PROJECT REPORT 332

OCHRATOXIN A (OTA) IN CEREALS: DEVELOPMENT OF A RAPID TEST; SPECIES AND CONDITIONS FAVOURING DEVELOPMENT

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OCHRATOXIN A (OTA) IN CEREALS: DEVELOPMENT OF A RAPID TEST; SPECIES AND CONDITIONS FAVOURING DEVELOPMENT

by

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This is the final report of a 3-year project that started in April 2000. This work was part-funded by the HGCA with a grant of £203,916 (Project 2320) as part of an £1.6 million EU project 'Prevention of ochratoxin A in cereals'

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ABSTRACT

The occurrence of ochratoxin A (OTA), which is produced mainly from the fungi *Penicillium verrucosum* and *Aspergillus ochraceus*, in cereals is of particular concern to the EU. In response to this concern, recently maximum permissible levels of 5 ppb in raw cereals and 3 ppb in processed products have been set by the EU for OTA.

As a result of the legislation, there was the need for work to be done to identify the causative organism(s) for the production of OTA in cereals and also on the identification of critical control points so that the limits can be met. In addition, it was necessary to develop rapid monitoring methods for OTA in cereals.

This work was part of an EU project on 'The prevention of ochratoxin A in cereals' (OTAPREV) for which the HGCA provided balancing financial support. The areas of the HGCA supported work included:

1. To determine which are the OTA producing fungi and what are the sources of infection
2. To determine differences between cereal species, farming methods and climates on OTA production
3. Development of rapid immunoassay systems for OTA producing fungi and OTA

The only fungus that was found to produce OTA in Europe was *P. verrucosum* and it was surprising that *A. ochraceus* was not found in warmer parts. Even with global warming it is not expected that a change in organism is likely, at least in the short term that could affect the storage of grain in the UK. It is concluded that *P. verrucosum* appears not to be present in the field. The low levels sometimes found at harvest could be where the grain has been inoculated from other sources e.g. from spores and other fungal material left over in machinery from the previous harvest. This would emphasise the importance of machinery and store hygiene.

All evidence points to the prompt and effective drying of cereals at harvest being the major Critical Control Point for preventing the formation of ochratoxin A. Therefore, rapid and effective drying of grain after harvest remains the number one priority for farmers and store keepers.

Prototype laboratory and on-site systems have been developed to detect *Aspergillus* sp and *Penicillum* sp. in culture and grain and also determine if they are toxigenic strains. The methods show sensitivity at least down to 10^4 CFU/g and are thus able to detect the numbers of organisms that could pose a potential problem in grain. Prototype rapid assay kits for OTA (for laboratory or on-site use) have a range of detection from 0.5 to 25 ppb and can be carried out, including extraction, in about 20 minutes. These assays are important additions to the techniques currently available. They provide the tools and therefore the opportunity, for the rapid and inexpensive monitoring of grain for spoilage organisms and the detection of OTA in line with EU legislation at 5 ppb for raw and 3 ppb for processed cereals.

SUMMARY

Introduction

The aim of this project supported by the HGCA was the prevention of ochratoxin A (OTA) in cereals. that was part of a much larger (about £1.6M or 2.53M Euros) EU project. This project was clustered with 2 other EU projects on the prevention and detection of mycotoxins (value about £3.5M or 5.4M Euros) in cereals and started early in 2000. The cost of the work by CSL and ADGEN in the EU project was 50% funded by the EU with additional support from HGCA funds.

In the EU project, CSL was participating in determining the critical control points for ochratoxin A in cereals and CSL and ADGEN were both involved in the development of rapid monitoring methods that are sensitive enough for the impending EU legislation at 5 ppb in raw cereals.

The work of the project was split in to a number of Work Packages (WPs) to be carried out by the partners in conjunction with the EU project. These WPs, within a number of Tasks, consist of:

Task 1 Determination of critical control points (CCP)

WP1: To determine which are the OTA producing fungi and what are the sources of infection. This work involved the collection of positive samples so that the OTA producing fungi could be isolated and characterised. Fungal isolations, characterisation and OTA analysis was carried out at CSL, and in Sweden and Denmark.

WP2: To determine the effect of differences in various cereal species, farming methods and climates on OTA production in Europe through a detailed summary. This was carried out to assist in the determination of CCPs for the production of OTA

Task 3 Development of rapid monitoring methods

WP 7: Development of rapid biosensor system for OTA. This was being developed at Cranfield University (at no cost to the HGCA) but it was planned that the antibody reagents produced in the project would be made available for this. However, the reagents were not required by Cranfield University.

WP8: Development of rapid immunoassay systems for OTA producing fungi and OTA that are sensitive enough to match the EU legislation on OTA which was set at 5 ppb for raw cereals and 3 ppb for cereal products. End user requirements in the UK for an assay were assessed in a study (by questionnaire) to assist in the design of the assays that were developed.

For this Summary Report, the various WPs are reported individually below.

WP1 Which are the OTA producing fungi and what are the sources of inoculum

Materials and methods

Samples

Over the course of the project about 105 OTA samples were collected for analysis from the UK.

Many of the samples were obtained from the HGCA survey for OTA in stored grain carried out in 2000.

Farm investigational samples were also collected. These were pre-combined ears hand threshed grain and chaff, soil and plant roots, dust from grain tanks, elevators, augers, combines, cutter bars and store and grain store sweepings. In total, there were more than 105 samples taken for analysis from the UK.

All of the above samples were sent to the Technical University of Denmark who was also a partner in the EU project along with other fungi that been isolated and in the CSL culture collection. The purpose of this work was to isolate and identify the fungus or fungi responsible for the production of OTA in the samples from the UK and also from others collected across Europe.

Mould isolation and identification and ochratoxin A analysis

Mould isolations and species identifications were carried out at both CSL and in Denmark and results exchanged for confirmation. This was achieved by using well-established standard methods, including the use of a medium optimised for *P. verrucosum.*

Analysis for ochratoxin A was carried out using a fully validated HPLC (High Pressure Liquid Chromatography) method that provided reliable quantitative results.

Mapping of the distribution of OTA producing fungi : P. verrucosum, A. ochraceus

The mapping of the distribution of these organisms throughout Europe in relation to possible climatic conditions was planned. A preliminary map of the distribution of the positive samples used from the HGCA funded survey of 2000 was carried out. However, as only *P. verrucosum* had been shown to be responsible for the production of OTA in the European samples it was not possible to carry out the full mapping exercise.

Results

The only fungi found to produce OTA in European cereals was confirmed as *P. verrucosum*. This organism was not found in soil or in field grain but on rare occasions was found on freshly harvested material at very low amounts.

Discussion

At the start of this work it was thought that in OTA positive samples perhaps *P. verrucosum* would be found in northern Europe and *A. ochraceus* in warmer more southern regions and that there would be a transition line between the sites where these two different fungi were found. Depending on where this line was, it could have had consequences in respect for continued global warming. For example, if the transition line had been

in northern France, then with continued global warming, both *P. verrucosum* and *A. ochraceus* could have become important in the production of OTA in UK cereals. It was surprising that only *P. verrucosum* was found to be responsible for OTA in European cereals and therefore there was no transition line. It was envisaged that the different species would be plotted against environmental (particularly temperature and humidity) factors so that predictions on later distribution of these species arising from global warming could be made.

In field samples, *P. verrucosum* was not generally found in both UK samples and also those from other parts of Europe. It is thus concluded that *P. verrucosum* appears not to be present in the field although it may have been at too low a level to detect. The low levels sometimes found at harvest could be where the grain is inoculated e.g. From spores and other fungal material left over in machinery from the previous harvest. This would emphasise the importance of good hygiene in machinery and store.

WP 2. Differences depending on various cereal species, farming methods and climate

Materials and methods

A questionnaire was devised to gather information on the farming methods practiced in Europe and was sent out to contacts in the member states. The questionnaire was formatted as a Microsoft Excel document that could be sent via email and completed electronically.

Some data such as meteorological information and grain production figures are widely available and were entered in the forms before sending.

Cereal experts in most of the Member States contacted together with Hungary and Bulgaria supplied responses to the questionnaire addressing the production, harvesting and storing of cereals within the EU.

The data were tabulated and interpreted in relation to the occurrence of ochratoxin A.

Results

About 200 million tonnes of the main cereals are grown within the Community.

Drying methods across Europe varied from none, drying in the sun to ambient and hot air systems. The moisture contents at drying also varied widely from about 13 to >25% and there were reports of bottle necks of grain at 20% MC standing for > 7 days.

Few countries carry out monitoring for OTA so it was difficult to say what levels were present or the percentage of samples of contaminated grain that were higher than the EU limits. However in countries where surveys are carried out, such as in the UK, generally there is low but persistent occurrence of OTA and in the UK this is in the order of about 3% of samples with levels greater than the EU limits.

Discussion

Because all evidence points to the prompt and effective drying of cereals at harvest being the major Critical Control Point for preventing the formation of OTA, the evaluation of data concentrated on information about climate, harvest, drying and storage. In regions where the cereal harvest is at greatest risk, measures to avoid mould and toxin problems are often most effective, while areas normally at less risk may not be the best prepared to avoid storage problems when unusual conditions occur. A significant problem arises where conditions at harvest are unpredictable as it may not be economic to have expensive drying machinery idle some years while in others the supply of damp grain may exceed the drying capacity available. Delays in drying may then put the grain at risk. Another problem arises when the infrastructure is such that sufficient funds and expertise are unavailable to advise on and ensure best storage practice. This is likely to prove a major problem to some prospective Member States in regards to OTA.

The existence of about 7,000,000 farms within the present Community, the majority of which are involved at least in part with cereal production, means that many different working practices and standards exist in addition to different situations arising from the climatic variation across Europe.

WP8: Development of rapid immunoassay systems for ochratoxin A producing fungi and ochratoxin A

WP8.1 Defining the performance and design parameters

Materials and methods

Selection of participants

Potential end-users of the on-site tests to detect ochratoxin A were selected from ADGEN's customer base, from internet searches to identify a representative cross-section of relevant organisations and direct contact with known members of grain associations.

A total of 204 UK contacts were identified. The sectors of the grain and related industries included millers, grain merchants, animal feed producers, food producers and testing laboratories.

Design of the questionnaire

The questionnaire comprised of 14 questions, most of which were in yes/no or simple multiple choice format. However, there were opportunities to give specific responses regarding such items as critical testing points and acceptable per test cost.

Results

A total of 38% of recipients responded. There was a high awareness of the dangers of mycotoxins (97%) and 79% were conducting some level of mycotoxin testing. The key point identified for mycotoxin testing was on receipt of commodities.

Respondents indicated a requirement for both qualitative and quantitative data. A threshold test was most attractive although a quantitative test was considered important in the event of any positive results with qualitative or threshold methods.

Current testing regimes were relatively infrequent with 43% only testing on a monthly basis. Acceptable per test costs and analysis turn round times varied greatly. Suggestions for per test cost ranged from £1 to £60. However, the majority suggested £5. Required turn round times varied from 10 minutes to 2 weeks. The most requested times were less than 1 hour. The survey identified those who were interested in being involved with the on-site validation of test kits (68%).

Discussion

The questionnaire generated an unusually high response rate perhaps reflecting the increased interest in mycotoxins. The level of knowledge of legal requirements was greater than expected, as was the percentage of respondents who already were testing. However, the frequency of testing and the numbers of tests conducted, in many cases, would not satisfy legislative or due diligence requirements. The interest in testing at intake clearly indicated the need for rapid and simple tests. The technologies on which this project is based were seen to offer a real opportunity to meet the needs of the industries concerned.

WP8: Development of rapid immunoassay systems for ochratoxin A producing fungi and ochratoxin A

WP 8.2: Development of rapid immunoassay systems for ochratoxin A producing fungi

Materials and methods

Preparation of Soluble Fungal Antigens

Shake cultures of OTA producing and non-producing fungal cultures were produced by methods established at CSL. From these cultures, soluble antigen preparations were made for the production of antibodies and subsequent testing these antibodies for their reactivity to different fungi.

Monoclonal antibody (Mab) and assays produced

Monoclonal antibodies were developed using standard techniques developed and established at CSL.

All antibodies produced were selected and screened by an indirect ELISA (Enzyme Linked Immunosorbent Assay) system.

Selected Mabs and those that were accessed, were tested for cross-reactivity against a range of different fungi and those with the desired reactivity taken forward for the development of prototype assays such as ELISA and or Lateral Flow Device (LFD).

Results

Many Mab cell lines were produced but 2 in particular and were studied further. Unfortunately, these Mabs proved to be either isolate specific or cross reacted with other fungi not associated with OTA production**.**

However, a highly specific *Aspergillus* sp and *Penicillum* sp Mab producing cell line (AF-CA2-CH8) was sourced. It was shown to have a high specificity to all OTA producing and non producing isolates of *P. verrucosum* and *A. ochraceus* with little or no cross reactions with other commonly associated field or storage fungi genera.

Prototype ELISA and LFD were developed using this antibody and both methods were compared to traditional plate count methods The ELISA had a sensitivity of 1×10^4 CFU / g with a good correlation with the plate count method (R^2 of 0.9446). The LFD had a similar sensitivity but it was thought that this could be further improved with more development.

Discussion

Prototype ELISA and LFD systems have been developed that detect *Aspergillus* sp and *Penicillum* sp. in culture and in grain. Both methods show sensitivity at least down to 10^4 CFU/g and thus able to detect the numbers of organisms that could pose a potential problem in grain.

Thus both methods provide the tools and therefore the opportunity, for the rapid and inexpensive monitoring of grain for spoilage organisms that have the potential for OTA production.

WP 8: Development of rapid ELISA systems for OTA producing fungi and OTA

WP 8.3.1: The production of monoclonal antibodies (Mab) to ochratoxin A (OTA) and their incorporation into Lateral Flow Devices (LFDs) in line with the EU legislative limits for ochratoxin A.

Materials and methods

Synthesis of OTA conjugates

Because OTA is a small molecule (hapten) and not a protein (and therefore not immunogenic) this hapten needed to be conjugated to carrier proteins before immunisation. As a result, conjugates of this toxin were synthesised and purified with carrier proteins (BSA: Bovine Serum Albumin, KLH: Keyhole Limpet Haemocyanin) and enzymes (AP : Alkaline Phosphatase and HRP: Horse Radish Peroxidase) in order to raise antibodies and develop specific detection assays.

Cereal Extraction Procedure for the OTA ELISA

Representative cereal samples were taken, ground in a laboratory blender and thoroughly mixed prior to proceeding with the developed extraction procedure. For this 5 g of ground sample was weighed and added to a suitable container with 12.5 ml of 70% methanol and shaken vigorously for three minutes. The extract was allowed to settle for three minutes and a 1ml aliquot taken from the settled supernatant and diluted with 1ml distilled water. This diluted supernatant was passed through a disposable filter syringe and 50 µl per well of filtrate used for ELISA testing.

Monoclonal antibodies (Mab) and Enzyme Linked Immunosorbant Assay (ELISA) to OTA

Monoclonal antibodies were developed using standard techniques developed and established at CSL.

All antibodies produced were selected and screened by an indirect ELISA (Enzyme Linked Immunosorbent Assay) system

Selected Mabs were tested for cross-reactivity against a range of different molecules (e.g. the carrier protein, other mycotoxins etc.) and those with the desired reactivity taken forward for the development of prototype assays (ELISA or Lateral Flow Device : LFD). The selected antibodies were then used for the development of a competitive assay which allowed for the quantitative measurement of OTA.

Competitive LFD to OTA production

Antibodies were incorporated in to one step and rapid LFDs that only take a few minutes to perform.

The LFD for OTA was developed for several applications. These were for:

1. Visual identification of OTA producing or toxigenic fungi

Suspected contaminated grain samples were plated onto agar plates following conventional culturing techniques and allowed to grow over a period of time. The cultures were then extracted and the extract tested by LFD for OTA. . The method was tested in a ring trial with 13 different institutes.

2. Testing of grain samples for OTA with visual examination, with or without immunoaffinity column (IAC) sample clean up / concentration

For detection directly from grain, a representative sample was extracted and the extract applied to the LFD and read visually after 15 minutes.

For Immunoaffinity column (IAC) Sample clean-up / concentration method the extract was passed down an IAC prior to applying the extract to the LFD.

3. Reading the colour intensity of the line

For reading the intensity of the line to make fully quantitative, the extract from a representative sample was applied to the LFD and the colour intensity of the line was read with a reader after 15 minutes.

Results

Mab production

Many cell lines were produced and one in particular, (Y49), produced a very specific Mab to OTA. The ELISA developed had a sensitivity down to 0.02 ppb of OTA with a working range of 0.02 to 16 ppb. A second Mab was sourced (AF-12) and worked well in an ELISA (described in 8.2.1) but not in LFD format.

LFD for the determination of OTA producing fungi

Monoclonal antibody Y49 was successfully incorporated in to an LFD and used for the detection of OTA producing fungi in culture. In addition a successful ring trial was carried out with about 94% of positive and 96% of negative samples correctly identified.

LFD for the detection of OTA in grain

For the visual reading of the LFD without IAC sample concentration, the sensitivity was about 250ppb but with IAC clean up and concentration this improved to about 20ppb. With the use of the reader to measure the colour intensity of the line, without IAC sample concentration, the sensitivity was to <1ppb with a 50% inhibition value of about 8ppb.

Discussion

The OTA LFD has been shown to be a very rapid and very reliable method for the identification of OTA producing fungi. This is mainly a research tool and should greatly aid work on the prevention of OTA.

The main use of course, is using it directly on grain and prototype assays for this purpose have been developed within the project. For this purpose suitable extraction procedures have been devised some with IAC sample clean up and concentration steps with devices being read visually or with a reader to give a fully quantitative results.

Currently sensitivities are about 250ppb for the visually read devices. With an IAC concentration step and visual reading of the devices, the sensitivity was improved to about 20ppb. With the use of the reader and no IAC concentration step, sensitivities were about the same level as that for the ELISA (<1ppb) which is well within the range of the EU limits for OTA at between 3 and 5ppb.

WP 8: Development of rapid ELISA systems ochratoxin A (OTA) producing fungi and OTA.

WP 8.3.2 The development of an ELISA kit to match the EU legislative limits for OTA

Materials and methods

Antibodies to OTA

The monoclonal antibodies (Mab) used were as described in 8.2.1. In brief the final Mab used for testing comprised of one that was produced (Y49) and another that was accessed (AF-12) by the partners.

Spiked flour samples

Flour produced from OTA free grain was spiked with various amounts of OTA and used for the evaluation of the assays produced.

Conjugation of Ochratoxin A

Ochratoxin A was conjugated to Horse Radish Peroxidase, Bovine Serum Albumin (BSA) Bovine Gamma Globulin (BGG) for use in the assay being developed.

ELISA format and Mab performance

Two possible assay formats for the detection of OTA were investigated depending on whether the OTA was conjugated to HRP or to some other carrier to give an indirect or competitive system. Both of the Mab mentioned above were evaluated in these ELISA formats.

Extraction of OTA from grain samples

A method employing a 50% methanol solution was developed and used for the extraction of OTA from the samples prior to analysis by ELISA.

Testing validation and comparison of the developed ELISAs and ELISA kit

Spiked and naturally contaminated samples were analysed by both the ELISA and standard HPLC method and comparisons made between the 2 methods.

The ELISA kit was also sent to another partner with coded samples of known OTA concentration for analysis and the results were compared to the assigned value.

Results

Choice of Mab

As stated in the materials and methods, 2 Mabs were tested and found to be most suitable. These were Y49 and AF-12. Of these 2, AF-12 appeared to perform slight better than Y49 in the ELISA system and was therefore taken forward for further evaluation.

The ELISA performance

The competitive ELISA proved to be the best format and the assay had a sensitivity down to 0.5ppb and up to 25 ppb without dilution of the sample.

With spiked grain, the recovery of OTA ranged from between 95 to 120%. With the comparison of the methods, the results generated by the ELISA, were generally between 87 and 118% of that by the HPLC method.

Discussion

During the course of this project, a rapid and sensitive immunoassay in ELISA format for the detection of OTA in cereal grains and cereal products was developed. The assay compared well with the reference HPLC method and the sensitivity and range (0.5 to 25ppb) was well within that required by the EU legislation of 5ppb in raw and 3ppb in processed cereals. A complete methodology and kit has been developed that can be carried out in a total assay time including extraction of about 30 minutes.

Overall conclusions from the project

From the work it is clear that there are some key messages:

- *Only Penicillium verrucosum* has been found to be responsible for the occurrence of ochratoxin A in UK cereals (and cereals from other parts of Europe).
- Rapid and effective drying of grain after harvest remains the number one priority for farmers and store keepers.
- The interest shown by the cereal trade for testing at intake clearly indicated the need for rapid and simple tests for ochratoxin A.
- Prototype rapid immunoassays in ELISA kit format for the detection of ochratoxin A down to <1 ppb have been developed. It is likely that these can be developed further and become commercially available and be sensitive enough to meet the EU legislation.
- Prototype Lateral Flow Devices (LFDs) for detection of ochratoxin A have been developed. These assay kits enable the one step and very rapid (a few minutes) detection of ochratoxin A. With the use of a reader (palm held models are now available) the sensitivity is as good as the ELISA and therefore match the EU limits.
- Later other information will be available from the EU project. For example, the drying model developed by one of the other partners and information that can be included in the HGCA 'Grain Storage Guide'.

TECHNICAL REPORT

Prevention of ochratoxin A in cereals (as part of an EU project by this title)

General introduction

There is increasing awareness of the incidence and potential damage arising from the presence of fungi and mycotoxins in materials destined for animal and human consumption.

One such mycotoxin of particular concern is ochratoxin A (OTA) which is produced mainly from the fungi *Penicillium verrucosum* and *Aspergillus ochraceus*. The former organism is a common mould that thrives in the cool, moist conditions of northern Europe and the latter mainly in warmer conditions although their distribution may be changing.

Recently, maximum permissible levels of 5 ppb in raw cereals and 3 ppb in processed products have been set by the EU for OTA.

Surveys of stored UK cereals show that a small but persistent percentage of samples examined exceed this level (2-3%) despite a knowledge good storage practice. This represents a large tonnage of grain and a potential serious economic loss.

As a result of the legislation, there is the need for work to be done to identify the causative organism(s) for the production of OTA in cereals and also the critical control points so that that the limits can be met.

Also because of the legislation, there is the need for reliable testing methods. Often results are required quickly e.g. on receipt of grain which probably only allows 15 to 20 minutes to conduct a test.

Conventional analysis of OTA is by laboratory methods such as High Pressure Liquid Chromatography (HPLC) which is expensive (£60 to £100 per sample) and does not offer a turnaround time (usually several days at best) that is compatible with the needs of the industry.

Immunoassays, on the other hand, offer a real alternative for rapid and inexpensive laboratory analysis (e.g. by ELISA) and on site testing that can be carried out in minutes using Lateral Flow Devices (LFDs). Commercial kits to detect OTA were first introduced in the late 1980's by the Neogen Corporation in the USA. However, they were optimised to meet the then USA requirements of around 20 ppb which far exceeds current EU limits of 3 ppb in processed and 5 ppb in raw cereals

The Central Science Laboratory and ADGEN were involved in an EU project on 'The prevention of ochratoxin A in cereals' (OTAPREV) for which the HGCA provided balancing financial support.

This project worth £1.65M (2.53M Euros), started 01/02/2002 and finished on 31/10/2003 and was 'clustered' with 2 other projects on mycotoxins the total worth of which was in the region of £3.5 M (5.4M Euros).
The total cost of the contribution for CSL and ADGEN was £503,499 with half of the funds coming from the EU (£251,750) but the total cost to the HGCA was only £203,916.

The objectives and expected achievements of the EU project were divided into 4 different tasks which are the all important steps in a HACCP managing programme for OTA in cereals.
These tasks consisted of:
Task 1. Determination of critical control points (CCP)
Task 2. Specification of critical limits for the CCP's
Task 3. Developing rapid monitoring methods
Task 4 Establishment of corrective action.
Within each of these tasks there were a number of Work Packages to achieve the objectives.

CSL was involved in all of the following work packages (WPs) below and ADGEN in WP8 as set out in the original proposal

WP1 : *To determine which are the OTA producing fungi and what are the sources of infection*
WP2 *: To determine differences on various cereal species, farming methods and climates on OTA production*
WP7*: Development of rapid biosensor systems for OTA. CSL will be developing reagents, as part of this work, for inclusion in to biosensors along with molecular imprinted polymers which is being carried out by another UK contractor (Cranfield University). However, the antibody reagents were not in the end required for this WP.*
WP8 *: Development of rapid immunoassay systems for OTA producing fungi and OTA. In addition to ELISA systems, rapid (minutes), one step, field type systems will be examined as has been developed by CSL for other analytes.*
Commercial laboratory kits are available in the UK for the detection of OTA but the sensitivity of these products is generally only around 8ppb. One kit is claimed to go down to 4ppb but the test time is 45 - 60 minutes, involves laboratory skills and hazardous reagents and it is unlikely to be quantitative at that level. Both work packages 7 and 8 are aimed at
producing rapid methods that are sensitive and have the required degree of quantification low enough to match the impending EU legislation on ochratoxin A which is likely to be set
at between 4 ppb.

The report gives and discusses the results of the above work that was carried out as part of the EU project and supported by the HGCA. For this purpose, the Technical Report is written in sections corresponding to the individual WPs mentioned above.

WP1 Which are the ochratoxin A (OTA) producing fungi and what are the sources of inoculum

Introduction

It was planned that other EU partners in the project would screen in excess of 300 cereal samples (as many as possible containing OTA for OTA producing fungi collected from all over Europe.

To be able to find contamination sources and critical control points, AFLP and phenetic methods were developed by the other partners to identify the fungi and strains of OTA producers found in positive grain samples. From the data based on all the identifications of OTA producers and a detailed analysis for other mycotoxins and other secondary metabolites by these same strains, it was planned to produce maps of these fungi in relation to climate and geographic origin on maps of Europe. For this purpose potential contamination sources, such as field-soil, dust in storage rooms etc. were also examined for OTA producers.

CSL collected isolates of OTA producing fungi, carried out taxonomic identifications in association with a partner from Denmark and 'fed' these in to this important part of the project.

Materials & Methods

Samples

Over the course of the project about 105 OTA samples were collected for analysis from the UK.

Many of the samples were obtained from the HGCA survey for OTA in stored grain carried out in 2000. Farm investigational samples were also collected. These we pre-combined ears, hand threshed grain and chaff, soil and plant roots, dust from grain tanks, elevators, augers, combines, cutter bars and store and grain store sweepings. In total, there were more than 105 samples taken for analysis from the UK.

All of the above samples were sent to Dr Jens Frisvard (Technical University of Denmark,) who was also a partner in the EU project along with other fungi that been isolated and in the CSL culture collection. The purpose of this work was to isolate and identify the fungus or fungi responsible for the production of OTA in the samples from the UK and also from others collected across Europe.

To avoid changes in mould and toxin levels during transportation of the grain samples, they were sent for analysis as soon as possible after each sampling time. On arrival at the laboratory the samples were split; half for mould analysis (stored at 4°C before processing) and the other portion for toxin determination (stored at -30°C).

Mould isolation and identification and ochratoxin A analysis

Mould isolations and species identifications were carried out at both CSL and in Denmark and results exchanged for confirmation. This was achieved by using well-established standard methods, including the use of a medium optimised for *P .verrucosum.*

For the mycological examination, a 40 g of sample were taken from each grain sample, weighed directly into a stomacher bag, 360 ml of 0.1 % bacteriological peptone (Unipath) water was added and the grain soaked for 30 minutes. After soaking, the grain was stomached for 1 minute and the resulting suspension labelled as the 10^{-1} dilution. This suspension was serially diluted down to 10^{-6}. For each dilution, two DG18 agar plates

(31.5 g commercial dehydration formulation; 220 g glycerol; 50 mg chloramphenicol; 1000 ml distilled water) were labelled and 0.1 ml dilution suspension pipetted onto the agar surface. The inoculum was spread over the surface of the agar and incubated at 25°C for 10 days. Colonies on each plate were identified and counted.

Ochratoxin A analysis

The whole sample of each grain portion stored for OTA analysis was milled and mixed thoroughly. The remainder was then re-frozen and stored for future reference. Analysis for ochratoxin A was carried out using a fully validated HPLC method based on that tested by Scudamore and MacDonald 1998 that provides fully quantitative results. Pre-harvest grain was not analysed for OTA as no potential OTA producing fungi were isolated from these samples. The total error would comprise that due to sampling and that inherent in OTA analysis.

For the analysis, 25 g of ground sample was extracted in 100 ml of acetonitrile:water (60:40). The mixture was blended using a homogeniser for 3 minutes and filtered the through filter paper by gravity and 5 ml of the filtrate was added to 55 ml phosphor buffered saline solution (PBS). This was transferred ≥ 55 ml in to a pre-labelled plastic sample tube, and placed on an ASPEC (automated sample preparation equipment) for automated immunoaffinity column clean-up and analysis.

The immunoaffinity clean-up column was conditioned with PBS, the sample added (50 ml), the column washed with PBS and then air-dried with OTA then being eluted with 2% acetic acid in methanol. The eluate was diluted with water before HPLC analysis.

A Spherisorb ODS2 HPLC column (25cm x 4.6mm) was used, with an inline filter, C18 guard column or equivalent and a mobile phase consisting of acetonitrile:water:acetic acid (99:99:2) flowing at 1 ml/minute. The product was detected using fluorescence with excitation set at 333nm and emission at 477nm.

Mapping of the distribution of OTA producing fungi : P. verrucosum, A. ochraceus

The mapping of the distribution of these organisms throughout Europe in relation to possible climatic conditions was planned. A preliminary map of the distribution of the positive samples used from the HGCA funded survey of 2000 was carried out. However, as only *P. verrucosum* had been shown to be responsible for the production of OTA in the European samples (see results and discussion below) it was not possible to carry out the full mapping exercise.

Results

Over the course of the project about 105 OTA samples were collected for analysis from the UK.

Many of the samples were obtained from the HGCA survey for OTA in stored grain carried out in 2000. Of the stored grain samples, those positive for OTA were selected and these ranged in OTA concentration from

0.5 to 231 ppb. See Table 1.1. In the samples containing OTA, the only toxigenic fungi found were *P. verrucosum* not only in the samples from the UK but also in those from the rest of Europe. Figure 1.1 shows the distribution of OTA positive samples examined in the UK and Figure 1.2 shows the distribution of the OTA positive samples examined in the rest of Europe.

In addition to *P. verrucosum* being the only OTA producing fungi found to be associated with positive samples in Europe, this organism was not found in soil or in field grain but on rare occasions was found on freshly harvested material at very low amounts.

At the start of the project it was envisaged that as well as *P. verrucosum*, *A. ochraceus* would also be an important producer of OTA in European cereals. However, the only fungi found to produce OTA in European cereals was confirmed as *P. verrucosum* whereas in the past a number of different fungal genera/species were thought to be responsible. The mapping of the distribution of these organisms in relation to possible climatic conditions has therefore proved unnecessary although distribution maps where positive samples were found were produced (see figures 1.1 and 1.2).

Discussion

At the start of this work it was thought that in OTA positive samples perhaps *P. verrucosum* would be found in northern Europe and *A. ochraceus* in warmer more southern regions and that there would be a transition line between the sites where these two different fungi were found. Depending on where this line was, it could have had consequences in respect for continued global warming. For example if the transition line had been in northern France, then with continued global warming, both *P. verrucosum* and *A. ochraceus* could have become important in the production of OTA in UK cereals. It was surprising that the only fungus found to be responsible for OTA in European cereals was *P. verrucosum* and therefore there was no transition line. It was envisaged that the different species would be plotted against environmental (particularly temperature and humidity) factors so that predictions on later distribution of these species arising from global warming could be made. This scenario may have had important implications for the safe storage of cereals in the UK had there been signs of the emergence of another organism implicated in OTA production but fortunately this does not seem to be happening.

In field samples, *P. verrucosum* was not generally found in both UK samples and those from other parts of Europe. It is thus concluded that *P. verrucosum* appears not to be present in the field although it can not be discounted as it may have been at an amount too low to detect. The low levels sometimes found at harvest could be where the grain has become inoculated e.g. From spores and other fungal material left over in machinery from the previous harvest. This poses the question of where inoculation of the grain occurs and the general consensus from other aspects of the EU project and other work carried out by CSl is that a major source could be machinery used in the field (e.g. combines) and in store (e.g. augers). This reiterates the importance of machinery and store hygiene.

References

Scudanore, K. A., and MacDonald, S. J., 1998, A collaborative study of an HPLC method for the determination of ochratoxin A in wheat using immunoaffinity column clean-up. Food Additives and Contaminants, **15** 401-410.

Table 1.1. Samples collected and analysed for OTA concentration and OTA producing

Reference	Description	Source	Date Sent	OTA HPLC	Origin	Isolate
PIL 253	A. ochraceus	IMI 16247	22/3/00	No		Sent
PIL 657	A. ochraceus, Rice	W. Yin	22/3/00		China	Sent
PIL 661	A. ochraceus, Rice	W. Yin	22/3/00		China	Sent
00195	A. ochraceus Wheat	HGCA 2000	29/09/00	Low	Kent, Canterbury	Sent
CBS 263.67	A. ochraceus	CBS	27/11/00	High		Sent
B002	Barley	HGCA 2000 survey	10/5/00	0.5	Map ref. sent	
B015	Barley	"	10/5/00	1.1	"	
B036	Barley	"	10/5/00	3.9	"	
B063	Barley	"	10/5/00	0.5	"	
B065	Barley	"	10/5/00	1.7	"	
B066	Barley	"	10/5/00	1.3	"	
B075	Barley	"	10/5/00	3.1	"	
B079	Barley	"	10/5/00	8.5	"	
B081	Barley	"	10/5/00	13.8	"	
B090	Barley	"	10/5/00	15.6	"	
B096	Barley	"	10/5/00	0.7	"	
B104	Barley	"	10/5/00	24.5	"	
W001	Wheat	"	10/5/00	19.9	"	
W013	Wheat	"	10/5/00	15	"	
W014	Wheat	"	10/5/00	1.1	"	
W017	Wheat	"	10/5/00	1.6	"	
W021	Wheat	"	10/5/00	0.8	"	
W023	Wheat	"	10/5/00	2.8	"	
W031	Wheat	"	10/5/00	3.3	"	
W032	Wheat	"	10/5/00	1.5	"	
W049	Wheat	"	10/5/00	2.8	"	
W059	Wheat	"	10/5/00	0.5	"	
W066	Wheat	"	10/5/00	1	"	
W067	Wheat	"	10/5/00	0.9	"	
W075	Wheat	"	10/5/00	3.7	"	
W106	Wheat	"	10/5/00	1	"	
W114	Wheat	"	10/5/00	1.1	"	
W123	Wheat	"	10/5/00	50	"	IBT 22625
W139	Wheat	"	10/5/00	0.7	"	
W156	Wheat	"	10/5/00	4.6	"	
W161	Wheat	"	10/5/00	11	"	
W163	Wheat	"	10/5/00	231	"	IBT 22626
W165	Wheat	"	10/5/00	1.5	"	
W168	Wheat	"	10/5/00	0.6	"	
W171	Wheat	"	10/5/00	2	"	
W173	Wheat	"	10/5/00	3.6	"	

W176	Wheat	“	10/5/00	1.4	“	
W180	Wheat	“	10/5/00	1.2	“	
W181	Wheat	“	10/5/00	1.9	“	
W183	Wheat	“	10/5/00	3.2	“	
W192	Wheat	“	10/5/00	5.2	“	
B	Sweeping	OCS 2000	14/11/00	915	Rosemaund	
C	Sweeping	OCS 2000	14/11/00	1465	Rosemaund	
D	Sweeping	OCS 2000	14/11/00	1205	Rosemaund	
1	Sweeping	OCS 2000	14/11/00	221	Drayton	
A	Sweeping	OCS 2000	14/11/00	1260	Bridgets	Pv 40
A	Sweeping	OCS 2001	24/7/01	665	Rosemaund	Pv
B	Sweeping	OCS 2001	24/7/01	1.5	Rosemaund	Af
C	Sweeping	OCS 2001	24/7/01	486	Rosemaund	Pv
D	Sweeping	OCS 2001	24/7/01	437	Rosemaund	Pv
E	Sweeping	OCS 2001	24/7/01	2.5	Rosemaund	Af
F	Sweeping	OCS 2001	24/7/01	96.1	Rosemaund	
J	Sweeping	OCS 2001	24/7/01	649	Rosemaund	Pv/Ao
K	Sweeping	OCS 2001	24/7/01	639	Rosemaund	Pv
L	Sweeping	OCS 2001	24/7/01	29.1	Rosemaund	Pv/Af
2	Sweeping	OCS 2001	24/7/01	59.8	Bodenham	Pv
3	Sweeping	OCS 2001	24/7/01	35.1	Bodenham	Pv
4	Sweeping	OCS 2001	24/7/01	<0.2	Bodenham	Pv
6	Sweeping	OCS 2001	24/7/01	615	Bodenham	Pv
7	Sweeping	OCS 2001	24/7/01	428	Bodenham	An
8	Sweeping	OCS 2001	24/7/01	3.3	Bodenham	Pv
10	Sweeping	OCS 2001	24/7/01	133	Bodenham	Pv
Bin 1 Backlog	Wheat	OCS 2001	24/7/01		Yorkshire	PV 29
00336	Wheat	HGCA 2000	24/7/01	~44	Hampshire	PV 22
00337	Wheat	HGCA 2000	24/7/01		Hampshire	Ao
1	Pre-combine ears and hand threshed grain,	OCS 2001	28/9/01		Rosemaund	
2	Pre-combine ears and hand threshed chaff	OCS 2001	28/9/01		Rosemaund	
3	Whole crop+ soil	OCS 2001	28/9/01		Rosemaund	
4	Dust from grain tank	OCS 2001	28/9/01		Rosemaund	
5	Dust from front elevator	OCS 2001	28/9/01		Rosemaund	
6	Dust from inside unloading auger	OCS 2001	28/9/01		Rosemaund	
7	Dust from straw chopper at rear of combine	OCS 2001	28/9/01		Rosemaund	
8	Dust form straw walker inside combine	OCS 2001	28/9/01		Rosemaund	
9	Front elevator sample	OCS 2001	28/9/01		Rosemaund	
10	Unloading auger sample	OCS 2001	28/9/01		Rosemaund	
11	Sample from straw chopper at rear of combine	OCS 2001	28/9/01		Rosemaund	
12	Filter from mask used during combining	OCS 2001	28/9/01		Rosemaund	
13	Sample from straw chopper	OCS 2001	28/9/01		Rosemaund	
14	Sample from straw walker unloading auger	OCS 2001	28/9/01		Rosemaund	
15	Grain tank sample	OCS 2001	28/9/01		Rosemaund	
16	Whole crop+soil	OCS 2001	28/9/01		Drayton	
17	Pre-harvest grain store sweeping	OCS 2001	28/9/01		Drayton	

18	Pre-harvest combine cutter dust	OCS 2001	28/9/01		Drayton	
19	Pre-harvest combine sieve/cutter dust	OCS 2001	28/9/01		Drayton	
20	Combined grain	OCS 2001	28/9/01		Drayton	
21	Straw and chaff from rear of combine	OCS 2001	28/9/01		Drayton	
22	Weed and crop trash from headland	OCS 2001	28/9/01		Drayton	
23	Pre-combine ears hand threshed grain	OCS 2001	28/9/01		Drayton	
24	Pre-combine ears hand threshed chaff	OCS 2001	28/9/01		Drayton	
25	Post-harvest dust and chaff form cutter bar	OCS 2001	28/9/01		Drayton	
26	Post-harvest grain store sweeping	OCS 2001	28/9/01		Drayton	
1-6	Stored Barley	OCS 2002	16/7/02		Bodenham	
1-6	Stored Wheat	OCS 2002	16/7/02		Bodenham	
1-6	Stored Beans	OCS 2002	16/7/02		Bodenham	
1-6	Stored Barley	OCS 2002	16/7/02		Rosemaund	
1	Stored Wheat Hereward	HGCA 2001	18/7/02	5	Dover	Pv
2	Stored Wheat CONSORT	HGCA 2001	18/7/02	22.8	Dover	Pv
3	Stored Barley Pearl		18/7/02		Yorkshire	Pv
4	Stored Wheat Claire		18/7/02		Yorkshire	Pv

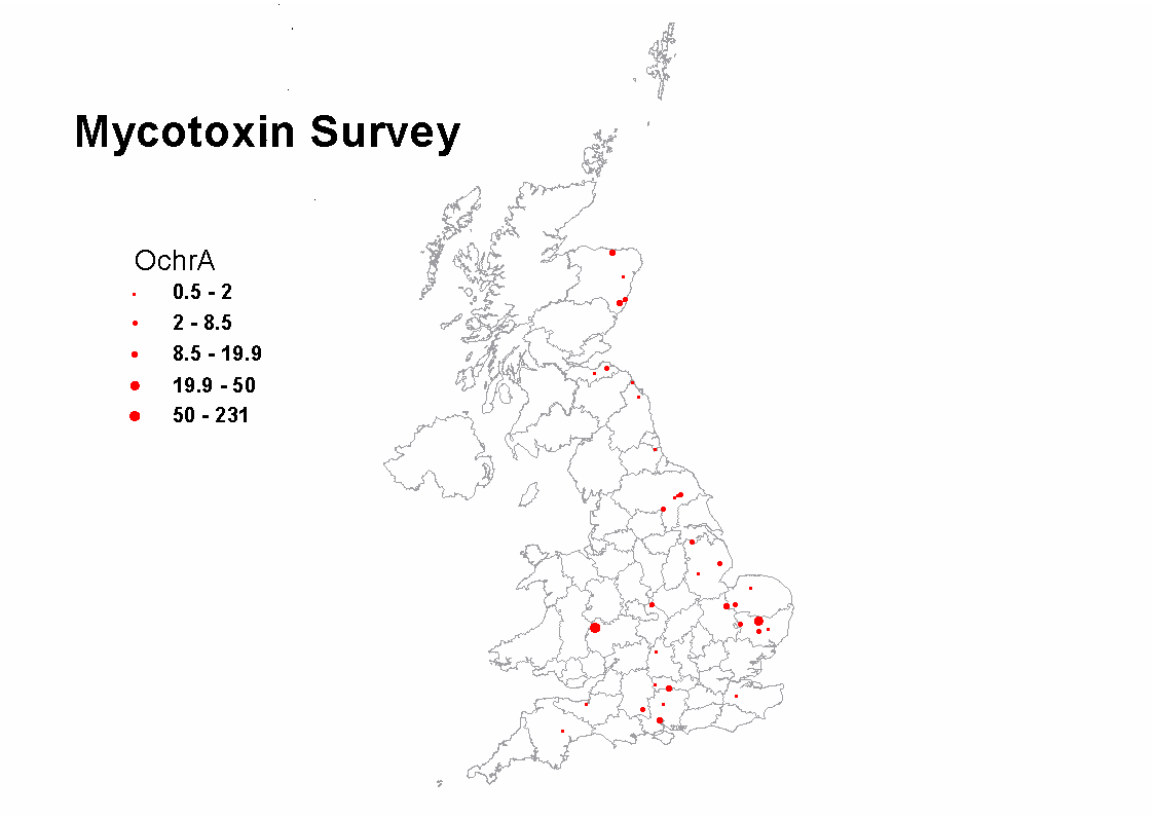

Figure 1.1. Distribution of OTA positive grain samples collected in the UK

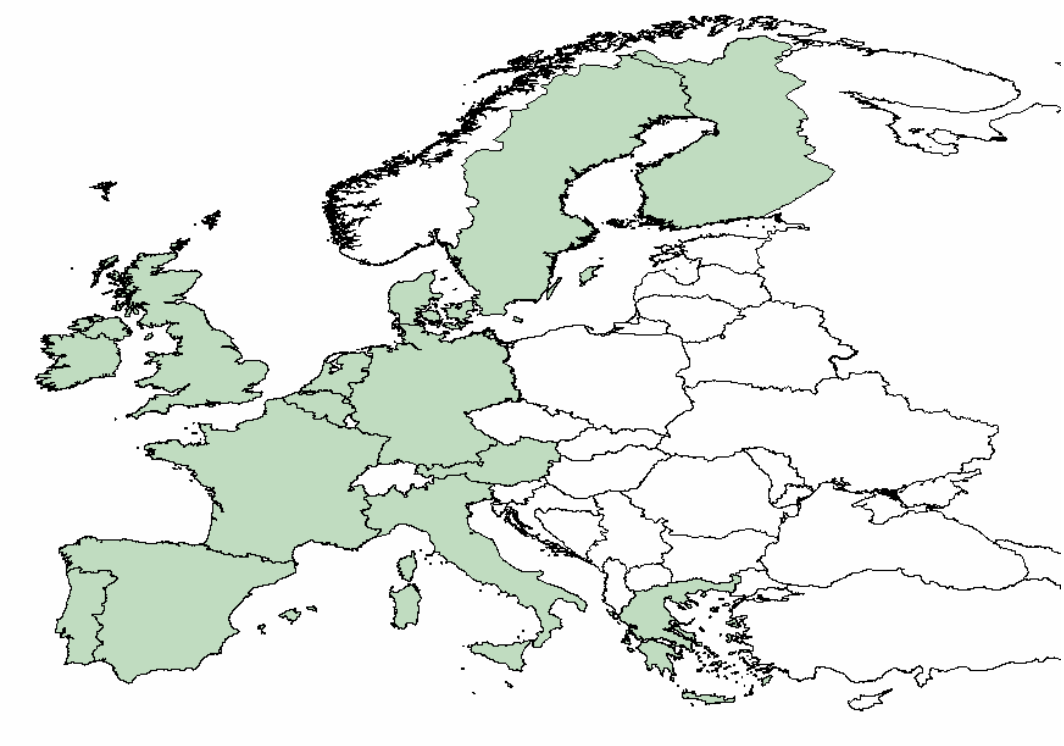

Figure 1.2. Shaded areas show the countries where OTA positive cereal samples were examined.

WP 2. Differences depending on various cereal species, farming methods and climate

Replies from cereal experts in Europe on a questionaire on the reduction, harvesting and storing cereals within the EU with particular reference to ochratoxin A.

Introduction

Recently, maximum permissible levels of 5 µg/kg in raw cereals and 3 µg/kg in processed products have been set for OTA within the EC. Surveys of stored cereals in Europe over many years (e.g. Olsen *et al*. 1993; Scudamore *et al.,* 1999; Wolff, 2000; Puntaric *et al*., 2001) show that samples examined sometimes exceed this level. Even if the percentage of samples contaminated at the statutory limits within the Community were as low as 3% this would represent a large tonnage of grain (approximately 6 million tonnes) and a potential serious economic loss. In monetary terms this would equate to a loss of 800-1000 million euros assuming that no alternative use for the grain was available. The high cost of monitoring programmes for preventing contaminated grain entering the food chain must also be considered.

The food and brewing industries are increasingly demanding high quality cereals for food and drink products and require grain at least conforming to the statutory limits set for OTA. There is thus a major incentive for European Cereal Industry to minimise OTA (and other mycotoxins) in grain to enable it to remain competitive worldwide and to reduce consumer risk as far as possible. Clearly there is an urgent need to understand the factors that encourage mycotoxin formation both pre and post harvest as this will assist in developing strategies to minimise mycotoxin formation.

Application of HACCP-like assessment of the cereal food chain supports the established knowledge that drying grain quickly at harvest is the most crucial factor in avoiding mould and mycotoxin formation during subsequent storage. Thus instigation of a suitable monitoring check system to ensure that grain is dried rapidly enough to safe moisture content, together with a regular inspection and monitoring of grain moisture, should eliminate the risk of ochratoxin A formation during storage. In addition, compliance with Good Agricultural Practice (GAP) will assists in the production of good quality grain.

However, the continued occurrence of OTA in grain shown by surveys suggests that either good practice is not or cannot, always be fully followed or that all the factors involved in the formation of OTA are not completely understood.

Thus, if grain cannot always be dried quickly enough it becomes important that all factors that affect the potential for formation of OTA are fully understood. Only by obtaining this information can sound and effective advice be made available in the field on how to minimise this risk.

This task within the EC and HGCA funded Project, 'Prevention of ochratoxin A', aimed to establish how cereal production, harvesting and storage procedures vary across the Community by compiling a small data base from the information provided in a questionnaire sent to cereal experts. The cooperation of 'grass root' experts means that the information is their view and not necessarily an official or Government view, especially where answers required may be sensitive or subjective, e.g. 'is OTA considered a problem in your Country'?

By examining climatic data, it has been possible to assess the relative risk of ochratoxin A formation at harvest across the Community and to examine how successful national recommendations and practice have been in minimising this risk to date. These data and this appraisal are intended to complement the results from the scientific studies of the many of factors investigated by other Partners within the EU Project. Moreover, information gathered within this project should be of value to other projects within the Mycotoxin Cluster.

Materials and methods

A questionnaire was designed to cover many aspects of the growing, harvesting, drying and storage of grain within the Community, with special reference to OTA (see WP2 Appendix). This was completed after seeking the views of several experts in cereal production. The questionnaire was formatted as a Microsoft Excel document that could be sent via email and completed electronically. For security, most fields not requiring data input were locked to prevent alteration of the layout. The form was only supplied in English because it was impracticable to translate into all the languages necessary and this may have contributed in a few instances to difficulties in encouraging a full response and may in some instances represented a barrier in reaching the most appropriate contact. In summary, the questionnaire consisted of 9 sections. The first gave contact details of the respondent and the last two enquired about legal or advisory limits existing at the time including monitoring schemes for OTA and the possibility of any follow up visits if required. Sections 2 to 7 addressed the following subjects with particular relevance to OTA, climatic conditions (2), cereal production and use (3), crop production (4), harvesting (5), drying (6) and storage (7).

Some data such as meteorological information and grain production figures are widely available and were entered in the forms before sending.

Contacts were sought in each Member State and also in several prospective Member countries. This involved considerable effort and perseverance. The objective was to encourage experts in cereal production and storage with direct practical involvement with grain to complete the questionnaires. Experts were sought with the assistance of international and national cereal bodies including CoCeral, IRTAC, HGCA and the help of individuals known personally to the author or reached with help from some of the other participants in the current project. Direct approach to official government bodies was avoided as far as possible as past

experience suggested that this could be a lengthy and convoluted pathway even then with no guarantee of reaching the most appropriate expert.

Completed forms were received from Austria, Bulgaria, Denmark, Finland, Germany, Greece, Hungary, Ireland, The Netherlands, Sweden and the UK and a response, although only with very little information, from Portugal. No information was received from Belgium/Luxembourg, France, Italy and Spain. In the absence of replies from some states limited data for cereal production and climatic data were available from reliable sources and these were added to the information received.

The data have been tabulated and are discussed here in relation to the occurrence of OTA.

Results

Information obtained from the survey questionnaires and from associated searches is given in tables 1-8 while figure 2.1 shows the variation in the size of farms across Europe. Mean monthly maximum and minimum temperatures, rainfall and sunshine hours for the 30-year period 1961-1990 for 0.5º x 0.5º latitude/longitude grids were obtained from CRU European Gridded Climatology tables, Hulme *et al.*, 1995, adjacent to 30 European cities in 18 countries. These cities were selected so as to be close to major cereal growing areas. However, it is accepted that the climate of any area in close proximity to a large city may be modified by its very presence.

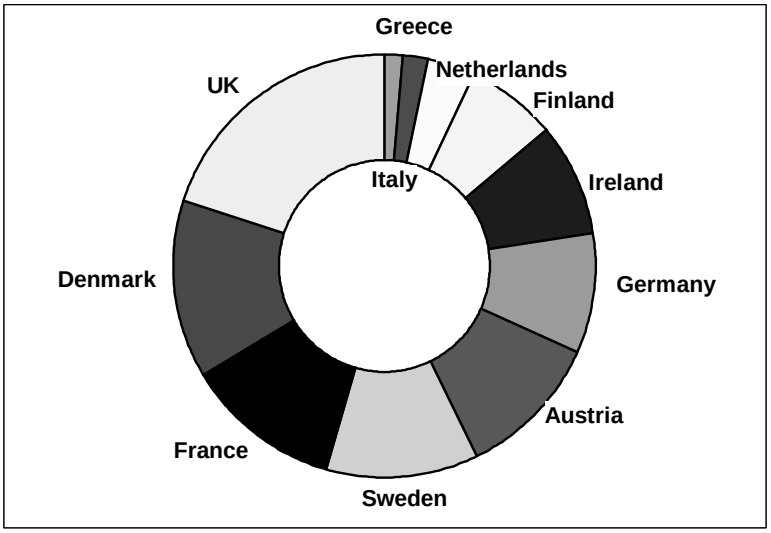

Figure 2.1: Relative average size of farms within the EC (UK 69 ha to Greece/Italy 7)

This figure was constructed by information from the questionnaire and supplemented with data from the IIASA website (International Institute for Applied Systems), ERD database (European Rural Development). www.iiasa.ac.at/Research/ERD/ DB/data/eco/agr/agr_eu_3.htm

Table 2.1 shows the mean annual tonnage of wheat, barley, oats, rye and maize grown within the EC in the years 1999-2000 taken from data published by CoCeral. In addition, triticale is produced in most Member

states as is also sorghum, millet and rice particularly in Italy, Spain, France and Greece. The data shown are the totals for all wheat, barley and oats that thus consist of the combined amount of Winter, Spring and Durum wheat, Winter and Spring barley and Winter and Spring oats. Separate data are available for each type of cereal but are not given here. The productivity achieved for each cereal on a country basis is shown in table 2.2 in which yield is presented as tonnes/hectare.

Table 2.1: Cereal production (data from the questionnaire and Coceral crop forecasts (http://www.coceral.com/main.html)

	Cereal, million tonnes					
Country	Wheat	Barley	Oats	Rye	Maize	Total
Austria	1.35	1	0.15	0.1	1.45	4.05
Belgium/Luxemburg	1.7	0.38	<0.1	<0.1	0.24	2.32
Denmark	4.6	3.83	0.19	0.27	0	8.89
Finland	0.43	1.76	1.17	<0.1	0	3.36
France	37.24	9.73	1.2	0.18	15.9	64.25
Germany	20.65	12.6	1.2	4.24	3.25	41.94
Greece	1.38	0.41	<0.1	<0.1	1	2.79
Ireland	0.68	1.37	0.13	0	0	2.18
Italy	6.27	1.26	0.4	<0.1	10.45	18.38
Netherlands	1.01	0.34	<0.1	<0.1	0.14	1.49
Portugal	0.48	<0.1	<0.1	<0.1	0.8	1.28
Spain	6.1	9.32	0.75	0.21	3.8	20.18
Sweden	2.04	1.76	1.1	0.2	0	5.1
UK	15.9	6.4	0.58	<0.1	0	22.88
TOTAL EC	99.8	50.2	6.9	5.2	37.0	199.1
Bulgaria	2.64	0.65	0.06	0.03	1.7	5.1
Hungary	2.64	1.04	0.18	0.08	7.15	11.1

Some of the important varieties of cereals grown are shown in table 2.3. Respondents were invited to list up to 4 of the most used varieties of each cereal currently used, if possible listing them in order of popularity. The different methods of drying the grain at harvest are given in table 2.4 and these need to be considered together with the range of moisture content typical at harvest in each State, table 2.5 and the delays in drying that may be experienced from time to time, table 2.6. This information is not broken down here for individual cereals.

The management of grain during storage will in part depend on the storage method or structure. Table 2.7 shows that these range from individual bags of about 25kg to huge on-floor heaps that may be 1000 tons or more. Most common however are metal or concrete silos and bins between about 10 and 1000 tons. Each will present its own problem in monitoring and control of the storage conditions. The principal uses of cereals are for human food, animal feed, malting and brewing, seed and industrial purposes, table 2.8. In addition cereals may be produced for home use or export.

Table 2.2: Cereal yields (data from the questionnaire and Coceral crop forecasts (http://www.coceral.com/main.html)

	Cereal, tonnes/hectare				
Country	Wheat	Barley	Oats	Rye	Maize
Austria	5.0	4.3	3.9	3.7	9.4
Belgium/Luxemburg	7.5	5.4	5.6	5.4	8.4
Denmark	7.2	5.2	5.1	5.0	-
Finland	3.2	3.1	3.6	2.4	-
France	7.3	6.3	4.5	4.5	8.9
Germany	7.4	5.9	4.7	5.3	8.3
Greece	2.4	2.5	2.1	2.2	7.9
Ireland	9.4	7.1	6.4	-	-
Italy	5.5	4.3	3.0	2.5	9.8
Netherlands	8.4	6.5	5.5	5.7	8.0
Portugal	1.7	2.2	1.0	0.9	4.1
Spain	3.6	2.9	1.9	1.8	9.0
Sweden	6.0	3.9	3.7	5.1	-
UK	8.0	5.7	5.6	5.4	-
Bulgaria	2.7	2.6	-	-	3.5
Hungary	3.6	3.1	-	-	6.4

Wheat may be used for all of the purposes shown although in most Member States a major use was for production of flour for bread, cakes, breakfast cereals and pasta (durum wheat). On the world scale about 65% of wheat is used for human food that is only exceeded by rice of which nearly 90% of this cereal is eaten by man. (FAO Food outlook.
http://www.fao.org/WAICENT/faoinfo/economic/giews/english/fo/fotoc.htm

By contrast, very little barley goes for human consumption its main usage being animal feed and malting. Rye is used for bread and breakfast cereals particularly in Germany, Austria, Finland and Greece and commonly for animal feed through the EC. Oats is destined primarily for animal feed but a varying amount is used for breakfast cereals. The major use of European grown maize is for animal feed. Whole plant maize is used for the production of silage. However, there is a substantial breakfast food and snack food market.

Discussion

Farms and farming practice prior to harvest

It is clear from the responses to this survey that the size of the farm, the individual fields and farming practice have traditionally varied greatly across the Community, often being influenced by the cultural background of the people of each particular region or community and as well as the prevailing climate. Figure 2.1 shows that small farms of about 7 ha. are typical in Greece and Italy while, at the other end of the scale, the average size within the UK in 1997 was 69 ha.

Even within these averages there may be a large variation in individual farm size. For example, data on the distribution of farm size by area in England & Wales, 1996 (taken from DEFRA, UK, website http://www.defra.gov.uk/) shows that although 15% of farms are less than 5 ha, more than 16% cover areas larger than 100 ha. With an estimated 7,000,000 farm holdings across the 15 Member Community in 1997, this implies a potential enormous variation in farming practice both within individual States and across the whole Community. Standards in hygiene, cultivation, efficiency and formal codes of practice must also vary considerably.

Clearly with this huge numbers of farms, most of which are likely to produce some cereals, the dissemination of research findings and the subsequent provision of guidelines for avoiding or minimising OTA occurrence represents a major logistic problem that must be effectively tackled to fully exploit the findings of this project.

Farm size is also measured in European Size Units (ESU), where one ESU is defined as 1200 European Currency Units (average value 1987-89) of SGM (Standard Gross Margin). It is a measure of the economic size of holdings in terms of the value they add to variable inputs and thus differs from physical measures, such as area, which take no account of the intensity of production. Five size groups are defined ranging from very small size group less than 8 ESU to very large that is greater than 200 ESU. The farm ESU size may also affect the method and effectiveness of dissemination.

Studies on the formation of OTA have consistently suggested that it is a post-harvest problem. While this is probably so, it is still not clear whether specific factors prior to harvest (other than moisture) may influence the extent and rate of its occurrence after harvest in risk situations. For example, a recent study funded by the UK Food Standards Agency 2003 includes research that shows that broken grains are much more susceptible

to invasion by *P. verrucosum* with more rapid growth and higher levels of OTA ultimately formed. The susceptibility of individual cereal kernels to mechanical damage during harvest for example, might be affected by many factors such as the variety, protein content, nutrient content and disease pressure. Drought stress is well known to cause cracking of grains and invasion of insects and fungi that can cause problems with aflatoxin. The need to use some irrigation for cultivation of maize in Greece and Bulgaria and limited use for cereals in parts of Austria are cited in the questionnaire and suggest that drought stress may be a problem at times in part of Europe. While proper and prompt drying at harvest should theoretically suitably address this Critical Control Point, this realistically cannot always be achieved and the possible influence of other pre-harvest and during- harvest factors must be considered.

The completeness of the data supplied on the use of fertilizers, herbicides, insecticides and fungicides (both on seed and on the growing crop) varied considerably, ranging from comprehensive to nil returns. Not surprisingly there was a general suggestion of a trend towards 'ecological systems', with reduced inputs. Fungicide use tends to be greatest in the wetter parts of Europe and relates to the pressure stemming from a range of fungal diseases. In some countries 2 or 3 fungicide applications are sometimes required using anything from a ¼ dose to the fully recommended commercial dose. Because the pre-harvest data are not comprehensive and the factors addressed probably do not influence the formation of ochratoxin in store to a major extent, the data are not discussed in further detail here but are archived for future reference.

Influence of climate on the conditions at harvest

Climate across Europe shows a large variation and this means that each area and Country will be faced with different advantages or problems in the production, handling and storage of cereals. It will also determine where each type of cereal can be grown. The introduction of new varieties with properties optimised for specific growing conditions or tailored towards the needs of the cereal industry and end users means that the area suitable for growing each particular cereal crop is continually changing and expanding. This is also likely to be affected by future changes in the global climate in addition to economic considerations.

Temperature is probably most relevant to this assessment during 2 periods, that close to and at harvest and then after harvest during storage. Prior to and during the harvest period, temperature together with sunshine, rainfall and wind speed will influence the rate of evaporation of moisture from soil and crops. Then throughout the subsequent storage period both the outside and internal storage unit temperature will affect the rate of any fungal growth in those circumstances that the grain is stored, or becomes, moist.

The highest mean maximum temperatures often exceed 30˚C during the period June to September in areas of Spain, Greece, Portugal and parts of Italy while daytime temperatures in the mid 20's occur in Bulgaria, Hungary, Romania, parts of central Europe and Southern France. In contrast many parts of North Western Europe expect temperatures no higher than 20˚C although it can be considerably warmer but usually only for short periods. In many of the areas where the highest temperatures occur, these are often accompanied by

low rainfall and a high number of hours of sunshine. Conversely, much of Western and Northern Europe have higher rainfall and less sunshine. For example, during the period July to September, Portugal and Spain normally record nearly 1000 hours of sunshine and rainfall as low as 30 mm in contrast to parts of Ireland and the UK which suffer as little as 400 hours of sun while some areas of Scotland receive over 320 mm of rainfall over the same period. Other parts of northwest Europe similarly expect high rainfall and low temperatures relative to southern and eastern regions.

During winter when grain is in store, mould development will be slowed or halted completely by the temperatures of –15˚C or below such as is experienced in Finland and other parts of Scandinavia. In contrast, areas of Spain, Portugal, Italy and France expect outdoor temperatures to rarely fall below 5˚C. At this temperature *Penicillium verrucosum* can grow if grain is moist enough. In typical winters, Denmark, UK, Germany, Austria and Netherlands also have prolonged periods when temperatures inside bulk grain will support mould growth if the moisture content is allowed to rise too high. However, there are also other factors that must be considered. Storage systems must be clean and sound, free from leaks and protected from undue exposure to the weather as factors such as this may assist in raising moisture levels and encouraging mould growth. For example, when large structures such as steel silos are exposed to strong direct sunshine, evaporation of moisture will occur and this will be translocated to cooler parts of the silo where it can condense and locally raise grain moisture. This is well known and has been studied recently in Sweden.

There are thus 3 main climatic factors that determine the moisture content of cereal grains at harvest: rainfall, temperature and sunshine. High temperature, prolonged sunshine and low rainfall will produce the driest grain while low temperature, high or persistent rainfall and dull weather will result in the grain of highest moisture content. The rate of evaporation of moisture will also be affected by the strength, humidity and direction of the wind. While the control of these factors is mostly out of human control, long-term meteorological averages can be used to identify those areas that face the biggest potential problems due to growth of *P. verrucosum* and other moulds and subsequent development of ochratoxin A initiated by difficulties in drying high moisture content grain at harvest time. It must be recognised that unpredictable exceptional weather may occur anywhere from time to time and that long-term climatic averages can only be used as a guide.

For illustrative purposes, an empirical scoring system using the published data for temperature, sunshine and rainfall that can be expected over the period prior to and during harvest has been used to produce a map showing the potential for ochratoxin A to occur in stored grain across Europe, figure 2.2. ‘The potential for ochratoxin A’ formation has been estimated on an empirical basis. In summary, this is based on the simple concept that rain will raise moisture levels in soil, plants and grain seeds, sunshine will reduce them by evaporation while the higher the temperature, the higher the rate of evaporation is likely to be. Each site has been scored on the average long-term data for temperature, rainfall and sunshine hours in the 3-month period

up to and including the usual harvest time. In addition wind speed will also accelerate evaporation especially in exposed locations and this factor will also depend on the humidity of the prevailing air mass.

When the long-term climate indicates that dry and warm conditions are the norm prior to and during the harvest period there will be less problem in drying grain to levels suitable for safe storage, minimal mould problems and the least potential for the formation of OITA. In contrast when conditions are likely to be wet and cool, attention to drying is more important and must be done thoroughly as the potential for mould and mycotoxin problems is much higher.

The data for the potential for OTA at harvest across Europe estimated by this empirical system is shown in figure 2.2.

The treatment of the climatic data shows that the areas currently recognised as being at risk are quite well represented by this map. Hence most of northwest Europe has a very high inherent risk of contaminated cereals while Spain. Portugal, Greece, Southern France, most of Italy and Bulgaria should have a very low risk of mycotoxin problems occurring. There is a corridor from the Benelux countries across Germany, Austria, and Northern Italy to Eastern Europe where the model suggests that there is a medium potential for ochratoxin A. However, the actual incidence of OTA will depend on further additional factors. In areas where hot dry weather is the norm, drying installations will either not exist or will have a relatively low capacity. If exceptionally wet weather should then occur, (as is understood to have occurred in large parts of the Central and Eastern Mediterranean in 2002) management of the grain would need special attention to avoid moist grain going into store still wet and suffering subsequent problem with moulds and possibly OTA and other mycotoxins.

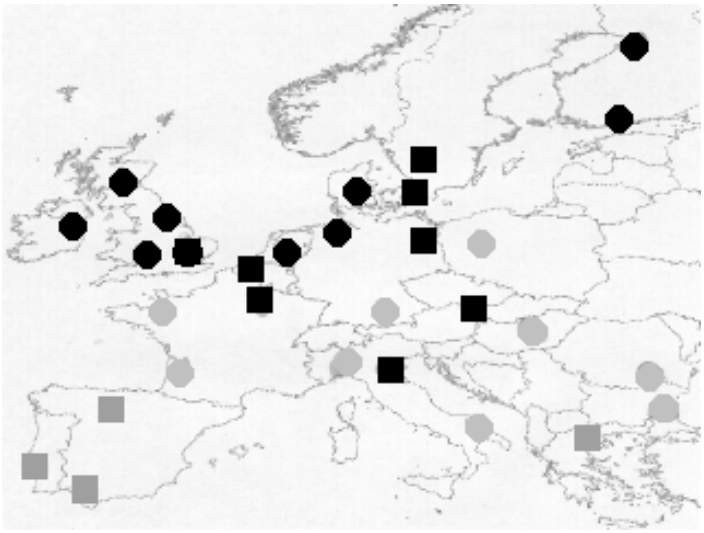

Figure 2.2: Estimated risk of potential OTA formation existing at harvest, based on 30-year climatic data for each city, in the period up to harvest

● = highest risk ■ = moderate risk ● = lower risk ■ = extremely low

Where climate is less predictable, the drying capacity available will depend on many factors so that the frequency with which harvesting of damp grain exceeds the ability to dry quickly must be balanced against the cost of having expensive or sophisticated drying installations lying idle. This is discussed further later.

Cereal production and use

About 200 million tonnes of the main cereals are grown within the Community, table 2.1. Hence, if only 3% were to be contaminated by OTA amounts above the statutory limit this would represent nearly 7 million tonnes lost with a monetary value approaching 1 billion euros. Examination of figure 2.2 and table 2.1 suggests that well over half of the grain grown in the EC may be harvested in conditions that have the potential for OTA formation if not managed correctly.

The productivity achieved for each cereal, table 2.2, depend on the suitability of the climate for each crop, the nature of the agricultural land that is available and the improved efficiency possible by the applying new research and the investment in the agricultural industry. The need to improve productivity stems from many possible reasons as fundamental as the need to feed the indigenous population, to make optimal use of available agricultural land, where this is limited, and the need to obtain income by exporting. Increases in productivity will depend on investment in the industry both nationally and with EC support. Recent emphasis on increased food safety for the Consumer means that aspects of quality have become much more important especially when there are problems with over production of cereals. Thus it becomes increasingly essential to manage the grain more effectively and to increase its quality by ensuring that it is free from contaminants. Factors that may lead to deterioration of grain during long-term storage need to be fully understood and management systems must be optimised to supply high quality cereals free from mould contaminants.

It is clear that there are few varieties of cereals that are used universally and most countries appear to have their own popular cultivars, table 2.3. Because improved varieties are being continually introduced and older ones phased out, these data can only represent a snapshot in time. It is unclear to what extent variety affects mould infection in store and thus whether this factor plays any role in OTA formation. Other factors such as the presence of broken or stressed grains, the soundness of the seed coat or seed maturity at harvest may be more important variables. There appears to be little information published on the importance of these factors.

Methods and practice in drying grain

The wide variation in climate across greater Europe implies that the need for grain drying and the methods used, table 2.4, is going to be very different from Country to Country and has implications for the potential development of OTA. It is clear that drying facilities and handling in each country need to be matched to the conditions produced by the prevailing climate. Delays between harvesting damp grain and drying must be avoided or minimised as far as possible and cereals need to be monitored appropriately during storage.

Drying at harvest

The variation in climate across Europe has already been discussed and determines to a major degree how and to what extent grain should be dried after harvest prior to storage. The range of grain moisture expected at harvest, table 5, indicates that most States need to dry grain at least on some occasions although there are a lack of data from the hotter, drier countries. Moisture content quoted for wheat, barley, oats and rye are similar but maize is usually harvested at higher moisture contents. As a moisture content below 14% is necessary for safe long-term storage of all the cereals this suggests that drying of maize will be needed in most instances and the provision of enough capacity to cope with normal and exceptional conditions should be in place.

Table 2.3: Some of the principle cereal varieties used in Europe (data from questionnaire)

COUNTRY	Wheat 1	Wheat 2	Wheat 3	Wheat 4	Barley 1	Barley 2	Barley 3	Barley 4	Oats 1	Oats 2	Oats 3	Oats 4
Austria	Capo	Renan	Silvius	Belmondo	Barke	Virac	Virgo	Prosa	Monarch	Expander	Jumbo	
Bulgaria	Sadova	Katja	Todora	Zagorka	Karnobat	Obzor	Krasy	Ruen	N85	Kondor	Mina	
Belgium/Lux												
Denmark	Ritmo	Kris	Stakado	Baltimor	Hanna W)	Ludo (W)	Barke (S)	Otira (S)	Revisor	Corrado	Markant	Adamo
Finland	Tjalve	Manu	Vinjet	Bastian	Artturi	Arve	Erkki	Rolfi	Veli	Leila	Aarre	Salo
France												
Germany	Ritmo	Flair	Batis	Bandit	Theresa	Hanna	Regina	Marinka	Jumbo	Lutz	Fuchs	Flamingsnova
Greece	Simeto*	Mexikali*	Centauro	Gemini	Arda	Lesant	Caresse	Mucho	Kassandra			
Hungary												
Ireland												
Italy												
Netherlands	Reside	Vivant	Drifter	Napier	Barke	Reggae	Chalice	Prestig	Gigant	Valiant		
Portugal												
Spain												
Sweden	Kosack	Tarso	Kris	Vinjett	Otira	Barke	Pongo	Kinnan	Belinda	Stork	Sang	Freja
UK	Consort	Claire	Malacca	Savannah	Pearl	Halcyon	Jewel	Regina	Gerald	Jalma	Drummer	Banquo

*=durum

Table 2.3 (continued): Some of the principle cereal varieties used in Europe (data from questionnaire)

Country	Rye 1	Rye 2	Rye 3	Rye 4	Maize 1	Maize 2	Maize 3	Maize 4
Austria	Elect	Motto			Banguy	Raissa	Clarica	Monalisa
Bulgaria	Danae	Eros	Chulpan		Kneja	Pioneer	Plovdiv	Px
Belgium/Lux								
Denmark	Dominator	Avanti	Hacada	Picasso				
Finland	Akusti	Amilo	Kartano	Voima				
France								
Germany	Avanti	Hacada	Nikita	Esprit				
Greece	Local				Costanza	Eleonora	DK-ALP	
Hungary								
Ireland								
Italy								
Netherlands	Rapid	Esprit	Amilo	Halo	Simphony	Husar		
Portugal								
Spain								
Sweden	Amilo	Esprit	Picasso					
UK	Picasso	Ursus	Esprit	Hacada				

Table 2.4: Drying of cereals, and methods used (data from questionnaire)

Country	Wheat		Barley		Oats		Rye		Maize	
	Drying required?	Methods used	Drying required?	Methods used	Drying required?	Methods used	Drying required?	Methods used	Drying required?	Methods used
Austria	not usually	hot air	not usually	hot air	not usually	hot air	not usually	hot air	normally	hot air
Bulgaria	not usually	sun	not usually	sun	not usually	sun	not usually	sun	not usually	sun
Belgium/Lux										
Denmark	sometimes	hot air and ambient air	sometimes	hot air and ambient air	sometimes	hot air and ambient air	sometimes	hot air and ambient air	not grown	
Finland	normally	hot air	normally	hot air	normally	hot air	normally	hot air	not grown	
France										
Germany	sometimes	hot air and ambient air	sometimes	hot air and ambient air	sometimes	hot air and ambient air	sometimes	hot air and ambient air	normally	hot air
Greece	not usually	ambient air	not usually	ambient air	not usually		not usually		sometimes	hot air
Hungary	not usually	hot air	not usually	hot air	not usually	hot air	not usually	hot air	normally	hot air
Ireland	normally	hot air	normally	hot air	normally	hot air	not grown		not grown	
Italy										
Netherlands	sometimes	hot air and ambient air	not usually	hot air and ambient air	sometimes	hot air and ambient air	sometimes	hot air and ambient air	normally	hot air
Portugal										
Spain										
Sweden	normally	hot air	normally	hot air	normally	hot air	normally	hot air	not grown	
UK	sometimes	hot air and ambient air	sometimes	hot air and ambient air	sometimes	hot air and ambient air	sometimes	hot air and ambient air	not grown	

Table 2.5: Moisture contents,% of cereals at harvest (data from questionnaire)

Country	Wheat		Barley		Oats		Rye		Maize	
	Typical values	Maximum	Typical values	Maximum	Typical values	Maximum	Typical values	Maximum	Typical values	Maximum
Austria	14	18	14	18	14	18	14	18	32	36
Bulgaria	16-18	20	17-18	20	16-18	19	15-16	18	18-20	25
Belgium / Lux										
Denmark	15-18	24	15-18	22	15-18	22	16-19	22		
Finland	17-25	28	17-25	28	17-25	28	17-25	28		
France										
Germany	14	19	14	19	14	19	14	19	35	38
Greece	10	12	11	13	10	13	10	13	18	25+
Hungary	13	16	13	15	13	15	13	16	23	30
Ireland	17-23	23+	17-23	23+	17-23	23+				
Italy										
Netherlands	15-17	20	14-16	18	15-17	20	15-17	20	25 +	40
Portugal										
Spain										
Sweden	19-22	25	19-22	30	19-22	30	19-22	30		
UK	13-19	25	13-18	27	13-18	25	13-18	25		

Although these data for moisture are subjective, they do present a picture very similar to that shown in figure 2.2 showing the potential risk from ochratoxin across Europe. Quite predictably the need to dry was greatest in northern and western Europe, with this being the norm in Sweden, Finland and Ireland while UK, Denmark, Germany, Netherlands quite often needed to dry grain at harvest. Among those countries that normally need to dry cereals, Finland almost exclusively uses hot air dryers to reduce moisture rapidly to safe storage conditions close to harvest. Grain there is not usually left damp and the extremely cold temperatures during winter effectively halt mould growth and associated mycotoxin development. The method of choice for drying appears to be hot air in Austria, Hungary, Ireland and in Sweden for wheat and rye. Denmark, the Netherlands, Germany and the UK (and Sweden for barley and oats), operate both hot air and ambient air drying systems. Bulgaria relies mainly on sun drying.

Table 2.6: Delays in drying at harvest (data from questionnaire)

		Possible time before grain is dried, days		
		At moisture content		
COUNTRY	Are there sometimes delays in drying grain at harvest?	<18%	18-22%	>22%
Austria	no	-	-	-
Bulgaria	yes	6	4	4
Belgium/Lux				
Denmark	yes	14	7	4
Finland	no	-	-	-
France				
Germany	yes	<4	<4	<4
Greece	yes	14	7	4
Hungary	no	-	-	-
Ireland	yes	7	7	7
Italy				
Netherlands	yes	14	7	<4
Portugal				
Spain				
Sweden	yes	14	7	<4
UK	yes	14	7	<4

Implication of delays in drying damp grain

All recent research and reasoned analysis of known information suggests that a major Critical Control Point for preventing the development of OTA is drying grain to a safe storage moisture at harvest with minimal delay. In most of Europe there may sometimes be a delay before drying can be undertaken, table 6. The responses received suggest that it is normal practice to dry all grain immediately in Austria, Finland and Hungary. This information is in line with the suggestion that OTA is not a major problem in Finland and Austria. However, a number of publications from workers in Hungary suggest that OTA can be a problem in this country, at least in animal feed, and this would tend to suggest that storage conditions are not always effective in keeping grain dry over prolonged storage periods. Analytical limits of detection quoted for OTA in some scientific papers are also sometimes lower than elsewhere so this does not allow direct comparison on the same basis with the situation within an expanded Europe.

At least 8 respondents admit that on occasion damp cereals may be held for up to 7 days with moisture contents above 20% before drying can be carried out. This is not usually done intentionally but as a result of local weather conditions or a lack of drying capacity. Results of a number of laboratory experimental studies show that on some occasions significant amounts of OTA can be produced within this time and under these conditions but it must be considered that the temperature will also be important. However, the results reported (and also the personal experience of the author) show that the formation of OTA is very unpredictable and depends on many factors including temperature, moisture content, whether or not the grain was artificially inoculated in the laboratory or whether *P. verrucosum* was already present naturally. There is a strong indication that the availability of sources of *P. verrucosum* is an important factor in the commercial situation.

This information shows that over a large part of Europe, there are occasions and conditions at harvest that may be expected to initiate the formation of OTA. With education about the specific problem of OTA it is suggested that at these ‘crisis ‘ situations grain might be managed more effectively by farmers and grain store managers.

Table 2.7: Typical type and size of storage systems, tonnes (data from questionnaire)

Country	Metal silo	Concrete silo	Bin	Flat store	Heap	Bags
Austria	100-200	250-500	yes	2000	yes	
Bulgaria	50	yes	300	100	20	yes
Belgium/Lux						
Denmark	100-1000	yes	yes	100-500	10-100	
Finland	yes	yes				
France						
Germany	yes	yes	yes	yes	yes	
Greece	6000	20000		2000	1000	yes
Hungary	600	1000	40	2500	100	yes
Ireland	500	yes	100	5000	1-100	
Italy						
Netherlands	200	200	1	1000+	100	yes
Portugal						
Spain						
Sweden	400	5000	100	yes	yes	
UK	100-5000	100-1000	30-50	500	10 - 10000	

Management of grain during storage

Only Denmark, Germany, Ireland and the UK stated clearly that they have a Code of Practice for the safe storage of grain. In addition, storage facilities vary significantly across Europe, table 2.7, so that any Code of Practice must be relevant to the storage system in use. However, most countries appear to have guidelines for storage that include recommendations for regular monitoring of temperature and moisture content. Insect and pest management was cited in most replies. The reply from Bulgaria suggested that on private farms there is little control of grain moisture. This is often accompanied by insect invasions of *Sitophilus* and *Sitotroga* and fungal deterioration is sometimes obvious due to the high moisture of grain at harvest or lack of control during storage. This is consistent with reports of ochratoxin A problems in that Country. In practice it is likely that some farmers in Member States at times do not fully follow recommendations for a variety of reasons. This may be more likely where the grain never leaves the farm and so is not subjected to commercial pressures. Improved dissemination of information emphasising the need to minimise the risk from mycotoxins that result from poorly stored grain should assist in improving the quality of grain storage across the Community.

Cereal usage

There are a few specific observations that can be made concerning cereal use (see table 2.8) in connection with the OTA problem. Firstly in processes such as milling, OTA will not be equally distributed in milled fractions as demonstrated in the processing work package of this project. Careful attention must thus be paid to the uses of each fraction, particularly those in which OTA is concentrated (commonly residues from cleaning, and the bran and offal fractions). It has been common practice to use such fractions for animal feed without careful consideration of any consequences for animal health, or indirectly for human health and safety. It is known that there is some small percentage transfer of OTA from barley to beer but the work package in this project suggests that the presence of the producing mould (*P. verrucosum*) in the barley may be more important because in some situations OTA can be generated during certain stages. One possible use for contaminated cereals such as wheat and maize is for industrial purposes such as alcohol or starch and if the economics are acceptable and starch is proved to be clean this would seem a possible outlet for rejected grain. Another interesting use for wheat germ in the production of antibiotics, pharmaceuticals and skin conditioners while wheat bran may be used as a carrier of enzymes, antibiotics and vitamins. It is unclear whether there has been any safety evaluation of wheat bran contaminated with OTA in relation to this use (usage figures for this purpose have not been sought so no indication of the potential problem can be made).

Regulations and guidelines

The questionnaire was initiated prior to the introduction of statutory limits for OTA in cereals so section 8 was partly out of date by the time the given answers were assessed. It was interesting that in general, OTA was not considered to be a problem except in Denmark although it must be emphasised that this was the view of the respondent and did not necessarily represent an official or commercial viewpoint. Few countries currently carry out regular surveys for OTA although the EC SCOOP Project has recently gathered all relevant reliable data together.

Table 2.8: Main uses of cereals, A to D in perceived order of importance (data from questionnaire)

Country	Wheat A	Wheat B	Wheat C	Wheat D	Barley A	Barley B	Barley C	Barley D	Oats A	Oats B	Oats C	Oats D
Austria	bread	export	animal fd	seed	animal fd	export	malt	seed	animal fd	industrial		
Bulgaria	bread	breakfast	export	animal fd	animal fd	industrial	malt	other	animal fd	industrial	snacks	breakfast
Belgium /Lux												
Denmark	animal fd	bread	seed		animal fd	export	malt	seed	animal fd	breakfast	seed	
Finland	bread	animal fd	industrial	seed	animal fd	malt	industrial	other	export	animal fd	seed	breakfast
France												
Germany	bread	export	animal fd	industrial	animal fd	industrial	export	malt	animal fd	export	seed	
Greece	export	bread	animal fd	seed	animal fd	malt	seed	other	animal fd	breakfast		
Hungary	bread	animal fd	export	seed	animal fd	industrial	export	seed	animal fd	seed	export	breakfast
Ireland												
Italy												
Netherlands	animal fd	bread	industrial	breakfast	malt	animal fd	industrial	seed	animal fd	breakfast	seed	
Portugal												
Spain												
Sweden												
UK	bread	animal fd	breakfast	export	animal fd	malt	export	seed	animal fd	breakfast	snacks	seed

Table 2.8: Main uses of cereals, A to D in perceived order of importance (continued) (data from questionnaire)

Country	Rye A	Rye B	Rye C	Rye D	Maize A	Maize B	Maize C	Maize D
Austria	bread	animal fd			animal fd	industrial	export	snacks
Bulgaria	animal fd	bread	breakfast	snacks	animal fd	industrial	snacks	breakfast
Belgium /Lux								
Denmark	export	animal fd	bread	seed				
Finland	bread	seed	other					
France								
Germany	bread	export	animal fd	industrial	animal fd	export	industrial	
Greece	bread	animal fd			animal fd	industrial	snacks	bread
Hungary	animal fd	industrial	export	seed	animal fd	export	industrial	seed
Ireland								
Italy								
Netherlands	animal fd	bread	breakfast	seed	animal fd			
Portugal								
Spain								
Sweden								
UK	animal fd	bread	seed					

Conclusions

In drawing these responses together, results from other work packages in the EU project have been considered as well as other information available to the author.

Ochratoxin A is generally accepted as a post-harvest problem and is rarely found before harvest in soundly conducted studies and surveys except in trace amounts. Similarly *Penicillium verrucosum*, the principle (if not only) fungal species responsible is rarely found in grain at harvest. However most of western, central eastern and northern Europe is potentially at risk from OTA in stored cereals. Only the hot south should normally have little risk as the climate is unlikely to provide favourable conditions for its formation under normal circumstances. The wetter and cooler western and northern areas are at greatest risk but the actual apparent severity of the problem is also affected by local practices and circumstances.

In principle, if grain from the field at harvest is dried immediately and quickly to an acceptable moisture content that is guaranteed to be uniform through the storage bulk and moisture and temperature is subsequently monitored throughout the bulk during the whole storage period, OTA cannot occur unless conditions at any point have deviated from 'the acceptable range'. Drying at harvest is clearly the most important Critical Control Point so that if this is controlled according to agreed criteria and the grain is subsequently properly monitored, OTA should not be an issue.

Indications from the responses to this questionnaire suggest that where hot air drying is used and capacity can cope with the intake of grain even in poor weather conditions, OTA is less likely to be a problem. The use of on floor ventilation systems is in principle sound if the installation is engineered to cope with all normal conditions, is well maintained and operated efficiently. However, it may not be able to reduce moisture content quickly enough under unusually wet and cool conditions. This makes it important that the store keeper should ideally understand the factors that may lead to the formation of OTA and be able to undertake immediate correction if things start to go wrong.

Safely dried grain may subsequently be affected by moisture translocation, by effects of sun on grain bins, insect activity or leakages. These need to be eliminated as far as possible or immediate action taken to deal with such occurrences. Management must thus be on-going right through storage.

Acknowledgement

The interest and assistance in providing contacts and information by many scientists in a range of organisations across the Community was fundamental to the successful completion of this task. The current author, responsible Partner and Project Coordinator express their sincere thanks to all who helped and pay tribute to the linguistic ability of those who so expertly responded in a language other than their own.

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Appendix

Prevention of ochratoxin A in cereals (as part of an EU project by this title): Project 2320

WP 2. Differences depending on various cereal species, and farming methods climate

Questionnaire devised as part of this WP

Survey of cereal production and storage in Europe on behalf of the EC

Section 1: General Information

Contact name: | **Country:**

Address: | **Tele. No:**

Fax No.:

E-mail:

Post code:

Official position:

Section 1a: Guidance for completing this questionnaire

Correspondence will normally be by E-mail unless this is impossible. Text can be entered into each box and should normally automatically scroll from the top. Do not try to change the size of the text boxes. If necessary continue in a box on the additional sheet provided.

Data is available for section 2. In setion 3, values may already be entered from published data. These can be changed if they are considered incorrect or out of date. For manual returns use the duplicate pages included.

Please use **Y** for yes and **N** for no, or other characters specified.

In the general information boxes, please give a brief summary of the normal situation or practice and describe any extreme situations which can sometimes occur. Because this project aims to identify the factors which may cause ochratoxin A formation, it is important to supply information, especially about the unusual situations which can sometimes arise.

If anything is unclear or problems arise in using this electronic form please contact the author.

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Survey of cereal production and storage in Europe on behalf of the EC

Section 2a: Climate

(This information is widely avalable. Do not complete this section)

Region 1

Mean temperature	year		J-M		A-J		Jy-S		O-D
Mean max. temp.	year		J-M		A-J		Jy-S		O-D
Mean min. temp.	year		J-M		A-J		Jy-S		O-D
Rainfall, mm	year		J-M		A-J		Jy-S		O-D
Sunshine, hours	year		J-M		A-J		Jy-S		O-D

Section 2b: Climate

Region 2

Mean temperature	year		J-M		A-J		Jy-S		O-D
Mean max. temp.	year		J-M		A-J		Jy-S		O-D
Mean min. temp.	year		J-M		A-J		Jy-S		O-D
Rainfall, mm	year		J-M		A-J		Jy-S		O-D
Sunshine, hours	year		J-M		A-J		Jy-S		O-D

Please comment on problems which may be caused by the climate during cereal production

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Section 3: Cereal production and use

Which of the following cereals are grown? Y=yes, N=no

Wheat ☐ Barley ☐ Oats ☐ Rye ☐ Maize ☐

What is the approximate annual production, in million tonnes, to 1 decimal place or use<0.1?

Wheat ☐ Barley ☐ Oats ☐ Rye ☐ Maize ☐

What is the approximate area of cereal under cultivation, in million hectares, 1 place or use <0.1?

Wheat ☐ Barley ☐ Oats ☐ Rye ☐ Maize ☐

Please list the most used varieties of each cereal (up to 4).

wheat	barley	oats	rye	maize
1	1	1	1	1
2	2	2	2	2
3	3	3	3	3
4	4	4	4	4

What are the main uses for each cereal, choose from:

Export=1, bread=2, breakfast cereals=3, malt=4, snack products=5, animal feed=6, industrial products=7, seed=8 other=9. (Please list in order of importance)

wheat	barley	oats	rye	maize
A	A	A	A	A
B	B	B	B	B
C	C	C	C	C
D	D	D	D	D

If you wish to provide further information about cereal production and use do so here:

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Section 4: Growing the cereal

Please describe the normal practice or situation in your country. The keywords are only given as a guide. If there is a significant difference between cereals please specify.

a) Sowing the crop

keywords	
Typical farm/field size	
Soil types:	
eg. sandy, loam, etc.	
Soil preparation	
Sowing period	

b) Assisting the crop

Keywords	
Herbicide use	
Insecticide use	
Irrigation use	
Fertilizer use	

c) Disease control

Keywords	
Fungicide use	
Doses used	
Fungal diseases that occur	

Section 5: Harvesting

Please descibe normal procedures for harvesting cereals, including the machinery, handling of grain and transport of the cereal to store. Indicate the normal harvest period and how long does in last? Give examples of problem conditions that occur. Please indicte any differences between the types of cereals.

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Section 6: Drying

Is drying required after harvest? Use **U** = usually>75% of the time, **S**=sometimes 25% to 75%, **R**=rarely <25%

wheat	barley	oats	rye	maize

If yes, what methods of drying are used? Use **H**=heated air, **A**=ambient air, **S**=sun, **0**=other

wheat	barley	oats	rye	maize

Note: use more than one letter per box if necessary and list the most important first

What are the moisture contents, %, at harvest?

	wheat	barley	oats	rye	maize
typical range					
maximum					

Is there sometimes a delay between harvest and drying the grain? Y/N ☐

If yes, how long could these delays be in days at the moistures given below?

moisture	<4	4 to 7	7 to 14	>14	days
>18%					
18-22%					
<22%					

Section 7: Storage

Where is grain stored? Please use Y=yes, N=no

on farm ☐ central depots or cooperatives ☐ other ☐

specify: _____

How long is grain stored in each situation?, months

	farm	depot	other
typical			
maximum			

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Section 7: Storage continued

How often are each of the following methods of storage used? Use numbers as follows: 3= commonly used, 2=sometimes used, 1=rarely used, 0=not used

	wheat	barley	oats	rye	maize
metal silo					
concrete silo					
bin					
flat store					
heap on floor					
bags or sacks					
other (please specify)					

Please summarise the typical capacity in tonnes of the storage systems listed above

metal silo		concrete silo	
bin		flat store	
heap on floor			
*other		* other:	

Management of grain during storage. Describe the recommended normal practice adopted for the management of grain duing storage, eg temperature and moisture measurement and control, rodent and insect control, etc.

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Section 8 : Regulations/guidelines

Is ochratoxin A considered to be a problem in your country? Y/N ☐

Is there a legal/advisory limit for ochratoxin A in cereals? legal ☐ advisory ☐

If yes, what is the limit, µg/kg ☐

Is there regular monitoring of ochratoxin A in cereals? yearly ☐ less frequently ☐

Is there a code of practice for safe storage of cereals? N/Y ☐

If yes, is it possible to send a copy? ☐

Section 9: Follow up

If required, would you be able to assist in arranging on-site visits to representative stores or farms? ☐

or, would you be able to introduce a contact who could arrange these on-site visits? ☐

Information supplied by: ☐ Please print

Signature: ☐

Date: ☐

******THANK YOU FOR YOUR TIME AND INTEREST IN COMPLETING THIS QUESTIONNAIRE*****

Survey of cereal production and storage in Europe on behalf of the EC

Sheet for additional information

Reference to section and subject Section no. Subject

Reference to section and subject Section no. Subject

Reference to section and subject Section no. Subject

Questionnaire on crop production and storage practice in Europe

Conducted as part of EU project QLRT-1999-00433, prevention of ochratoxin A in cereals

REQUEST FOR ASSISTANCE

Because the amount of ochratoxin A (OTA) permitted in cereals is soon to be controlled by legislation within the Community, a project entitled 'prevention of OTA in cereals' has been funded under the 5th Framework, Quality of Life Programme. This project is to examine many of the factors thought important for the occurrence of ochratoxin A.

One task within this Project is to produce a database of information about cereal production and storage across the Community. This will be achieved by means of a questionnaire. This data will be used to assess which are the critical points that control ochratoxin A formation. This will assist International Organisations in assessing the risk to consumers from ochratoxin A in all cereals products (from wheat, barley, rye, oats and maize).

It is hoped that each Member State will complete a questionnaire. This will need a broad practical knowledge of cereal production, drying and storage by the experts completing this form.

The form requests information about cereal production, but especially drying and storage. Some information is readily available, for instance that on climatic data and tonnage of cereals produced. These sections will not require any action. Although there are quite a few questions, it is hoped that the form will not take too long to complete. This can be sent either electronically, as an 'Excel' document, or by post as a hard copy. It is regretted that only an English version can be supplied as it is being sent to all current Member States and, in addition, to selected Candidate Member States.

<u>If you are unable to assist I would be grateful if you could let Mr Keith Scudamore know this as soon as possible, preferably using the E-mail address given in the attached letter.</u>

WP 8: Development of rapid ELISA systems ochratoxin A producing fungi and ochratoxin A

WP8.1: Defining the performance and design parameters

Introduction

The increasing concern regarding mycotoxins in food and feedstuffs has resulted in the introduction of EU legislation setting very low permitted maximum residue levels (MRLs) in cereals and other commodities. Of immediate concern to cereal growers and grain processors is the MRLs of 3-5ppb for ochratoxin A (OTA) in cereals. The indications are that this type of legislation will in coming years be extended to include Fusarium toxins such as deoxynivalanol, zearalenone, fumonisin and T-2. Testing for mycotoxins at these low levels poses technical challenges in itself. However, the customer requirement is for tests that are not only sensitive but simple to use on-site by operators with limited technical skills.

Diagnostic tests based on immunological techniques such as ELISA and lateral flow were considered most appropriate. The challenge was not only to meet the technical performance regarding sensitivity, but to format tests that are robust enough to be used in the factory environment and simple enough to be used reliably by staff with limited technical skills.

It was considered essential to determine as far as possible at the outset what the potential end-users required. This was to establish a customer – defined specification so that any product emerging from the project would relate directly to the expressed needs of the industry.

Material and Methods

Selection of participants

Potential end-users of the on-site tests to detect OTA were selected from ADGEN's customer base, from internet searches to identify a representative cross-section of relevant organisations and direct contact with known members of grain associations etc.

A total of 204 UK contacts were identified. The industry sectors that they represent are shown in Table 8.1.1.

Table 8.1.1. End-user survey – participants by industry sector

Industry sector	Number who received questionnaire
Animal feed producers	64
Brewers	3
Distillers	3
Food producers	14
Grain merchants	41
Maltsters	9
Millers	49
Poultry feed producers	3
Pet food producers	3
Testing laboratories	4
Unknown	10
Associations	1
Total	204

Design of the questionnaire

The questionnaire comprised of 14 questions (see below) most of which were in yes/no or simple multiple choice format. However, there were opportunities to give specific responses regarding such items as critical testing points and acceptable per test costs.

Results

A total of 38% of recipients returned their questionnaire. The responses to each question are presented below.

1. Are you familiar with mycotoxins such as aflatoxin and ochratoxin A and the potential dangers to humans and animals from consuming contaminated grain and grain products?

Yes 95% No 5%

2. Are you aware of EU and UK Government legislation setting limits for the level of contamination of cereals and cereal products by aflatoxin?

Yes 82% No 18%

3. Are you aware of impending EU legislation that sets limits for the level of contamination of cereal and cereal products by ochratoxin A?

Yes 62% No 38%

4. Does your company currently carry out any testing for the presence of mycotoxins?

Yes	79%	No	21%

5. If yes, which toxins do you test for?

Aflatoxin	96%	Ochratoxin A	71%
Vomitoxin	33%	T-2toxin	20%
Zearalenone	29%	Fumonisin	16%
Other	10%		

6. Is testing conducted:

In the factory	0%
On-site laboratory	23%
Sub-contracted to laboratory	51%
Rely on supplier's certificate	4%
Combination of above	23%

7. At what point (s) in the processing/production do you carry out, or would like to carry out, testing?

On receipt of commodities/ingredients	31%
During/after storage	1.5%
During processing	1.5%
Finished products	6%
Other	1.5%
Combination	57%

8. What would be your ideal turn round time for analysis?

Responses ranged from 10 minutes to 2 weeks

9. Are there any critical points in production/processing that a simple – to – use rapid test (e.g. 10 to 20 minutes) would be particularly beneficial?

The majority of respondents stated that intake would be the critical testing point.

10. With reference to Q9, what would be the likely skills of the operatives who may use a rapid on-site test kit?

Unskilled	18%
Skilled but no formal qualifications	53%
GSE/HNC/HND	7%
Graduate	7%
Combination	17%

11. Are you aware of the EU requirements for the sampling of cereals and cereal products for ochratoxin analysis?

Yes 40% No 60%

12. What would you consider to be a reasonable price to pay for an on-site, rapid and sensitive diagnostic test?

Responses ranged from £1 to £60. Over 30% suggested £5.

13. What is the frequency and volume of your current testing?

Daily 6%
1-5 samples 25% 5-10 samples 25% more than 10 samples 25% not stated 25%

Weekly 13%
1-5 samples 62% 5-10 samples 0% more than 10 samples 25% not stated 13%

Monthly 43%
1-5 samples 63% 5-10 samples 4% more than 10 samples 15% not stated 18%

Other 16% - mainly quarterly

No comment 20%

14. What type of on-site test result would you find most useful?

Quantitative	34%
Qualitative	17%

Either/both	41%
Not specified	7%

Discussion

A total of 38% of the questionnaires were returned. An important finding was that 97% of respondents are aware of the potential dangers of mycotoxins and some 79% already are performing some mycotoxin testing. These figures are higher than expected and clearly reflect the growing concern about mycotoxin in food and feedstuffs. The majority of testing is for aflatoxin (97%) and OTA (71%). This is as expected as aflatoxin in nuts, maize and dried fruits has been for several years the subject of legislation. Ochratoxin A is now of particular concern in Europe as it is produced on cereals by fungi that thrive in the northern European climate. Also, OTA is the subject of recent EU legislation.

The survey responses have given valuable indications of the specification for the new diagnostic tests. There is a desire to test on-site at intake. Thus a rapid test (10-20 minutes) is required. It must be user friendly as over 70% of the operatives using the tests will have no formal technical/scientific qualifications, although many will be skilled workers who should have no difficulty following a set protocol. A threshold or qualitative test may be adequate and technically is probably the limit of what can be developed in a 10 minute test. However, where a fully quantitative test is preferred this probably can still be provided as an on-site test where a sample turn round time of 20 minutes is acceptable.

The increase in the need to test for mycotoxins carries financial penalties. First there is the direct cost of conducting the tests, particularly if a traditional analytical laboratory is used. Second, there is the potential for product rejection or product downgrading where the legislative or customer specifications regarding mycotoxins are not met.

Thus there is an expressed need for a rapid test that can provide real time data to identify potential problems at the earliest point in processing grains or products derived there from. Due to the increased level of testing required this must be done at the lowest possible unit cost. These requirements provide further evidence that the immunoassay technology used in this project is the correct one to meet the majority of the end user specification.

It was encouraging to find that 68% of respondents wished to have further involvement in the project.

WP8: Development of rapid immunoassay systems for ochratoxin A producing fungi and ochratoxin A

WP 8.2: Development of rapid immunoassay systems for ochratoxin A producing fungi

Introduction

The protection of the consumer's health by decreasing the amount of ochratoxin A (OTA) in food especially cereals produced in Europe, by 50% or more is a major objective of all EU member states. Fungal contamination of agricultural commodities is commonplace both in the field (e.g. *Fusarium sp.)* and during storage (*Penicillium and Aspergillus* species). These attacks can render a partial or a total loss upon the crop, as well as presenting a health threat by the production of mycotoxins and allergenic spores.

A HACCP programme for OTA in cereals has been developed where the focus resides upon a need to test commodities quickly and efficiently to ensure that all food from field to table has an assured quality. One of the key elements is to provide effective tools and this could be made possible by using rapid immunodiagnostic detection techniques. Broad-spectrum antibodies that react with a wide range of different fungi are available but there is a need for specific monoclonal antibodies to OTA producing fungi. Generally suitable antibodies are developed in to ELISA systems but in certain applications, other formats such as Lateral Flow Devices (LFDs) may be more suitable. These are one-step rapid systems that only take a few minutes to complete, and can be performed by unskilled personnel and have been developed and are commercially available for a number of plant pathogens (Danks & Barker 2000). Having both laboratory (e.g. ELISA) and on-site rapid systems (such as LFDs) increases the options available for the successful monitoring for OTA producing fungi. This report describes efforts to develop a specific Mab to the main OTA producing fungi i.e. *P. verrucosum* and *A. ochraceus* and the incorporation of a pre-existing *Aspergillus* and *Penicillum* species specific monoclonal antibody into an ELISA and LFD.

Materials and Methods

All fungal cultures (*Penicillum verrucosum, Aspergillus ochraceus, Mucor, Alternaria, M. nivale* etc) were prepared from the Central Science Laboratory culture collection.

Preparation of Soluble Fungal Antigens

(i) *Shake culture for preparation of soluble antigens* of OTA producing and non-producing fungal cultures were produced as described by (Banks *et al*. 1996). Spore suspensions of fungal culture were prepared and seeded into conical flasks containing 100 ml of liquid medium (Burrell *et al.,* 1966) and incubated in the dark on a rotary shaker at 140 rev min^{-1} for 7 days at 25^{o} C. Fungal pellets from each flask were collected by vacuum filtration and washed with 30 ml of sterile, distilled water followed by 30 ml of sterile phosphate-buffered saline (PBS). The fungal material was then freeze-dried, snap frozen in liquid nitrogen and ground to a fine powder with a pestle and mortar. The powdered mycelium was suspended in PBS (200 mg of fungal

material in 1.5 ml PBS) and centrifuged, and the supernatant, representing the soluble part of the antigen, was saved and stored at –20°C for immunization.

For cross-reactivity studies, soluble antigens were prepared as above from fungal material collected either from shake culture or scraped directly from PDA agar plates, which were then snap frozen in liquid nitrogen and processed in a similar fashion as soluble antigens.

(ii) *Soluble antigen preparation on whole wheat grains*

Moistened wheat grains (22% moisture content) were treated with spore suspensions of both *Penicillum verrucosum* and *Aspergillus ochraceus* and incubated at 20°C and 25°C respectively until appreciable amounts of fungal growth was visible.

Prior to use, all soluble antigen preparations were assayed for their total protein estimation, using a modified Bradford assay (Banks *et al*., 1992).

Monoclonal Antibody (Mab) production

Immunization schedule:

Adult female Balb/C mice were immunized with 0.1 ml of a 1 mg ml^{-1} initial innoculm of each immunogen and mixed with an equal quantity of Freund's adjuvant (Freund 1956). The first injection was in Freund's complete adjuvant given via the subcutaneous (SC) route and subsequent injections were made at 2, 4 and 6 weeks in Freund's incomplete adjuvant intraperitoneally (IP). Sera samples were collected at 6 weeks after the start of the immunizations and tested by ELISA to monitor the immune response. Mice that had responded well, as shown by their sera containing strong polyclonal (Pab) antibodies to the antigen, were selected for Mab production. Selected mice were given a final boost injection at least 8 weeks after the start of the immunisation schedule, with the antigen in PBS. Four days later the mice were sacrificed by cervical dislocation under anesthesia and their spleens removed for fusion. (Kane & Banks 2000, Banks et. al. 1996).

In total twelve mice (4 boxes) were immunized with each of the fungal immunogens; *Penicillum verrucosum* and *Aspergillus ochraceus.* Two of the three boxes were immunized with soluble extract antigens and the remaining two boxes were immunized with the soluble antigens prepared on wheat.

Cell Fusion

Splenocytes from the immunized mice were fused at a ratio of 3:1 with myeloma cell line SP_2/0.Ag 14 (ECACC). Fusion was carried out by the gentle addition of 1 ml of 30% (w/v) polyethylene glycol (PEG) over 60 seconds. Then 6 ml of warm serum-free RPMI 1640 medium (Gibco) was added over the next 4 minutes with gentle stirring. A further 43 ml of warm RPMI was added before centrifugation at 2000rpm for 5 min at room temperature. The resulting pellet of cells was re-suspended into 50 ml of growth medium (RPMI 1640 + 20% FCS with 1ml HAT (Gibco) and 0.5ml HES (Sigma)) and distributed into five 96-well microplates, 100 µl per well.

Hybridoma production

Fused cells were incubated at 37°C with 5% CO_2. All wells were fed on days 3, 6 and 10 days. On day 3, 100µl of growth medium was added to each well. On days 6 and 10, 100µl of growth medium was removed from each well and replaced with 100µl of fresh growth medium containing HAT and HES. Supernatants were screened by indirect ELISA at approximately day 13. Hybridomas that produced antibodies reacting with the immunogen were bulked into 24 well plates and subsequently cloned twice by limiting dilution (Harlow & Lane, 1988). Cloned cells were then bulked to 25cm^2 tissue culture flasks and supernatants were collected for screening when cell densities had become high and the medium was showing signs of acid production (i.e. turning yellow). All cloned hybridoma cells of interest were frozen in a mixture of 90% foetal calf serum and 10% DMSO at -80°C for 24 h and then transferred to -150°C.

Isotyping

All Mabs were isotyped for the determination of the immunoglobulin isotype class and subclass, using ISO-*strip* isotyping kit (Roche Diagnostics), following the manufacturer's instructions.

Purification

Hi-Trap Protein G affinity columns (Amersham Biosciences) were used to purify culture supernatants and protein estimations were calculated using a commercial protein assay reagent (PerBio).

Fungal antibody screening by ELISA

An indirect ELISA was used to screen for antibodies to both soluble fungal antigens of OTA producing fungi. Ninety-six well microtitre plates (Maxisorp, Nunc) were coated overnight at +4°C with the soluble antigen extracts of a selection of OTA producing and non-producing fungal antigens species e.g. *Penicillum verrucosum* and *Aspergillus ochraceus*, other *Penicillum* and *Aspergillus* species and other fungal genera e.g. *Mucor* sp, *M. nivale*, and *Alternaria* sp. These antigens were produced by either the shake culture method or from the grain washings method. Dilutions of all antigens were made to 5µg/ml in 0.05M sodium carbonate buffer pH 9.6 and coated on to the ELISA plate. The following day the plates were washed three times with Phosphate buffered saline plus 0.05% Tween 20 (PBS-T). This washing step was performed after each of the subsequent steps. Non-specific blocking was carried out by adding 250µl per well of 5% Skimmed Milk (Marvel), for 1 hour at 33°C. Culture supernatants diluted 1:2 in PBS-T were added to the wells (100µl) and incubated again, for 1 h at 33°C. After washing an alkaline phosphatase conjugated anti-mouse IgG specific antibody (100µl per well) diluted in PBST, was applied and incubated at 33°C for 1 h. Finally 100µl of AP substrate at 1mg ml^{-1} (p-nitrophenol, Amersham Biosciences) diluted in a 0.1M Diethanolamine substrate buffer was added to each well and incubated at room temperature for 1 h. The absorbance was measured at 405 nm on a Molecular Devices microtitre plate reader. Cell lines producing antibodies of interest were identified and selected for cloning.

Aspergillus /Penicillium specific competitive LFD Production

Specific Aspergillus/Penicillium monoclonal (AF-CA2-CH8) was bound by passive adsorption to dyed latex particles. Sensitised latex particles were then applied onto a release pad, to produce a stable particle reservoir for release onto a nitro-cellulose-based membrane. Two lines of reagent were immobilized onto the membrane using a reagent dispenser. The target reference or test line was comprised of Aspergillus/Penicillium antigen and the control line is a line of goat anti-mouse antibody. The release pad and membrane are assembled together with an absorbent pad into a plastic housing Fig 8.2.2.

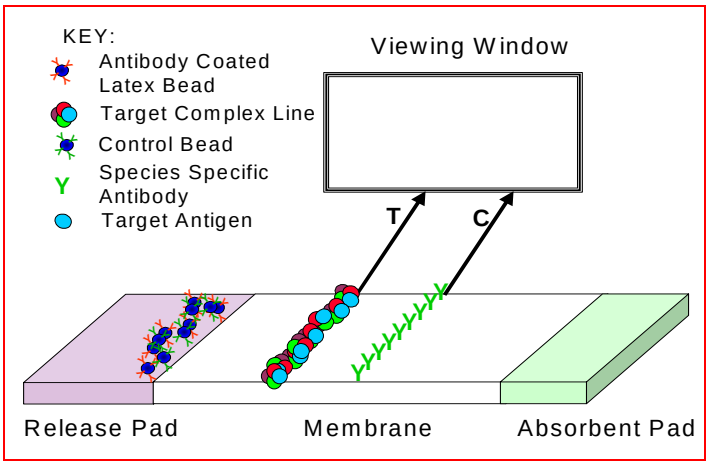

Fig 8.2.2. A schematic diagram of the OTA competitive LFD

Results

Monoclonal antibody Development

Tail bleeds were taken of all mice immunized with soluble antigen preparations of both *Penicillum verrucosum* and *Aspergillus ochraceus* and tested by indirect ELISA for Pab reactivity towards the immunogen and other OTA producing and other fungi, Table 8.2.1. Selection of the most hyperimmune mice was made for fusion and Mab development.

Table 8.2.1. The ELISA absorbance values of the cross-reactivity testing of Pab in tail bleeds for the purpose of selection for Mab production is given in the table below.

Tail Blood ID	**Soluble Fungal Antigen ID**			
	PIL 333 *P. AURANTIOGRIESIUM VAR MELANOCONIDIUM*	**M115** *P. VERRUCOSUM*	**PIL 253** *F.CULMORUM*	**A. niger**
178.3	0.325	0.418	1.400	-
179.1	0.286	0.330	0.950	-
184.3	1.320	1.176	0.453	0.151
186.2	0.190	0.176	1.044	0.128

Many fusions were performed on the hyper immune mice as indicated in fig 8.2.2. Fusion No 164 and 168 yielded 10 promising hybrids of which two exhibited good Mab characteristics. Prototype ELISA formats were devised in order that extensive cross-reactivity testing against a wide range of taxonomically similar and dissimilar OTA producing fungal genera. Two Mab cell lines were subsequently selected. Cell line 168/4.G6.F8.C5 showed a high specificity toward all *Penicillin verrucosum* species that were known producers and non-producers of Ochratoxin A. A second cell line 164/2.A11.D10.A10 raised against *Aspergillus ochraceus*, displayed a high specificity toward all OTA producing and non-producing isolates. These cell lines were successfully cloned twice, fully characterized, weaned bulked and column affinity purified.

The Mab raised against *P. verrucosum* (168/4 G6.F8.C5) showed a high specificity towards a range of both OTA producing and non-producing isolates. This Mab was fully characterised as an IgG1 antibody type and affinity column purified to provide enough Mab to enable extensive cross-reactive studies to be carried out. Subsequent detailed ELISA evaluations showed that this MAB was isolate specific. For the Mab selected that was raised against *Aspergillus ochraceus* (164/2.A11.D10.A10), although showing significant specificity towards *Aspergillus ochraceus* isolates (both OTA producing and non-producing isolates) it also showed some recognition of other fungal genera i.e. *Fusarium* sp, *Alternaria* Sp and *Mucor* Sp. it was therefore decided that this monoclonal was unsuitable for further investigations.

At the EU OTA Project Meeting (Bari, January 2002) it was agreed that *Penicillin verrucosum* only was considered to be the most important fungus responsible for OTA production in European cereals. All partners and the EU Project Officer agreed that an assay for *A. ochraceus* was not appropriate and that work should concentrate on *Penicillin verrucosum*. To this aim, the 2 promising Mab producing cell lines (*Aspergillus/Penicillum* specific and *Penicillum verrucosum* specific) were selected and evaluated further.

Table 8.2.2. Example of antibody cross-reactivity testing with Mabs raised against *A. ochraceus, P. verrucosum,* Aspergillus/Penicillum species and a general mould Pab

Fungi Tested	**Monoclonal and Cell lines Tested**					
	Aspergillus ochraceus (164)		***Penicillum verrucosum (168)***		***Asp/Pen*** Specific AF-CA2-CH8	Pab 75 General mould
	A	**B**	**C**	**D**		
***P. Verrucosum* OTA Producer**						
M115/15	+++	++	++	+++	+++	+++
HGCA 00394	+++	+++	+++	+++	+++	+++
***P. Verrucosum* OTA Non-Producer**						
HGCA 00488	+++	++	+	+++	+++	++
HGCA 00545	+++	++	++	+++	+++	++
***Aspergillus* OTA Producer**						
A. niger (HGCA 00260)	-	++	+	-	-	-
A. ochraceus	+++	++	++	-	+++	++
***Aspergillus* OTA Non-Producer**						
A. terrus	++	++	++	-	+	-
E. amstelodami	+++	+++	+++	-	+++	+++
OTHERS						
M. nivale	+	++	+	-	+	-
F.graminearum	-	-	-	-	-	-
Mucor sp	-	+++	+++	-	-	++
Alternaria sp	-	+++	+++	-	-	-
Cladosporium sp	-	++	+	-	-	+

+++ strong reaction ++ medium reaction + weak reaction

A highly specific *Aspergillus* sp and *Penicillum* sp Mab producing cell line (AF-CA2-CH8) was sourced providing an alternative supply of monoclonal antibody and ELISA evaluations were performed in parallel with cell line 164/2.A11.D10.A10 for comparison. It was clearly demonstrated (Table 8.2.2) that the sourced Mab had a high specificity to all OTA producing and non producing isolates of *P. verrucosum* and *A. ochraceus.* Investigations were carried out upon both pure fungal cultures and contaminated cereal samples with little or no cross reactions observed with other commonly associated field or storage fungi genera.

This sourced antibody was characterised as an IgM isotype and concentration techniques (Tangential Flow Filtration) employed to provide bulk quantities of this antibody to allow for standardised assay development.

ELISA Development

The ELISA data has been compared against traditional methods of fungal detection e.g. Colony Forming Units (CFU) and good correlation with an R^2 of 0.9446 and a sensitivity of 1 x 10^4 CFU / g achieved (Fig 8.2.3).

Considering the inherent inaccuracies associated with the CFU method, this correlation was considered to be very good.

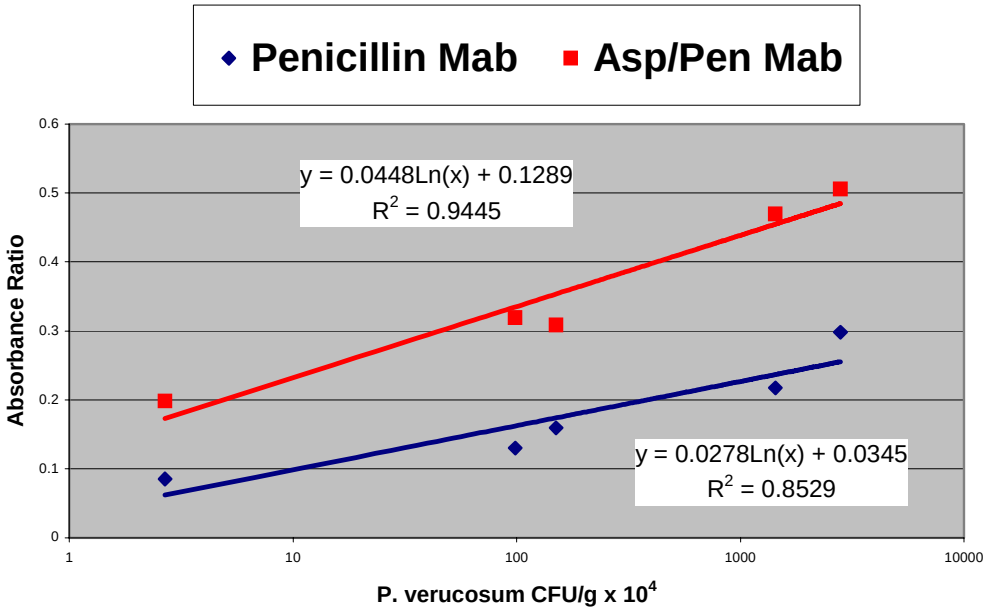

Fig 8.2.3 Relationship between ELISA absorbance values and CFU for a range of levels of *P. verrucosum* contamination in barley.

Aspergillus and Penicillium specific LFD development

The MAB raised against *P. verrucosum* (168/4 G6F8C5) was found to be isolate specific, and therefore potentially only offered limited use for the detection of *P. verrucosum*. As a result no more work was carried out with this Mab but the sourced Mab (AF-CA2-CH8) has been successfully incorporated in a competitive LFD and showed a good correlation when compared against traditional dilution plating techniques. The limit of detection was investigated by direct comparisons between fungal biomass and visual assessment of the LFD on infected cereal samples the results of which are illustrated in figure 8.2.4. The sensitivity of the assay appears to be at least down to 10^4 CFU/g, as different colour intensities of positive lines are clearly visible across the fugal densities tested, with complete "wipe out" of the line At 10^6 CFU/g. This would be even more apparent using a reader and likely to increase the sensitivity of the assay over a broader range of CFUs and likely that the LFD will result in being more sensitive than the ELISA. It hoped to continue the work in this direction even after the end of the project.

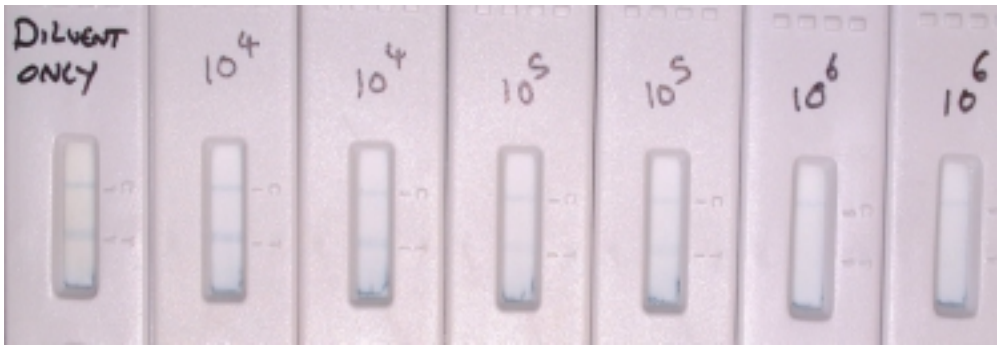

Fig 8.2.4 Duplicate Asp/Pen specific LFD Detection of an *Aspergillus* sp artificially infected cereal sample

Discussion

Prototype ELISA and LFD systems have been developed that detect *Aspergillus* sp and *Penicillum* sp. in culture and in grain. Both methods show sensitivity at least down to 10^4 CFU/g and thus able to detect the numbers of organisms that could pose a potential problem in grain.

It was surprising that with only visual reading of the LFDs, it appeared to be potentially more sensitive than the ELISA. It is hoped that further work will be carried out on the LFDs and with the use of a reader to measure the colour intensity of the line and it is anticipated that the sensitivity can be greatly improved. Such a system would offer a tremendous opportunity for the rapid and inexpensive monitoring of grain for spoilage organisms that have the potential for OTA production. Thus it would enable checks to made on the quality of grain at intake and all through the supply chain and assist in the application of a HACCP control system.

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WP 8: Development of rapid ELISA systems for ochratoxin A producing fungi and ochratoxin A

WP 8.3: Development of rapid immunoassay systems for ochratoxin A

Introduction

There is increasing awareness of the incidence and potential damage arising from the presence of mycotoxins in materials destined for animal and human consumption.

Even with a good understanding of storage conditions and the causative organism(s), ochratoxin A (OTA) is still found regularly in stored grains in Europe. This mycotoxin, as the UK Committee on Toxicity (COT) advise, is a human genotoxic carcinogen, accumulates in the kidney and its nephrotoxic effects have been demonstrated in all mammalian species tested (Harwig *et al.* 1983).

Increasing pressure to reduce contamination levels has resulted in the European Commission's legislation to harmonise limits on mycotoxins (under Council Regulation EC/315/93) with the limits for OTA set as 5 ppb for raw and 3 ppb in processed cereals.

Conventional analysis of OTA is by laboratory methods such as High Pressure Liquid Chromatography (HPLC) which is expensive and does not offer a turnaround time that is compatible with the needs of the industry. However, rapid immunodiagnostic methods offer a real alternative.

The concept of using Immunological systems to detect mycotoxins is not new and was first reported in 1975 by Aalund *et al*. Commercial kits to detect OTA were first introduced in the late 1980's by the Neogen Corporation in the USA and clearly demonstrate that assays can be made user friendly and rapid (around 20 minutes). However at the start of the project, currently available immunoassays had generally been optimised to meet the then USA requirements of around 20 ppb and at best were only sensitive down to about 8 ppb which far exceeds the EU limits of 5 ppb for raw and 3 ppb in processed cereals.

In addition, the only assays that were available were in ELISA format which are generally more suited to laboratory use.

Lateral Flow Devices (LFDs), one step rapid systems that only takes a few minutes to complete, have been developed for a wide variety of anaytes such as plant pathogens (Danks & Barker, 2000). Such LFDs are now being developed for a range of small molecules, such as OTA, using a competitive format, as opposed to the double antibody sandwich format, often adopted for qualitative detection of protein targets (Danks *et al*. 2001, 2003).

This report describes the work carried to produce and obtain better antibodies to OTA that could be used in assays that match the EU legislative limits for this mycotoxin. In addition, the incorporation of these antibodies in to Later Flow Devices is described.

The report on this work on the immunoassays developed for OTA within WP8 is split in to two halves:

8.3.1. The production of monoclonal antibodies (Mab) to OTA and their incorporation in to LFDs in line with the EU legislative limits for ochratoxin A

8.3.2 The development of an ELISA kit to match the EU legislative limits for OTA

8.3.1. The production of monoclonal antibodies (Mab) to OTA and their incorporation in to LFDs in line with the EU legislative limits.

Materials and Methods

All inorganic chemicals and organic solvents were reagent grade or superior and purchased from Sigma Chemical Company (St. Louis, Mo.,), unless otherwise noted.

Synthesis of OTA Conjugates

Ochratoxin A has a low molecular weight (MW) of 403 Da and is therefore not immunogenic unless conjugated to a carrier protein to raise its MW. As a result, conjugates of this toxin were synthesised with carrier proteins (BSA, KLH) and enzymes (AP and HRP) in order to raise antibodies and develop specific detection assays. Various approaches including the use of (i) photoactivatable, bifunctional linker sulfo-HSAB (Pierce, UK), (ii) bifunctional linker ASBA (Pierce, UK), (iii) Synthetic carrier poly-l -lysine were employed.

The conjugates were purified by gel-filtration chromatography and / or extensive dialysis and thee concentration of hapten-protein solutions assayed by the Coomassie-blue method (Bradford, 1976). The ratio of conjugation in some soluble preparations was determined as the difference in the number of free amino groups in proteins before and after conjugation (Habeeb, 1966). It was not possible to calculate the molar ratios of conjugation for the OVA and KLH conjugates due to their low solubility .

For the conjugation of OTA to KLH via the free carboxyl (-COOH) group the initial reaction was with ASBA, after activation of the carboxyl group by EDC, followed by linkage of the KLH (keyhole limpet haemocyanin) imitated by irradiation with short-wave UV light for 10 minutes. The synthesized OTA-KLH conjugate was used as the immunogen and the OTA-BSA conjugate was used in monoclonal selection in a competitive ELISA format and during LFD development.

OTA ELISA Cereal Extraction Procedure

Representative cereal samples were taken, ground in a laboratory blender and thoroughly mixed prior to proceeding with the extraction procedure. Five grammas of ground sample was weighed and added to a suitable container with 12.5 ml of 70% methanol and shaken vigorously for three minutes. The extract was allowed to settle for three minutes and a 1ml aliquot taken from the settled supernatant and diluted with 1ml distilled water. This diluted supernatant was passed through a disposable filter syringe and 50 µl per well of filtrate used for ELISA testing.

Mab Production

Immunization schedule

Adult female Balb/C mice were immunized with 0.1 ml of a 1 mg ml^{-1} solution of each immunogen mixed with an equal quantity of Freund's adjuvant (Freund 1956). The first injection was in Freund’s complete

adjuvant given via the subcutaneous (SC) route and subsequent injections were made at 2, 4 and 6 weeks in Freund's incomplete adjuvant intraperitoneally (IP). Sera samples were collected at 6 weeks and tested by ELISA to monitor the immune response. Mice that had responded well, as shown by their sera containing strong polyclonal antibodies (Pab) to OTA, were selected for Mab production. Selected mice were given a final boost injection at least 8 weeks after the start of the immunisation schedule, in PBS and four days later the mice were sacrificed by cervical dislocation under anesthesia and their spleens removed for fusion (Kane & Banks 2000, Banks et. al. 1996).

Cell Fusion

Splenocytes from the immunized mice were fused at a ratio of 3:1 with myeloma cell line SP_2/0.Ag 14 (ECACC). Fusion was carried out by the gentle addition of 1 ml of 30% (w/v) polyethylene glycol (PEG) over 60 seconds. Then 6 ml of warm serum-free RPMI 1640 medium (Gibco) was added over the next 4 minutes with gentle stirring. A further 43 ml of warm RPMI was added before centrifugation at 2000rpm for 5 min at room temperature. The resulting pellet of cells was re-suspended into 50 ml of growth medium (RPMI 1640 + 20% FCS with 1ml HAT (Gibco) and 0.5ml HES (Sigma)) and distributed into five 96-well ELISA plates at 100 µl per well.

Hybridoma production

Fused cells were incubated at 37^{o}C with 5% CO_2. All wells were fed on days 3, 6 and 10 days. On day 3 100µl of growth medium was added to each well. On days 6 and 10, 100µl of growth medium was removed from each well and replaced with 100µl of fresh growth medium containing HAT and HES. Supernatants were screened by indirect ELISA at approximately day 13. Clones that produced antibodies reacting with immunogen were bulked into 24 well plates and subsequently cloned twice by limiting dilution (Harlow & Lane, 1988). Subclones were then bulked to 25cm^2 tissue culture flasks and supernatants were drawn for screening when growth was confluent and the medium was showing signs of acid production (i.e. turning yellow). All subclones of interest were frozen in a mixture of 90% foetal calf serum and 10% DMSO at -80^{o}C for 24 h and then transferred to -150^{o}C.

Isotyping

All Mabs were isotyped for the determination of the immunoglobulin isotype class and subclass, using ISO-*strip* isotyping kit (Roche Diagnostics), following the manufacturer's instructions.

Purification

Hi-Trap Protein G affinity columns (Amersham Biosciences) were used to purify culture supernatants and protein estimations were calculated using a commercial protein assay reagent (PerBio).

Selection by ELISA of Mab to OTA

Induced cell lines to OTA synthesized conjugates were screened by indirect ELISA. Ninety-six well microtitre plates (Maxisorp, Nunc) were coated overnight at +4^{o}C with OTA – BSA Conjugate (EDC method, Conjugate No 80); diluted to 5µg/ml in 0.05M sodium carbonate buffer pH 9.6. The following day the plates were washed three times with Phosphate buffered saline plus 0.05% Tween 20 (PBS-T). This washing step was performed after each of the subsequent steps. Non-specific blocking was carried out by

adding 250µl per well of 5% Skimmed Milk (Marvel), for 1 hour at 33°C. Culture supernatants diluted 1:2 in PBS-T were added to the wells (100µl) and incubated again, for 1 h at 33°C. After washing an alkaline phosphatase conjugated anti-mouse IgG specific antibody (100µl per well) diluted in PBST, is applied and incubated at 33°C for 1h. Finally 100µl of PNPP (p-nitrophenyl phosphate, the AP substrate Amersham Biosciences) at 1mg ml^{-1} diluted in a 0.1M Diethanolamine substrate buffer was added to each well and incubated at room temperature for 1 h. The absorbance was measured at 405 nm on a Molecular Devices microtitre plate reader. Positive cell lines were identified and selected for cloning.

OTA Standard Preparation

Ochratoxin A in a mixture of Benzene:Acetic acid (99:1) at 10 µg/ml was measured by UV spectrometer. A maximum absorbance at approx. 333nm was taken and the concentration was calculated from:

µg/ml OTA = (MWt x 1000 x A) / Extinction coefficient where MWt = 403, Ex = 5550

An accurately measured volume of this was evaporated and re-dissolved in methanol to make a solution at 2.5 µg/ml. This was diluted with methanol to make a Stock Solution at 0.25 µg/ml. The UV estimation of this procedure can be carried out using Toluene: Acetic rather than benzene, in which case E= 5440. Further dilutions of this stock solution were performed in 35% Methanol solution for both ELISA and LFD applications.

OTA Competitive ELISA

ELISA plates (Maxisorp, Nunc) were coated overnight at +4°C with an OTA: KLH conjugate (Conjugate No 80), diluted to a concentration of 4.5µg ml^{-1} in coating buffer (0.05M Sodium hydrogen Carbonate/Sodium Carbonate buffer (pH 9.6), 100µl per well). See Fig. 8.3.1.1. The following day the plates were washed three times in PBST (0.15M Phosphate Buffered Saline pH 7.2 with 0.05% Tween 20) and blotted dry. Blocking was carried out by the addition of 250 µl of 5% Bovine Serum Albumin for 1 h at 33°C. 50 µl of OTA standards or 50 µl of OTA extracted cereal sample, with 50 µl of OTA Mab (Y49) (diluted 1:10,000 in 35% Methanol) was added to the wells in triplicate, and incubated at room temperature with shaking for 1 h. The plates were further washed with PBST followed by the addition of 100 µl rabbit anti-mouse immunoglobulin (Sigma) conjugated to horseradish peroxidase (HRP) diluted 1:8000 in PBST. The plates were incubated once more for 1 hour at 33°C. 100µl of a one-step HRP (TMB) substrate solution substrate was added per well and incubated at room temperature for 15 minutes. 50µl of Stop Solution (20% Sulphuric Acid) was added per well and absorbance measurements were read at 450 nm on a microtitre plate reader (Molecular Devices).

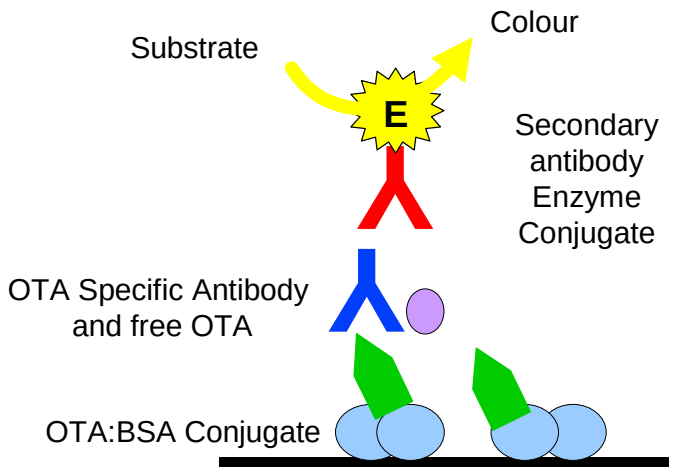


Fig 8.3.1.1 Diagrammatic representation of an OTA Competitive ELISA format.

OTA specific LFD Production

The competitive LFD relies upon the competition for binding sites on sensitized latex particles. Specific OTA monoclonal Y49 was bound by passive adsorption to dyed latex particles. An additional rabbit serum sensitized latex particle was produced as an internal control bead. Both sensitized latex particles were then applied, using an immersion procedure, onto a release pad, to produce a stable particle reservoir for release onto a nitro-cellulose-based membrane. Two lines of reagent are immobilized onto the membrane using a reagent dispenser. The target reference or test line is comprised of OTA-BSA conjugate. The control line is a line of goat anti-rabbit antibody. The release pad and membrane are assembled together with an absorbent pad into a plastic housing. Fig. 8.3.1.2

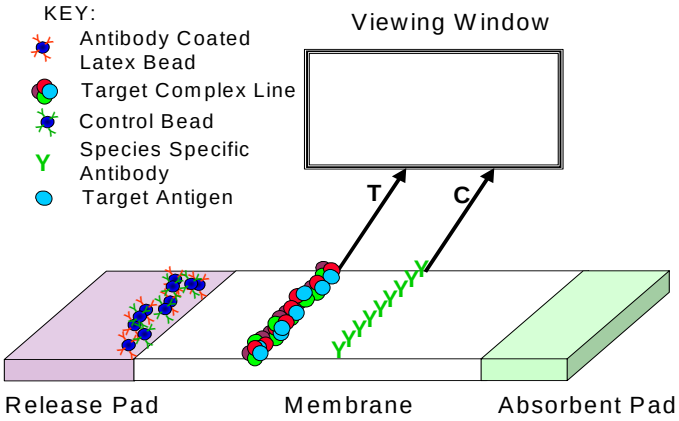


Fig. 8.3.1.2 A schematic diagram of the OTA competitive LFD

The LFD for OTA was developed for several applications. These were for:

1. Visual identification of OTA producing or toxigenic fungi
2. Testing of grain samples for OTA with visual examination, with or without immunoaffinity column (IAC) sample clean up / concentration
3. Reading the intensity of the line using a reader to make fully quantitative.

(i) OTA LFD Test Procedure for the identification of OTA producing fungi – Fungal Plate Cultures and Extraction

Suspected contaminated grain samples were plated onto agar plates following conventional culturing techniques and allowed to grow over a period of time. The cultures were then extracted and the extract tested by LFD for OTA. .

Fungal Plate Culturing

Strains of toxigenic and non-toxigenic fungi were grown on CYA or malt extract agar (MEA) plates using standard mycological techniques and incubated at 25^{o}C in the dark for an optimum time period of 8 to 10 days for OTA production.

Fungal Plate Extraction

After the plates had been grown up, a 1 cm diameter plug was removed from the centre of the fungal colony using a sterile, size 6-core borer. The plug was placed into an extraction bottle containing 5 steel ball bearings and 0.5 mls 70% methanol solution. The bottle was shaken vigorously for 30 seconds until the plug was macerated. To each bottle 1.5 mls of sterile PBS solution was added and shaken for a further 10 seconds. Finally 70 ul of extracted sample was dispensed onto the sample well of the LFD. The device was read after 5 minutes and the results interpreted from the number of lines present.

Detection of OTA in grain by LFD

Direct Grain Detection

A 5g representative sample of grain to be analysed was collected and added to 12mls of 70% methanol in a glass screw-tight bottle and shaken vigorously for 5 mins. The suspension was diluted 1:4 in sterile PBS buffer and 70ul of this extract applied to the LFD and read visually after 15 minutes.

Immunoaffinity column (IAC) Sample clean-up / concentration method

A representative 50g sub sample of grain was collected and added to 200mls of 60% acetonitrile. This mixture was blended at high speed for 2 minutes and then the sample was centrifuged for 10 minutes (at 16000g (av)). Four ml of the resulting supernatant (equivalent to 1g of food matrix) was taken and diluted with 44ml phosphate buffered saline pH 7.4 (PBS). The whole of the diluted extract was applied to the OchraPrep® Column and the sample was allowed to pass through the column at a flow rate of 2-3 ml/min. The column was washed by passing 20ml of PBS through the at a flow rate of 5ml/min, ensuring that the liquid was removed from the column by using downward pressure from the syringe. The bound OTA was slowly eluted from the column by using 1.5ml of desorption buffer (acetic acid:methanol (2:98 v/v) and

allowing this to pass through the column under gravity with collection in to a sample vial. Backflush of the column with this desorption solution was carried out 3 times to ensure complete elution. Air was also pushed through the column to ensure collection of the last drop. Finally, 1.5ml of distilled water was passed through the immunoaffinity column and collected in the sample vial to give a 3ml total volume. This eluted extract was dilutes 1:5 with PBStT buffer, mixed well and 70ul was added to the LFD and read visually after 10 minutes.

A Biodot LFD reflectance photometer used in conjunction with QuadScan Reduction Companion Program (Version 1.20) when available was used and allowed an accurate reading of the degree of latex accumulation at both the target and control lines to be made. This data was plotted into an Excel spreadsheet and the data interpreted.

EU Ring Test on the visual identification of OTA producing or toxigenic fungi using the OTA LFD

A ring trial was carried out with participants form 13 different institutes. Each institute received 20 LFDs and all the components required to carry out the test as described above. They received 20 fungal plates labelled only with a sample identification letter (A-T), each plate containing different toxigenic or non-toxigenic fungi, commonly found in grain stores. The plates had been inoculated from the same stock cultures and incubated for three days before dispatch to the participants. On arrival the pre-inoculated agar plates were placed at 25^{o}C for 3 days, and fungus growth was observed. If after this time, approximately 10 days from initial inoculation, samples had grown and appeared uncontaminated then the participants carried out an LFD test on each culture plate. The results were then returned to CSL for analysis. All the tests were carried out within 14 days of original dispatch. A plate of each fungal culture, 10 days post inoculation was verified at CSL by ELISA and HPLC and this result was used as the definitive identification of OTA production.

Results

Mab Development

Indirect ELISAs were carried out to test the reactivity of Pab (tail bleeds at 6 weeks) towards the hapten for all the mice immunized with synthesized conjugates of OTA. Testing included pre-absorbtion with 0.2% BSA to remove interference from carrier protein i.e. BSA mediated antibody response. Fusion 170 resulted in six specific and sensitive Mab raised toward the immunogen OTA-KLH (Conjugate No 80). Extensive competitive ELISA optimisation studies upon these cell lines have enabled the development of a prototype OTA specific ELISA. This assay is capable of detecting OTA in solution to very low parts per billion (ug/Kg) / high parts per trillion (ng/Kg). The assay has been validated ‘in-house’ against ‘real’ cereal samples and the sensitivity has been proven to be well within the range of EU legislation of 3-5 ppb in cereals and cereal products. Figure 8.3.1.3 shows a typical prototype ELISA kit OTA using OTA standards prepared in accordance to the AOAC Official Methods Book (15th edition) with 50% inhibition of the OTA Mab at achieved at an OTA concentration of 4-5ppb

Further details of the ELISA however, are given in Part 2.2 of the report.

The cell line 170/2.C12.G5.B7 (Y49) was characterized as an IgG1 antibody, weaned off FCS and bulked upon the Techno-mouse system (Integra Biosciences). When this antibody was in sufficiently large quantities i.e 2 litres equating to approximately 20mg of antibody, it was affinity column purified (Protein G), to produce large quantities of standardized reagents to enable assay optimisation, cross reactivity and cereal validation studies to be carried out.

The resulting ELISA was shown to have a sensitivity of OTA detection down to 0.02 ppb with a working range of 0.02 and 16 ppb and showed that cross-reactions to OTB were at 0.4 ppb and with coumarin and citrinin at concentrations greater than 100 ppm.

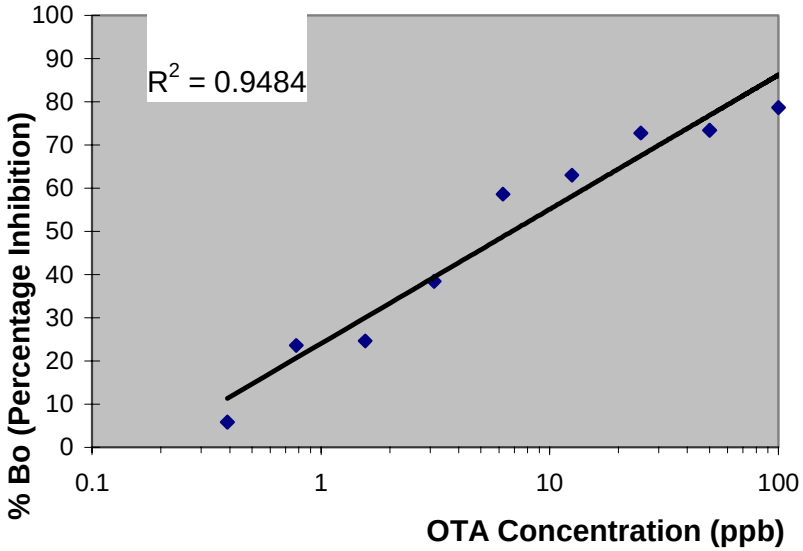


Figure 8.3.1.3. Typical graph showing inhibition of purified Mab 170/2 by standards in the range of 0.39-100 ppb (0.39-100 ng/ml)

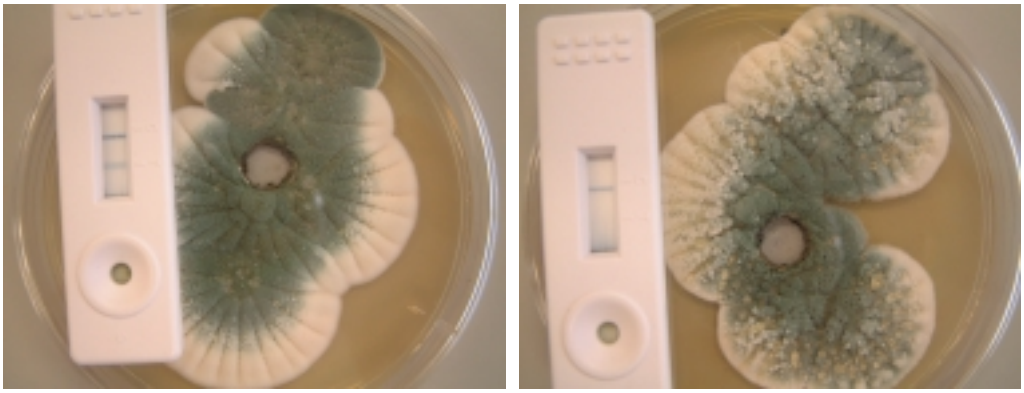
The remainder of this section of the report concentrates on the LFD development while a separate section (8.3.2) deals with that for the ELISA.

Ochratoxin A specific LFD Development

The OTA monoclonal antibody, Y49 has been successfully incorporated into a competitive Lateral Flow Device (LFD) format, which allow for the rapid detection of OTA. One of the uses of this one-step competitive LFD allows for visual identification of OTA producing and non-producing fungi on plate cultures and from contaminated cereal samples, directly with and without sample clean up / concentration with immunoaffinity columns (IAC). In addition to visually reading the LFD, a reader has also been employed which measures the intensity of the line thus making the test fully quantitative.

OTA LFD detection of OTA producers from plate culture

Typical LFD results are shown in Fig.8.3.1.4. The absence of OTA in a sample is indicated, if after 5 minutes, two lines are visible which indicates a non-OTA producing culture. Only one visible line indicates that OTA is present in the sample, *i.e.* the culture is an OTA producer.



Non-OTA Producer OTA Producer

Figure 8.3.1.4. Example LFD results, after 5 minutes. Two samples from two different strains of P. verrucosum, one producing and one not producing OTA

Time course studies on 10 strains of *P. verrucosum* were conducted and results indicated that detection of OTA was detected in 4 out of 5 toxigenic strains at day 5 and for all 5 strains 8 days post inoculation. No detection of OTA was shown from the 5 non-producing strains even after 14 days post inoculation. The results obtained from the 12 strains of *Aspergillus ochraceus* showed 3 out of 9 producers were detected 8 days post inoculation, and 9 out of 9, 10 days post inoculation. The 3 strains, which did not produce OTA, showed little or no detection after 14 days.

EU Ring Test Validation

The ring test results are shown in Table 8.3.1.1. The results are presented as the number of participants, out of 13, which correctly identified OTA production or non-production for each culture.

Table 8.3.1.1. A compilation of all the ring trial results

			Observed Result	
Sample	Culture	Actual Result	Correct	Incorrect
A	*P. verrucosum*	OTA producer	13	0
B	*P. verrucosum*	non-producer	13	0
C	*P. verrucosum*	OTA producer	11	2
D	*P. verrucosum*	non-producer	13	0
E	*P. verrucosum*	OTA producer	13	0
F	*P. verrucosum*	non-producer	12	1
G	*P. verrucosum*	OTA producer	11	2
H	*Cladosporium sp.*	non-producer	13	0
I	*Aspergillus niger*	non-producer	11	2
J	*Mucor racemosus*	non-producer	12	1
K	*Absidia corymbifera*	non-producer	13	0
L	*Fusarium culmorum*	non-producer	13	0
M	*Alternaria sp.*	non-producer	13	0
N	*Aspergillus flavus*	non-producer	13	0
O	*P. martensii*	non-producer	13	0
P	*P. expansum*	non-producer	12	1
Q	*P. verrucosum*	non-producer	13	0
R	*A. ochraceus*	non-producer	13	0
S	*A. ochraceus*	OTA producer	12	1
T	*Eurotium amstelodami*	non-producer	12	1
			(249)	**(11)**

Statistical analysis was carried out on the ring trial results. For the positive samples this is known as sensitivity and it is the percentage of samples that were correctly identified as positive. Using the data provided, 61 out of 65 or 93.9 %, with a 95 % confidence interval of 85.2 %-97.6 %. It is assumed that all supposedly positive samples do in fact contain OTA. The specificity is the proportion of samples correctly identified as being negative. This value is 188 out of 195, or 96.4 %, with a confidence interval of 92.8 % - 98.3 %. These approximate confidence intervals have been created using standard methods for binomial data. The intervals are valid as there is no heterogeneity (large differences) between laboratories.

To assess within-laboratory repeatability, accordance measures were computed. Accordance is the percentage chance that two samples sent to the same laboratory under standard repeatability conditions will both be given the same result. It is calculated by averaging the probability that two samples will be given the same result over all laboratories. The accordance measure for the five positive samples was 89.2 %, with a 95 % confidence interval of 86.2 %-96.9 %. For the 15 negative samples the accordance was 93.3 %, with a 95 % confidence interval of 90.0 %-97.1%.

Inter-laboratory reproducibility was measured by concordance. This is the percentage chance that two samples sent to different laboratories will both be given the same result. Using a similar method to that for accordance, the concordance percentage for the positive samples was 88.2% with a 95% confidence interval of 79.5%-96.9%. The concordance for the 15 negative samples was 93.0% with its 95% confidence interval being 88.3%-97.0%

Development of an LFD for the detection of OTA in grain

A competitive Lateral Flow Device (LFD) for the rapid detection of OTA in contaminated cereal samples was developed. Work was carried out using MAB Y49 (produced by Partner 4) as the MAB accessed by Partner 10 (AF-12) proved unsuitable in LFD format.

Visually read LFD development

Representative and replicated samples (1-10) of a range of known OTA contaminated grain samples were extracted as above and the sample applied to the LFD. These samples had previously been analysed by HPLC and sample 3 and 5 had OTA concentration levels of 451 ppb and 284 ppb respectively. Sample numbers 3 and 5 were correctly identified as having high concentrations of OTA by visual examination by LFD. See Fig. 8.3.1.5.

The sensitivity was not low enough for the EU legislation (5ppb) with visual reading of the LFD. However, it is envisaged that identification of possible “hot spots” in a grain bulk by use of this method could indicate if there was a risk of the whole bulk exceeding the legislative limits.

Fig 8.3.1.5. Examples of duplicate LFD results of direct OTA LFD testing with LFD numbers 3 and 5 showing high positive results

The same samples used above were passed down an Immunoaffinity Column (IAC) to increase the sensitivity. Contaminated grain samples were processed according to the IAC clean-up method (see Appendix 1 of this section) and applied to the LFD

After 10 minutes, all samples containing OTA at a concentration of greater than 20ppb (as identified by HPLC) gave a positive result by the LFD with IAC method for sample numbers 3919, 3812, 4012, 3817 and 3816 (see table 8.3.1.2). There appeared to be a false positive results for sample 3815 but it was not sure of that was due to a fault in the HPLC method or if fungal growth and OTA production had continued in the sample. There was also appeared to be a possible false negative point (4021). Unfortunately the range of samples tested did not include samples around the EU limits, and hence it is difficult to say if the LFD with IAC can be read visually at levels less than 20ppb.

Table 8.3.1.2 Comparison of HPLC and OTA LFD with IAC sample concentration results.

Sample ID	4014	4013	3815	4011	4021	3919	3812	4012	3817	3816
HPLC OTA Conc. ppb	n.d	0.4	0.49	0.9	15.3	21.6	22.3	189	284	451
LFD Result	-	-	+	-	-	+	+	+	+	+

Sample extract 3919 (approximately 20ppb) was serially titrated in 17% methanol solution and applied to LFD and read after 10 minutes. A line is clearly visible at 1/100 dilution (equivalent to 0.2ppb) but not at 1/10 (i.e. 2ppb) and indicates that the test may be sensitive to EU limits (see Fig. 8.3.1.6). However, many more “real” samples are needed to test this hypothesis with direct comparisons made of LFD results with those obtained from HPLC.

Neat 1/10 1/100 1/1000

Fig 8.3.1.6 Serial Dilution of Sample 3191

On OTA standards, without matrix and IAC clean-up, a sensitivity of 250ppb was reported previously for the LFD and with optimisation this has now been improved to between about 50ppb. This is shown when different OTA standards ranging from 0 to 10 ppm were added to the LFD and “wipe out of the line occurred around 50ppb (see Fig. 8.3.1.7).

Fig 8.3.1.7 Indicating the visual end point at a about 50ppb where the test line is not visible

LFD for OTA in grain using a reader to make fully quantitative

Recently a reader (See Fig. 8.3.1.8) for measuring the colour intensity of the test line in LFD formats has become available. Further work has been carried out using this reader to determine if the LFD format can be used to obtain very rapid and sensitive quantitative results. It is envisaged that with the use of a reader, some standards would always have to be run with the test to validate the colour intensity of the line.

Fig. 8.3.1.8. Reader for measuring the colour intensity of the test line in LFD formats

Standards containing differing amount of OTA were made up in 20% methanol as usual. These were run on LFDs and read after exactly 15 minutes using the BIODOT reader to measure the intensity of the colour development in the line. Percentage inhibition of the line was calculated; 100-(OD standard /OD of Solvent

Blank x100). The results are presented in Figure 9 below and indicate that the sensitivity is down to <1ppb with a 50% inhibition value of about 8ppb. (without IAC sample concentration).

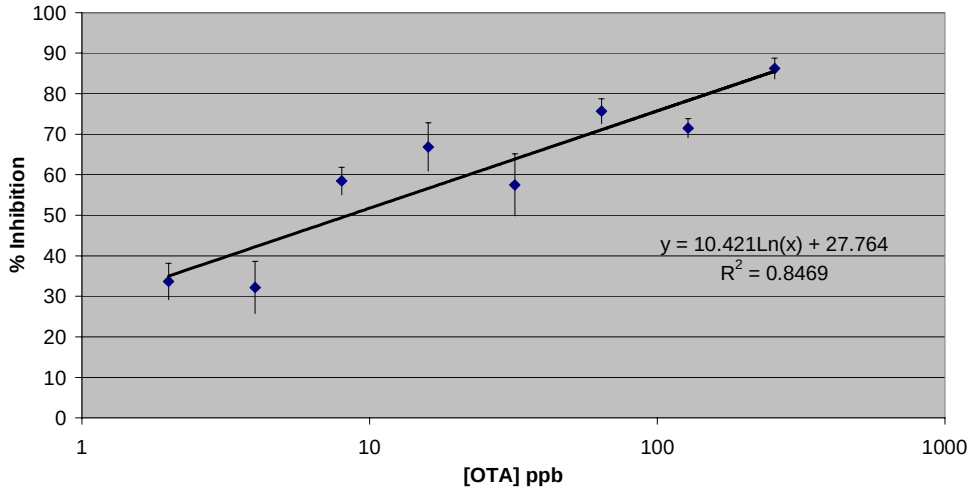


Fig 8.3.1.9. Percent inhibition of differing amounts of OTA in standards.

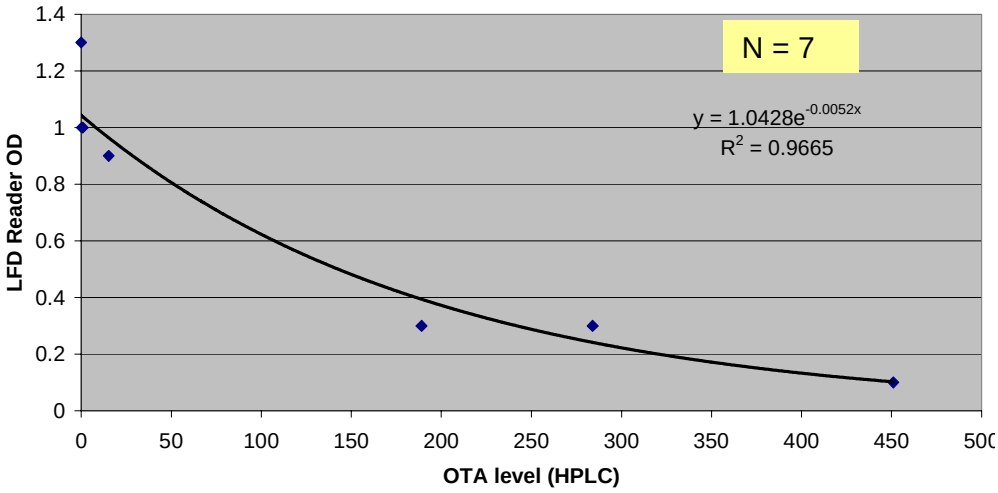


Fig 8.3.1.10. Comparison of LFD reader OD and OTA (in ppb) results in grain samples obtained by HPLC analysis.

The Grain samples containing different amounts of OTA (see table 8.3.1.1) were also analysed by LFD with the reader and compared to the results from HPLC analysis. The results are given in Fig. 8.3.1.10 and show a

good relationship (R^2 = 0.9665) over the range of OTA concentrations ranging from about 0.5ppb to 450ppb although the number of samples available for analysis was limited. Only seven of the sample were compared the three samples removed were 4021 (false negative) 3815 (false negative) and 4014 (OTA not detected by HPLC).

Discussion

A sensitive, reliable Mab has been produced to OTA which has successfully been incorporated into two application types. The first, an ELISA method was developed in order to select the monoclonal antibody from fusion and cloning steps. This assay is both robust and reliable and has been shown to be satisfactory in the determination of OTA concentration levels from contaminated grain and has been further evaluated alongside another ELISA developed by Partner 10 (ADGEN) using a sourced Mab. This work is not reported fully in this section (see Part 8.2.2) but the development of the ELISA using Mab Y49 also greatly aided the development of the LFD assay kit. This was also aided by the "unlimited" supply of the Mab through the use of the Technomouse (Integra Biosciences). The OTA LFD has been shown to be a very rapid and very reliable method for the identification of OTA producing fungi. It enables users to carry out OTA analysis on plate cultures 10 days post inoculation and takes only a few minutes to obtain an unambiguous result. The components of the test are relatively inexpensive and the method requires little or no previous experience to successfully carry it out. The results indicate that OTA produced from either *Penicillium* or *Aspergillus* species can be determined, with a longer culture time recommended for *Aspergillus* species cultures. The use of selective media would enable more specific identification of target cultures and would also reduce the number of tests required and also possibly the culturing times required. For example DYSG media Fig 8.3.1.11 developed by Partner 9 (Technical University of Denmark), enables a user without aseptic technique, to plate out contaminated grain samples as this only allows the growth of *P. verrucosum.* It is then easily identified and confirmed by OTA LFD as an OTA producer.

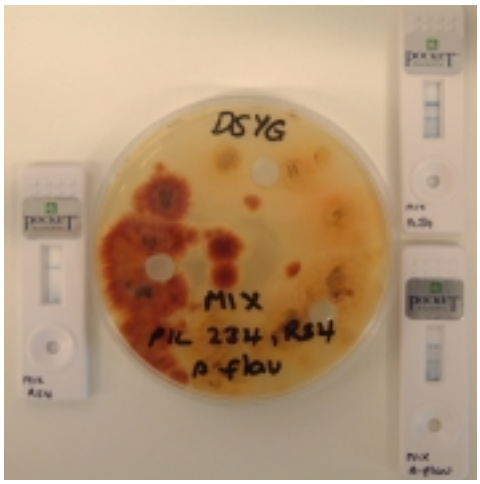


Figure 8.3.1.11. Use of DSYG Media for selection of *P. verrucosum* isolates for use with the OTA LFD

Alternative uses of the OTA LFD have been tried and tested by many partners involved within the Mycotoxin Cluster, these include:

(i) **Ludwig Niessen (Technische Universistaet) Muenchen -** Investigated OTA production in liquid cultures. Toxin detected at Day 6 post inoculation. 13 strains of *A ochraceus* plate cultures tested for OTA activity – Day 9 gave optimum results.

(ii) **Maria Teresa Gonzalez (University of Madrid - Spain) -** 36 different isolates of *Aspergillus* and *Penicillium* species originating from wine/grapes.

(iii) **Giancarlo Perrone (Inst. Of Sciences and Food Production) Italy -** 10x *Aspergillus* (Grapes) strains supplied including *A.ochraceus, A. alliaceus, A. niger, A. niger, A carbonarius.*

(iv) **Rob Samson (Holland) & Jens Frisvad Biocentrum (Denmark) -** 100 tests supplied survey *Aspergillus* strains isolated in Europe.

(v) **John O'Callaghan (University College Cork) Ireland -** Mutant Selection screening for atoxigenic mutants of *Aspergillus ochraceus.* Comparisons with Fluorescence with Coconut Milk Agar (CMA) selection and by Thin-Layer Chromatotography (TLC). See Fig. 8.3.1.12

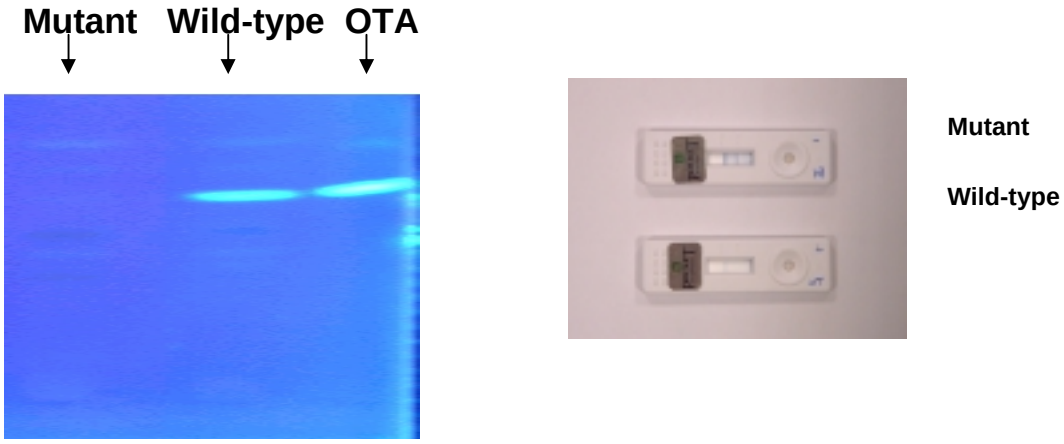


Figure 8.3.1.12. Mutant Selection using OTA LFD

A video presentation of the LFD Detection kit was made at Poznan, Poland; September 2002 (De-Tox Fungi Project Meeting) and for Uppsala, Sweden; September 2002 (OTA-PREV project meeting). Thirteen independent European laboratories took part in a ring test of the LFD assay for OTA producing fungi in October 2002. Six positive samples and fourteen negative fungal cultures were selected for use in the ring-test and these were composed of a selection of *Penicillium, Aspergillus* and *Eurotium spp*. Statistical analysis of the Ring-Test results showed that 93.9% of positive samples were identified accurately and 96.4% of the negative samples were correctly identified. A COR (Concordance Odds Ratio) value of 1.1 and 1.0 was achieved for all positive and negative samples respectively (Concordance is a measure of inter laboratory

reproducibility and Accordance is a measure of intra-laboratory reproducibility), a value of 1.0 would indicate concordance and accordance are equal indicating that the assay performed well.

Favourable and interesting comments were reported back to CSL via a questionnaire following the Ring-Test and some examples are included here:

"…a useful intermediary processing step for OTA determination before proceeding with more time consuming tests…",

"…a rapid assay for OTA production…..a means of monitoring…faster than TLC or HPLC for qualitative work…. extremely useful and easy to use.."

"..low expertise necessary..",

"..possible use for farmers during storage..",

"…useful in food micro labs…occurrence of ochratoxigenic fungi in food commodities…".

Further interest in the use of the LFD has been shown by the FAO for use in Third World countries. In such countries there is a lack of resources and the only way to test commodities for OTA is to look for OTA producing fungi though culturing techniques. Of course, culturing techniques can only give an indication of a possible problem because it would still not be known if the fungi isolated are OTA producing / toxigenic strains. This uncertainty could be overcome by the use of the LFD and because of the relatively low cost of the devices, not be too much of a drain on the limited resources.

As the LFD detects the toxin then the main use of course, is using it directly on grain. and prototype assays for this purpose have been developed within the project. For this purpose suitable extraction procedures have been devised some with IAC sample clean up and concentration steps with devices being read visually or with a reader to give a fully quantitative results.

Currently sensitivities are about 250ppb for the visually read devices with methanol extraction but without IAC concentration. With an IAC concentration step and visual reading of the devices, the sensitivity was improved to about 20ppb. With the use of the reader and no IAC concentration step, sensitivities were about the same level as that for the ELISA (<1ppb).

It envisaged that further work will be carried out and possibly further funding sought to make these assays more available perhaps through demonstration activities.

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WP 8: Development of rapid ELISA systems ochratoxin A producing fungi and ochratoxin A

WP 8.3.2: The development of an ELISA kit to match the EU legislative limits for OTA

Materials and Methods

Ochratoxin A (OTA) as a dry powder (5mg) (0-1877), tris-HCl (T 3253.), tris base (T 1503.), bovine gamma globulin (G 7516.), hydrochloric acid (H7020), sodium lauryl sulphate (L-4390), sodium chloride (S-9625), sodium azide (S-2002), thimerosal (cat no), sodium cyanoborohydride (S-8628), 1,1' carbonyldiimidazole (CDI) (C-7625), 1-ethyl-3- (3-dimethylamino-propyl) carbodiimide (EDC) (E-1769), peroxidase (polymerised and maleimide activated) and sodium hydroxide (S8045) were purchased from Sigma-Aldrich Company limited. Certified Ochratoxin A standards were purchased from Supelco (50µg/ml in benzene acetic acid) (4-6912). Bovine serum albumin (BPE-1600-100.) and sodium phosphate (di-basic)(S/4480/53) were purchased from Fisher UK. Methanol (ACS grade)(291926G), potassium chloride (10198) and potassium dihydrogen orthophosphate (10203) were purchased from BDH Ltd. Commercially available ELISA kit (Veratox - quantitative Ochratoxin Test kit) (#8610) for the detection and quantification of OTA and enhanced K-blue TMB peroxidase substrate (308177) were purchased from Neogen Corporation. Peroxidase was purchased from Biozyme laboratories (HRP4). Samples of wheat and barley were obtained from Gibbs Yard Farm, SAC, Auchincruive and analysed using a commercial ELISA kit (Neogen Corporation) and HPLC analysis and confirmed as free from OTA contamination, This supply of grain was subsequently employed in spike recovery experiments.

Preparation of OTA stock and working solutions

OTA (Sigma-Aldrich O-1877) was supplied as 5 milligrams (mg.) of powder (minimum 98%) and dissolved in 200µl of absolute ethanol to produce a stock solution of 25 mg/ml. This stock solution was stored at 4^{o}C in a tightly sealed amber glass vial stored in a sealed tube containing a tissue soaked in Ethanol to minimise evaporation of the OTA solvent. A working stock of OTA at a concentration of 50µg/ml was prepared from this stock and stored at 4^{o}C (in a tightly sealed amber glass vial stored in a sealed tube containing a tissue soaked in 50% methanol) by the addition of 10µl of stock OTA to 4990µl of 50% Methanol. A working dilution of OTA was prepared from this working stock by adding 100µl of working stock to 900µl of 50% methanol. This working dilution was made fresh every week and used to prepare dilutions of OTA for use in spiking experiments.

Ochratoxin A– free grain

Samples of grain were taken from the OTA-free wheat and barley samples, were ground in a laboratory blender using the standard procedure and 25 g of these ground samples were sealed in individual foil packets (catalogue number etc) and sent to two separate companies for independent OTA analysis by High Performance Liquid Chromatography (HPLC) (Valenta, 1998) (Cereals Research Non-profit Company

(CEREIPB) Alsokikotosor 9; 6726 Szeged; Hungary.; Fundacion Gaiker (GAIKER) Parque tecnologico, Edificio 202, 48170 Zamudio (Bizkaia) Spain.)

Preparation of spiked grain samples

Samples of OTA-free wheat and barley (25g) were placed in 100 ml capacity polystyrene containers fitted with screw caps (Fischer FB 51499). To each sample was added known amounts of OTA in a 200µl volume of 50% MeOH. The spiked grain samples were allowed to air dry overnight at room temperature in a fume cabinet. Spiked samples were either used immediately on preparation or stored for up to a week at 4^{o}C for later analysis. Grain samples were ground and extracted in 50% Methanol as detailed below and analysed for OTA content.

Preparation of spiked flour samples

Samples of OTA-free wheat and barley (100g) were finely ground using the standard procedure and then placed in 100-ml capacity polystyrene containers fitted with screw caps (Fischer FB 51499). Samples of OTA for spiking prepared from working dilutions of OTA such that the desired amount of OTA for the appropriate spiking level was present in 2.0 ml of 50 % MeOH. To the prepared flour was added 2.0 ml of the appropriate OTA spike, in an even distribution through the flour sample. The spiked flour was left to air dry overnight at room temperature in a fume cabinet. The entire flour sample was used for the extraction of OTA.

Consensus samples for the evaluation of ELISA

For part of the evaluation of the ELISA, consensus samples were obtained. These were part of the FAPAS (Food Analysis Performance Assessment Scheme, Sand Hutton, York.YO41 1LZ) proficiency testing rounds, or consensus samples were independently purchased from FAPAS for analysis (Anon.,1999; Anklam et al., 2002; Trucksess et al., 1995)

Conjugation of OTA

A. <u>Conjugation of OTA to Horse Radish Paroxidase (HRP).</u>

- CDI coupling. (Xiao et al., 1995; Williams et al., 1981)

A 1mg/ml solution of OTA in acetone was freshly prepared, and 1,1'-carbonyldiimidazole (CDI) was prepared as a 50-mg/ml solution in acetone. 125 µl of the fresh OTA solution was mixed with 7.5 µl of the CDI solution and left to incubate for 10 minutes in the dark at room temperature. HRP (10g) was up to 2ml with 0.1M $NaHCO_3$ buffer pH 9.6. The prepared HRP solution (1ml) was added to a 5-ml reaction tube and the OTA/CDI mixture added drop wise with continual stirring. Once all of the OTA/CDI mix was added the

reaction tube was sealed and the reaction was allowed to continue for 2 hours at room temperature. The resulting conjugate was extensively dialysed against 0.05M carbonate buffer to remove unused reactants, and finally against dH_2O to remove the carbonate salts. The conjugate was stored at 4^oC in the dark until needed. Ranges of different molar ratios of OTA to HRP were used to try to optimise the conjugation reaction of OTA to HRP. Long term storage of the conjugate at -20^oC was also carried out.

- DEPC (1-ethyl-3, 3-dimethylaminoprpyl carbodiimide) coupling. (References)

A fresh solution of OTA was prepared by dissolving 1.0 mg of OTA in 0.125 ml of ethanol. The HRP is freshly prepared as a 2mg/ml solution in 0.1 M NaCl. 0.75 ml of 0.1M Sodium Phosphate buffer (pH 7.0) is added to the OTA solution and the resultant mixture mixed well. To this solution is added 1 ml of the HRP solution, immediately followed by the addition of 35 mg of EDPC. The reaction tube is sealed and the resultant mixture allowed to react for 24 hours at room temperature in the dark with constant stirring. After the reaction is complete, the resultant conjugate is extensively dialysed against a large excess of 0.1 M sodium phosphate buffer (pH 7.0). The conjugate was stored at 4^oC for short periods, or for longer-term storage at -20^oC. Ranges of different molar ratios of OTA to HRP were used to try to optimise the conjugation reaction of OTA to HRP. Long term storage of the conjugate at -20^oC was also carried out.

B. Conjugation of OTA to Bovine Serum Albumin (BSA)/Bovine Gamma Globulin (BGG).

Ochratoxin A can be coupled to carrier proteins by both of the protocols outlined above for CDI and EDPC conjugation. Simply replace the HRP with either BSA or BGG and carry out the conjugation procedure as detailed above. Ratios of OTA to carrier protein (w/w) from 1: 5 to 1:20 were prepared and tested for use in the development of the OTA assay.

C. Conjugation of AF-12 monoclonal antibody to peroxidase (Tsang et al., 1984, 1995; Harlow et al. 1988)

Peroxidase (HRP) (Biozyme Laboratories) (16g) was dissolved in 4ml of dH_2O. To this was added 0.8ml of freshly prepared 0.1 M sodium periodate (Sigma-Aldrich). This mixture was stirred for 20 minutes at room temperature (RT) in the dark. To the resultant mixture was added six drops of ethylene glycol and the resultant mixture was stirred for a further 5 minutes at RT in the dark.. The activated HRP was filtered through a PD-10 column (Sephadex G-25 - Amersham Biosciences) with a 1 mM acetate column running buffer (1 mM sodium acetate, pH 4.2), and the coloured fractions collected. To the pooled HRP-containing fractions was added 10 mg of IgG, and then 1M carbonate buffer (carbonate: bicarbonate buffer 1.0 M pH 9.5) was added dropwise to raise the pH to between 9.0 and 9.5. The IgG - activated peroxidase mixture was stirred at room temperature in the dark for 2 hours. After the 2-hour incubation 0.2 ml of freshly prepared

sodium tetraborate (4 mg/ml) was added and incubated for 2 hours at room temperature in the dark, with constant stirring. The conjugate was extensively dialysed against PBS, and then aliquoted and stored at -20^{o}C in PBS/glycerol (50% glycerol v/v). Conjugate is diluted in TBS/BSA for use in the OTA ELISA.

Decision on assay format

Two possible assay formats were possible for the detection of OTA depending on whether the OTA was conjugated to HRP or to some other carrier protein (Candlish et al., 1988; Morgan, 1989).

Assay Format A: OTA conjugated to HRP.

Monoclonal antibody against OTA was dissolved in tris-HCl Buffer, pH 9.0 and 100µl added to the wells of microtitre plates (Nunc Immulon 4). The plates are covered and left to incubate at 4^{o}C overnight. The plates were then washed three times in TBS/Tween (tris buffered saline pH 7.5 with 0.05% tween 20) and the remaining binding sites on the plate blocked by the addition of 300µl per well of a 5% BSA solution in TBS (tris buffered saline pH 7.5). The plates were covered and incubated at 37^{o}C for 2 hours. The blocking solution was washed from the wells by three washes with TBS/Tween and the plate blotted dry. Dilutions of OTA: HRP conjugate freshly prepared in TBS, and standard dilutions of OTA was prepared from working stocks of OTA. OTA: HRP (150µl) added to the appropriate wells of a mixing plate (untreated Immulon 4 microtitre plates), followed by 150 µl of the appropriate OTA standard. The contents was mixed using a pipette and 100µl of this mix is dispensed in duplicate wells of the antibody coated reaction plate. The plate was covered and incubated for 1 hour at 37^{o}C in the dark. Free OTA in the sample/standard competes with binding of the OTA: HRP to the coated anti- OTA monoclonal antibody. Unbound reactants were removed from the reaction wells by washing three times in TBS/tween and blotting dry. K-Blue enhanced TMB substrate (Neogen Corporation) was then added (100µl/well), and the plate covered and left to incubate for 10 minutes in the dark at room temperature. The reaction between the bound HRP (in the OTA; HRP conjugate) gave rise to a blue colour, the intensity of which was inversely proportional to the concentration of OTA in the sample or standard. The reaction between the HRP and the substrate is stopped by the addition of 50 µl of 10% H_2SO_4 and the optical density (OD) read on a plate reader at 450 nm. (Thermo Labsystems Multiskan Ascent).

Assay Format B. OTA conjugated to BSA or BGG

OTA coupled to a carrier protein (8.1.9) was suspended in tris-HCl buffer pH 9.0 and 100µl of the diluted conjugate added to the wells of a microtitre plate. The plate was covered and incubated at 4^{o}C overnight. The plates were then washed three times in TBS/Tween and remaining binding sites on the plate blocked by the addition of 300µl per well of a 5% BSA solution in TBS The plates were covered and incubated at 37^{o}C for 2 hours. The blocking solution was washed from the wells by three washes with TBS/tween and the plate blotted dry. Monoclonal anti-OTA antibody was diluted in TBS, and 150µl added to the appropriate wells of a mixing plate, followed by 150 µl of the appropriate OTA standard, and the contents mixed using a pipette. This mix was dispensed (100µl/well) in duplicate wells of the antibody-coated reaction plate. The plate was

covered and incubated for 1 hour at 37°C in the dark. Free OTA in the standard competed with binding of the monoclonal antibody to the OTA coated to the wells of the reaction plate. Unbound reactants were removed from the reaction wells by washing three times in TBS/Tween and blotting dry. To the wells of the reaction plate was added 100µl of HRP-anti mouse IgG, diluted in TBS, the reaction plate was covered and incubated at 37°C for 1 hour. The reaction plate was washed three times with TBS/tween and blotted dry. To the appropriate wells of the reaction plate was added 100 µl of K-Blue enhanced TMB substrate (Neogen Corporation), and the plate covered and left to incubate for 10 minutes in the dark at room temperature. The reaction between the HRP and the substrate was stopped by the addition of 50 µl of 10% H_2SO_4 and the optical density (OD) read on a plate reader at 450 nm. (Thermo Labsystems Multiskan Ascent).

Assessment of monoclonal antibody performance

A number of commercially available and specially produced antibodies were acquired for evaluation for potential application in both of these assay formats. These antibodies were assessed in both assay formats outlined above at various different concentrations.

Extraction of OTA from grain samples

The sample to be tested was collected according to accepted sampling procedures, such that a representative sample was obtained and collected into a sample container, which was tightly sealed. The sample thus collected was stored at 2 - 8° until used for analysis. A solution of 50% methanol was prepared by mixing equal amounts of ACS grade methanol with dH_2O. The entire collected sample was finely ground using the standard procedure. A 25g portion of the ground sample was blended with 100 ml of 50% MeOH in a high-speed laboratory blender, or alternatively, 10 grams of the ground sample could have been weighed into an extraction pot (Fischer FB 51499), and to this added 40 ml of 50% MeOH. The extraction pot was tightly sealed and shaken vigorously for 5 minutes. The sample was allowed to settle for 3 minutes and particulate matter removed either by centrifuging the supernatant at 3000g for 5 minutes, or by filtering through a Whatman No. 1 or similar filter. The particle-free supernatant or filtrates was collected for later analysis (Trucksess et al., 1995; Wood, 1999; Morgan, 1989).

Analysis of spiked and naturally contaminated grain samples by OTA-ELISA

See Appendix of this section: Protocol for quantitative OTA test for grain and cereal products

ELISA and HPLC comparison

Samples of grain, which were naturally contaminated, and previously tested for OTA content by HPLC were obtained from partner 6 of Finland from the EU project. The samples of grain were extracted and the supernatant collected for assaying by the OTA ELISA kit developed as part of this project. Duplicate samples were assayed for OTA content and the values compared to those found by HPLC analysis. Also, five spiked samples of grain were prepared, as were five replicates of each spike level. Three of these were stored

at -20^{o}C as duplicate samples, one set of samples was extracted and tested by the ELISA system, and the other samples sent for HPLC analysis.

Trial of OTA ELISA kits

Ochratoxin A ELISA kits were made that contained all the reagents necessary to carry out OTA analysis. Eighteen samples of wheat and barley flour contaminated with known amounts of OTA were coded. These coded samples and the ELISA kits were despatched to CSL for analysis. The results reported for the OTA levels found in the samples using the developed ELISA kit were compared to the assigned value in the spike.

Results

Confirmation of uncontaminated grain

Samples of grain were taken and ground in a laboratory blender to the consistency of a finely ground coffee From these ground samples, 25 g were sealed in individual foil packets and sent for analysis by HPLC in two independent testing laboratories. The results from these analyses showed that the both the wheat and barley stocks contained less than the minimum detectable limit (less than 0.2 μg/kg) of OTA

As all the individual samples of the stock grain tested negative for the presence of OTA, it was possible to utilise these stocks of wheat and barley for the preparation of spiked samples.

EU legislative limits and CEN requirements for Ochratoxin detection.

Commission regulation (EC) No. 466/2001 8th March 2001 setting maximum levels for certain contaminants in foodstuffs, as amended by Commission Regulation (EC) No. 472/2002 of 12th March 2002, establishes maximum levels for OTA in raw cereals, grains, all products derived from cereals and dried vine fruits. CEN report on Food Analysis-Biotoxins-Criteria of analytical methods of mycotoxins (CEN report was approved by CEN on 23rd December 1998. CEN/TC 275 CR 13505) outlines performance characteristics for analytical methods for mycotoxins. These performance criteria are outlined in table 8.3.2 1.

Table 8.3.2.1. CEN performance criteria for analytical methods for mycotoxins.

Level (μg/kg)	Ochratoxin A		
	RSD_r%	RSD_R%	Recovery
<1	< 40	<60	50 to 120
1 - 10	< 20	< 30	70 to 110

RSD_r% = Relative within laboratory standard deviation

RSD_R% = Relative between laboratory standard deviation

For these characteristics, the limit of detection/quantitation should be at least 1 ppb, with a range of quantitation from 1 to 25 ppb. The recovery percentage and reproducibility should exceed those outlined in

the CEN requirements, with recovery in the range of 80 -110% over the entire range of the assay, and with the reproducibility being within 20%.

Assessment of monoclonal antibody performance.

A number of commercially available and specially developed monoclonal antibodies were obtained for evaluation. These antibodies were employed in both assay formats, and the suitability of these antibodies was determined. The suitability of the antibodies tested is outlined in table 8.3.2.2.

Table 8.3.2.2. Assessment of monoclonal antibody performance.

Antibody	Source	Description	Performance
Immunolab	Immunolab	polyclonal anti Ochratoxin A	++
5014	Biogenesis	polyclonal anti Ochratoxin A	+
170/2	Central Science Laboratory(CSL)	monoclonal anti Ochratoxin A	++++
170/3	CSL	monoclonal anti Ochratoxin A	++
170/4	CSL	monoclonal anti Ochratoxin A	_
AF-12	Adgen	monoclonal anti Ochratoxin A (IgG1)	++++

Of the antibodies tested, two were performing well enough to be investigated further and optimised for use in the development of the OTA ELISA. The monoclonal antibodies CSL 170/2 and AF-12 were selected for further evaluation. These two antibodies were optimised for both assay format A and assay format B. Typical standard curves obtained with standard levels of OTA, utilising these two monoclonal antibodies in both assay format A and B, are shown in figures 8.3.2.1a to 8.3.2.1d.

Both antibodies gave good performance with standard quantities of OTA, when tested against plates coated with OTA conjugated to a carrier protein. In this instance, the OTA was conjugated to BSA (assay format B) and the assay did not perform as well in when HRP labelled OTA was competing for binding to monoclonal antibody coated plates (Assay format A). Since performance of the assay is significantly superior when utilised in assay format B, this was the format chosen for further development of the OTA ELISA. Since we had access to larger quantities of the AF-12 antibody, and the performance of this antibody was slightly superior to that of 170/2, it was decided to concentrate on antibody AF-12, in assay format B for the future development of a highly sensitive and rapid ELISA for the detection of OTA in grain and grain products.

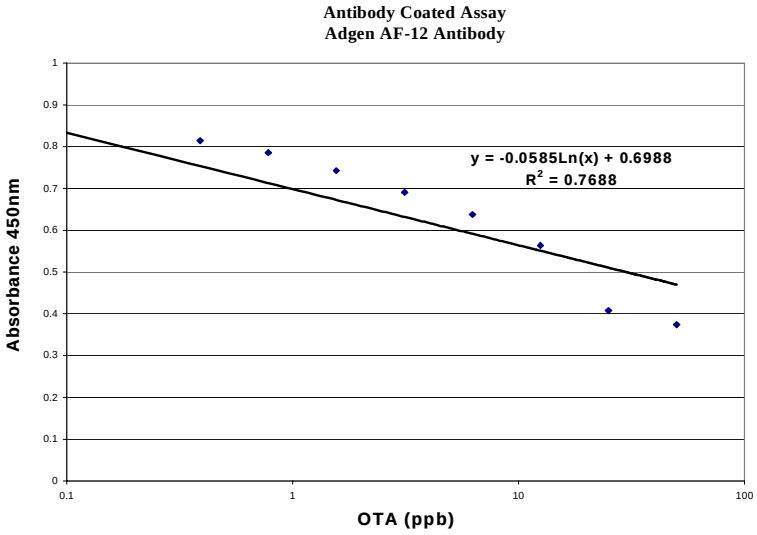

Figure 8.3.2.1a. Standard curve of OTA. Competition ELISA format A, employing AF-12 antibody

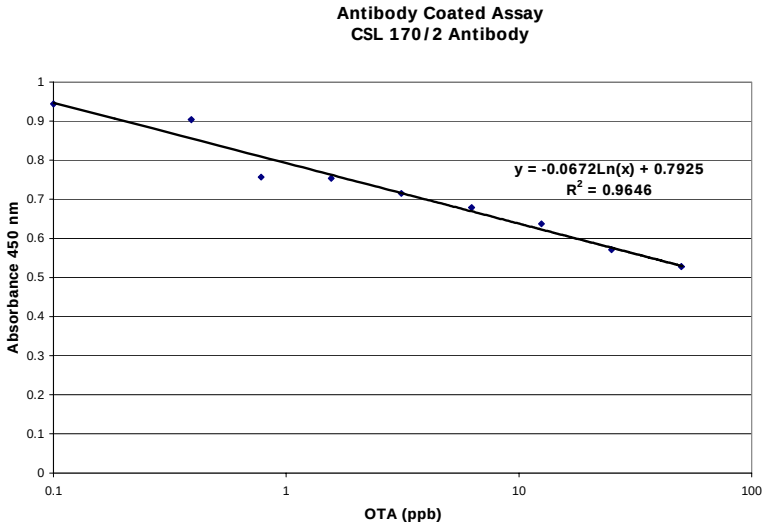

Figure 8.3.2.1b. Standard curve of OTA. Competition ELISA format A, employing 170/2 antibody

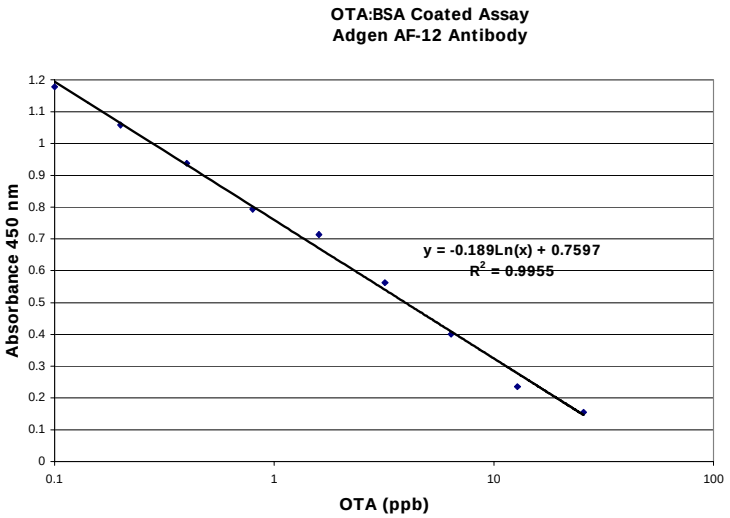

Figure 8.3.2.1c. Standard curve of OTA. Competition ELISA format B, employing AF-12 antibody

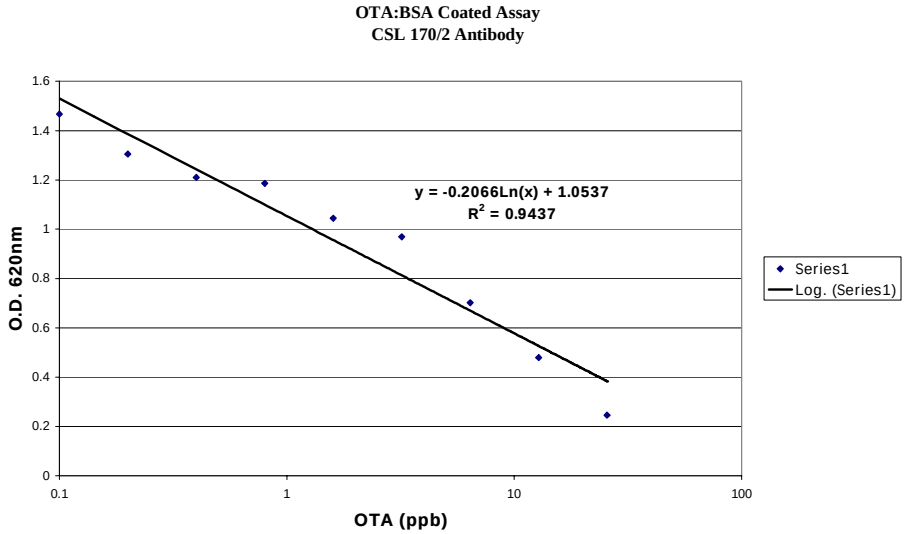

Figure 8.3.2.1d. Standard curve of OTA. Competition ELISA format B, employing 170/2 antibody

Mass production of Monoclonal antibody AF-12, and direct conjugation to HRP

The AF-12 cell line has been sent to a company (OEM concepts) that specialises in the large-scale production and purification of monoclonal antibodies. We have now received over 1 gram of purified antibody, for use in the development of the OTA ELISA. This has enabled us to conjugate the AF-12 antibody directly to HRP, resulting in a significant reduction in the time required to carry out the ELISA and making the assay simpler to carry out. Initial conjugations were conducted employing Poly-HRP (peroxidase, polymerised and maleimide activated for direct conjugation to antibodies. Unfortunately the conjugates produced by this technique, which it was hoped would lead to an improvement in the sensitivity and performance of the assay did not produce any functional conjugates. A more conventional peroxidase conjugation technique was employed, by which good quality, functional AF-12: HRP conjugates were

produced. Using these conjugates the ELISA for the detection of OTA has been simplified to a rapid 20-minute test, without any loss in accuracy or sensitivity. (See appendix 8.3.2: Ochratoxin A ELISA protocol). As can be seen from figure 8.3.2.2, the conjugated AF-12 antibody gave good performance in the OTA ELISA over a range of OTA concentrations from 0.1 to 25 ppb, with an acceptable correlation coefficient to allow sample values to be determined accurately.

Optimisation of OTA to carrier protein conjugation

Ochratoxin A contains a free carboxyl group, (-COOH) through which it can be linked directly to a carrier protein. A range of different carrier proteins and conjugation techniques for the covalent linkage of OTA to carrier protein are available. The conjugation of Ochratoxin A has been carried out to both BSA and bovine gamma globulin (BGG) employing two different conjugation techniques; EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) conjugation and CDI (1,1'-carbonyldiimidazole) conjugation and the OTA to carrier protein ratio has been optimised. Both of these techniques produced viable conjugates, however, conjugates produced by the CDI conjugation technique proved to be relatively unstable, and only had a short (less than 1 month) shelf life. It was therefore decided that the EDC conjugation protocol would be employed in all future studies. The best conjugates were produced employing BSA, at a ratio of 1mg of OTA to 10mg of BSA.

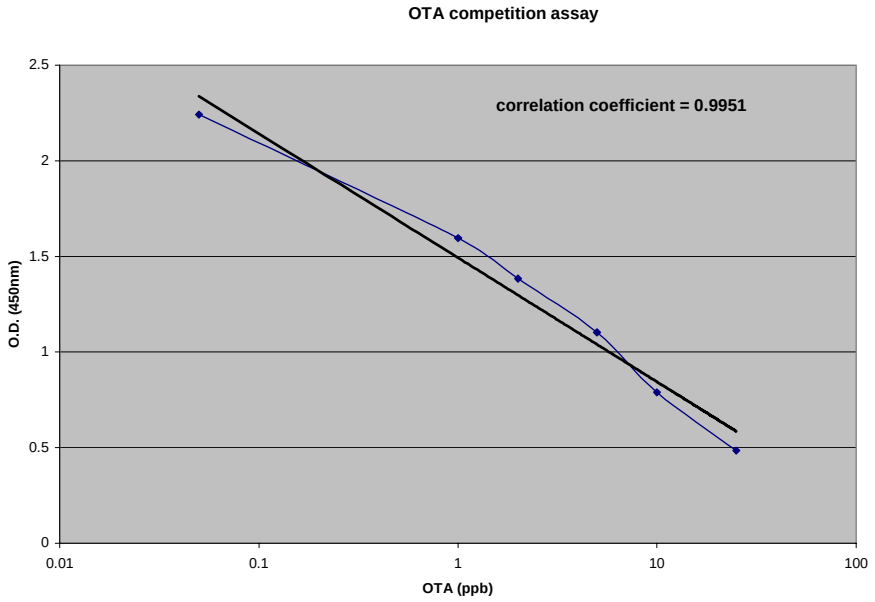

Figure 8.3.2.2. Performance of the OTA ELISA utilising AF-12: HRP conjugated monoclonal antibody.

Performance of the assay with spiked grain extracts

Samples of spiked grain were prepared and the particle free extract for both wheat and barley samples obtained. The sample extract was subsequently spiked with known levels of OTA and the levels of OTA were then determined using our assay system. Tables 8.3.2.3a and 8.3.2.3b gives the correlation between calculated values and the value of the spike and figure 8.3.2.3 an example of a standard curve obtained during this work.

Table 8.3.2.3a: Spiked wheat test.

Standard	**O.D.450 nm**	**Result (%)**	**Spike Level**	**O.D.450 nm**	**Result (%)**
0	1.936	0.0 (N/A)	Wheat		
1	1.376	1.0 (100%)	20	0.512	19.1 (95%)
2	1.156	2.2 (110%)	10	0.713	9.2 (92%)
5	0.951	4.3 (86%)	5	0.968	4.1 (82%)
10	0.705	9.4 (94%)	1	1.358	1.1 (110%)
25	0.427	27.4 (109%)	0	1.928	0.0 (N/A)

Table 8.3.2.3b: Spiked barley test.

Standard	**O.D.450 nm**	**Result (%)**	**Spike Level**	**O.D.450 nm**	**Result (%)**
0	1.936	0.0 (N/A)	Barley		
1	1.376	1.0 (100%)	20	0.486	21.2 (106%)
2	1.156	2.2 (110%)	10	0.695	9.8 (98%)
5	0.951	4.3 (86%)	5	0.972	4.0 (80%)
10	0.705	9.4 (94%)	1	1.394	1.0 (100%)
25	0.427	27.4 (109%)	0	2.031	0.0 (N/A)

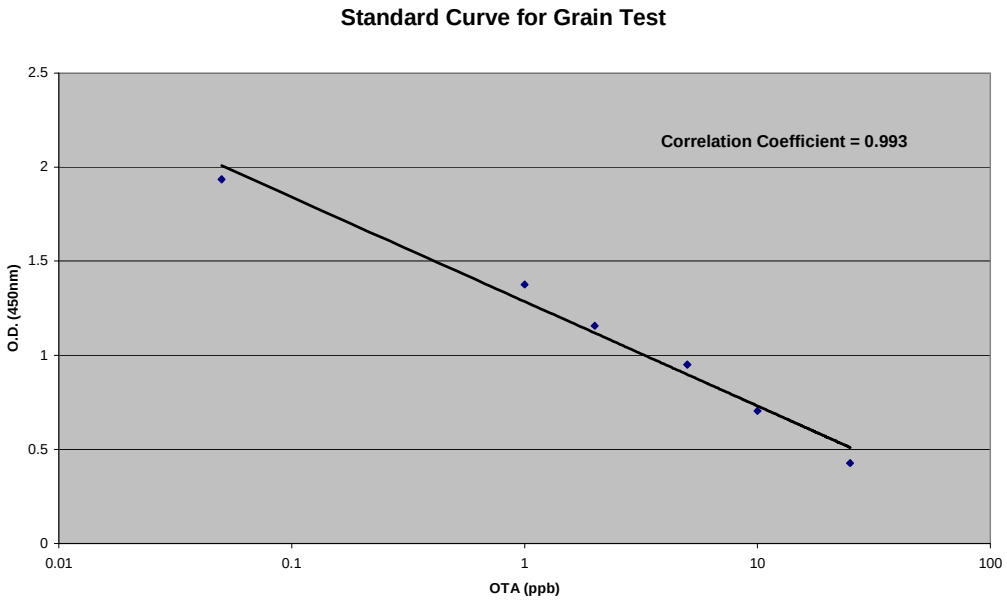

Figure 8.3.2.3. Standard curve for testing of spiked wheat and barley extracts.

Recovery of OTA from Spiked Grain Samples

Spiked grain samples were prepared and extracts of the grain prepared according to the assay protocol (See appendix of WP 8.3.2). The spiked grain samples were subsequently tested using the assay system. As can be seen from the data presented in tables 8.3.2.4a and 8.3.2.4b, there is a good level of agreement between the assigned spike level, and the calculated value. These levels are well within the performance characteristics agreed upon for the assay as regards percent recovery.

Table 8.3.2.4.a: Recovery of OTA from spiked barley

Standard	**O.D Value**	**Result**	**Spike**	**O.D Value**	**Result**
25	0.285	24.4	6.25	0.581	6.0 (96%)
10	0.467	9.7	1.25	0.927	1.4 (112%)
5	0.595	5.6	0.25	1.181	0.3 (120%)
2	0.861	1.9	0.00	1.312	0.0 (N/A)
1	0.958	1.2			
0.5	1.081	0.6			
0.1	1.248	0.1			
0.0	1.342	0.0			

Table 8.3.2.4b : Recovery of OTA from spiked wheat

Standard	O.D. Value	Result	Spike	O.D. Value	Result
25	0.334	25.4	25	0.344	24.2 (96.8%)
10	0.579	9.3	10	0.593	8.9 (89%)
5	0.730	5.4	5	0.734	5.3 (106%)
2	0.982	2.0	2	0.981	2.0 (100%)
1	1.062	1.3	1	1.106	1.0 (100%)
0.5	1.214	0.4	0	1.319	0.0 (N/A)
0.1	1.244	0.3			
0.0	1.326	0.0			

*Comp**arison of ELISA results with HPLC analysis***

A large number of naturally contaminated samples of flour (including a number of uncontaminated samples) were obtained from Partner 6 of Finland from the EU project. These samples were supplied with data on the level of OTA contamination as determined by HPLC. This allowed a direct comparison between the ELISA and HPLC analysis to be made. Unfortunately a large proportion of the supplied samples were quite heavily colonised by mould fungi, which could have affected the OTA levels present in the sample. The grain samples, which were free from visual fungal contamination, were prepared and extracted and analysed for OTA content using the OTA ELISA system (see table 8.3.2.5). For levels of OTA that were within the range of the OTA ELISA kit performance, there was close agreement between the levels detected by ELISA and by HPLC analysis. The agreement for the heavily contaminated samples was not as accurate as that obtained for OTA levels below 25 ppb. This was to be expected, as these highly contaminated samples were outside the range of the assay (0 to 25 ppb). However, all these high levels of OTA contaminated samples still gave very high results in the ELISA showing that this method can still indicate when there were very high amounts of OTA. Dilution of heavily contaminated samples would generally be carried but, in the practical situation, the majority of naturally contaminated samples would fall within the range of the kit.

Further spiked grain samples were prepared with known levels of OTA. Five replicates of each spike level were prepared and three of these were stored at -20^{o}C as duplicate samples, one set of samples was extracted and tested by our ELISA system, and the other samples despatched for HPLC analysis. (See tables 8.3.2.6)

Table 8.3.2.5. Comparison of ELISA results with HPLC data.

HPLC Result	ELISA Result	**% Correlation**
90.5	106.8	118
13.4	13.6	101
1.38	1.2	87
0.73	0.8	109
<0.2	0.3	N/A
<0.2	0.0	N/A
0.33	0.5	151
12.2	13.6	111
29.21	31.2	107
4.29	3.6	84
1798	357.2	Over-Range
12220	462.8	Over-Range
2052	440.0	Over-Range

Table 8.3.2.6. Comparison of OTA ELISA and HPLC for recovery of OTA from spiked grain samples.

Sample Name	**HPLC result (µg/kg)**	**ELISA result (µg/kg)**
Barley 0 µg/kg	<0.2	0.0
Barley 1 µg/kg	1.02	1.1
Barley 3 µg/kg	4.12	3.8
Barley 5 µg/kg	7.17	6.5
Barley 10 µg/kg	11.17	9.8
Barley 25 µg/kg	29.15	26.7
Wheat 0 µg/kg	<0.2	0.1
Wheat 1 µg/kg	1.11	0.80
Wheat 3 µg/kg	2.19	2.2
Wheat 5 µg/kg	3.92	4.4
Wheat 10 µg/kg	11.49	10.7
Wheat 25 µg/kg	30.95	27.6

FAPAS proficiency Testing programmes

Registration for participation in the series 17 FAPAS proficiency testing programme was made and the OTA ELISA was used in the analysis of FAPAS samples as part of this proficiency-testing scheme. Ranges of different matrices were included in this testing series, and include a number of grain samples, which were analysed for OTA content utilising the ELISA. The results for FAPAS series 17 round 24 are given below in table 8.3.2.7.

Table 8.3.2.7 FAPAS Series 17 round 21 Ochratoxin analysis.

April 2003.

Report Number 1721.

Cereal Test Material.

Assigned Value = 6.61 g/ml (ppb).

Adgen are laboratory number **067**

Laboratory Number	Result µg/ml (ppb)	Z-Score	Laboratory Number	Result µg/ml (ppb)	Z-Score
001	6.1	-0.4	026	10.047	2.4
002	7.6	0.7	027	5.8	-0.6
003	3.16	-2.4	028	5.62	-0.7
004	10.6	2.7	029	5.7	-0.6
005	7.4	0.5	030	7.93	0.9
006	6.39	-0.2	031	5.4	-0.8
007	6.63	0.0	032	5.04	-1.1
008	6.7	0.1	033	9.6	2.1
009	5.23	-0.9	034	8.48	1.3
010	8.9	1.6	035	3.1	-2.4
011	6.8	0.1	036	6.57	0.0
012	5.8	-0.6	037	5.0	-1.1
013	8.41	1.2	038	6.89	0.2
014	6.01	-0.4	039	8.06	1.0
015	5.68	-0.6	040	9.0	1.6
016	8.0	1.0	041	3.6	-2.1
017	7.1	0.3	042	8.6	1.4
018	5.56	-0.7	043	5.9	-0.5
019	9.2	1.8	044	4.3	-1.6
020	6.49	-0.1	045	5.54	-0.7
021	8.4	1.2	046	10.72	2.8
022	3.25	-2.3	047	6.75	0.1
023	8.2	1.1	048	6.32	-0.2
024	9.6	2.1	049	3.4	-2.2
025	6.0	-0.4	050	8.6	1.4

Laboratory Number	Result µg/ml (ppb)	Z-Score	Laboratory Number	Result µg/ml (ppb)	Z-Score
051	5.81	-0.5	069	8.0	1.0
052	3.03	-2.5	070	4.4	-1.5
053	6.20	-0.3	071	5.53	-0.7
054	7.86	0.9	072	7.8	0.8
055	7.11	0.3	073	8.0	1.0
056	5.80	-0.6	074	6.0	-0.4
057	7.88	0.9	075	8.6	1.4
058	7.1	0.3	076	7.1	0.3
059	7.2	0.4	077	7.1	0.3
060	5.91	-0.5	078	3.75	-2.0
061	6.11	-0.3	079	3.77	-2.0
062	6.89	0.2	080	6.0	-0.4
063	12.8	4.3	081	11.1	3.1
064	5.78	-0.6	082	4.95	-1.1
065	7.2	0.4	083	5.6	-0.7
066	6.9	0.2	084	6.7	0.1
067	**5**	**-1.1**	085	6.9	0.2
068	4.5	-1.5			

The satisfactory range for the Z-score value is +/- 2.0

As can be seen from the data given above, the ELISA system gave results which agreed with the assigned value of OTA within the acceptable range of reported results.

Ring trial of the OTA ELISA kit

Samples of spiked grain were prepared and coded and sealed in individual foil packets. Duplicate samples were sent for analysis by Partner 4 using the OTA ELISA kit. Duplicate samples were retained by Partner 10 in case further analysis was required. As can be seen from these results (see table 8.3.2.8), at least for test A, there was reasonably consistent agreement with the actual spike values used in this experiment. Unfortunately, in test B there seems to have been a reduction in the performance of the ELISA kit. Further investigation into the possible causes of this loss of assay performance was carried out using duplicate test kits that had been produced at the same time as those employed in this experiment. In these experiments it was noted that there was a marked deterioration in the actual OTA standards employed in the assay. This deterioration of the standards was reflected in the poorer correlation coefficient observed in test B. It is believed that this deterioration in the standards was due to the treatment and nature of the glass vials used for

the storage of the standards. For future ring trials, type I borosilicate amber glass vials will be used and should prevent a repeat of this breakdown of the standards.

Table 8.3.2.8. Results for ring trial of ELISA kit.

OTA std	**Average OD value**				**Calculated result**	
	Test A	**Test B**			**Test A**	**Test B**
0.0	1.053	1.212			0.0	0.0
0.5	0.957	0.879			0.2	0.7
2.0	0.577	0.659			1.9	1.6
5.0	0.323	0.278			5.4	6.8
10	0.199	0.183			10.7	11.6
25	0.107	0.115			23.2	20.3
Correlation Coefficient A = 0.9966 Correlation Coefficient B = 0.9697						
Sample designation	**Average OD value**		**Actual Value**		**Calculated value**	
	Test A	Test B	Test A	Test B	Test A	Test B
1	0.239	0.344	12.5	12.5	8.4	5.0
2	1.058	1.201	0.50	0.50	0.0	0.0
3	0.589	0.915	1.5	1.5	1.8	0.6
4	0.426	0.626	3.0	3.0	3.4	1.8
5	0.649	0.847	1.5	1.5	1.4	0.8
6	0.993	1.513	0.0	0.0	0.1	0.0
7	0.302	0.446	6.25	6.25	6.0	3.3
8	1.053	1.259	0.0	0.0	0.0	0.0
9	0.986	1.184	0.0	0.0	0.1	0.0
10	0.990	1.135	0.0	0.0	0.1	0.1
11	0.747	0.497	0.75	0.75	0.9	2.8
12	0.261	0.213	6.25	6.25	7.4	9.6
13	0.194	0.245	12.5	12.5	11.1	8.0
14	0.619	1.065	1.5	1.5	1.6	0.2
15	0.397	0.666	3.0	3.0	3.9	1.5
16	0.128	0.178	25	25	18.7	12
17	0.143	0.225	25	25	16.4	9.0
18	0.760	1.048	0.75	0.75	0.8	0.3

Performance Characteristics

Over the course of this project numerous tests have been carried out on both spiked and naturally contaminated grain samples (data not shown). From these data it has been possible to calculate the percentage of false positives and false negatives. The actual level set will depend on what value of OTA is to be counted as a positive and a negative value. For the purposes of this calculation, a value of less than 0.5 ppb is taken as being negative. Therefore, an uncontaminated grain sample with a result for OTA of less than 0.5 ppb is regarded as a negative, and not as a false positive. Samples containing 0.5 ppb OTA or greater are regarded as being positive. Using these criteria the rate of false positives was calculated as 0%, with no cases of uncontaminated wheat or barley reporting as positive. The rate of false negatives was calculated as

less than 2%. In each case, the occurrence of false negatives involved OTA levels of less than 1.0 ppb, with all instances of OTA over 1 ppb reporting as positive. Recovery rates for our ELISA system are generally between 80% and 120% over the range of 0.5 to 25 ppb, with occasional calculated values giving lower recoveries (although the majority of these values gave over 70% recovery).

Discussion

During the course of this project, a rapid and sensitive immunoassay for the detection of OTA in cereal grains and cereal products was developed. A range of different monoclonal antibodies for application in this assay format were investigated. Two antibodies (AF-12 and CSL 170/2) were considered suitable for further evaluation. These two monoclonal antibodies were further investigated in two different assay formats. Due to the overall good performance of the AF-12 antibody and the availability of large quantities of purified antibody, it was used for the development of the ELISA assay. A complete methodology has been developed, including a full extraction protocol, which allows the recovery and detection of OTA in small grains and products derived there from in a total assay time (grinding the sample, extraction, and detection) of about 30 minutes (see appendix of this section). The sensitivity of the assay system is as low as 0.1 ppb, a limit of quantitation of 0.5 ppb and a range of detection from 0.5 to 25 ppb. This range of detection and sensitivity exceeds the regulatory requirements and matches or exceeds the performance characteristics observed with competitor products.

A good correlation has been found between OTA ELISA and HPLC. The kit has also been used in the FAPAS proficiency-testing scheme and has given acceptable correlations with the consensus results obtained in this accreditation programme. A small ring trial was set up to test the performance of the completed kit at a different laboratory. Duplicate test kits and spiked samples were sent from Partner 10 to Partner 4 where the spiked samples were analysed for OTA content. The initial trial gave an acceptable correlation between the theoretical spike level and the calculated value from the assay. Unfortunately, the second test gave significantly lower results, and an unacceptable correlation. On further investigation, it was determined that the standard solutions of OTA supplied with the assay were unstable. This problem has since been solved, and further independent ring trials are planned after the end of this project as part of the commercialisation process.

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WP 8: Development of rapid ELISA systems ochratoxin A producing fungi and ochratoxin A

WP 8.3.2 The development of an ELISA kit to match the EU legislative limits for OTA

Appendix

Quantitative Ochratoxin A test kit For Grain and cereal products.

Read instructions carefully prior to starting the assay

Store at 2 – 8°C.

Intended Use.

This quantitative Ochratoxin A (OTA) test kit is a competitive direct enzyme linked immunosorbent assay (ELISA) is intended for the detection and quantitation of OTA in food commodities.

Intended User.

This test kit is intended for use by personnel familiar with food and feed testing procedures. As technique can significantly effect the results, operators should be familiar with ELISA based assay formats. If any problems arise or are anticipated contact your supplier who will arrange appropriate training in the use of this assay.

Assay Principle.

This quantitative assay for OTA is a direct competitive ELISA in a micro-titre plate format, which allows the quantitative determination of OTA in food samples, given as parts per billion (ppb). Free OTA in the sample extract (sample) competes with enzyme labelled anti ochratoxin antibody (conjugate) for bound OTA on the assay wells. After a wash step (to remove unbound antibody conjugate), substrate is added. The substrate reacts with the bound conjugate to produce a blue colour. (The intensity of the colour is inversely proportional to the amount of OTA present in the sample or standard. i.e. the more blue colour develops the less OTA is present). Once sufficient colour has developed, the reaction is stopped by the addition of stop solution (the colour changes from blue to yellow) and the optical densities of samples and controls are measured in a micro-well reader. The optical density readings from the controls form the standard curve, from which the optical densities of the samples are plotted to calculate the concentration of ochratoxin A in parts per billion (ppb).

Materials Provided.

1. Assay strips coated with OTA.
2. Mixing wells.
3. Strip Holder and lid.
4. 6 bottles of 1.5 ml each. Labelled 0, 1, 2, 5, 10 and 25 ppb OTA controls (see materials hazard section for handling of OTA controls).
5. 1 bottle of HRP labelled Anti-OTA Antibody solution.
6. 1 bottle of Substrate solution.
7. 1 bottle of stop solution.
8. 1 bottle of wash buffer concentrate.
9. Assay recording Sheet. (feel free to photocopy this sheet)

Materials Required But Not Provided.

1. Extraction Materials.
    - ACS Grade Methanol.
    - Measuring Cylinders. (250ml, 20ml.)
    - Extraction pot.
    - Filter syringes, Whatman No.1 filter paper (or equivalent).

2. Blender.
3. Laboratory balance capable of weighing 1 to 25 grams.
4. Micro-well reader with a 450-nm filter.
5. Multi-Channel pipette.
6. 100 µl pipette.
7. Tips for Multi-Channel and 100 µl pipette.
8. 20 ml Universal.
9. Paper Towels.
10. Wet Waste container.
11. Timer.
12. Water Proof Marker.
13. Wash Bottle (or plate washer).
14. Reagent boats.
15. Distilled water.

Sample Preparation and Extraction Protocol.

The sample to be tested should be collected into a container that can be tightly sealed. Sample container should be sealed and stored at 2 – 8 ^{o}C until used for analysis.

1. Prepare a solution of 50% Methanol by mixing equal volumes of ACS grade Methanol with Distilled or deionized water. (i.e. Add 100 ml Methanol to 100 ml dH_2O)
2. Obtain a representative sample of the grain. Grind the entire sample in a laboratory grinder, such that at least 75% of the ground material passes through a 20-mesh sieve. (approximately the particle size of a finely ground coffee)
3. Blend 25 grams of the ground sample with 100 ml of the methanol/water solution, or add 10 grams of the ground sample to 40 ml of the methanol/water solution in an extraction pot. Tightly seal the extraction pot and shake the sample vigorously for 5 minutes.
4. Remove particulate material from the sample either by centrifugation at 3000g for 5 minutes or by passing the sample through a filter syringe or Whatman No. 1 filter paper (or similar) and collect the particle free filtrate as the sample.

Wash Buffer Preparation.

The wash buffer is prepared by diluting the wash buffer concentrate supplied with distilled water. The wash buffer concentrate should be stored at 4^{o}C until used. Allow the wash buffer concentrate to come to room temperature and ensure that the wash buffer salts have fully dissolved prior to use. Dilute 10 ml of wash buffer concentrate to 200 ml with distilled water. Seal the bottle and return unused wash buffer to 4^{o}C storage.

Assay Procedure

Allow all reagents to warm to room temperature (18^{o} – 30^{o} C) before starting the assay procedure.

1. Mix the contents of each reagent bottle by swirling prior to use of any of the reagents.
2. Remove one mixing well foe each sample to be tested, plus 6 mixing wells for the controls. Place the wells in the strip holder.
3. Remove an equal number of OTA coated wells and place in strip holder. (return unused mixing and sample wells to the reseal-able bag and re-seal the bag)
4. Pipette 100 µl of the appropriate controls and samples into the relevant mixing well, using a fresh pipette tip for each sample and control. Record the position of controls and samples on the assay recording sheet.
5. Transfer 150 µl of conjugate for each well employed in the current assay into a pipette boat, and using the multi-channel pipette transfer 100 µl of the conjugate to the mixing wells.

   (Take Care not to touch the sample/control present in the wells with the pipette tips, if this should occur discard the tips and continue using fresh tips)

6. Using the multi-channel pipette mix the contents of the mixing wells. Use a fresh tip for each well.
7. Using fresh pipette tips for each well transfer 100 µl from each mixing well to the appropriate Ochratoxin coated sample well.
8. Discard the mixing wells.
9. Cover the plate with the lid provided, and incubate at room temperature for 10 minutes.
10. Wash the assay plate 3 X with wash buffer and blot dry.
11. With new tips on the multi-channel pipette add 100 µl to each well of the assay plate. Mix the contents of the wells by gentle agitation of the plate.
12. Cover the assay plate with the lid provided and incubate for 10 minutes at room temperature.
13. After the 10 minute incubation using fresh tips on the multi-channel pipette add 100 µl of stop solution to each of the assay wells. Mix the contents of the wells by gentle agitation.
14. Read Optical Density in a micro-well reader using a 450 nm filter. (N.B. Air bubbles should be eliminated as they could effect the accuracy of the O.D. readings obtained)
15. Record results and analyse data.

Precautions.

Methanol solution. (the OTA standards are made up in 50% methanol).

Highly flammable. Keep tightly closed and avoid sources of combustion.

Toxic if swallowed or if inhaled. Avoid contact with skin. The methanol solution contains OTA and Should not be disposed directly (see Ochratoxin for disposal)

Ochratoxin A.

Highly toxic. Toxic by inhalation, skin contact and ingestion. Avoid contact with skin. Gloves and other protective lab-ware should be worn at all times. IN case of contact with skin, wash affected area with soap and water and rinse with copious amounts of water. In case of contact with eyes, rinse with copious amounts of water and seek medical advice.

Disposal. Mix with flammable solvent and dispose of by incineration. Alternatively, small quantities of OTA can be de-activated by placing in a sodium hypo-chloride solution (Chloros tablets or similar)) and disposed to waste drain with copious quantities of water.

Stop Solution. (10% H_2SO_4)

Corrosive, avoid contact with skin and eyes. Gloves and other protective lab-ware should be worn at all times. In case of contact with skin, flush with copious amounts of water. In case of contact with eyes, flush with copious amounts of water and seek medical attention. Spillage, mop up with paper towels and copious amounts of water. Wear gloves and Lab-coat at all times when handling.

Disposal. Discard to waste drain with copious quantities of water.