red wine	< 0.01	No Data	No Data	No Data	No Data
	0.19	12	93	5.5	9.9
	0.81	14	90	9.9	12
	1.7	14	No Data	11	13
	2.5	15	85	8.9	13
Beer	< 0.01	No Data	No Data	No Data	No Data
	0.07	14	No Data	19	20
	0.19	13	95	10	18
	0.7	15	87	7.2	18
	1.4	13	94	4.6	16

Proficiency testing

The data reported thus far have been validated by collaborative trial. Proficiency testing provides a means to compare the result of a "blind" test sample against the best estimate of the statistically derived assigned (true) value, for the analyte in question. The Food Analysis Performance Assessment Scheme (FAPAS, 2005) organizes proficiency testing on a regular basis. Participating laboratories submit OA analysis in a variety of matrices. A summary of 2004/5 data for OA in wheat and barley is provided in the table below.

Table 6: Results of FAPAS proficiency testing for barley and wheat (2004/5)

Report number	Matrix	Assigned OA Value ($\mu g kg^{-1}$)	Results submitted	Acceptable Scores[a]
1732	Barley	5.60	95	75 (79%)
1737	Wheat	24.50	79	66 (84%)

[a]Acceptable scores defined as z=±2

The most often used procedure was IMA cleanup followed by LC separation and fluorescence detection. There are little data of statistical significance from other methods of analysis. The data show that 1 in 5 barley and 1 in 6 wheat submissions did not fulfil the criteria for the test.

Immunochemical methods

All of the methods described so far:

- Are time consuming.
- Are labour intensive.
- Require significant capital outlay for sophisticated instrumentation.
- Have high maintenance costs.
- Need highly skilled/trained personnel.
- Are laboratory based.
- Cannot be used to conduct analysis *in situ*.

For the reasons stated, the collaboratively validated laboratory-based procedures are used for regulatory or official control purposes, or optionally in retrospective routine analysis of large batches. However, there are a number of situations, particularly in food commodity handling and industrial processing, which require a different analytical approach. This approach can be described as analytical surveillance/monitoring, and the methods available for use in such situations have yet

to be tested, either by an independent third-party, or by collaborative ring-trial. An easy-to-use field based technique would enable grain handlers and processors to determine the OA contamination rate *in situ*.

The major drawback to field-based techniques like ELISA, lateral flow devices and immunosensors is that they are prone to matrix interferences (cross-reactivites) which may give rise to false positive or false negative results (Lacey *et al.*, 1991; Visconti and De Girolamo, 2005). This was particularly the case in some of the older immunoassays which lacked the sensitivity of modern equivalents. They may also lack the sensitivity of HPLC-based tests.

There have, however, been some advances in sample preparation methods and the availability of commercial kits and more information is now being presented in the scientific literature.

The various rapid test approaches will now be described.

Enzyme-Linked Immunosorbent Assay (ELISA)

Enzyme Linked Immunosorbent Assays (ELISA) are immunochemical techniques based on specific antigen-antibody reactions. The test principle is based on competitive-ELISA principles. The wells of the microtiter plate are coated with antibodies specific to OA. An extract containing OA and OA-enzyme conjugate are added to the test well. The free and conjugated OA compete for the binding sites of the (well coated) OA antibodies. Unbound enzyme conjugate is removed by washing. Enzyme substrate and a chromogen are added to the wells and the mixture is incubated for a fixed time period. Bound enzyme conjugate converts the colourless chromogen to a coloured complex. Stopping solution is added, changing the colour once more and a measurement is made spectrophotometrically (at a wavelength determined by the chromogen used). The absorbance value measured is inversely proportional to the concentration of OA in the sample.

Gumus *et al.* (2004) used the Ridascreen Ochratoxin A ELISA kit to determine OA in barley, malt and beer. The lower detection limit (LOD)was 0.08 μgl^{-1} for beer and 0.4 μgkg^{-1} for barley and malt. Recovery rates were more than 85%. The sensitivity of

this method appears comparable to LC methods but analysis is only cost-effective when conducted on multiple samples, i.e. a batch.

Zheng *et al.* (2005) spiked OA-free corn to final OA concentrations levels of 0, 2, 5, 10, 20, 30, 40 and 80 μgkg^{-1} and compared the AgraQuant ELISA Ochratoxin A Assay test kit (Romer Labs Asia) with HPLC. The ELISA had an LOD of 1.9 -3.8 μgkg^{-1} depending on the commodity tested (corn, milo, wheat, barley, soybeans and green coffee). The kit could detect both ochratoxin A and B, but underestimated levels of toxin in barley and soybeans at the higher (80 μgkg^{-1}) spiked levels.

Wang *et al.* (2007) raised their own polyclonal antibodies and developed both a competitive direct (cd) ELISA and a membrane–based colloidal gold flow-through device. For the cdELISA the recovery rate was 74-110% and correlations between this and HPLC results were good (R^2 = 0.984). They found that matrix effects of beer could be removed by diluting it 25-fold in PBS. The concentration of analyte causing 50 % inhibition of colour development was 0.07 $ngml^{-1}$. The membrane-based colloidal gold assays had a visual detection limit of 1.0 $ngml^{-1}$ and a detection time of less than 10 minutes. Both methods were considered by these authors as being suitable for OA detection in foods.

Commercially available immunochemical methods provide:

- Fully quantitative and rapid analysis.
- A simple analysis protocol.
- Potential cost advantages when compared to classical approaches.
- Enhanced sensitivity over classical procedures.
- Specificity.
- Low operator qualification.
- Portable analysis formats.
- An ability to conduct analysis *in situ.*

Commercially available ELISAs are conveniently packaged in the form of a "Test Kit", providing:

- A 48 or 96 well (antibody-coated) microtiter-plate.
- Enzyme-labelled OA toxin (Conjugate).

- Enzyme substrate.
- A series of standards.

Additionally, analysis may require:

- A sample grinder (fitted with an 800µm screen).
- Items of glassware.*
- Organic solvent (methanol) for the extraction.
- Plate-reader or microwell reader.

*Some suppliers provide an extraction kit.

The above items are purchased separately.
A summary of ELISA based test kits is provided in Table 7.

The r-Biopharm Group (Darmstadt, Germany) also supplies an IMA clean-up column which can be used in conjunction with ELISA based systems. Monoclonal antibodies (specific to OA) are covalently bonded to mini-column support material. OA (in an applied extract) binds to the monoclonal antibodies. Addition of methanol denatures the antibodies, releasing the OA for analysis.

Lateral Flow Devices (LFD)

Lateral flow or "dipstick" technology is one of the fastest growing areas in diagnostic testing. Early work resulted in a lateral flow device for OA with a sensitivity of 4 μgkg^{-1} which was used to test samples of wheat, rye, maize and barley (De Saeger *et al.*, 2002). This was suitable as a screening device but did occasionally give false positive or false negative results.

A LFD consists of an inert, porous membrane supporting immobilized antibodies. Specific OA-antibodies, sample and enzyme-labelled OA (enzyme conjugate) are added sequentially to the porous strip. The OA specific antibodies bind to the immobilized antibodies. On addition of an OA containing extract, the sample wicks along the membrane, the OA binding to the OA-specific antibodies. Enzyme–labelled OA is then added which binds to any "free" OA-specific antibodies. Unbound enzyme conjugate is removed by washing.

Table 7: Supplier information on ELISA based test kits for OA

Supplier	Web address	Test Kit/ No tests	LOD (ppb)	Range (ppb)	Cross-reactivity (%)
BioControl Systems Diagnostics Ltd	www.biocontrolsys.com (previously found at www.raisio.com)	Ochratoxin-A EIA (96 wells) (16 standards/controls plus 40 samples)[a]	1.0[b]	0.25 – 5.0	100 Ochratoxin A 9.3 Ochratoxin B <0.1 Ochratoxin α[f] <0.1 Coumarin <0.1 4-Hydroxy-coumarin
Neogen Europe	www.neogeneurope.com	Veratox for Ochratoxin (48 wells) (10 standards/controls plus 19 samples)	1.0	2.0 –25.0	100 Ochratoxin A 18 Ochratoxin B
r-Biopharm-rhône	www.r-biopharmrhone.com	Ridascreen Ochratoxin A (96 wells) (10 standards/controls plus 19 samples)	0.63	0 - 2.03[d] 0-50[e]	100 Ochratoxin A 14 Ochratoxin B 44 Ochratoxin C[g] <0.1 Ochratoxin α
		Ridascreen Ochratoxin A 30/15 (96 wells) 12 standards/controls plus 36 samples)	1.25[c]	0 – 1.8[d] 0-36[e]	
Romer Labs	www.romerlabs.com	AgraQuant Ochratoxin Assay 2/40 48 and 96 wells (10 standards/controls plus 19/38 samples)	1.9	2.0 – 40.0	100 Ochratoxin A 108 Ochratoxin B

[a]Based on duplicates
[b]Based on ±3SD
[c]2.5µgKg^{-1} for rapid screening
[d]Stated
[e]Accounting for dilution
[f]Metabolite of Ochratoxin A
[g]Metabolite of Ochratoxin A

A chromogen is added which reacts with the bound enzyme to form a coloured complex. After a short incubation period, a coloured band appears in the result window, which can be interpreted either:

1. Visually against a card.
2. Inserted into a portable reader (purchased separately).

The colour of the test band is inversely proportional to the concentration of OA in the sample.

The benefits of LFDs (over and above microtiter plate based ELISAs) are:

- Single samples or small batches can be analysed rapidly.
- Qualitative and quantitative formats are available.

A summary of LFD test kits for OA is provided in Table 8.

Table 8: Supplier information on LFD-based test kits for OA

Supplier	Web address	Test kit/No Tests	LOD (ppb)	Screening Range (ppb)
Charm Sciences Inc	www.charm.com	ROSA Ochratoxin (Quantitative) Test[a] 20/100/500 LFD Tests	<1.0	0 - 12
BioControl Systems Diagnostics Ltd	www.biocontrolsys.com	Ochratoxin-A Flow-Through Rapid Test 10 LFD Tests	N/A[b]	4[c]

[a]GIPSA approved (Certificate No. FGIS 2008-104)
[b]Screening test
[c]Screening level

Rapid test methods based on IMA columns

One manufacturer, r-Biopharm-rhône, has used IMA technology to refine and simplify sample preparation in rapid test formats. Two options are available:

1. Ochracard: following extraction using PBS, the sample is passed through an IMA column. A portion of the eluent is applied to the Ochracard, which contains monoclonal antibodies specific to OA. OA present in the sample is retained by the antibodies. Further addition of enzyme-conjugate binds to "free" antibody sites. Excess conjugate is removed by washing. Addition of substrate containing the chromogen, followed by a stopping reagent causes the development of a purple stain where the conjugate has bound to the antibody. The intensity of the coloured spot is compared to a colour comparator card, providing a semi-quantitative estimate of OA in the sample.

2. Ochrascan: following a simple extraction using an aqueous solution of sodium bicarbonate, a portion of the extract is applied to the Ochrascan IMA column, containing bound monoclonal antibodies. OA in the sample is retained on the IMA column. Application of chloroform to the IMA column elutes bound OA to be retained by a florisil tip. The tip is viewed under UV light at 366nm, the OA showing up as a fluorescent band. The intensity of the band is compared with a comparator card, providing a qualitative estimate of OA in the sample.

Additional details are supplied in Table 9.

Table 9: Supplier information on rapid test kits based on immunoaffinity (IMA) columns

Supplier	Web address	Test kit/No Tests	LOD (ppb)	Screening Range (ppb)
r-Biopharm-rhône	www.r-biopharmrhone.com	Ochracard P48 20 Tests	N/A[a]	3 - 10
		Ochrascan 25 Tests	2	0 - 20[b]

[a]Screening test
[b]Dependent on extract volume analysed

Other methods

A number of alternative methods of detection have also been reported and examples of these are discussed below.

Array biosensors

The OA toxin is simply extracted from the ground sample with methanol, and then added to the array biosensor.

Ngundi *et al.* (2005) used an array biosensor based on a competitive immunoassay format. The array consisted of an immobilised OA-derivative in competition with the toxin (in solution), for a fluorescent anti-OA antibody, introduced (spiked) in the sample mixture. The method sensitivity was claimed to be similar to that observed in traditional immunoassay and ELISA formats and varied according to the source of the sample. The benefit was the reduction in sample preparation, although it was claimed that pre-concentration and sample clean-up lowered the limit of detection.

In a refinement of the assay, the same authors developed regenerable arrays using anti-OA antibodies in a multiplex reaction for several mycotoxins (Ngundi *et al.*, 2006). Here fluorescently-labelled antibodies to OA and DON compete with sample to bind antibodies on an array. Fluorescent signals decrease with increasing concentrations of free mycotoxins in methanol extracts. The limits of detection of this array were 60 µg OA /kg barley. This limit is higher than for ELISA-based techniques and above the maximal levels for malt, but given that the technology is fairly new it is likely to improve in sensitivity with time. These authors are also investigating ways to make the surfaces regenerable, which would reduce the cost of the test.

Alarcon *et al.* (2005) described an immunochemical technique which combined the selectivity of a competitive immunoassay with the sensitivity claimed by electrochemical screen-printed electrodes of pure carbon construction. OA extracts from wheat were prepared in aqueous acetonitrile and assayed directly (i.e. without sample clean-up). The limit of detection was 0.4 $\mu g kg^{-1}$. Results correlated positively with those obtained (on the same wheat extracts) using LC with fluorescence detection following IMA column cleanup.

Time-resolved fluoroimmunoassay

Other antibody-based methods have been developed. For example an indirect competitive time-resolved fluoroimmunoassay (TRFIA) with a recovery rate of 82-104% has been shown to be capable of detecting OA within a working range of 0.02-400 μgl^{-1}. In this study, the concentration of analyte causing 50 % inhibition of colour development was 1.018 $ngml^{-1}$ (Huang *et al.*, 2006). This suggests that the TRFIA test was less sensitive than the cdELISA developed by Wang *et al.* (2007).

Fluorescence polarisation

Shim *et al.* (2004) describe a competitive fluorescence polarisation immunoassay for OA in standard solutions covering a concentration range of 5.0 - 200.0μgl^{-1}. The observed limit of detection was 3.0μgl^{-1}. Recoveries of OA extracted from barley spiked with 50.0 - 100.0μgl^{-1} (and compared with ELISA) were in excess of 90% (for each procedure), although there were reported matrix effects which created a disparity between data sets.

Methods based on spectroscopy

More recently Adányi *et al.* (2007) reported the use of optical waveguide lightmode spectroscopy (OWLS), a chip-based technique for the detection of OA in competitive immunoassays. The OWLS technique compared well with ELISA formats using the same antibodies and had a detection range between 0.5 and 10 μgl^{-1} in buffer samples. In barley samples, the matrix effect could be eliminated by preparing standard solutions in the matrix and constructing a calibration curve. The regression coefficient (R^2) was calculated as 0.96.

Capillary Zone Electrophoresis-Laser Induced Fluorescence (CZE-LIF)

Corneli and Maragos (1998) reported a method for OA isolation and quantification based on CZE-LIF in a variety of commodities including maize. The sample was processed using silica followed by IMA column cleanup. Reported sensitivity was comparable to LC with fluorescence and was free from matrix effects. Using analyte recovery (spiked) in the range 0.2 – 10 μgkg^{-1}, the mean recovery for maize was 99%. The method has a number of benefits over LC with fluorescence detection

including limited sample requirement, inexpensive capillaries and absence of toxic organic solvents.

Molecular imprinted polymers (MIP)

Technology exists to polymerise monomers which mimic antibodies. The MIP is formed from a monomer in the presence of a cross linker, radical initiator and a template molecule (i.e. OA in this case). Post-polymerisation, the template molecule is extracted, forming gaps with specific binding sites for the target molecule (i.e. OA analyte). Zhou and Lai (2004) described a procedure using the monomer N-phenylacrylamide (PAM) and the cross-linker trimethylolpropane trimethacrylate (TRIM). Once formed, the MIP was used to prepare SPE micro columns. Wheat extracts applied to the micro column, spiked with 100μgl^{-1} OA, were detected using fluorescence. The reported limit of detection was 5.0μgl^{-1}. Using the analyte recovery method, the average was 103%, demonstrating superior affinity for the MIP.

Liquid Chromatography-Mass Spectrometry (LC-MS)

Chung and Kwong (2007) reported a method to quantify OA in cereals (rice) using aqueous acetonitrile containing Ochratoxin B (OB) as an internal standard. The buffered extract was applied to an IMA column prior to reversed-phase LC-MS analysis. Two transitions were measured: the identification of OA and the quantification of OA. The method was demonstrated to be very sensitive, with a limit of detection of 0.021 μgkg^{-1}. Using the method of analyte recovery in the range 0.05-0.15 μgkg^{-1}, the average recovery was 104%. This is the first fully validated method using IMA and LC-MS for the detection of OA in cereals, and there are plans to extend the analysis to cover other cereal commodities.

Standard reference materials

Standardisation is important in the analysis of OA due to its heterogeneous distribution (particularly in cereal commodities). The use of traceable standard reference materials (SRM) provides confidence in the analytical approach. For LC based methods, pure OA standards are available from r-Biopharm-rhône (Ochrastandard) and Biopure (Ochratoxin A). Standards are supplied with a certificate of conformance. There is, however, an absence of matrix specific contaminated

reference materials for OA. One of the key elements in the assessment of analytical performance is the extractability of the target (OA) analyte. Use of pure standards in analyte recovery (spike) mode will not provide a true measure of analyte recovery, due to the fact that the contaminant is in unbound form.

Conclusions

Official and fully validated methods based on LC with fluorescence detection are in existence for the measurement of OA in cereals. IMA clean-up using monoclonal antibodies may additionally be used to increase selectivity and sensitivity by removal of matrix interferences. These methods, used for official control purposes, involve specialist equipment and require highly–skilled and trained personnel if a high level of consistency is to be achieved. However, the methods are not suitable for routine and *in situ* screening and monitoring programmes, due to their inflexibility and time-consuming nature.

In routine surveillance situations (where an estimation of contamination is more important), rapid test procedures based on immunochemical techniques provide a realistic alternative analytical option to classical, validated instrumental techniques. The commercial availability of rapid test kits based on antibody-antigen reactions is the most feasible option for the proposed evaluation. The analysis platforms available are:

- Microtiter-plate type assays with and without IMA clean-up.
- LFD (or dipstick) type assays.
- IMA column type assay.

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