CHAPTER 10

Methodology Development

INTRODUCTION

A large number of publications has appeared on chemical assays for the determination of ochratoxin A (OTA) in a wide range of commodities. All of them involve extraction, cleanup, separation, detection, quantitation and confirmation of identity (van Egmond, 1991). A shortcoming in most of these methods is that they were developed for the analysis of OTA alone. The other ochratoxins, especially ochratoxin B (OTB) have different physical properties e.g. different solubilities in organic solvents and the percentage recoveries for these ochratoxins, are quite low when traditional methods are used. The aim of this chapter is to develop a suitable method for the analysis of all the known ochratoxins in wheat (artificially infected) with a very high ochratoxin content, as well as for the analysis of very low levels of the ochratoxins in coffee, wine, urine and blood samples. A basic background on the LC-MS technique is provided in this chapter since it was used as one of the main techniques in the analysis of the ochratoxins.

This chapter comprises the following sections:

10.1 The development of a LC-MS method for the analysis of the ochratoxins in blood and urine

10.2 Determination of the ideal time, for harvesting the ochratoxins after innoculation with *Aspergillus ochraceus* Wilh.

10.3 The selection of the optimal solvent for the extraction of the ochratoxins from wheat

10.4 Determination of the best solid phase extraction (SPE) columns for sample cleanup

10.5 Standard curves and internal standards

10.6 Determination of the extraction efficiency and percentage reproducibility of the aminopropyl (NH_2) SPE columns for the analysis of the ochratoxins

MATERIALS AND METHODS

Reagents and Solvents

Methanol, acetic acid (HPLC grade) and deionised water were used for the HPLC analyses. AR grade methanol, acetone, dichloromethane, ethyl acetate, chloroform, hexane and sodium carbonate were obtained from Merck. Whatman no. 5 filter paper was used for filtering the fungal extracts. SPE columns were purchased from Supelco.

HPLC

A Hewlett Packard 1090 High Performance Liquid Chromatography (HPLC) system equipped with a quadrupole pump, autosampler and diode array detector was used. The mobile phase used was methanol/water/acetic acid (60:40:2) with a 4,6 mm x 150 mm, 5 μm reversed phase column (Discovery C_{18}, Supelco) applying a flow rate of 1 ml/min. The UV detector was set at a wavelength of 332 nm and ChemStation software was used.

TLC

TLC was done on 20x20 silica plates (Merck) with toluene/acetic acid (4:1) as mobile phase (R_f OTA 0.5; R_f OTB 0.35; R_f OTα 0.23). The ochratoxins separated effectively on this system.

LC-MS

A VG Quattro II - HPLC 1090 system was used with positive ionisation.

10.1 INTRODUCTION TO LIQUID CHROMATOGRAPHY – MASS SPECTROMETRY (LC-MS)

Individual components in a mixture can usually be separated by the process of chromatography, depending on the physico-chemical properties of the substances. Chromatography involves the selective distribution of components between two heterogeneous phases; the stationary and the mobile phase. In gas chromatography (GC) the mobile phase is a gas whereas in liquid chromatography the mobile phase is a liquid. The

ochratoxins are insufficiently volatile and labile, and pre-column derivatisation is necessary if these have to be analysed by a gas chromatograph (GC) or GC-MS. No derivatisation is needed for LC-MS. This method comprises the separation of samples into its chemical components on a liquid chromatograph and the detection of these components by an MS detector. The advantage of the MS detector is that in addition to quantitative information on the components in a sample, structural information on the molecular ions of compounds can also be obtained. A sample can also be introduced directly into the MS detector with the syringe pump (Gaskell, 1997). The MS used, consists of an atmospheric pressure ionisation source (API), ion optics, mass analyser and ion detection system. The API source produces both positively and negatively charged ions. The type of ions transmitted by the ion optics into the mass analyser is determined by the polarity of the potentials applied to the lenses of the API source and ion optics. The mass-to-charge ratios of the ions produced in the API source are measured by the mass analyser, from where selected ions are ejected to the ion detection system where a signal is produced. The LC-MS apparatus in use can be operated in an electrospray ionisation (ESI) or an atmospheric pressure chemical ionisation (APCI) mode. ESI is a softer ionisation technique and is used to analyse polar compounds, whereas APCI is used to analyse compounds of medium polarity that have some volatility. In the ESI mode the sample enters a needle to which a high voltage is applied which sprays the sample solution into a fine mist of electrically charged droplets (see **Figure 1**). As the solvent evaporates from the droplets, the electrical charge density at its surface increases to a critical point known as the Rayleigh stability limit (Bruins, 1998). At this limit the droplets divide into smaller droplets due to electrostatic repulsions exceeding the surface tension. This process is repeated many times until sample ions are ejected into the gas phase and analysed by the MS detector. The ESI mode can be used either in the negative or positive ion polarity mode depending on the polarity of the preformed ions in solution. Acidic molecules form negative ions in solution and basic molecules form positive ions. Samples can carry a single charge or multiple charges depending on the structure of the analytes and the type of carrier solvent. Droplet size, surface charge, liquid surface tension, solvent volatility and ion solvation strength are all factors that influence the ESI process. Volatile organic solvents like methanol and acetonitrile in conjunction with volatile weak acids and bases are superior solvents to water and strong acids and bases for ESI (Bruins, 1998).

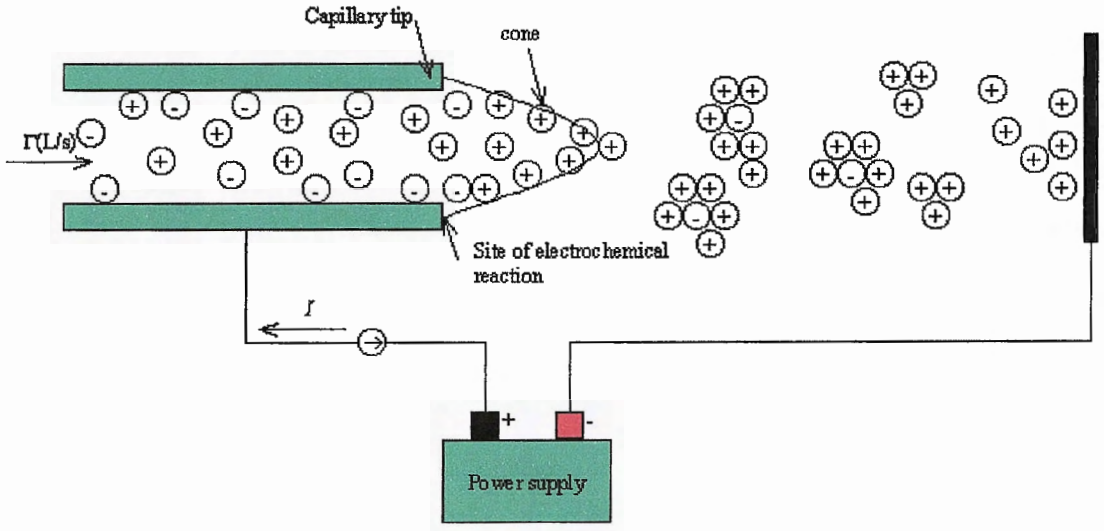


Figure 1 : Illustration of several aspects of the electrospray ionization process (Enke, 1997).

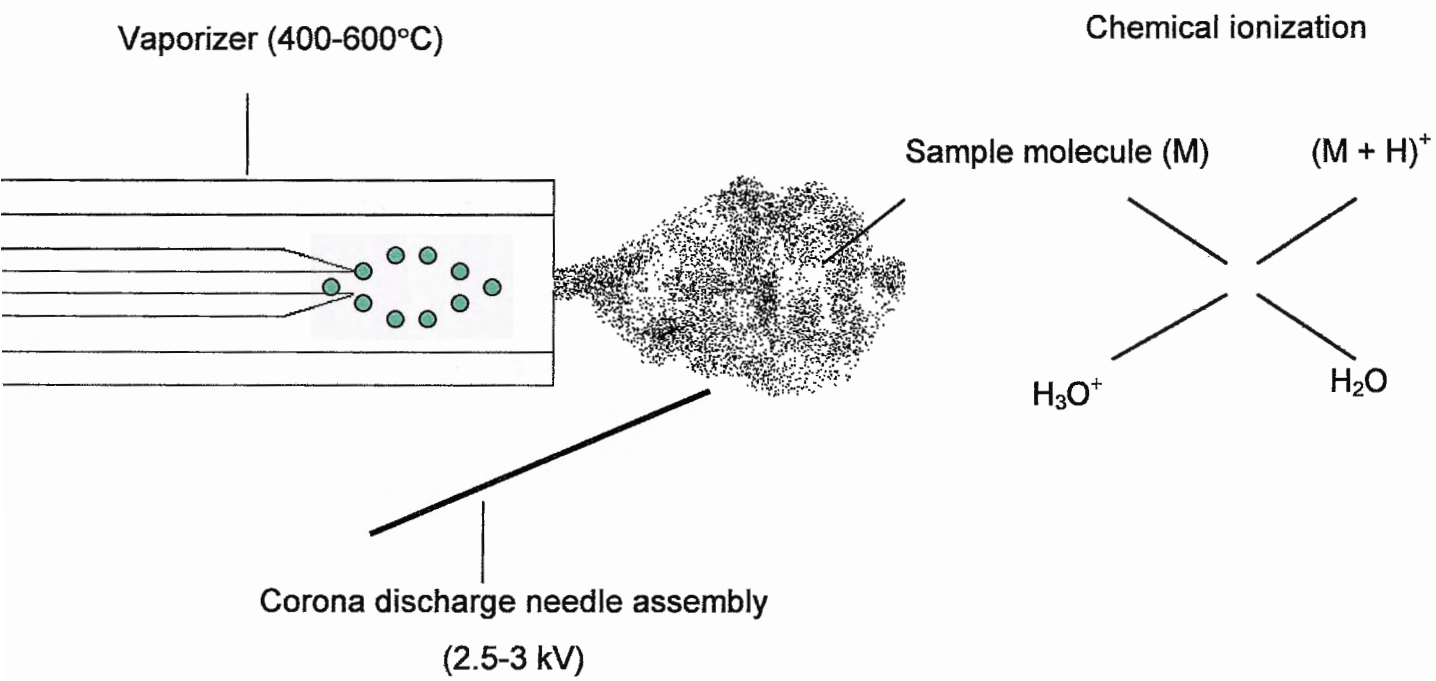


Figure 2: Illustration of atmospheric pressure chemical ionisation

Ions are produced in APCI by spraying the sample solution into a fine mist of droplets by the APCI nozzle in a high temperature tube that vaporises the droplets and by applying a high voltage to a needle located near the exit of the tube (see **Figure 2**). A corona discharge is created by the high voltage and reagent ions are formed through a series of chemical reactions with solvent molecules and nitrogen sheath gas. The sample molecules react with these reagent ions to form sample ions that enter the MS detector and are then analysed. APCI can be used in positive and negative ion polarity mode. Most molecules especially those with one or more basic atoms like nitrogen produce a stronger ion current in the positive ion mode (Bruins, 1998).

Molecules which have acidic sites such as carboxylic acids and acid-alcohols produce more negative ions. The negative ion polarity mode usually generates less chemical noise and has thus better selectivity than the positive ion mode (Bruins, 1998).

Determining the best ionisation mode for ochratoxin analysis

The MS parameters were separately optimised for OTA and the IS by direct infusion of the compounds into the source. Positive and negative ion electrospray ionisation (ESI) as well as positive- and negative ion atmospheric pressure chemical ionisation (APCI) mass spectrometry was evaluated. Negative ion electrospray ionisation was found to the best ionisation method for OTA since the highest sensitivity was obtained with the lowest amount of secondary fragmentation peaks (See **Chapter 9**).

10.2 Determination of the ideal harvest time for maximum production of OTA

Procedure for the inoculation of wheat

A frozen culture of *A. ochraceus* Wilh. (MRC 10582) kept at -70 °C, obtained from the CSIR Culture Collection, Pretoria, was plated onto potato dextrose agar plates. The petri dishes were incubated at 25 °C for three days in the dark. Erlenmeyer flasks (8 x 500 ml) were filled with Durum wheat (80 g) and distilled water (25 ml) autoclaved for 30 min and cooled down to room temperature. The wheat was then inoculated with a spore suspension (2 ml) of three

day old cultures of *A. ochraceus*. The flasks were placed on a rotary shaker (350 rpm). Two of the flasks were removed after 10 days on the rotary shaker, two flasks after 15, two after 20 and two flasks were removed after 25 days after which they were harvested for analysis. This mouldered wheat was kept at -10°C, prior to analysis.

Sample preparation

Methanol/chloroform (1:1, 200 ml) was added to the flasks and the samples milled at 1000 rpm for 10 minutes, filtered and washed with methanol/chloroform (1:1, 20 ml). The solvent was evaporated and the extract resuspended in methanol/water (95:5, 50 ml) and hexane (50 ml). The two layers were separated, after adequate mixing by shaking. The methanol layer was evaporated to dryness and the extract resuspended in chloroform (50 ml) and 0.5 M sodium carbonate (50 ml), the latter layer containing the ochratoxins. The layers were separated and the aqueous layer was acidified to pH 2 with 1 M hydrochloric acid and re-extracted into chloroform (50 ml). The chloroform layer was evaporated to dryness and the extract was resuspended in methanol (50 ml), diluted ten times with water (the solution remained clear) and injected onto the HPLC (10 µl) (Each sample was treated absolutely identically).

Results and discussion

A period of fifteen days seemed to be ideal for ochratoxin production (see **Figures 3-5**). The amount of OTA produced raised for the first 15 days (to ± 7 mg OTA per gram wheat) after which it declined as the OTA became metabolised by the fungal enzymes (see **Table 1**).

Table 1: OTA production by *A. ochraceus* after different growth periods on Durum wheat.

Growth period	**OTA produced**
10 days	2.1 mg/g wheat
15 days	7.1 mg/g wheat
20 days	6.3 mg/g wheat
25 days	5.5 mg/g wheat

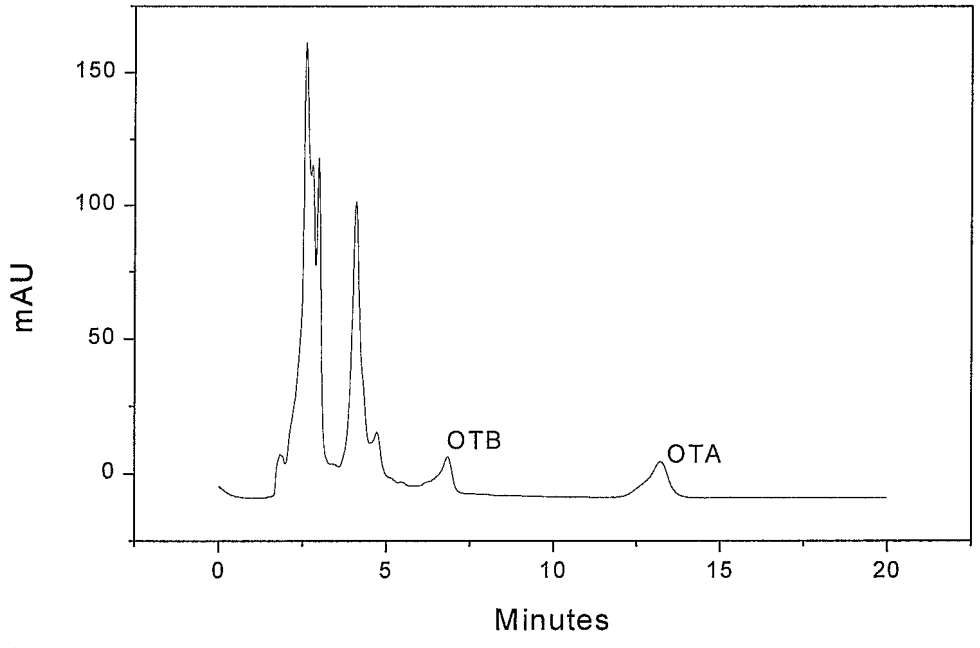


Figure 3: An HPLC chromatogram of a cultivated wheat extract after a growth period of 10 days by *A. ochraceus* with the peak of OTB appearing after 6 minutes and that of OTA at 13 minutes.

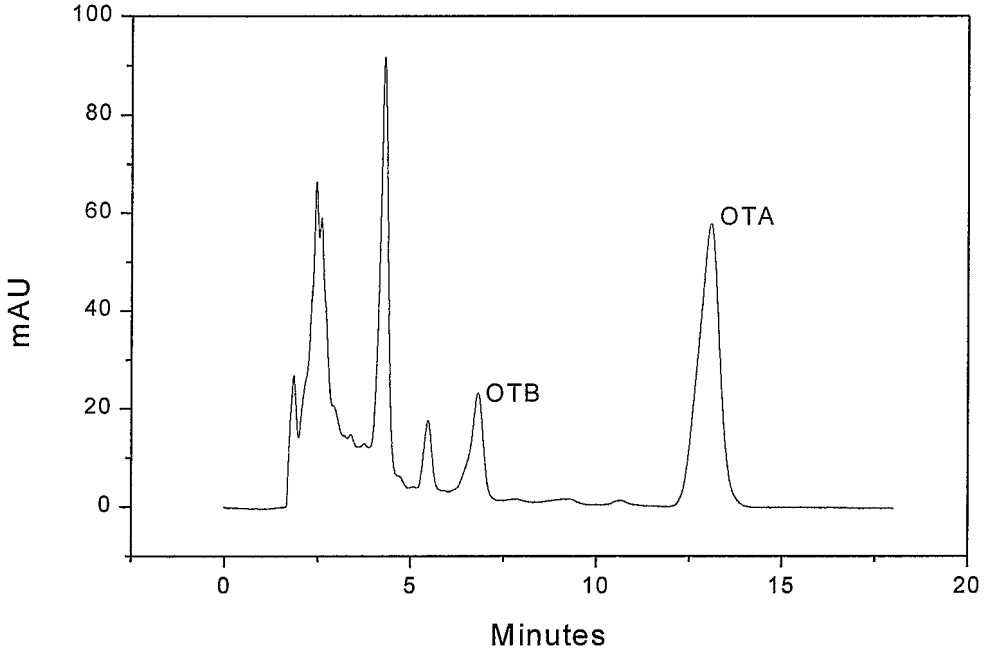


Figure 4: An HPLC chromatogram of a cultivated wheat extract after a growth period of 15 days by *A. ochraceus* with much higher OTA and OTB peaks than after a growth period of 10 days (**Figure 3**).

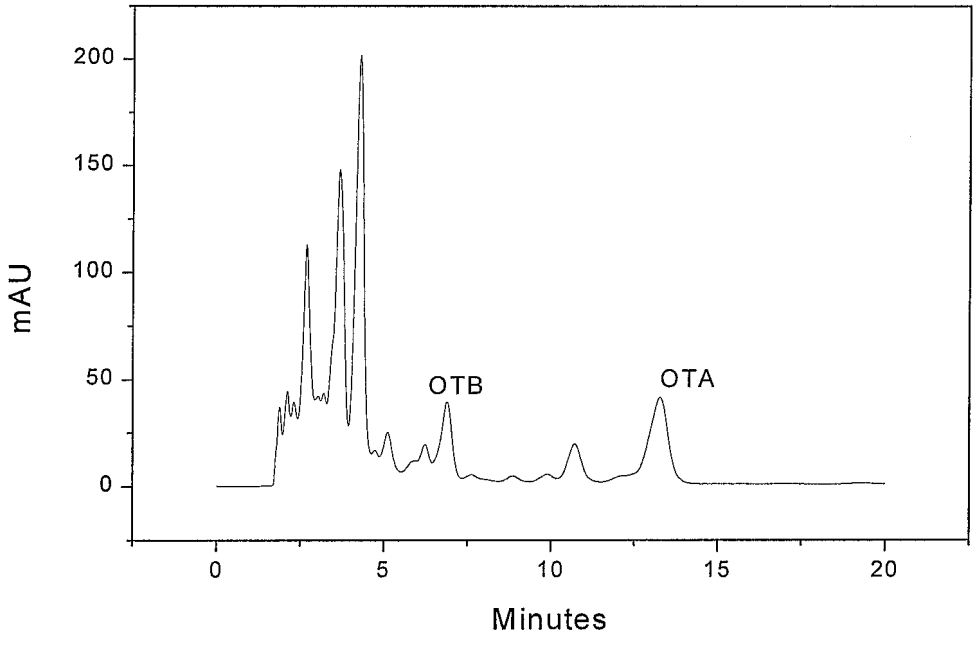


Figure 5: An HPLC chromatogram of a cultivated wheat extract after a growth period of 25 days by *A. ochraceus*. The OTA and OTB peaks are weaker and there are also more ochratoxin-type substances than in **Figure 4.**

10.3 The determination of the best solvent for extracting ochratoxin from wheat

A wide range of solvents was used in the literature for the extraction of the ochratoxins. These solvents can be divided roughly into three groups: acidified organic solvents e.g. chloroform/*ortho*-phosphoric acid (Patel *et al.*, 1997); organic solvents e.g. ethyl acetate (Breitholz *et al.*, 1991), and mixtures of organic solvents e.g. chloroform/methanol and aqueous water miscible organic solvents e.g. aqueous sodium bicarbonate/methanol (Micco *et al.*, 1989). Aqueous water miscible organic solvents are usually also used in immunochemical methods in order to allow the immunochemical reaction to take place in an aqueous environment (Ramakrishna *et al.*, 1990).

Procedure for the inoculation of wheat

A frozen culture of *A. ochraceus* Wilh. (MRC 10582) kept at -70 °C, obtained from the CSIR Culture Collection, was plated onto potato dextrose agar plates. The inoculated agar plates were incubated at 25 °C for three days in the dark. Erlenmeyer flasks (10 x 500 ml) which

contained Durum wheat (80 g) and distilled water (25 ml) were autoclaved for 30 min and cooled to room temperature. The wheat was then inoculated with a 2 ml spore suspension of three day old cultures of *A. ochraceus*. The flasks were placed on a rotary shaker (350 rpm) for 14 days, harvested and the mouldered wheat was kept at -10°C, until it was analysed.

Procedure for the extraction of the ochratoxins

The content of the above flasks was mixed well and transferred to beakers (20 g in each of 40, 600 ml beakers), the extraction solvents (300 ml, see **Table 2**) were added and milled at 2000 rpm for 15 minutes. The samples were left for 24 hours to soak, milled for 15 minutes at 2000 rpm, filtered and washed with extraction solvent (20 ml). Water (100 ml), was added to all the beakers that contained acetic acid to extract the acetic acid. The two layers were separated and the water layer discarded. The organic solvents were subsequently evaporated to dryness and the extracts dissolved in methanol/water (95:5) to a total volume of 100 ml. Hexane (100 ml) was added to the wheat extracts to remove excess oils. The hexane layer was discarded and the methanol/water layer was diluted ten times and injected (10 µl) onto the HPLC (Each sample was analysed in exactly the same manner).

Table 2: Amounts of OTA and OTB extracted with different solvents

Solvent	OTA (mg/g wheat)	OTB (mg/g wheat)
Methanol/chloroform (1:1)	7.18	6.05
Acetone	3.29	1.75
Dichloromethane	6.50	3.57
0.5 M Sodium carbonate/chloroform (1:1)	5.26	1.90
Acetone/chloroform (1:1)	4.86	2.84
Ethyl acetate	7.04	5.64
Methanol/chloroform/acetic acid (48:48:4)	5.63	5.49
Toluene	6.33	0.563
Toluene/acetic acid (95:5)	7.40	0.844

Results and Discussion

The results from **Table 2** indicated clearly that the best solvent for the extraction of OTA was toluene/acetic acid (95:5), however, OTB was very poorly extracted by this mixture. It was thus decided to use methanol/chloroform (1:1) in all instances where both OTA and OTB were to be effectively extracted and determined.

10.4 Evaluating different sample cleanup methods

SPE is frequently used as a time-saving and inexpensive alternative for liquid-liquid extraction to clean-up and to concentrate analytical samples. SPE can be used, either to retain analytes of interest on the packing material, while contaminants pass through after which the analytes are washed from the packing: Alternatively the contaminants can be retained on the packing material while the analytes of interests pass through unretained by the packing material. The mechanisms of interaction for the matrix analytes and packing materials are similar to those in liquid chromatography. In this study the following types of SPE packing materials were used:

Ion-exchange: The surface of the packing material contains ionisable functional groups that retain analytes with ionic charges opposite to those of the packing material. Organic solvents or solutions containing counter-ions of greater ionic strength are used to elute the analytes of interest from the packing material.

Normal phase: The packing material is polar and these columns will retain polar analytes in a non-polar matrix while non-polar materials will pass through.

Reversed phase: These columns contain a non-polar stationary phase, that will retain non-polar analytes in a polar matrix while letting polar compounds pass through.

The aim of these experiments was to find a suitable SPE column, which has the capacity to retain the ochratoxins from the extraction solvent matrix. Since the extraction solvent consists of an organic solvent component (non-polar) and the ochratoxins has non-polar and ionisable functional groups, it was decided to test the ion-exchange and normal phase columns first. LC-SCX (containing aliphatic sulfonic acid with Na^+ acting as the counterion) and LC-SAX (containing quaternary amine groups with Cl^- as the counterion) were the ion-exchange columns investigated. LC-NH_2 (containing aminopropyl functional groups), LC-Diol (containing diol groups), LC-Si (silica) and LC-CN (containing cyanopropyl groups) were the normal phase columns tested. Of these columns only the silica (Jiao *et al.,* 1992), reversed

phase LC-C18 (Nesheim *et al.*, 1992) and cyano (Cohen and Lapointe, 1986) columns were previously employed for ochratoxin analysis.

Procedure for the inoculation of wheat

A frozen culture of *A. ochraceus* Wilh. (MRC 10582) kept at -70 °C, was plated onto potato dextrose agar plates. The inoculated agar petri dishes were incubated at 25 °C for three days in the dark. Erlenmeyer flasks (5x 500 ml) were filled with Durum wheat (80 g) and treated in a similar way as **Section 10.3**.

Procedure for the extraction of the ochratoxins

The content of each of the above wheat flasks was transferred to a beaker (600 ml). Methanol/water (60:40, 200 ml) was added to three of the beakers and chloroform (200 ml) was added to the remaining two. The wheat was milled at 2000 rpm for 15 minutes and the samples left to soak for 24 hours. It was subsequently milled for 15 minutes at 2000 rpm, filtered and washed with the extraction solvent (20 ml). The extracts were transferred to volumetric flasks (5 x 250 ml) and the flasks filled with methanol/water (60:40).

Procedure for the extraction of the ochratoxins with normal phase and ion exchange columns

The SPE columns were put on a vacuum manifold and solvents were passed through the columns with the help of a vacuum pump. A flow rate of 1 ml/min was used throughout these experiments. The samples that eluted after each step, (including transferring samples onto the column, washing and eluting the analytes), were collected and analyzed on HPLC (in all instances where these were in a methanol, water or methanol/acetic acid medium) or on TLC (in the instances where the matrix comprised hexane or hydrochloric acid or chloroform).

Ion-exchange columns

The methanol/water, wheat extracts were used for these analyses. Note that the LC-Diol and LC-NH_2 columns can act as an ion exchange or a normal phase column.

Experiment 10.4.1

The ion-exchange SPE columns were at first conditioned with methanol/water (60:40, 2.5 ml), and the wheat extract (2.5 ml) subsequently added. The columns were washed with hexane (2.5 ml), followed by chloroform (2.5 ml). The analytes were eluted with 0.5 M hydrochloric acid (2.5 ml) followed instantly by methanol (2.5 ml). The eluant was collected after each step and analysed either by HPLC or by TLC.

Results and Discussion

TLC showed intense spots for all of the ochratoxins in the hexane extracts of the LC-Diol and LC-SCX columns, much lighter spots in the LC-SAX and only OTC spots in the hexane extracts of the LC-NH_2 columns.

The chloroform extracts and the hydrochloric acid extracts of all the columns were investigated by TLC on silica gel. None of the extracts revealed the presence of OTA, OTB, or ochratoxin C (OTC). The LC-NH_2 and LC-SAX columns retained the ochratoxins effectively, while the LC-SCX and the LC-Diol columns did not retain these at all (see **Table 3**). The LC-SAX column was not as effective in retaining the ochratoxins in the washing step; therefore, it was decided to investigate the necessity of the washing step.

Table 3: HPLC results of experiment 10.4.1 indicating relative ochratoxin concentrations of the methanol/water and methanol extracts, after it passed through the different columns, and the percentage of the total area in the chromatograms occupied by other compounds.

	MeOH/water			MeOH		
Column	**OTA**	**OTB**	**Other peaks**	**OTA**	**OTB**	**Other peaks**
No column	2310	916	75%			
LC-SCX	694	343	72%	0	0	100%
LC-NH_2	0	0	100%	1957	755	17%
LC-Diol	1524	610	66%	0	0	100%
LC-SAX	0	0	100%	66	0	76%

Experiment 10.4.2.

The columns (LC-NH_2 and LC-SAX) were at first conditioned with methanol/water (60:40, 2.5 ml), and the wheat extract (2.5 ml) subsequently added. In this experiment the washing

step was left out. Hydrochloric acid (0.5 M, 2.5 ml) was sent through the column followed by two subsequent additions of methanol (2.5 ml) to elute the ochratoxins.

Results and Discussion

No ochratoxin spots were observed on the TLC of a chloroform extract of the foregoing hydrochloric acid extraction. It is evident that washing decreased the amount of impurities present in the final eluant (see **Table 4**). The LC-SAX column does not retain the ochratoxins as well as the LC-NH_2 column. The next step was to investigate ways to improve the final eluting step.

Table 4: HPLC results of experiment 10.4.2 indicating ochratoxin concentrations (in absorbancy units) of the methanol/water and methanol extracts, after it passed through the different columns, and the percentage of the total area in the chromatograms that other compounds occupy.

	Methanol/water			**First methanol step**			**Second methanol step**		
SPE	**OTA**	**OTB**	**Other**	**OTA**	**OTB**	**Other**	**OTA**	**OTB**	**Other**
None	2310	916	75%						
$LCNH_2$	0	0	100%	1922	794	27%	0	0	100%
LCSAX	157	149	95%	1279	365	21%	0	0	100%

Experiment 10.4.3

The columns (LC-NH_2 and LC-SAX) were at first conditioned with methanol/water (60:40, 2.5 ml), and the wheat extracts (2.5 ml) transferred onto the columns. The ochratoxins were eluted with methanol/acetic acid (4:1) without washing, followed by methanol (2.5 ml).

Results and Discussion

The importance of the washing step is evident from the high amounts of impurities in the eluting step (see **Table 5** under "Other"). The use of methanol/acetic acid instead of hydrochloric acid followed by methanol seems to be very successful. No ochratoxin is left after this step on the LC-NH_2 column and very little on the LC-SAX as is evident from the methanol extract which was sent through the column after the eluting step.

Table 5: HPLC results of experiment 10.4.3 indicating relative ochratoxin concentrations (in absorbance units) of the different extracts, after it went through the different columns as well as the percentage of the total area in the chromatograms that other compounds occupy.

	Methanol/water			Methanol/acetic acid			Methanol		
Column	**OTA**	**OTB**	**Other peaks**	**OTA**	**OTB**	**Other peaks**	**OTA**	**OTB**	**Other peaks**
None	2310	916	75%						
LC-NH_2	0	42	88%	2294	931	61%	0	0	0
LC-SAX	247	138	92%	2150	813	59%	0	109	0

Normal phase columns

Experiment 10.4.4

The wheat extracts obtained upon extraction by chloroform were used for this experiment. The normal phase SPE columns were at first conditioned with chloroform (2.5 ml), followed by transferring the wheat extract (2.5 ml) onto the columns. The columns were washed with chloroform (2.5 ml), the ochratoxins eluted with methanol/acetic acid (4:1) and the columns washed with methanol to ensure that all the ochratoxins were removed from the columns.

Results and Discussion

The ochratoxins were effectively retained by the LC-Si column, however, some of the toxins were washed off in the chloroform-washing step (see **Table 6** and **7**). The LC-Si column, retained also OTC, which is an advantage above the LC-NH_2 column, which did not retain OTC because OTC does not have a carboxylic group that can interact with the amino groups on the packing material. The LC-CN and LC-Diol columns proved to be ineffective. The importance of the washing step involving chloroform was evaluated in the next experiment.

Table 6: HPLC results of experiment 10.4.4 indicating relative ochratoxin concentrations of the different extracts, after it passed through the different columns and the percentage of the total area in the chromatograms that other compounds occupy.

	Methanol/acetic acid(4:1)			Methanol		
Column	**OTA**	**OTB**	**Other peaks**	**OTA**	**OTB**	**Other peaks**
No column	1582	1049	84%			
LC-CN	0	0	100%	0	0	100%
LC-Si	738	1244	78%	0	0	100%
LC-Diol	0	0	100%	96	45	78%

Table 7: TLC results of experiment 10.4.4 indicating the presence of the ochratoxins in the chloroform used as extracting solvent and the chloroform used for washing after it passed through the different columns.

Column	Chloroform (extraction solvent)	Chloroform (washing)
LC-CN	Intense spots of OTA, OTB and OTC	Intense spots of OTA, OTB and OTC
LC-Si	No spots	Intense spots of OTA, OTB and OTC
LC-Diol	Intense spots of OTA, OTB and OTC	Intense spots of OTA, OTB and OTC

Experiment 10.4.5

The wheat extracts obtained upon extraction by chloroform were used for this experiment. The columns were at first conditioned with chloroform (2.5 ml), followed by addition of the wheat extract (2.5 ml). The ochratoxins on the columns were eluted with methanol/acetic acid (4:1) and washed with methanol.

Results and Discussion

It is evident that although the ochratoxins were well retained by the LC-Si column (see **Tables 8** and **9**), the amount of impurities retained was also high, when no washing step was used. The LC-CN and LC-Diol columns did not retain the ochratoxins sufficiently. The efficiency of partially purifying ochratoxin extracts with LC-NH_2 columns is illustrated in **Figures 6-8**.

Table 8: HPLC results of experiment 10.4.5 indicating relative ochratoxin concentrations of the methanol/acetic acid and methanol extracts, after it passed through the different columns as well as the percentage of the total area in the chromatograms that other compounds occupy.

	Methanol / acetic acid (4:1)			Methanol		
Column	**OTA**	**OTB**	**Other peaks**	**OTA**	**OTB**	**Other peaks**
No column	1582	1049	84%			
LC-CN	872	576	83%	0	0	100%
LC-Si	1586	1045	78%	0	0	100%
LC-Diol	835	523	84%	0	0	100%

Table 9: TLC results of experiment 10.4.5 indicating the presence of the ochratoxins in chloroform which was used as extracting solvent after it passed through the different columns.

Column	Chloroform
LC-CN	Intense spots of OTA, OTB + OTC
LC-Si	No spots
LC-Diol	Intense spots of OTA, OTB + OTC

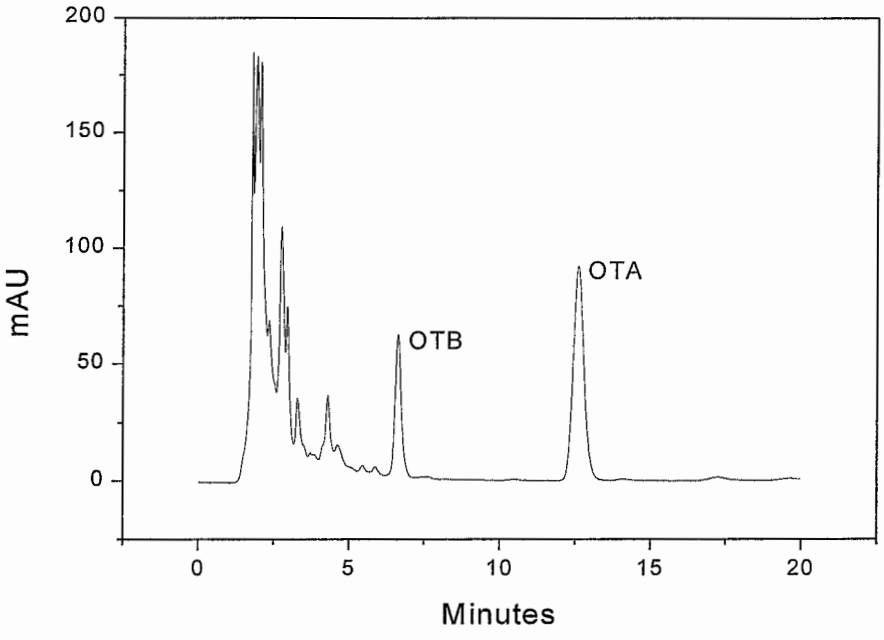


Figure 6: An HPLC chromatogram of a methanol/water wheat extract prior to SPE cleanup.

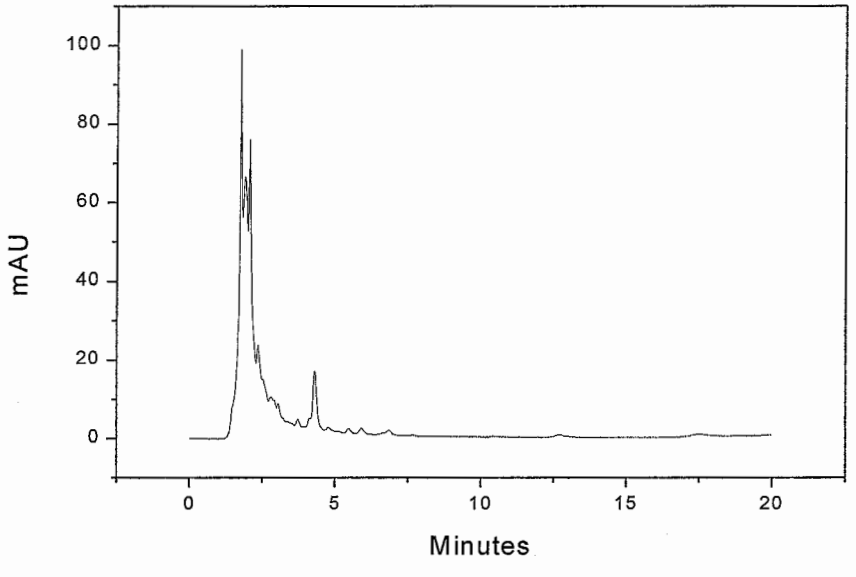


Figure 7: An HPLC chromatogram of a methanol/water wheat extract, after it passed through an LC-NH_2 SPE column (Ochratoxins A and B were retained on the column).

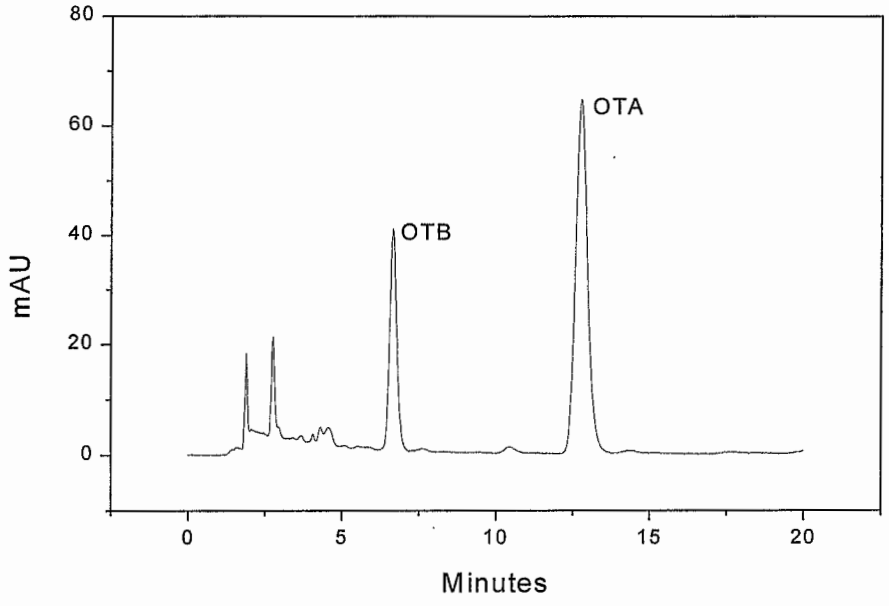
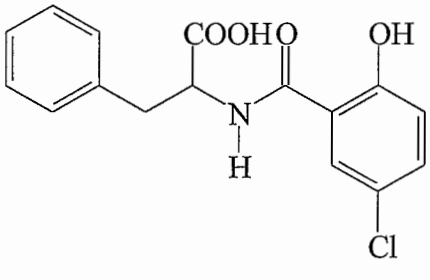


Figure 8: An HPLC chromatogram of the ochratoxins present in a wheat extract, after it was cleaned-up with a LC-NH_2 SPE column.

10.5 Standard curves and internal standards

Intractable problems were experienced with the reproducibility of the HPLC analysis of the ochratoxins. It was, therefore, decided to rather use an internal standard (IS) than an external standard curve. 5-Chlorosalicylic acid coupled to phenylalanine produced N-(5-chloro-2-hydroxybenzoyl)-phenylalanine a very stabile compound with a structure very similar to OTA. The HPLC-retention time of N-(5-chloro-2-hydroxybenzoyl)-phenylalanine lies between OTA and OTB and its UV spectrum is also very similar, making it detectable at the same excitation and emission wavelengths as the ochratoxins.



N-(5-chloro-2-hydroxybenzoyl)-phenylalanine

A stock solution of N-(5-chloro-2-hydroxybenzoyl)-phenylalanine (1 mg/ml in methanol) was diluted ten times with methanol to produce the internal standard (IS). Stock solutions for OTA, OTB, Br-OTB and OTα were made by dissolving the pure compound (20 mg) in methanol (10 ml). A dilution series (1.0; 0.2; 0.02; 0.4; 0.1 and 0.01 mg/1.5 ml) of the stock solutions were made, IS was added and used for injection onto the HPLC. The mobile phase of the HPLC method was changed to methanol/water/acetic acid (60:50:2) to lengthen the retention times and to obtain better separations (see **Figure 9**).

A non-linear standard curve was obtained for all four of the compounds (see **Figure 10** as a typical example). It is of importance to note here that these standard curves were constructed to quantify relatively high levels of ochratoxins present in the *A. ochraceus* cultivated wheat extracts (up to 10 mg/g wheat see **Chapter 4**).

Linear standard curves were constructed for the quantification of the ochratoxins in later Chapters (see **Figure 11**). The limit of detection was 0.5 ng OTA per injection with a signal to noise ratio (S/N) of 3 and the limit of quantitation was 2 ng OTA. The limits of quantitation were determined by adding 10 standard deviations of the blank to the blank signal and adjusting for the standard deviation of the standard curve.

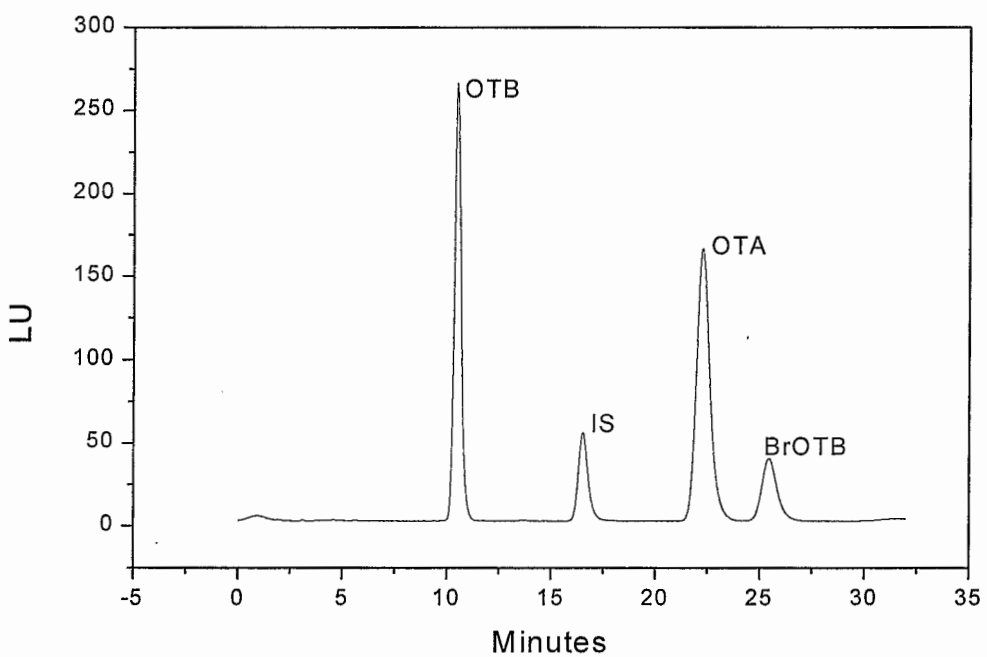


Figure 9: An HPLC chromatogram of a mixture of OTB, IS, OTA and Br-OTB with a mobile phase of methanol/water/acetic acid (60:50:2).

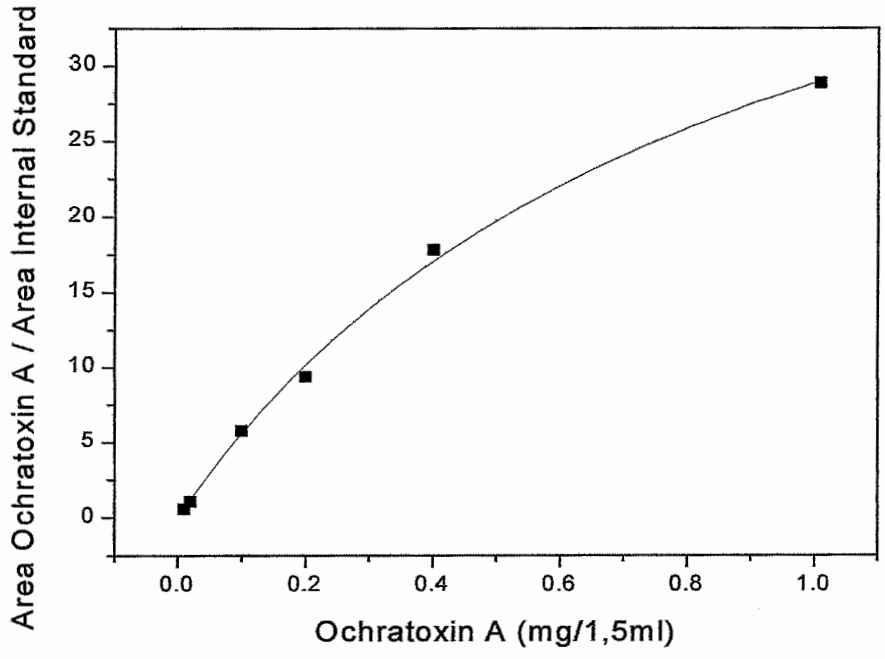


Figure 10: Standard curve of OTA for HPLC analysis with N-(5-chloro-2-hydroxybenzoyl)-phenylalanine as internal standard.

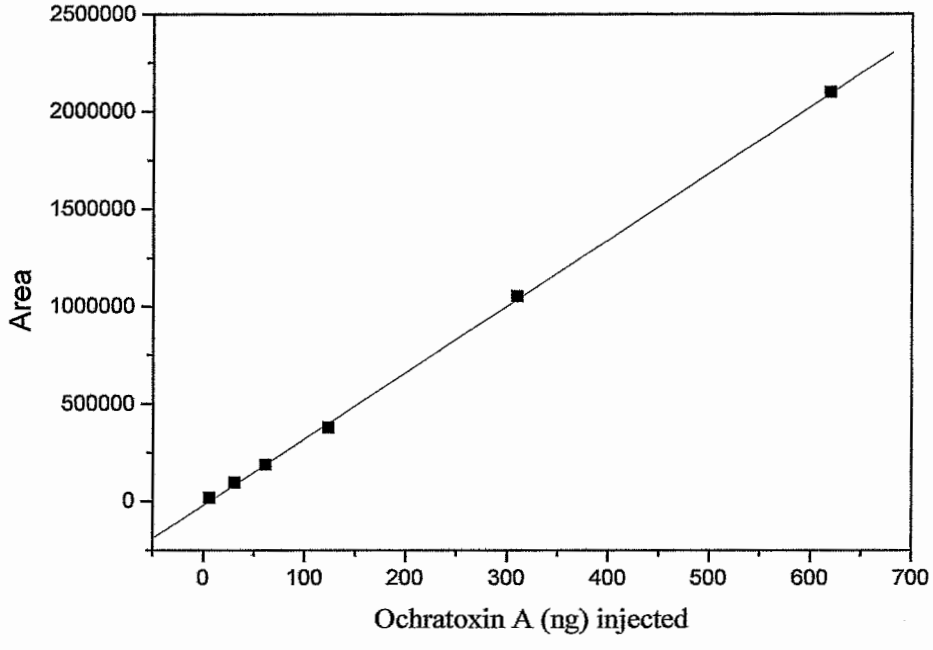


Figure 11: Linear standard curve of HPLC-peak areas versus the amount of OTA injected.

Determination of the percentage recovery

There are two ways to determine the percentage recovery of the analyte in question from a matrix. Wheat with no OTA or with a known level of OTA can be spiked with an OTA standard and the OTA can be extracted and the levels determined; alternatively wheat can be cultivated with OTA producing fungi and the OTA levels determined. The latter seems to be the more scientifically correct way, owing to the similarity of this method to the one used in our experiments. If wheat is spiked with OTA, it stays on the surface of the wheat kernels and it is recovered much easier, and unnaturally high recoveries may be obtained.

Procedure

Cultivated wheat (see above for procedure), (2 x 40g) was transferred to two beakers (600 ml), methanol/chloroform (1:1, 200 ml) was added and it was milled for 10 min at 3000 rpm sealed and left for 24 hours. The wheat extracts were vacuum filtered with Whatman No. 1 filter paper. The flask and filter pad were rinsed with methanol/chloroform (1:1, 40 ml). The filter cake and filter paper were quantitatively transferred to a specially cleaned beaker.

The extracts were then transferred to volumetric flasks (250 ml) and filled with methanol/chloroform (1:1). The LC-NH_2 SPE columns were conditioned with methanol /chloroform (1:1, 2.5 ml), the filtrates (2 ml) were placed on the columns and allowed to flow at ± 2 drops per second. The SPE columns were washed with chloroform (2.7 ml) and allowed to run completely dry. The ochratoxins were eluted from the columns with methanol/acetic acid (4:1, 2.5 ml) and collected into test tubes. The eluant (1.5 ml) of the SPE extraction was transferred to the autosampler vials and the IS solution (100 µl, see above) was added prior to HPLC analysis.

Repeat extraction: Methanol/chloroform (1:1, 200 ml) was added to the filter cake and filter paper, it was milled, left for 24 hours, filtered, the filter paper and filter cake transferred to a new beaker and the whole extraction process was repeated. This re-extraction process was repeated four times, and all four of the extracts were analysed for OTA.

The percentage recovery was determined as follows:

$$[OTA]_{Total} = [OTA]_{First\ extraction} + [OTA]_{Second\ extraction} + [OTA]_{Third\ extraction} + [OTA]_{Fourth\ extraction}$$

% Recovery = $\{[OTA]_{First\ extraction} / [OTA]_{Total}\}$ x 100

Results

The results showed an exponential decrease in OTA concentration (See **Figure 12**). The ochratoxin content of the fifth extraction was too low to detect under the current HPLC conditions. A percentage recovery of 78.4% was obtained for the first set of data and 79.7 % for the repeat of the experiment. It was concluded that only one extraction of OTA was sufficient; this protocol was followed throughout the study.

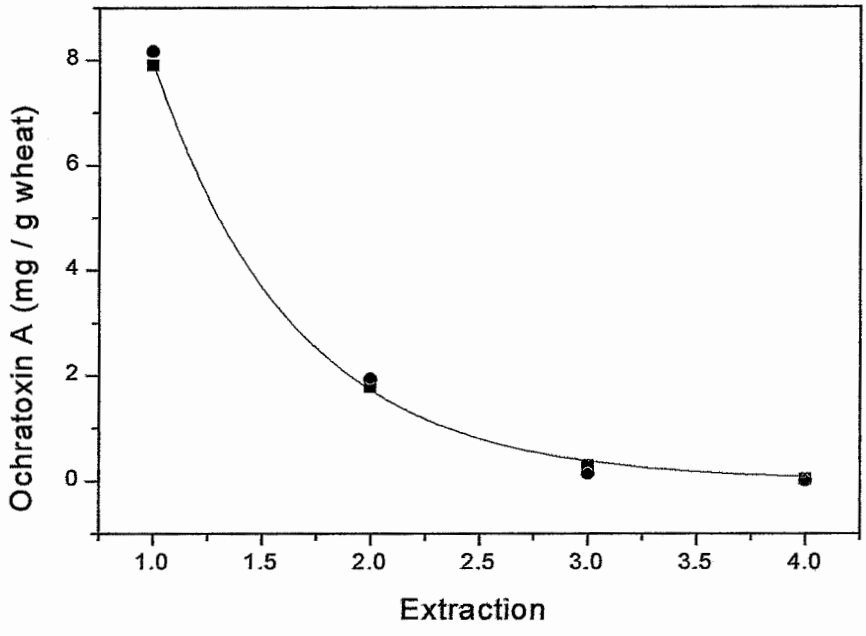


Figure 12: Exponential decrease of OTA versus the number of extraction of the wheat.

Determination of the percentage recovery of the SPE columns

Procedure

The LC-NH_2 SPE columns were conditioned with methanol/chloroform (1:1, 2.5 ml), the standard OTA solution (2 ml, 0.026 mg/ml, n = 6) were placed on the columns and allowed to flow at ± 2 drops per second. The SPE columns were washed with chloroform (2.7 ml) and allowed to run completely dry. OTA was eluted from the columns with methanol/acetic acid (4:1, 2.5 ml) and collected into test tubes. The eluant (1.5 ml) of the SPE extraction was

transferred to autosampler vials and IS stock solution (100 µl) was added prior to duplicate analysis on HPLC.

Results

The recovery for OTA was 97%± 2% relative standard deviation (n = 6).

CONCLUSIONS

The following conditions were decided on for ochratoxin analysis of *A. ochraceus* cultivated wheat:

- One extraction with methanol/chloroform (1:1)
- Cleanup with LC-NH_2 SPE columns by using chloroform in the washing step and methanol/acetic acid (4:1) in the elution step
- HPLC analysis on a reversed phase column (C_{18}), employing N-(5-chloro-2-hydroxybenzoyl)-phenylalanine as internal standard

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