Studies on the Metabolism of Ochratoxin A

A thesis submitted in fulfilment of the requirements for the degree Ph.D. (Chemistry) of the Potchefstroom University for Christian Higher Education.

by

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Aan my ouers

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Opsomming

Sleutelterme: Ochratoksien A, Karboksipeptidase A, Bromo-ochratoksien B, Toksikokinetika, detoksifisering, elektrosproei-ionisasiemassaspektrometrie, Aminopropiel-soliedefase-ekstraksie.

Die ochratoksiene is sekondêre metaboliete van verskeie *Aspergillus* en *Penicillium* spesies en is die eerste groep mikotoksiene wat ontdek is na die opspraakwekkende ontdekking van aflatoksien. Ochratoksien A (OTA) is 'n belangrike mikotoksien omdat dit dikwels in die natuur voorkom en niersiektes in varke (Danish porcine nephropathy) en pluimveë veroorsaak. OTA word ook geïmpliseer as die oorsaak van soortgelyke siektes in mense ('Balkan endemic nephropathy' en urienweg-gewasse in Noord-Afrika). **Hoofstukke 2** en **3** beklemtoon die belangrikheid van OTA en die navorsing wat tans op mikotoksiene gedoen word. Daar word gefokus op die molekulêre genetika van fungi; die meganisme van aksie van die mikotoksiene; verskille in die metabolisme en farmakokinetika van verskillende diere; kwantifisering van mikotoksiene; die beraming van die risiko wat blootstelling aan mikotoksiene op mens en dier kan hê en regulasies vir die beheer van mikotoksienkontaminasie.

Metodes is in **Hoofstuk 10** beskryf om die toksien in lae vlakke in verskillende matrikse te meet deur gebruik te maak van omgekeerde fase hoëdruk-vloeistofchromatografie met fluoresensie deteksie en tandem vloeistofchromatografies-massaspektrometriese tegnieke. Aminopropielsoliedefase-ekstraksiekolomme is vir die eerste keer gebruik in die monstervoorbereidingsstappe van ochratoksienanalises. Hierdie tegnieke en metodes is toegepas in 'n opname om die omvang van OTA-kontaminasie in koffies op die Suid-Afrikaanse mark te bepaal. Die voorlopige resultate dui daarop dat die vlakke van OTA effens hoër is op die Suid-Afrikaanse mark as op die Europese mark (**Hoofstuk 5**).

'n Studie is onderneem om verskillende halogeen-ochratoksienderivate biologies te produseer en om die invloed van verskillende halogeensoute op die produksie van die ochratoksien deur *Aspergillus ochraceus* te ondersoek. Broom-ochratoksien B, die broombevattende analoog van OTA is vir die eerste keer biologies geproduseer. Daar is gevind dat verhoogde vlakke van kaliumchloried in die groeimedium die produksie van OTA deur *Aspergillus ochraceus* verhoog. Hierdie ontdekking kan die opbrengste van OTA in die kommersiële produksie van ochratoksiene vir gebruik in biologiese navorsing as standaarde aansienlik verhoog. Die

verryking van die koringmedium met kaliumfluoried en kaliumjodied het die skimmel vergiftig en geen jodo- of fluoro-ochratoksien B is geproduseer nie (**Hoofstuk 4**).

Die struktuur-funksie verwantskappe van OTA is ondersoek deur die kinetika van die hidrolise van die molekuul en struktuuranaloë deur karboksipeptidase A, te vergelyk deur van 'n vloeistofchromatografies-massaspektrometriese tegniek gebruik te maak. Daar is gevind dat die hidrolise baie meer effektief is in die *des*-halogeen verbindings en dat daar nie 'n groot onderlinge onderskeid in die kinetika van hidrolise van die verskillende halogeenbevattende verbindings is nie (**Hoofstuk 8**).

Die toksikokinetika van OTA is vir die eerste keer in blou-apies bepaal. Die eliminasie van die toksien in die plasma dui op 'n tweekompartement-model en die eliminasiehalfleeftyd is vasgestel as 19-21 dae vir blou-apies. Die halfleeftyd van OTA in die mens is wiskundig bereken as 46 dae en daar is tot die gevolgtrekking gekom dat die inname van OTA-gekontamineerde voedsel oor lang tydperke, 'n kumulatiewe opbou van potesieel gevaarlike gifstowwe in die liggaam kan veroorsaak (**Hoofstuk 9**), dié hipotese word gesubstansieer deur die voorkoms van OTA in die bloed van verskeie bevolkingsgroepe.

Daar is ondersoek ingestel na moontlik maniere om ochratoksienkontaminasie biologies deur giste, skimmels of lipases te bekamp deur die OTA-molekule na nie-giftige afbraakprodukte te metaboliseer. Daar is vir OTA-afbraak getoets op 323 giste, 8 skimmels en 23 lipases. 'n Lipase van *Aspergillus niger* is die eerste bewys van 'n lipase wat OTA kan afbreek (**Hoofstuk 7**). Vier giste is ook gevind wat OTA kan afbreek waarvan, een spesie, *Trichosporon mucoides* in 'n groeikultuur die OTA aansienlik afbreek binne 48 uur. (**Hoofstuk 6**). Hierdie is ook die eerste bewys van giste wat OTA kan afbreek. Daar is gevind dat die fungi, *Cochliobolus sativus*, *Penicillium islandicum* en *Metarhizium anispoliae* ook in staat is om OTA af te breek. In al die gevalle is OTA na die nie-giftige ochratoksien α en die aminosuur, fenielalanien afgebreek.

Summary

Keywords: Ochratoxin A, Carboxypeptidase A, Bromo-ochratoxin B, Toxicokinetics, Decontamination, Electrospray ionization-mass spectrometry, Aminopropyl solid phase extraction.

The ochratoxins, metabolites of certain *Aspergillus* and *Penicillium* species are the first group of mycotoxins discovered subsequent to the epoch-making discovery of the aflatoxins. Ochratoxin A (OTA) is a very important mycotoxin owing to its frequent occurrence in nature, its established role in Danish porcine nephropathy and in poultry mycotoxicoses and its implicated role in Balkan endemic nephropathy and urinary system tumors among population groups in North Africa. **Chapters 2** and **3** highlight the importance of OTA and the research currently being done on mycotoxins. These efforts are focused on the molecular genetics of toxinogenic fungi; the mechanism of their action; species differences in metabolism and pharmacokinetics; quantification of mycotoxins; risk assessments on the exposure of man and animals to mycotoxins and regulations for the control of mycotoxin contamination.

Methods developed to analyse OTA in different matrices by using reversed phase high performance-liquid chromatography with fluorescence detection and tandem liquid chromatography-mass spectrometry techniques are described in **Chapter 10**. Amino propyl solid phase extraction columns were used for the first time in cleanup steps of ochratoxin analysis. These techniques and methods were applied to the first survey on the levels of OTA in coffee on the South African retail market (**Chapter 5**). The results suggest that the levels of OTA in the coffee on the South African market are somewhat higher than the levels of OTA in coffees on the European market.

The possibility to biologically produce different halogen-ochratoxins by supplementing the growth medium of *Aspergillus ochraceus* with halogen salts was investigated. Bromo-ochratoxin A was produced for the first time in this way. Supplementation of inoculated wheat with potassium iodide and –fluoride resulted in the poisoning of the yeast and no iodo- or fluoro-ochratoxin B was produced. It was found that *Aspergillus ochraceus* produced OTA in higher yields at elevated levels of potassium chloride. This finding has important commercial applications in the production of OTA (**Chapter 4**).

The ochratoxins are hydrolyzed *in vivo* by carboxypeptidase A. The hydrolysis of the ochratoxins and analogues by carboxypeptidase A was measured *in vitro* in a structure-function relation study by employing mass spectrometric techniques. The kinetic data of the ochratoxins were compared to the values of a number of synthesized structural analogues. It was found that the halogen containing analogues had lower turnovers than their *des*-halo analogues. There were no substantial differences in the kinetic data between the different halogen containing analogues (**Chapter 8**).

The toxicokinetics of OTA in vervet monkeys were determined for the first time. The clearance of OTA from the plasma suggested a two-compartment model and the elimination half-life was determined to be 19-21 days. The half-life of OTA in humans was determined by allometric calculations to be 46 days. We came to the conclusion that the long term consumption of OTA contaminated foods will lead to potentially hazardous levels of the toxin in the body (**Chapter 9**). This hypothesis can be substantiated by the incidence of OTA in the blood of various population groups.

Possible ways to decontaminate OTA contaminated foods by degrading the compound biologically with yeast; moulds or lipases to non-toxic compounds were investigated. Eight moulds, 323 yeasts and 23 lipases were screened for ochratoxin degradation. A lipase from *Aspergillus niger* is the first lipase that was proven to degrade OTA (**Chapter 7**). Four yeasts were found to degrade OTA of which one, *Trichosporon mucoides* degraded OTA substantially within 48 hours in a growing culture (**Chapter 6**). In addition to this first report of yeasts which have the ability to degrade OTA, the fungi *Cochliobolus sativus*, *Penicillium islandicum* and *Metarhizium anispoliae* also proved to degrade OTA. OTA was degraded in all instances to the non-toxic ochratoxin α and the amino acid phenylalanine.

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List of Acronyms and Abbreviations

APCI	Atmospheric pressure chemical ionization
BEN	Balkan endemic nephropathy
Br-OTB	Bromo-ochratoxin B
C_{max}	Maximum measured value
C_p	Plasma concentration
CSIR	Council for Science and Research, Pretoria
ES-MS	Electrospray mass spectrometry
GC-MS	Gas chromatography-mass spectrometry
HPLC	High performance liquid chromatography
IEF	Iso-electric focussing
IS	Internal standard
JECFA	Joint Expert Committee on Food Additives
LEM	Leucoencephalomalacia
LC-MS	Liquid chromatography-mass spectrometry
MeOH	Methanol
Me-OTA	Ochratoxin A methyl ester
Me-OTB	Ochratoxin B methyl ester
MRC	Medical Research Council, Tygerberg
OTα	Ochratoxin α
OTβ	Ochratoxin β
OTA	Ochratoxin A
OTB	Ochratoxin B
OTC	Ochratoxin C
Phe	L-β-Phenylalanine
PMSF	Phenylmethylsulfonyl fluoride
RSD	Relative standard deviation
S/N	Signal to noise ratio
SDS	Sodium dodecyl sulphate
SPE	Solid Phase Extraction
$t_{1/2}\beta$	Elimination half-life
$t_{1/2}\alpha$	Distribution half-life
TLC	Thin layer chromatography
Tris	Tris(hydroxymethyl)aminomethane
UV	Ultra violet

Papers that emanated from this dissertation

- Steyn, P.S., and Stander, M.A. (2000). Mycotoxins with special reference to the carcinogenic mycotoxins: aflatoxins, ochratoxins and fumonisins. In Ballantyne B, Marrs TC and Syversen T (eds): *General and Applied Toxicology*, MacMillan Reference Ltd, London, pp. 2145-2176.
- Steyn P.S. and M.A. Stander, (1999). Mycotoxins as causal factors in diseases of humans, *J.Toxicol.-Toxin Reviews*. **18** (3,4), 229-244.
- Stander, M.A., Steyn, P.S., Lübben, A., Mantle, P.G., Miljkovic, A., and Marais, G. (2000). Influence of halogen salts on the production of the ochratoxins by *Aspergillus ochraceus* Wilh., submitted to *Journal of Agricultural and Food Chemistry*.
- van der Westhuizen, F.H., Stander, M.A., Steyn, P.S., and Payne, B.E. (2000). A Kinetic study into the Hydrolysis of the Ochratoxins and Analogues by Carboxypeptidase A, submitted to *Toxicology and Applied Pharmacology*.
- Stander, M.A., Nieuwoudt T.W., Steyn, P.S., Shephard, G.S., Creppy E.E. and Sewram, V. (2000). Toxicokinetics of ochratoxin A in vervet monkeys, submitted to *Toxicology and Applied Pharmacology*.
- Stander, M.A., Bornscheuer, U., Henke, E. and Steyn, P.S. 2000, Screening of commercial lipases for the degradation of ochratoxin A, submitted to *Toxicology and Applied Pharmacology*.
- Stander, M.A. and Steyn, P.S. Survey of ochratoxin A content in coffee on the South African retail market, to be submitted.
- Steyn, P.S., Stander, M.A., van Rooyen, T., and Smit, M.S. The metabolic degradation of ochratoxin A by yeasts, to be submitted.

Conferences

- Presented a poster at Franck Warren Conference on ochratoxin A (Natal, 1997).
- Presented a paper at the NABSA Conference on ochratoxin A (Gaborone, 1997).
- Will present papers at the X International IUPAC Symposium on Mycotoxins and Phycotoxins (Guarujá, Brazil, May 2000) on Chapters 4 and 9.

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