CHAPTER 11

Final conclusions: Studies on the metabolism of Ochratoxin A

The aims of the research work presented in **Chapter 1** were achieved in the following ways:

❖ Comprehensive reviews with special emphasis on the toxic effects which are of relevance for the understanding of the human pathologies and risk associated with the occurrence of mycotoxins in food commodities form the substance of **Chapters 2** and **3**. **Chapter 2** is primarily dedicated to the carcinogenic mycotoxins: Aflatoxins, ochratoxins and fumonisins. This chapter is an updated form of a review article that was published by Macmillan in the second edition of *General and Applied Toxicology*, pp. 2145-2176 edited by Ballantyne, Marrs and Syversen in 1999. **Chapter 3** concentrates on toxic effects, biochemical mechanism of action and the diseases caused by these mycotoxins viz. primary liver cancer (aflatoxins), Balkan endemic nephropathy and Chronic interstitial nephropathy (ochratoxin A), human oesophageal cancer (fumonisins) and also mycotoxicoses caused by other mycotoxins *viz.* alimentary toxic aleukia (T-2 toxin), ergotism (ergotamine and related alkaloids) etc. This chapter was communicated by Prof. Steyn at the Third Symposium of Monastir, Tunisia: Environmental Toxicants and Pathologies and was published in *Journal of Toxicology - Toxin Reviews* **18**, 229-244 (1999).

❖ In **Chapter 4** the first time biosynthesis of bromo-ochratoxin B was related by fermentation of *Aspergillus ochraceus* inoculated Durum wheat, supplemented with high levels of potassium bromide (1.5 g / 40 g wheat). Elevated chloride concentrations (1 g / 40 g wheat) increased the production of OTA by *Aspergillus ochraceus*. This technology could improve the commercial production of OTA markedly. It was found that supplementation of wheat with potassium iodide and potassium fluoride (>50 mg halogen salt / 40 g wheat) inhibited the growth of the fungi and subsequently the production of the ochratoxins. In addition, a number of minor metabolites e.g. ochratoxin α (OTα), ochratoxin β (OTβ), (4*R)*-4-hydroxyochratoxin B [(4*R*)-OH-OTB], (4*R*)- and (4*S*)-4-hydroxyochratoxin A [(4*S*)-OH-OTA and (4*R*)-OH-OTA], and citrinin, were identified in the *Aspergillus ochraceus* inoculated Durum wheat, supplemented with high levels of

potassium bromide (>1.5 g / 40 g wheat). Analysis included amino-propyl solid phase extraction column cleanup, followed by quantitative analysis on reversed phase HPLC using fluorescence detection and employing N-(5-chloro-2-hydroxybenzoyl)-phenylalanine as internal standard, whereas high field NMR spectroscopy, mass spectrometric and retention time comparison studies on HPLC enabled the identification of metabolites. This chapter was accepted for publication in the *Journal of Agricultural and Food Chemistry*, a publication of the American Chemical Society.

❖ The preliminary results of a survey on the ochratoxin A content of coffee on the South African retail market are presented in **Chapter 5**. Soluble coffee and roasted coffee beans were purchased on the local market and analysed for OTA using aminopropyl- solid phase extraction column clean-up and quantitation with reversed phase HPLC. This is to our knowledge the first report on the use of this type of solid phase extraction columns for the analysis of ochratoxin A. Preliminary results suggest that the levels of OTA in coffee on the South African market are slightly higher than on the European market. The levels varied between undetectable to 40 ng OTA per gram coffee with the highest levels of OTA found in some of the less expensive brands of instant coffee. The detection limit of the HPLC method was 0.5 ng per injection. The average level of OTA for all the coffees analysed was 6 ng/g. It is of importance to note that these findings constitute the first report on the natural occurrence of OTA on a food commodity in South Africa, despite the early discovery of OTA by the South African group of van der Merwe and coworkers in 1965.

❖ Yeasts (323 different strains) were screened as a resting cell suspension for OTA degradation, and the results reported in **Chapter 6**. Seven of these yeasts proved to degrade OTA, of which a growing culture of *Trichosporon mucoides* degraded OTA most effectively within 48 hours to OTα. The degradation of OTA by the yeast, *Trichosporon mucoides* was studied using HPLC with fluorescence detection. The success achieved with the studies involving yeasts, particularly *Trichosporon mucoides* stimulated us to extend the study to a few other fungi, *Cochliobolus sativus* (MRC 10870), *Penicillium islandicum* (MRC 1583) and *Metarhizium anispoliae* (MRC 11853) [obtained from the culture collection of the CSIR, Pretoria]. All three of the fungi showed a promising ability to degrade OTA although the reaction time is unacceptably long.

❖ Twenty-three commercial lipases were screened for the ability to degrade OTA to non-toxic compounds. The lipase from *Aspergillus niger* from the supplier, Amano proved to have the ability to metabolically degrade OTA to the substantially non- toxic OTα and phenylalanine, as illustrated by HPLC with fluorescence detection. The lipase was also partially purified using anion-exchange column chromatography. This project was undertaken at the Institut für Technische Biochemie, Universität Stuttgart, Stuttgart, Germany, under supervision of dr. Uwe Bornscheuer. The results of **Chapter 7** were submitted for publication to *Toxicology and Applied Pharmacology*.

❖ **Chapter 8** focussed on the hydrolysis of OTA and its halogenated analogues by carboxypeptidase A. A number of OTA analogues was synthesized by coupling reactions with the amino acid, phenylalanine and different halogen containing salicylic acid derivatives. The synthesized compounds were fully characterized with ^{1}H and ^{13}C NMR spectroscopy, infrared- and UV spectroscopy and mass spectrometry. The halogen containing analogues had lower turnovers than their des-halo analogues. This confirmed the results of Doster and Sinnhuber (1972, *Fd. Cosmet. Toxicol.* **10**, 389-394) that OTA is hydrolysed much slower by carboxypeptidase A than its, less toxic des-halogen analogue, OTB. No significant differences were observed in the rate of hydrolysis of OTA and Br-OTB by carboxypeptidase A that could provide insight in the difference of their toxicity. This chapter was submitted for publication to *Toxicology and Applied Pharmacology*.

❖ The toxicokinetics of ochratoxin A was investigated in vervet monkeys (*Cercopithecus aethiops*) in **Chapter 9**. The project was done in conjunction with the PROMEC group at the Medical Research Council, Tygerberg, Dr. G. Shephard, Ms. T.W. Nieuwoudt, Dr. J. Seier and Dr. V. Sewram; and Prof. E.E. Creppy, University of Bordeaux II, France. Three female monkeys were intravenously administered with 0.8 mg, 1.5 mg and 2 mg ochratoxin A per kg body weight. Blood and urine were collected over a period of 21 days. Kidney function of the monkeys remained unaffected as monitored by measuring the chemical pathology parameters of the plasma. Plasma and urine extracts were analysed by liquid chromatography coupled to negative ion electrospray ionisation mass spectrometry and reversed phase high performance liquid chromatography equipped with fluorescence detection. The elimination half-life of OTA in the monkeys was determined to be 19-21 days and the average total body clearance was 0.22 ± 0.7 ml/h.kg and, the

average apparent distribution volume of the central compartment 59 ± 9 ml/kg and of the peripheral compartment was 59 ± 20 ml/kg. The toxicokinetic data obtained from the study into the metabolism of OTA in vervet monkeys contributed to the calculation of the biological half-life of OTA in humans. These results are important for the establishment of acceptable daily intake rates of OTA for humans and animals and shed some light on the occurrence of ochratoxin-related diseases in humans. This chapter was recently submitted for publication to *Toxicology and Applied Pharmacology*, part of the work will also be presented at the IUPAC Symposium on Mycotoxins and Phycotoxins in Brazil during May 2000.

❖ **Chapter 10** focussed on the methodologies developed for the analysis of the ochratoxins. A harvest time of 14 days following inoculation proved to be optimal for OTA production on Durum wheat under the growth conditions indicated in **Chapter 10**. Extraction with chloroform/methanol (1:1) followed by cleanup with LC-NH$_2$ SPE columns by using chloroform in the washing step and methanol/acetic acid (4:1) in the elution step proved the best extraction conditions for the ochratoxins in wheat. HPLC analyses were done on a reversed phase column (C$_{18}$), employing N-(5-chloro-2-hydroxybenzoyl)-phenylalanine as internal standard.

In conclusion, the dissertation, entitled Studies on the Metabolism of Ochratoxin A provided seminal findings on the development of techniques for the analysis of the ochratoxins and their metabolites at the nanogram level, the said analytical methodology was applied to the metabolism of ochratoxin in micro-organisms (fungi and yeasts), enzymes (carboxypeptidase A and lipase) and in non-human primates (vervet monkeys). In addition the bioproduction of bromo-ochratoxin B was achieved, as to serve as a model substance in the structure function studies. Attempts were consistently made to explain the findings in terms of the observed differences in toxicity of the various ochratoxins and their role in human ochratoxicosis.