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New Challenges In The Determination Of Food Contaminants

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Declaration

I, the undersigned, certify that this thesis submitted for the degree of PhD in chemistry is the result of my own research, except where otherwise acknowledged, and that this thesis (or any part of the same) has not been submitted for a higher degree to any other university or institution.

Signature Mohamed Attya Date 01/12/2009

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List of papers

1) Mohamed. Attya, Leonardo Di Donna, Bartolo Gabriele, Alessia Fazio, Fabio Mazzoti, Giovanni Sindona; A New method for the quantification of ochratoxin A in foods by HPLC-FLD and diastereoisomeric dilution assay; *manuscript (in preparation).*

2) Fabio Mazzoti, Leonardo Di Donna, Mohamed. Attya, Bartolo Gabriele, Alessia Fazio, , Giovanni Sindona; Isotope dilution method for the assay of rotenone in olive oil and river waters by liquid chromatography-multiple reactions monitoring tandem mass spectrometry; *Rapid Commun. Mass. Spectrometry, 2009, 23, 3803-3806*

3) Bartolo Gabriele, Mohamed Attya, Alessia Fazio, Leonardo Di Donna, Pierluigi Plastina, Giovanni Sindona; a new and expedient total synthesis of ochratoxin A and d_5-ochratoxin A; *Synthesis 2009;1815-1820.*

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1) Mohamed. Attya, Leonardo Di Donna, Bartolo Gabriele, Alessia Fazio, Fabio Mazzoti, Giovanni Sindona; Isotope dilution and Multiple Reaction Monitoring Mass Spectrometry in the identification and assay of rotenone; INTERNATIONAL CONGRESS “THE CENTENARY” Padova, August 31-September 4, 2009; p112

2) Attya M. , Di Donna L. , Gabriele B. , Fazio A. , Mazzotti F. , Sindona G. , " A new method for the quantification of ochratoxin A in foods by HPLC-FLD and diaetereoisomeric dilution method". Atti del convegno "XXIII Congresso Nazionale della Società Chimica Italiana", SORRENTO (NA), 2009, pp. 96-96.

3) Veltri L. , Gabriele B. , Salerno G. , Vetere M. , Attya M. , Mancuso R. , Plastina P. , " A novel synthesis of funcionalized pyrroles by a PdI_2-catalyzed oxidative carbonylation approach". Atti del convegno "XXIII Congresso Nazionale della Società Chimica Italiana", Sorrento (NA), 2009, 2009, pp. 277-277.

4) Gabriele B. , Attya M. , Fazio A. , Di Donna L. , Sindona G. , " New total synthesis of [$^2H_5$]- ochratoxin A". Proceedings "15th Workshop on the Synthesis and Applications of Isotopes and Isotopically Labelled Compounds", Bad Soden (Germany), 12-13 June, 2008.

5) Vetere M., Attya M., Gabriele B. , Mancuso R. , Plastina P. , Salerno G. , Veltri L. , " New synthesis of functionalized pyrroles-carbonyl heterocyclic by Pd-catalyzed oxidative ". Proceedings "IV Convegno Congiunto delle Sezioni Calabria e Sicilia della Società Chimica Italiana", RENDE (CS), 1-3 dicembre, 2008, pp. P79.

6) Gabriele B. , Attya M. , Fazio A. , Di Donna L. , Sindona G. , " Nuova sintesi totale di orcatossina e d5-ocratossina". Proceedings "7° Sigma Aldrich Young Chemists Symposium", Riccione (RN), October 22-24, 2007, pp. P7-P7.

Abbreviations

AOAC	Association of Official Analytical Chemists
ATP	Adenosine triphosphate
*a*w	Water activity
BEN	Balkan Endemic Nephropathy
CAC	Codex Alimentarius
CCFAC	Codex Committee on Food Additives
CCP	Critical Control Point
CEN	European Standardization Committee
CLSM	Confocal Laser Scanning Microscopy
EC	European Comission
EEC	Council Regulation
CICYT	Comisión Interministerial de Ciencia y Tecnología
CYA	Czapek Yeast Extract Agar
CZ	Czapek Dox Agar
DL	Detection Limit
DNA	Deoxyribonucleic acid
D.O.	Designation of Origin
DOCa	Qualified Designation of Origin
DRBC	Dichloran Rose Bengal Chloramphenicol agar
EC	European Commission
EEC	Council Regulation in the EU
e.g.	example given
ELISA	Enzyme-linked immunosorbent assay
et al.	From the latin phrase *et alii* meaning *and others*

EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FDA	Food and Drug Administration.
FD	Fluorescence detection
FB1	fumoni sin B 1
FB2	fumoni sin B2
FB3	fumoni sin B3
FB4	fumoni sin B4
FID	flame ionization detector
FLD	fluorescence detection
FUSX	fusarenone X
GC	gas chromatography
GAP	Good Agricultural Practices
HFB	heptafluorobutyryl
HFB 1	hydrolized fumonisin B 1
HOBt	hydroxyl benzo triazole
HMF	hydroxymethyl-2-furaldehyde
HPLC	high pressure liquid chromatography
HR	high resolution
IAC	immunoaffinity clean-up
IARC	International Agency for Research on Cancer
ICFM	International Commission on Food Mycology
ISO	International Organization for Standardization
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LD50	Lethal Dose which given all at once, will causes the death of 50 % of a group of test animals
LC	liquid chromatography
LDA	lithium di isopropyl amide

LOD	limit of detection
LOQ	limit of quantification
MAS	monoacetoxyscirpenol
MRM	multiple reaction monitoring
MS	Mass Spectrometry
NNT	Nordic Working Group on Food Toxicology and Risk Evaluation.
NOAEL	No Observed Adverse Effect Level
OTA	Ochratoxin A
OTB	Ochratoxin B
OTC	Ochratoxin C
Otα	Ochratoxin α
PEG	Polyethylene glycol
Phe	Phenylalanine
PCR	Polymerase Chain Reaction
MSPD	matrix solid phase dispersion
NCM	N-carboxymethyl
NEO	neosolaniol
OPA	orto-phthaldialdehyde
PB	particle beam
PDA	photodiode array
PFP	pentafluoropropionyl
PLE	pressurized liquid extraction
RSD	relative standard deviation
RP	Reversed phase
SCF	Scientific Committee for Food
SCOOP	Scientific Cooperation on Questions Relating to Food Projects
SPE	Solid Phase Extraction

SD	standard deviation
SIDA	stable isotope dilution assay
SIM	single ion monitoring
SRM	selected reaction monitoring
TLC	Thin Layer Chromatography
UNEP	United Nations Environment Programme
USA	United States of America
UT	Urothelial Tumours
UV	Ultraviolet radiation
VCPRD	Quality Wines Produced in a Specific Region
WHO	World Health Organization
TF	trifluoroacetyl
TFA	trifluoroacetic acid
TMS	trimethylsilyl
ZAL	zearalanol
ZAN	zearalanone
ZEN	zearalenone
ZOL	zearalenol

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Isotope dilution method for the assay of rotenone in olive oil and river waters by liquid chromatography-multiple reactions monitoring tandem mass spectrometry ***Rapid Commun. MASS. Spectro., 2009, 23, 3803-3806*** 107
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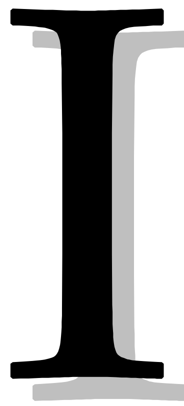

Objectives

1.1 Scope and objectives

1.1.1 general objectives

Cancer is a leading cause of death worldwide and diet is thought to play a substantial role in cancer etiology. A large number of scientific studies and reviews have addressed the potential for dietary components to influence the risk of developing cancer and many other grave diseases. One topic of particular interest has been the impact of food contaminants. Two complementary programs, among others, have reviewed and synthesized information on the carcinogenic potential of food contaminants and judged the degree of evidence linking different food contaminants to the risk of cancer in humans. These programs, the International Agency for Research on Cancer's *IARC* Monographs on the Evaluation of Carcinogenic Risks to Humans and the US National Toxicology Program's Report of Carcinogens have reviewed hundreds of chemicals, mixtures, and natural products and then graded the cancer risk posed to humans.[1,2]

There are 4 primary types of contaminants which can be potentially carcinogenic compounds, or have been directly correlated to adverse health effects in humans. The first are natural products that may be present in food and are unavoidable. Second, they are natural products that might be avoided but not easily such as the contamination of grain with the carcinogenic fungal metabolite myoctoxins (aflatoxins, ochratoxins). Third, naturally or anthropogenic chemicals may be present in food, which can be produced during the manufacture, or can be the result of pesticides residue; for instance rotenone which is a natural pesticide, and it has been investigated as a possible Parkinson-causing agent.[3] A fourth category of concern is anthropogenic chemicals intentionally added to foods, such as saccharin or food additives, but

these are not addressed always as contaminants because they are added intentionally.

The need to establish new rules for legalize the using of food additives or pesticides in agriculture, and to set limits for the assessment of dietary intake of food contaminants, has increased and forced several national and international organizations and agencies like FAO, WHO, USDA, NTP, and IARC to impose and set recommended guidelines, develop standardized assay protocols, and maintain up-to-date information on regulatory statutes.

The complete elimination of any natural contaminants from foods is an unattainable objective. Therefore, naturally occurring toxins such as mycotoxins are regulated quite differently from food additives or pesticides residues. Many governmental agencies around the world test products for food contaminants and have established guidelines for safe doses, but there is a need for worldwide harmonization of these regulations.[4] Unfortunately, sometimes the regulatory community seems to be setting limits based more on current analytical capabilities than on realistic health factors.[5] This fact has stimulated the development of new precious analytical methods able to identify and quantify contaminants in food and feeds, and also to permit their rapid determination at the strict regulation limits established.

The development of new, reliable, applicable, and simple analytical methods for the quantification of toxins in food was the principle goal of my thesis work. In particular, the development of new methods for the quantification of ochratoxin A and rotenone in foods are my work interests.

Ochratoxin A, is a highly toxic metabolite produced by some species of Aspergillus and Penicillium, are commonly found in food and feeds. Ochratoxin A (OTA) is nephrotoxic, hepatotoxic, teratogenic, and carcinogenic in animals. It was recently classified by IARC as a class 2B, possible human carcinogen. There are numerous methods for the determination of OTA in food-stuffs, such as thin layer chromatography (the first applied method), enzyme-linked immunosorbent assay, high performance liquid chromatography, and liquid chromatography–mass spectroscopy. The most widely used technique for analysis of OTA is liquid chromatography with fluorescence detection (LC–FLD), but recently the use of HPLC/MS-MS (tandem mass spectrometry) by employing isotopomers of the analytes as internal standard (staple isotope dilution assay "SIDA")[6], has proved high sensitivity, accuracy, and acceptable results. The success of SIDA as an analytical method was the incentive to develop a new method can have advantage over SIDA. Due to the high cost of the instruments which is needed to carry out SIDA like MS-MS, and the need of special skills; and the high LOD and LOQ which are investigated by it comparing with the others which are investigated by LC–FLD methods. My presented method provides lower cost, simple, applicable method, and have the same advantage of SIDA. The using of identically structure internal standard enhances the specificity of the determination, and that gives SIDA advantage over all the other methods. My presented method, Diastereoisomeric Dilution Assay for the quantification of OTA has involved by using liquid chromatography with fluorescence detection (LC–FLD) by employing the 3S-diastereoisomer of ochratoxin A (3S-OTA) as internal standard. 3S-OTA was synthesized by a new total synthesis, and its synthesis will be reported in this thesis together with a new total synthesis of OTA and d_5-OTA. 3S-OTA shows almost the same chemical and physical properties of wild-type OTA but it can

be discriminated by HPLC by its retention time. The high sensitivity of LC–FLD, and the using of an identically structure internal standard; give my method many advantages over all the methods have been involved in the quantification of OTA in food and feeds including SIDA.

Rotenone is a naturally occurring chemical with insecticidal and pisticidal properties, and it has used for centuries as a selective fish poison and more recently as a commercial insecticide. Rotenone was classified by the USDA National Organic Program as a non-synthetic and was allowed to be used to grow "organic" until 2005 when it was removed from the list of approved substances due to concerns about its safety,[7] also it was investigated as a possible Parkinson-causing agent.[3] Rotenone also has been analyzed using the high techniques methods.[8] But all the methods used for the quantification of Rotenone,[9] were missing the using of SIDA or an identically structure internal standard, which enhances the specificity of the determination. My goal was to develop a new method for the analysis of rotenone having advantages over all the existing methods. The development of SIDA was the best and most reliable choice for the quantification of rotenone by using d_3-rotenone as internal standard, which was synthesized for the first time of the stable isotopomers of rotenone (d_3-rotenone), and it will be reported in this thesis. Due to the high sensitivity and accuracy of SIDA gives our investigated method many advantages over all previous methods already reported for the quantification of rotenone.

1.1.2 Specific objectives

1. Synthesis of ochratoxin A, d_5-ochratoxin A, and Their 3-(S)-diastereoisomer (3-(S)-OTA, 3-(S)-d_5-OTA).
2. Development of a new method for the quantification of OTA in foods and feeds, by the means of LC/FLD, using the 3-(S)-OTA as internal standard.
3. Synthesis of d_3-rotenone.
4. Development of a new method for the quantification of rotenone in olive oil and river water based on stable isotope dilution, using d_3-rotenone as internal standard.

1.2 Structure of the thesis

This thesis is divided in to two basic parts, studying two different food contaminants (Ochratoxin A and Rotenone). I will describe in introduction the different effects on the human health, explaining its chemical and physical properties. Also the toxicology and the mechanism of action in the human body will be shown. A background to give idea about the methods of synthesis and analysis will be described as an introduction before the results and my thesis work.

I will discuss some practical applications and present my original work. The thesis is based on papers published in international journals. These have been edited to provide uniform format and mathematical notation throughout the thesis. However, for practical reasons, the original organization of the references is maintained.

Formally, the thesis is divided into six chapters

- Chapter 1. Description of the thesis contains the justification of this thesis and provides a brief overview of its context. The objectives of the thesis are explained and the structure of its contents is described.
- Chapter 2. Introduction to ochratoxin A, contains a briefly background, its properties, production, toxicology, and the presence of OTA in food.
- Chapter 3. This will start by a background about the previous synthetic methods that involve the Synthesis of ochratoxin A and d_5-ochratoxin A, showing their advantages and disadvantages. Followed by my work results the synthesis of ochratoxin A, d_5-ochratoxin A, and Their 3-(S)-diastereoisomer (3-(S)-OTA, 3-(S)-d_5-OTA), which will be described by published paper form.
- Chapter 4. Introduction to the different analytical techniques and methods which have been reported for the determination of ochratoxin A. Followed by a manuscript form in which I will explain A new method for the determination of ochratoxin A based on high performance liquid chromatography and diastereomeric dilution.
- Chapter 5. Introduction contains a background of the rotenone and its uses, also its properties and toxicity. I will describe also briefly the quantification of rotenone and its analytical methods, coupled with some notes about rotenone residue in food and water. The results and my work will be presented as a paper form with the title ʻʻ Isotope dilution method for the assay of rotenone in olive oil and river waters by liquid chromatography-multiple reactions monitoring tandem mass spectrometry ʼʼ.

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II

Ochratoxin A, Properties, Production, and Toxicology

2.1 Background

Of the myriad of currently known mycotoxins only a handful have been adequately characterized. Even the name is a partial misnomer, originating from the ancient Greek "μνκηϬ" (fungus) "ζοςικου" (arrow-poison) [1]. These substances are secondary metabolites produced by a number of molds, including members of the Aspergillus, Penicillium, Fusarium, Claviceps, and Alternaria families. Of the many proposed reasons for their production, the suggestions of facilitated competition with other microorganisms for nutrients and space and the generation of favorable germination conditions for fungal spores[2,3] are arguably the most likely candidates. Whichever the case may be, mycotoxins have been responsible for large financial losses in conjunction with contaminated and thus unsafe agricultural products as well as being the cause of diseases in both humans and animals. Diverse range of toxicological effects, including renal toxicity, mutagenicity, teratogenicity, neurotoxicity, and immunotoxicity, to name but a few, in both animals and humans. Based on animal studies and epidemiological studies in human populations, OTA has been classified as a class 2B carcinogen (possible human carcinogen) by the IARC.[4] Maximum permissible OTA concentrations of 5 and 3 μg/kg in raw cereals and processed cereal products, respectively, are currently under debate by several countries including the United States[5] and the member states of the European Union.[6-8] Difficulties in the interpretation of the available data and conflicts with respect to standards for commodity products (e.g., coffee, beer, wine, etc.) in international trade are, however, making agreement difficult.

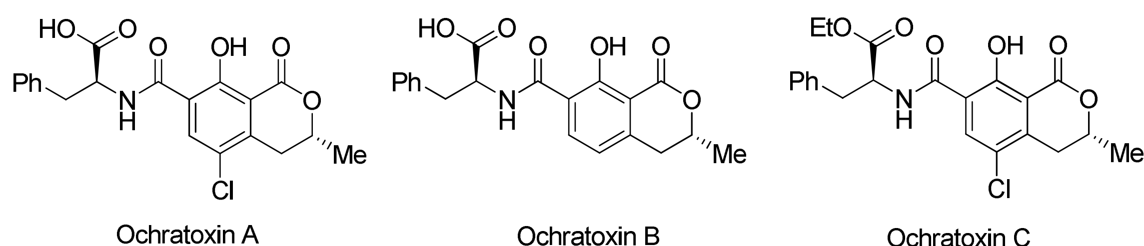
Several newspaper reports[9-11] and articles in popular science and consumer magazines[12-15] have raised public awareness of the potential health risks posed by mycotoxins and, in particular, by ochratoxin contamination of

human and animal foodstuffs, making the elucidation of the mechanisms of action and hence a more reliable risk assessment imperative.

2.2 Production and Properties

The family of ochratoxins consists of three members, A, B, and C (Figure 2.1), which are produced by several molds of the Aspergillus and Penicillium species (in particular Aspergillus ochraceus) under suitable conditions of temperature (21-28°C and 25-28°C for Penicillium and Aspergillus species, respectively) and humidity (aw > 0.7).[16-18] This can lead to field and storage contamination of, for example, of maize and grain products and via a carryover effect of meat, in particular pork. Ochratoxins are relatively heat stable; baking and roasting reduce the toxin content by a mere 20%, while boiling has no effect.[19,20] Due to the ubiquitous nature of the producing fungi, ochratoxins are found regularly as contaminants of animal fodder and human provisions as diverse as muesli, coffee, and wine.[21] Indeed, the consumption of certain red wines has been shown to surpass the recommended virtually safe dose by a factor of 20,[9] and total avoidance of ochratoxin consumption is practically impossible.

Figure 2.1: The structure of ochratoxins (A, B, C)



Chemically, ochratoxins are weak organic acids consisting of a dihydroisocumarin moiety joined by a peptide bond to L-phenylalanine (Figure

2.1). Structurally, the three toxins differ only very slightly from each other; however, these differences have marked effects on their respective toxic potentials, with ochratoxin A (OTA) being both the most commonly detected and the most toxic of the three. Substitution of chloride for a hydrogen atom in the isocumarin moiety yields ochratoxin B (OTB), which is significantly (10- to 20-fold) less toxic both in vivo and in vitro. Further structural alterations yield ochratoxin C (OTC), which is generally perceived as having little or no toxic potentia1.[22-24] However, a recent publication has reported OTC to possess a far greater toxic potential than either OTA or OTB in the human monocyte cell line THP-1.[25] In light of previously published data, this may be a cell-type specific effect. It appears, therefore, that strict structure activity relationships are a feature of ochratoxin toxicity. Due to its role as the most toxic of the family members, much research has focussed on OTA.

2.3 Toxicology of OTA

Often, a single mycotoxin can cause more than one type of toxic effect. The target organ of OTA toxicity in all mammalian species tested is the kidney, in which lesions can be produced by both acute and chronic exposure.[26] Animals can demonstrate variable susceptibilities to OTA depending on genetic factors (species, breedand, strain), physiological factors (age, sex, nutrition, other diseases) and environmental factors (climatic conditions, management, etc.). The LD_{50} is one way to measure the short-term poisoning potential (acute toxicity) of a compound. LD stands for ‘Lethal Dose’, and LD_{50} is the amount of a material, given all at once, which causes the death of 50 % (one half) of a group of test animals. Therefore, in acute toxicity studies, LD_{50} values of OTA, vary greatly among species, ranging from an oral LD_{50} of 0.20 mg/kg in dogs and 1 mg/kg in pigs, to more than 30 mg/kg in rats (Table 2.1). LD_{50} values are also strongly

influenced by the administration routes (oral feeding, intubation, intravenous or intraperitoneal injection), the solvents of toxins, the presence of other mycotoxins and the composition of the diet. Thus, data obtained in toxicological studies will be relative and not conclusive for the evaluation of the toxicological features of individual mycotoxins.

Table 2.1: LD_{50} values of OTA of different animal species (several sources).

Animal	LD_{50} (mg kg-1)	Administration way
Mice (female)	22	intraperitoneal
Rat (male)	30.5	oral
Rat (female)	21.4	oral
Rat (male)	12.6	intraperitoneal
Rat (female)	14.3	intraperitoneal
Chicken	3.3	oral
Turkey	5.9	oral
Quail	16.5	oral
Rainbow trout	4.7	intraperitoneal
Dog	0.2	oral
Pig (female)	1	oral

OTA is nephrotoxic, mutagenic, carcinogenic, teratogenic and immunosuppressive in a variety of animal species. It is a mitochondrial poison causing mitochondrial damage, oxidative burst, lipid peroxidation and interferes with oxidative phosphorylation. In addition, OTA increases apoptosis in several cell types. Much has been written about the possible role of OTA in

the etiology of these phenomena and detailed reviews on OTA toxicology have been published.[27-32]Although a complete review of the toxicology of OTA is beyond the scope and intention of this text, the most important points are outlined afterwards.

2.3.1 Carcinogenesis

Oral administration of OTA produced renal tumors in rats and mice.[33] Moreover, in mice OTA give rise to liver tumors in both sexes.[29] Nephrotoxic effects have also been demonstrated in other mammalian species. In the early 1970s, observers in Denmark noted a high incidence of nephritis in pigs,[34] a disease known nowadays as Danish porcine nephropaty, which was associated with the use of moldy rye, and particularly, with the presence of OTA in feed samples. Given that OTA is a kidney toxin in all mammals tested, it would appear prudent to assume it is also a kidney toxin in humans. Particularly, kidney failure rates in rural Scandinavian populations were proved high, and a possible cause was the ingestion of those pig tissues containing excessive levels of OTA.[35,36] Observational studies have associated OTA with two human disease states:

- **Balkan endemic nephropathy (BEN).**
- **Urothelial tumours (UT).**

The first was initially described in the 1950s as a human kidney disease, in a series of publications from different Easter Europe countries, where OTA is relatively high in the diet. Subsequent studies have also shown a high incidence of kidney cancer and cancer of the urinary tract in some BEN afflicted populations. The connection between human urinary tract tumors and OTA was postulated by a Danish study, based on regional coincidence of tumors of the

urinary tract in humans, human chronic kidney disease and, as an indication of regional OTA contamination of grain, the occurrence of nephropathy in pigs.[37] Studies carried out in several countries including Tunisia, Egypt and France, have also indicated a link between dietary intake of OTA and the development of renal and urothelial tumours.[38-42] To sum up, it is not possible to conduct studies in humans under controlled conditions but, the parallels between the pathological changes and functional deficits observed in pigs and those noted in human BEN/UT cases, suggest that OTA may play a role in human kidney and urothelial cancer. Recently, it has also been suggested that OTA can cause testicular cancer in humans, as positive associations have been found between the incidence of testicular cancer and the consumption of foods typically associated with OTA contamination. To adequately assess the human cancer risk of OTA, a variety of factors must be considered, such as specific exposure information, ample follow-up time, large sample sizes including adequate numbers of both males and females, control for confounding factors that may also affect cancer risk, etc. (FAO/WHO, 2003)[43]. The major difficulty with epidemiological studies on mycotoxins is obtaining data on historical exposure, since many of the effects observed are of a chronic nature. Even when using biomarkers, the estimate of exposure usually reflects only the recent past.[44] Furthermore, without thorough studies that take all these factors into account, it is not possible to conclude whether or not exposure to OTA increases cancer risk in humans.

In 1993, the International Agency for Research on Cancer (IARC) classified OTA as a possible human carcinogen (Group 2B) (Table 2.2), based on sufficient evidence of carcinogenicity in experimental animal studies and inadequate evidence in humans (IARC, 1993)[45]. In the subsequent years since the

IARC classification, studies have shown a tendency in the direction of group 2A toxicity,[46] as well as indicating the occurrence of synergistic multiple actions of diverse mycotoxins.

2.3.2 Mutagenesis/ Genotoxicity

Mutagenic or genotoxic chemicals are those capable of causing damage to DNA. For a long time, OTA was not considered to be genotoxic. However, in 1985, Creppy et al.[47] showed that OTA caused DNA single-stranded breaks in mice-spleen cells (in vitro) and in mouse spleen, kidney and liver, after injection of high OTA doses. Moreover, in 1991, Pfohl-Leszkowicz et al.[48] found several DNA adducts after oral application of OTA to mice. This discussion received considerable stimulus when it became known that cells from target organs of animals and also human ureter cells, react much more sensitively to changes in DNA.[49,50] However, there is still some disagreement about whether OTA reacts directly with nucleic acids or acts via an indirect mechanism to disrupt DNA.

2.3.3 Teratogenesis

OTA is a potent teratogen in rodents,[51,52] chickens,[53] and pig.[54] Both teratogenic and reproductive effects have been demonstrated. OTA causes birth defects in rodents. It is seen that OTA crosses the placenta and is also transferred to newborn rats and mice via lactation.[55] In the fetus, the major target is the developing central nervous system, thus OTA is also considered a neurotoxic compound. In addition, OTA-DNA adducts are formed in liver, kidney and other tissues of the progeny.[56,57] The mechanism of induced teratogenesis by OTA is still not clear, but it seems to affect both the progenitor and the embryo, in a direct way.[58] Thus, sufficient experimental evidence exists

in the scientific literature to classify OTA as a teratogen, affecting the nervous system, skeletal structures and immune system of research animals.

Table 2.2. Summary of the IARC evaluations and classification of mycotoxins on the basis of the carcinogenic risk to humans (IARC 1993, 1998).[45]

Mycotoxin	Risk carcinogenic to[a]		IARC[b] classification
	humans	animals	
Penicillic acid	AD	L	3
Aflatoxins	S	S	1
Aflatoxin B1	S	S	
Aflatoxin B2		L	
Aflatoxin G1		S	
Aflatoxin G2		I	
Aflatoxin M1	I	S	2B
Citrinin	AD	L	3
Cyclochlorotin	AD	I	3
Griseofulvin	AD	S	2B
Luteoskyrin	AD	L	3
OTA	I	S	2B
Patuline	AD	I	3
Rugulosine	AD	I	3
Sterigmatocytin	AD	S	2B
F. graminearum toxins	I		3
F. culmorum toxins	AD		
F. crookwellense toxins	AD		
Zearalenone		L	
Vomitoxin		I	
Nivalenol		I	
Fusarenone X		I	
F. sporotrichioides toxins	AD		3
T-2 toxin		L	
F. moniliforme toxins	I	S	2B
Fumonisin B1		L	
Fumonisin B2		I	
Fusarin C			L

[a] Evidence of carcinogenicity: (S) sufficient, (L) limited, (I) inadequate, (AD) absence of data;
[b] Classification criteria: Group 1: carcinogenic to humans; Group 2B: carcinogenic to animals and possible carcinogenic to humans; Group 3: non-classifiable for carcinogenicity to humans.

2.3.4 Immunosuppression

OTA is known to affect the immune system in a number of mammalian species. The type of immune suppression experienced appears to be dependant a number of factors, including the species involved, the route of administration, the doses tested, and the methods used to detect the effects.[59] OTA causes immunosuppression following prenatal, postnatal and adult-life exposures. These effects include reduced phagocytosis and lymphocyte markers,[60] and increased susceptibility to bacterial infections and delayed response to immunization in piglets.[61] Purified human lymphocyte populations and subpopulations are adversely affected by OTA in vitro.[62]

2.3.5 Action on different enzymes

Because of its structure, OTA was first shown to inhibit protein synthesis both in vitro and in vivo, by competition with phenylalanine. OTA might act on other enzymes that use phenylalanine as a substrate, such as phenylalanine hydroxylase,[28] and lower the levels of phosphoenolpyruvate carboxykinase, a key enzyme in gluconeogenesis.[63] Inhibition of protein and RNA synthesis is also considered another toxic effect of OTA.

2.3.6 Lipid peroxidation and mitochondrial damage

OTA enhance lipid peroxidation both in vitro and in vivo.[64,65] This action might have an important effect on cell or mitochondrial membranes. Several lines of experimental observations demonstrate that OTA effects mitochondrial function and causes mitochondrial damage.[66,67]

2.3.7 Apoptosis

OTA also induces apoptosis (programmed cell death) in a variety of cell types in vivo and in vitro.[68] The apoptosis is also mediated through cellular processes involved in the degradation of DNA.

2.4 Synergistic effects with other mycotoxins

Many toxicological studies have used pure OTA, free from the complex matrix of the biosynthesising fungus. In nature there are other microorganisms and their metabolites that increase the complexity of the matrix, which could protect from or enhance the effects of OTA. It appears logical to assume that exposure to several nephrotoxic substances could have more severe consequences than exposure to a single substance. But certain combinations of mycotoxins could be more toxic than the sum of their individual actions.[59] Accordingly, a hypothesis about synergistic effects between OTA and penicillic acid and possibly other fungal metabolites such as citrinin has emerged, and all together are suspected to be the responsible for the BEN.[69] The authors described differences in the renal pathologies resulting from OTA exposure alone and those observed following a combination of two or more other mycotoxins. One year later, Speijers and Speijers (2004)[70] confirmed the synergistic effect of combine both nephrotoxic compounds: OTA and citrinin.

2.5 OTA presence in food

2.5.1 Introduction

OTA is found in a variety of foods and beverages, including both plant-based products and animal products. Among the first ones, its presence in cereal grains (corn, wheat, barley, flour, oats, rye, rice, etc.), beans (coffee, cocoa, soy, etc.), spices, and beverages like coffee and wine must be highlighted. In 1983, OTA was reported in olive oil,[71] and recently it was detected again in this product.[72] OTA can be absorbed from contaminated feed by monogastric animals such as pigs, where it is accumulated in the blood and kidneys, and therefore it can be found in products made from them, such as black pudding, sausages, etc. Moreover, OTA has been detected in milk, cheese and other animal products. The presence of OTA in grape and its derivatives such as dried vines, grape juice, musts, wine, vinegar, etc. also has been reported. To sum up, OTA can be found in a wide range of raw commodities and also in processed foods made from contaminated resources, thus, it is difficult to avoid this substance.

2.5.2 OTA levels in contaminated food

Apart from measuring OTA in human fluids and tissues, exposure can also be estimated by measuring OTA levels in contaminated food that may have been consumed. Studies on some foods show that there are differences between the contamination level of different batches of food, and even within the batches, the mycotoxin might not be homogeneously distributed but be restricted to a small part of the batches.[73] Furthermore, the occurrence of mycotoxins can fluctuate considerably in time. Sometimes the mycotoxin

concentration can be high for a certain episode, whereas for another it might be negligible low. It is difficult to compare OTA levels between countries or between types of food, as data on the occurrence of OTA in food and beverages are not available for many commodities in many countries, and the data that are available are often out of date and/or incomplete. The consumption data used were mainly based on intake in Europe (Table 2.3). The European Commission (2000)[74] calculated and summarised intake figures for OTA. The total mean intake of OTA for Europe was estimated to be 3.7 ng/kg body weight per day, assuming a body weight of 60 kg.

Table 2.3. The relative contribution of different food categories to human OTA exposure (JECFA, 2001).[75]

Food category	OTA contamination (µg/kg)	Intake (g)	Daily intake of OTA (ng/kg body weight [a]/ day)	% of total intake
Cereals	0.94	230	3.58	57.8
Wine	0.32	240	1.23	20.8
Grape juice	0.39	69	0.44	7.3
Coffee	0.76	24	0.30	5.1
Pork meat	0.17	76	0.21	3.5
Beer	0.023	260	0.09	1.6
Dry fruits	2.2	2.3	0.08	1.1
Pulses	0.19	25	0.08	1.1
Cocoa	0.55	6.3	0.06	0.8
Poultry	0.04 1	53	0.06	0.8

[a] Body weight 60 kg.

Exposure assessments indicate that cereals and cereal products are the main contributors to the dietary intake of OTA (50-70 %), as almost all cereals seem to have the possibility.to contain OTA and their consumption is generally high (JECFA, 2001).[75] Grape juice and wines, were considered in a first approach

to be the second most prominent source of OTA intake for humans, with 7-20 %, respectively. Otteneder and Majerus (2000)[76] reduced this figure for wine to 2 % after new calculations, and more recently, Miraglia and Brera (2002)[77] estimated it to be 10 %. Other products contribute less to the dietary intake, but the incidence of contamination can be high in coffee, beer, raisins and spices. Therefore, if intakes are not greatly above what seems tolerable, why bother?. One reason is that average intake means that some individuals exceed this value and so some people may be at risk. Also, individuals may differ in their sensitivity to OTA. Further, OTA may be additive to, or synergistic with, other chemicals in food and the environment. Thus, the importance of human ochratoxicosis could be under-estimated because of the presence in our diet of substances such as phenylalanine, aspartame, vitamins, etc., which are capable of alleviating some of the effects of OTA, and could also change its profile of distribution and metabolism.[27] Indeed, the prevention of human ochratoxicosis could be achieved by using the sweetener aspartame, a structural analogue of OTA, which prevents the distribution of the toxin and accumulation in the organism by avoiding the binding to blood proteins.[78-80] It also greatly reduces the cytotoxic and nephrotoxic effects of OTA in the normal food contamination ranges.

2.6 Current status and future perspectives

The chlorophenolic mycotoxin OTA is prevalent in food sources and represents a potential human health hazard. OTA is a potent renal carcinogen in rodents and it has been linked to the fatal human kidney disease, BEN. Understanding the mechanism of OTA-mediated carcinogenesis is vital for the risk assessment community to place restrictions on the OTA content in human foods. The literature on OTA mediated toxicity and genotoxicity is dominated by

controversial evidence, making it difficult to place realistic restrictions on OTA in human food samples because we do not yet understand its threat to human health. One group of researchers report negative findings for the genotoxicity of OTA and argue that OTA does not undergo biotransformation to form electrophilic intermediates capable of reacting with biopolymers. This group of researchers argues that OTA-mediated genotoxicity through DNA adduction cannot account for the carcinogenicity observed in rodent models, and that some other unknown mechanism of action must be responsible for the carcinogenicity by OTA observed in rodents. A second group of researchers argue that OTA does undergo biotransformation to induce genotoxicity and that the adduct spots observed by ^{32}P-postlabelling represent attachment of an OTA-derived electrophile to DNA. Here an analogy to the established activity of other chlorophenol xenobiotics has been made and OTA-DNA adduct standards and synthetic metabolites, such as the hydroquinone OTHQ, have been prepared to support the OTA-mediated genotoxicity hypothesis. Because DNA adduction is strongly correlated with carcinogenesis, it is argued that DNA adduction coupled with oxidative DNA damage by OTA may play a key role in OTA-mediated carcinogenesis. These positive results for OTA-mediated genotoxicity provide convincing evidence that OTA should be viewed as a genotoxic carcinogen. Based on this classification we propose that the PTWI of OTA, currently set at 14 ng/kg bw/day based on nephrotoxicity in pigs, should be modified. The Virtually safe dose (VSD) of 1.8 ng/kg bw/day proposed by Kuiper-Goodman and Scott [103] that considers tumour formation by OTA as an endpoint would be a more prudent safety level to set for OTA intake. Further needing to new analytical methods to permit the rapid determination of OTA at the strict regulation limits established. Also the ease and the low cost of the method must be considered, to have an analytical method can be applied easily for its routine analysis.

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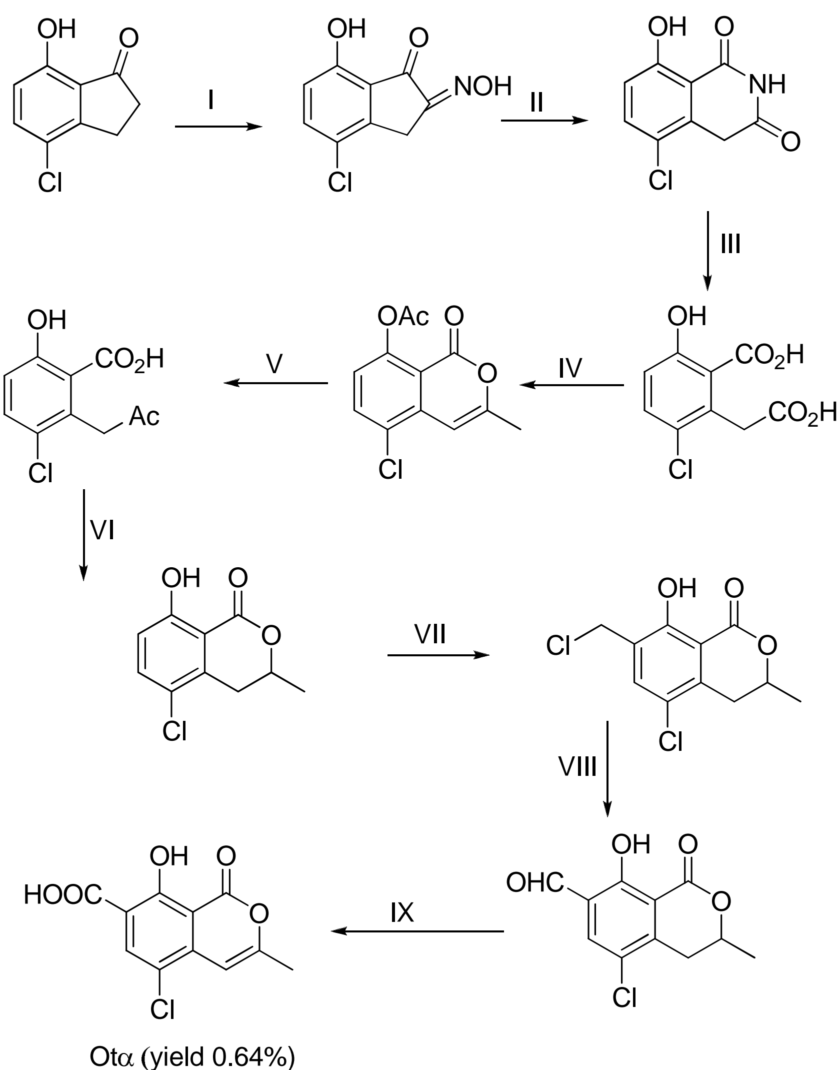
III

Synthesis of Ochratoxin A, and d_5-Ochratoxin A

3.1 Introduction

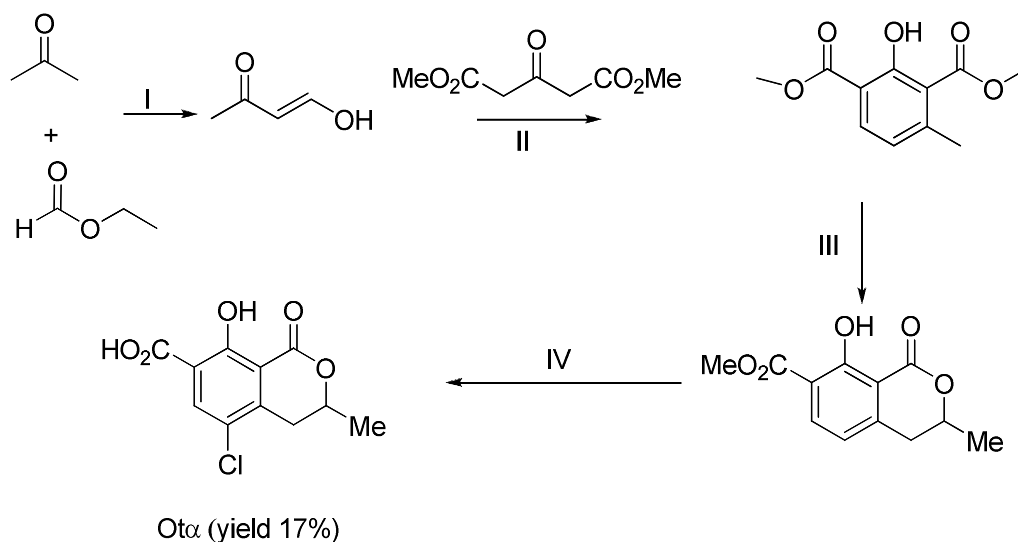
The first total synthesis of ochratoxin A was done in 1967 by Steyn et. al.,[1] after it had been isolated and identified in mild in 1960's. This method of synthesis gave a sufficient yield (10% for Otα) it was not easy to carry out. It was done to confirm the structure of OTA suggested by the same group in 1965.[2] The Steyn's method had confirmed the structure of OTA, and also was able to synthesize OTA for the first time. This method started by the synthesis of Otα in 8 steps with a yield of 10 %, and it was able to obtain the (-) Otα (80% optical purity) by fractional crystallization of the brucine salts. The coupling between Otα acid chloride and L-phenylalanine by the acyl azide method, produced OTA but it has not the sufficient optical purity. In 1970 Roberts and Woollven described an alternative, and substantially different, synthesis for this mycotoxin.[3] The synthesis of Otα started from 4-chloro-7-hydroxyindanone in 8 steps with a total yield 0.62% (scheme 3.1). The synthesis of OTA was done by a coupling reaction between (±) Otα and the protected L-phenylalanine using 2-ethoxy-1-ethoxy-carbonyl-1,2-dihydroquinoline (EEDQ), and the resulting mixture of the diastereoisomeric forms [(-, +) and (-, -)] of ochratoxin A, was then separated by PTLC to give OTA 90% optical purity with a yield of 35:50% depending on the method of crystallization. Several analogs of OTA have been prepared by applying Steyn's method by substitution of phenylalanine with other amino acids. Such as tyrosine, valine, serine, alanine, tryptophane, and glutamic acid, to study their toxicity effect comparing with OTA.[4] Also, Hadidane et. al.[5] have reported that the serine, hydroxyproline, and lysine analogs may occur in natural. Studies by Creppy et al.[6] have also demonstrated that OTA can be hydroxylated by phenylalanine hydroxylase to form the tyrosine OTA analog.

Scheme 3.1: Roberts and Woollven's method for the synthesis of Otα.



A facile synthesis of ochratoxin α, was reported by Kraus in 1981 [7] he was able to synthesize Otα with a yield of 17% over 4 steps starting from acetone and ethyl formate (scheme 3.2). It is a facile synthesis compared to the methods previously reported or comparing with Snieckus and coworkers (6% over 5 steps starting from 4-chlorophenol),[8] and Donner and Gill [who synthesized (*R*)-ochratoxin starting from (*R*)-propylene oxide over 9 steps with an overall yield of 10%].[9]

Scheme 3.2 : Kraus's method for the synthesis of Otα.



In 1995 it was reported new synthesis of analogs of ochratoxin A.[10] They were synthesized by obtaining Otα from the acid hydrolysis of naturally occurring OTA, following by a coupling reaction with the different substrates. The only method reported in the literature so far for the synthesis of d_5-OTA was based on a semi-synthetic sequence involving: acid hydrolysis of OTA to give Otα, followed by the conversion of Otα into the corresponding acyl chloride by the reaction with $SOCl_2$, and coupling reaction between the acyl chloride and d_5-L-phenylalanine methyl ester to give methyl protected d_5-OTA, which is deprotected with NaOH in MeOH to give d_5-OTA. The overall yield based on starting OTA was less than 5% over 4 steps.[11]

In this thesis I will present a new total synthesis of OTA, including a new synthesis of Otα prepared by a modification of the method originally proposed by Kraus.[7] Also the first total synthesis of d_5-OTA will be presented.

3.2 A New and Convenient Total Synthesis of Ochratoxin A and d_5-Ochratoxin A

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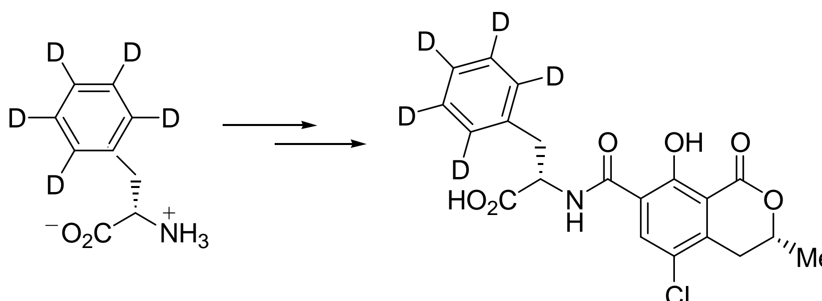


Abstract

A new total synthesis of the mycotoxin ochratoxin A (OTA) is presented, in which it is prepared in 9% overall yield from commercially available substrates. The key step consists of the condensation reaction between protected L-phenylalanine and 5- chloro-8-hydroxy-3-methy1-1 -oxoisochromane-7-carboxylic acid (ochratoxin α, Otα). The same strategy could be successfully applied to L-d_5-phenylalanine, leading to the first total synthesis of *d_5- OTA,* a molecular tracer for the detection and analytical quantification of the natural mycotoxin in food samples by means of stable isotope dilution assay (SIDA).

Key words: heterocycles, natural products, ochratoxin A, stable isotope dilution assay, total synthesis

Introduction

Ochratoxin A (OTA, **1a;** Figure 3.1) is a ubiquitous mycotoxin produced by some fungi of the *Aspergillus* and *Penicillium* species (such as *Aspergillus ochraceus* and *Penicillium verrucosum),* and is found in raw and improperly stored food products.[12,13] OTA has been shown to be nephrotoxic, mutagenic, genotoxic, teratogenic, hepatotoxic, neurotoxic, and immunotoxic, in both animals and humans,[14] and in 1993 was classified as a possible carcinogen to humans (Group 2B) by the International Agency for Research on Cancer (IARC).[15] toxin A (d_5-OTA, **2a),** and their (3S)-diastereomers 1b and 2b, respectively

OTA contamination occurs in a wide range of foods and beverages, including cereals, beans, dried vine fruits, coffee, wine, beer, grape juice, pork, poultry, spices, and chocolate.[12,13] The development of sensitive and accurate methods for the analytical determination of OTA in food products has therefore become increasingly important.[16] Stable isotope dilution assay (SIDA) is currently one of the most promising methods for the highly sensitive quantitative determination of microcomponents in food.[17] Thus, OTA can be detected and quantified in food samples by use of SIDA if, for example, a deuterated derivative, such as d_5-0TA **(2a;** Figure 3.1), is used as an internal standard.[16a]

Figure 3.1. Structures of (–)-ochratoxin A (OTA, **1a**), (–)-*d*$_5$-ochratoxin A (*d*$_5$-OTA, **2a**), and their 3-(*S*)-diastereoisomers (**1b** and **2b**, respectively)

1

a: R^1 = H, R^2 = Me
b: R^1 = Me, R^2 = H

2

a: R^1 = H, R^2 = Me
b: R^1 = Me, R^2 = H

In this paper, we report a new and efficient method for the total synthesis of OTA **(1a)** and d$_5$-OTA **(2a)**, together with their (3S)-diastereomers **1b** and **2b**, respectively, starting from but2-ynal and dimethyl 3-oxopentanedioate (3) (Schemes 3.3-3.5). The key step of the synthetic strategy consists of the condensation reaction between protected L-phenylalanine 7 (Scheme 3.4) or protected L-d$_5$-phenylalanine 9 (Scheme 3.5) and 5-chloro-8-hydroxy-3-methyl-1-oxoisochromane-7-carboxylic acid (ochratoxin α, Otα), which was prepared by a modification of the method originally proposed by Kraus (Scheme 3.3).[7]

Scheme 3.3. Synthesis of ochratoxin α (**6**)

3

1) NaH, THF, rt
2) Me—≡—CHO THF, −10°C

4 (48%)

1) LDA, −78°C
2) MeCHO, −78°C
3) AcOH

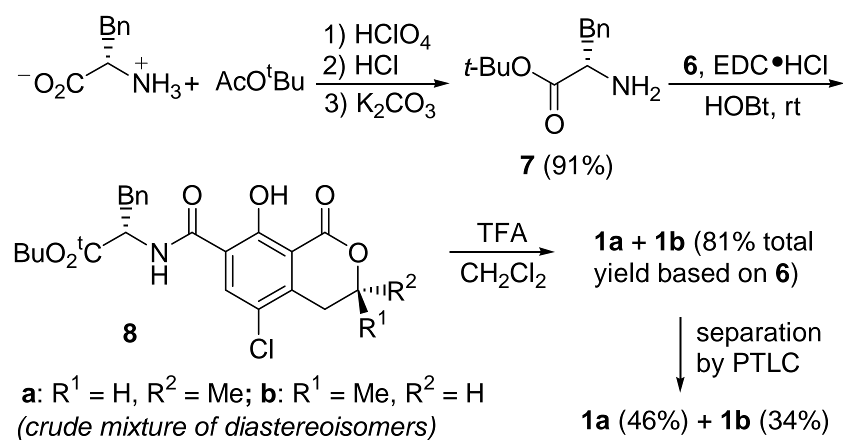
5 (70%)

1) SO_2Cl_2, CH_2Cl_2
2) LiOH, MeOH

6 (69%)

Thus, following the method recently disclosed by Covarrubias-Miliga,[18] crude but-2-ynal (readily available by quantitative oxidation of but-2-ynol with MnO_2 in CH_2C1_2)[19] was reacted with the sodium salt of commercially available dimethyl 3-oxopentanedioate (3) at –10 °C in tetrahydrofuran, to give dimethyl 2-hydroxy-4-methylbenzene-1,3-dicarboxylate (4) in 48% yield (Scheme 3.3). Kraus's method[7] was followed to provide lactone derivative (5): the methyl group of (4) was deprotonated with lithium diisopropylamide at –78 °C in tetrahydrofuran; addition of acetaldehyde and acidic workup followed; (5) was obtained in 70% yield. Chlorination of (5) with sulfuryl chloride in dichloromethane at room temperature, followed by hydrolysis of the ester group with lithium hydroxide in methanol finally gave OTa (6) in 69% yield. The overall yield of 6 was therefore 23% over three steps starting from commercially available (3), which is higher than that previously obtained by Kraus (17% over 4 steps starting from acetone and ethyl formate),[7] Snieckus and coworkers (6% over 5 steps starting from 4-chlorophenol),[8] and Donner and Gill [who synthesized (R)-Otα starting from (R)-propylene oxide over 9 steps with an overall yield of 10%] .[9]

Scheme 3.4. Synthesis of (–)-ochratoxin A (OTA, **1a**) and its 3-(*S*)-diastereoisomer (**1b**)



A condensation reaction between Ot*α* (6) and protected L-phenylalanine 7 was then performed in chloroform as the solvent at 25 °C for 20 hours in the presence of N-ethyl-yl-*N′*-(3-dimethylaminopropyl)carbodiimide hydrochloride and N-hydroxybenzotriazole (BtOH) as coupling agents, to give a mixture of protected diastereomers **8a,b** in ca. 86% total yield, based on (6) (Scheme 3.4). Deprotection of the crude mixture **8a,b** with trifluoroacetic acid in dichloromethane at room temperature for six hours led to a mixture of OTA **(1a)** and its (3S)-diastereomer **(1b)** in 81% total yield based on starting Ot*α* (6).[2] Separation of the two diastereomers by preparative TLC (PTLC) afforded pure **(1a)** and **(1b)** in 46% and 34% isolated yield, respectively. Both diastereomers were fully characterized by IR, ^{1}H NMR and ^{13}C NMR spectroscopy, HRMS, and specific rotation.

Scheme 3.5. Synthesis of d_5-(–)-ochratoxin A (OTA, **2a**) and its 3-(*S*)-diastereoisomer (**2b**)

Our strategy could be successfully applied to the first total synthesis of d_5-OTA **(2a)** and its (3S)-diastereomer (2b) (Figure 3.1), by use of L-d_5-phenylalanine as deuterated starting material, with essentially the same overall yields (Scheme 3.5). Thus, condensation of Otα (6) with protect ed L-d_5-phenylalanine (9), followed by deprotection and separation of the two resulting diastereomers **2a** and **2b** by preparative TLC led to pure **2a** and 2b in 47% and 36% isolated yield, respectively (Scheme 3.5). To our knowledge, this is the first example of the total synthesis of both d_5-OTA and its (3S)-diastereomer reported in the literature.[11] Both deuterated diastereomers **2a,b** were fully characterized by IR, ^{1}H NMR and ^{13}C NMR spectroscopy, HRMS, and specific rotation.

In conclusion, we have developed a novel and convenient total synthesis of ochratoxin A **(1a)** and its (3S)-diastereomer **lb,** with overall yields of ca. 9% and 6%, respectively, over only six steps, starting from commercially available starting materials. Our strategy has also allowed the first total synthesis of d_5-OTA **(2a)**, which can be used as an internal standard for the quantitative determination of the wild-type ochratoxin in foodstuff.

Melting points were determined on a Reichert Thermovar apparatus and are uncorrected. Optical rotations were measured on a Jasco DIP-1000 12 polarimeter equipped with a sodium lamp (589 nm) and a 10-cm microcell. ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra of samples dissolved in $CDC1_3$ or DMSO-d_6 were recorded on a Bruker DPX Avance 300 spectrometer at 25 °C; TMS was used as internal standard. IR spectra were recorded on a Perkin-Elmer Paragon 1000 PC FT-IR spectrometer. GC-MS spectra were obtained with a Shimadzu QP-2010 GC-MS apparatus (ionization voltage 70 eV). LC-MS analyses were carried out on a fractionlynx HPLC system composed of a autosampler/collector, a 600E pump

working in analytical mode, a 486 UV detector (set to 280 nm) and a ZMD mass spectrometer equipped with an ESI source; a 100 x 3.0 mm ONYX-C_{18} monolithic column was used [flow rate 1.5 mL/ min; run time 20 min; gradient elution with 0.5% HCO_2H in H_2O (solvent A) and Me0H (solvent B); solvent run: linear gradient from 95% A to 5% A in 14 min, linear gradient from 5% A to 95% A in 3 min, isocratic 95% A for 3 min. The MS conditions were the following: capillary voltage 3.15 kV, cone voltage 3 V, extractor 2 V, RF lens 0.2 V, source block and desolvation temperature 120, 250 °C respectively, ion energy 0.5 V, LM resolution 15.0, HM resolution 14.5 and multiplier 650 V. The nebulizer gas was set to 650 L/h. ESI-HRMS experiments were performed on a hybrid Q-Star Pulsar-i QqToF mass spectrometer equipped with an ion-spray ionization source. All samples were acquired at the optimum ion-spray voltage of 4.8 kV by direct infusion (5 µL/min) of a solution containing the appropriate compound dissolved in Me0H–H_20 (20 gg/mL). The N_2 gas flow was set at 20 psi and the declustering and the focusing potentials were kept at 50 and 220 V relative to ground, respectively. Commercially available flavonoids were used as calibration standard compounds. The accuracy of the measurement was within 5 ppm. MS^2 experiments were performed in the collision cell *q* on the isotopically pure (^{12}C) peak of the selected precursor ions by keeping the first quadrupole analyzer at unit resolution, and scanning the time-of-flight (ToF) analyzer. The collision energy was set to 20 eV, for each compound, while the gas pressure of the collision chamber was regulated at the instrumental parameters CAD 5, which corresponds to a pressure of the chamber of 6.86×10^{-3} Torr and a gas thickness of 9.55×10^{15} molecules/cm^2. All the acquisitions were averaged over 30 scans at a TOF resolving power of 7000. The molecular formula was evaluated by means of Analyst QS software. All reactions were analyzed by TLC on silica gel 60 F254 and by GLC on a Shimadzu GC-2010 gas chromatograph and capillary columns with polymethylsilicone with 5% phenylsilicone as the

stationary phase. Column chromatography was performed on silica gel 60 (Merck, 70-230 mesh). Preparatory TLC separations were carried out on Merck silica gel plates (20 x 20 cm, 0.25 mm thickness). $Mn0_2$, but-2-ynol, dimethyl 3-oxopentanedioate (3), NaH (95% purity), 2 M LDA in THF-heptane-ethylbenzene, acetaldehyde, SO_2C1_2, LiOH, L-phenylalanine, t-BuOAc, $HC1O_4$ (70%), EDC•HC1, BtOH, TFA, and L-d_5-phenylalanine (99% D) were commercially available and were used as received.

Dimethyl 2-Hydroxy-4-methylbenzene-1,3-dicarboxylate (4)

But-2-ynol (5.0 g, 71.3 mmol) was added to a suspension of MnO_2 (74.4 g, 860 mmol) in anhyd CH_2C1_2 (200 mL) at r.t. under N_2. After stirring at r.t. for 12 h, the mixture was filtered, and the solvent was removed by distillation at atmospheric pressure. The crude but-2-ynal thus obtained (still containing ca. 0.5 mL CH_2C1_2) was used as such for the next reaction. Dimethyl 3-oxopentanedioate (3; 11.5 g, 66.0 mmol) was added dropwise to a stirred suspension of NaH (95% purity; 1.9 g, 75.2 mmol) in anhyd THF (120 mL) at -10 °C under N_2, followed by crude but-2-ynal (obtained as described above). After additional stirring at -10 °C for 6 h, the mixture was poured into dilute aq HC1 (250 mL). The organic layer was separated, and the aqueous layer was extracted with EtOAc (3 x 50 mL). The combined organic phases were washed with brine (250 mL) and then dried (Na_2SO_4). After filtration of the mixture and removal of the solvent by rotary evaporation, the residue was purified by column chromatography (silica gel, hexane-EtOAc, 7:3); this gave pure **4**.

Yield: 7.1 g (48%); yellow crystals; mp 43-45 °C (Lit.[8] 44-46 °C). IR (KBr): ν = 2955 (m), 1730 (s), 1672 (s), 1259 (s) cm^{-1}.

^{1}H NMR ($CDCl_3$, 300 MHz): δ = 11.16 (s, 1 H, OH), 7.74 (d, J = 8.2 Hz, 1 H, H-5), 6.72 (d, J = 8.2 Hz, 1 H, H-4), 3.94 (s, 3 H, CO_2Me), 3.92 (s, 3 H, CO_2Me), 2.33 (s, 3 H, Me at C-3).

^{13}C NMR ($CDCl_3$, 75 MHz): δ = 170.2, 167.6, 159.0, 144.3, 131.0, 123.3, 121.1, 110.8, 52.3, 52.1, 20.1.

GC-MS: m/z = 224 (M^+, 32), 193 (38), 192 (100), 161 (93), 160 (28), 134 (48), 105 (17), 77 (27).

Methyl 8-Hydroxy-3-methyl-1-oxoisochromane-7-carboxylate ([5])

A solution of **4** (6.5 g, 29.0 mmol) in anhyd THF (30 mL) was added dropwise (15 min) to a 2 M solution of LDA in THF-heptane-ethylbenzene (36.3 mL, 72.6 mmol) maintained at -78 °C under N_2. After additional stirring of the mixture at -78 °C for 30 min, acetaldehyde (5.5 g, 124.9 mmol) was added, and the solution was stirred at -78 °C for 15 min and then at 0 °C for a further 15 min. After quenching of the mixture with glacial AcOH (10 mL) at 0 °C, H_2O (30 mL) and Et_20 (30 mL) were added, and the organic layer was separated. The aqueous layer was extracted with Et_20 (2 x 30 mL), and the combined organic layer was dried (Na_2SO_4). After filtration, the solvent was removed by rotary evaporation, and the crude product was crystallized from acetone; this gave pure **5**.

Yield: 4.79 g (70%); yellow solid; mp 108-109 °C (Lit.[7] 108110 °C).

IR (KBr): ν = 3419 (m, br), 1724 (s), 1660 (m), 1619 (m), 1431 (w), 1239 (m), 1217 (m), 808 (w), cm^{-1}.

^{1}H NMR ($CDCl_3$, 300 MHz): δ = 8.02 (d, J = 7.9 Hz, 1 H, H-6), 6.77 (dt, J = 7.9, 0.7 Hz, 1 H, H-5), 4.80-4.65 (m, 1 H, C*H*CH_3), 3.93 (s, 3 H, CO_2Me), 3.03-2.90 (m, 2 H,

CH_2), 1.53 (d, *J* = 6.4, 3 H, CHCH_3) (Note: the –OH signal was too broad to be detected).

^{13}C NMR ($CDCl_3$, 75 MHz): δ = 168.1, 166.2, 162.5, 145.2, 137.9, 117.6, 117.2, 110.2, 75.5, 52.1, 35.1, 20.6.

GC-MS: *m/z* = 236 (M^+, 59), 205 (52), 204 (18), 192 (28), 160 (100), 104 (24), 91 (6), 77 (30).

5-Chloro-8-hydroxy-3-methyl-1-oxoisochromane-7-carboxylic Acid (Ochratoxin *α*, Ot*α*, 6)

SO2C12 (6.9 g, 51 mmol) was added to a stirring soln of **5** (2.4 g, 10.2 mmol) in anhyd CH2C12 (50 mL) under N2 at 25 °C. After additional stirring of the mixture at 25 °C for 24 h, the resulting yellow solution was evaporated in vacuo to give methyl 5-chloro-8-hydroxy-3- methyl-1 -oxoisochromane-7-carboxylate as a yellow solid, which was suspended in Me0H (50 mL). LiOH (4.46 g, 186 mmol) was added, and the resulting mixture was allowed to reflux for 5 h. During this time a semisolid product separated from the mixture. After removal of the solvent under vacuum, H_2O (25 mL) and Et20 (25 mL) were added, and the organic layer was separated. The aqueous layer was acidified to pH 2 with 1 N aq HC1, and then extracted with Et20 (3 x 30 mL); then the combined organic layers were dried (Na_2SO_4). After filtration, the solvent was removed by rotary evaporation, and the crude product was crystallized from acetone; this gave pure ochratoxin *α* **(6).**

Yield: 1.8 g (69%); colorless solid; mp 245-246 °C (Lit.[7] 246 °C).

IR (KBr): ν = 3266 (m, br), 1732 (s), 1700 (s), 1680 (s), 1610, (s), 1440 (s), 1220 (m), 1200 (s), 1149 (s), 1100 (m) cm^{-1}.

^{1}H NMR (DMSO-d_6, 300 MHz): δ = 10.03 (s, br, 2 H, CO_2H + OH), 7.98 (s, 1 H, H-6), 4.87-4.60 (m, 1 H, C*H*CH_3), 3.20 (distorted dd, *J* = 17.1, 2.4, 1 H, C*H*H), 2.87 (distorted dd, *J* = 17.1, 11.6, 1 H, CH*H*), 1.46 (d, *J* = 6.1, 3 H, Me).

^{13}C NMR (DMSO-d_6, 75 MHz): δ = 167.0, 165.5, 160.4, 143.1, 136.0, 120.6, 118.1, 112.3, 74.4, 32.2, 20.1ESI-MS: *m/z* = 279 (100) [M + Na], 257 (80) $[M + H]^+$.

ESI-HRMS: *m/z* [M + Hr calcd for $C_{11}ll_{10}C10_5$: 257.0217; found: 257.0199.

MS/MS [M + Hr (ESI+, 30 eV): *m/z* = 239.00 (88.2) [M - H_2O + H]+, 220.99 (100.0) [M - $2H_20$ + H]+, 193.00 (59.7) [M - $2H_20$ -CO + H]+, 165.00 (15.3) [M - $2H_20$ - 2C0 + $H]^+$, 137.01 (17.4), 102.04 (10.5).

tert-Butyl L-Phenylalaninate (7)

Concd $HClO_4$ (70%; 1.5 mL, 2.5 g, 17.4 mmol) was slowly added to a suspension of L-phenylalanine (1.8 g, 10.9 mmol) in t-BuOAc (27.0 mL, 23.3 g, 200 mmol) under N_2 at 0 °C. After stirring of the mixture at 25 °C for 12 h, H_2O (55 mL) followed by 1 N HC1 (30 mL) were added. The mixture was basified to pH 9 by the addition of 10% aq K_2CO_3 solution, and then extracted with CH_2C1_2 (3 x 25 mL). The combined organic layers were dried (Na_2SO_4). After filtration, the solvent was removed by rotary evaporation, and the crude product was purified by column chromatography (silica gel, hexaneEtOAc, 1:1); this gave pure **7**.

Yield: 2.2 g (91%); colorless oil.

IR (film): ν = 1728 (s), 1458 (w), 1368 (m), 1154 (s), 847 (m), 740 (m), 700 (m)cm^{-1}.

^{1}H NMR ($CDCl_3$, 300 MHz): δ = 7.32-7.17 (m, 5 H, Ph), 3.60 (distorted dd, *J* = 7.6, 5.7, 1 H, C*H*NH_2), 3.02 (distorted dd, *J* = 13.6, 5.7, 1 H, C*H*H), 2.83 (distorted dd, *J* = 13.6, 7.6, 1 H, CH*H*), 1.51 (s, br, 2 H, NH_2), 1.42 (s, 9 H, *t*-Bu).

^{13}C NMR ($CDCl_3$, 75 MHz): δ = 174.3, 137.9, 129.5, 128.4, 126.7, 81.0, 56.5, 41.6, 28.1.

GC-MS: m/z = 221 (M^+, absent), 130 (16), 121 (28), 120 (100), 103 (19), 91 (45), 77 (25), 74 (97)

***tert-Butyl* N-{[(3R/3S)-5-Chloro-8-hydroxy-3-methyl-1-oxoisochroman-7-yl] carbony1}-L-phenylalaninate (8a,b)**

EDC·HCl (208 mg, 1.08 mmol) was added to a stirred soln of 7 (222 mg, 1.0 mmol), 6 (269 mg, 1.05 mmol), and BtOH (142 mg, 1.05 mmol) in anhyd $CHC1_3$ (5 mL) under N_2 at 0 °C. After additional stirring of the mixture at 0 °C for 15 min and at 25 °C for 20 h,
$CHC1_3$ (10 mL) and H_2O (10 mL) were added. The organic layer was separated, washed sequentially with 1 N HC1, H_2O, 5% $NaHCO_3$, and brine (10 mL each), and then dried (Na_2SO_4). After filtration of the mixture, the solvent was removed by rotary evaporation; this gave a crude mixture of diastereomers **8a,b,** which was used as such for the next step.

(-)-N-{[(3R)-5-Chloro-8-hydroxy-3-methyl-l-oxoisochroman7-yl]carbonyl}-L-phenylalanine (Ochratoxin A, OTA, la) and (+)-N-{[(3S)-5-Chloro-8-hydroxy-3-methyl-1-oxoisochroman7-yl]carbonyl}-L-phenylalanine (lb)

The crude mixture of diastereomers **8a,b,** obtained as described above, was added to a solution of TFA (12.3 g, 108 mmol) in anhyd CH_2C1_2 (20 mL) at 25 °C under N_2. The reaction mixture was stirred at 25 °C and monitored by TLC. When the reaction was completed (ca. 6 h), the solvent and excess TFA were removed by rotary evaporation. The resulting residual oil was dissolved in CH_2C1_2,

washed with H_2O (3 x 10 mL), and dried (Na_2SO_4). After filtration and removal of the solvent by rotary evaporation, a colorless solid was obtained, which was crystallized from benzene to give a mixture of **1a** and 1b.

Yield: 345 mg (81% from 6); colorless solid; mp 168-171 °C (Lit.[9] 169-172 °C).

Separation of la and lb

A mixture of the two diastereomers **1a** and 1b (100 mg) dissolved in $CHC1_3$ was then subjected to preparative TLC on plates coated with silica gel (20 plates, 20 x 20 cm, 0.25 mm thickness, benzene-acetone-HCO_2H, 79:20:1); this gave pure **1a** (R_f= 0.47) and pure **1b** (R_f= 0.43).

1a

Yield: 46 mg (46%); colorless solid; mp 110-112 °C; $[\alpha]^{25}_D$ ($CHCl_3$, c = 5 mg/mL) = – 31.5°.

IR (KBr): ν = 3029 (m), 2985 (m), 2928 (m), 1742 (m), 1674 (s), 1614 (m), 1534 (s), 1427 (m), 1391 (w), 1214 (m),1171 (m), 1139 (m) 809 (w), 758 (w), 702 (w).

^1H NMR ($CDCl_3$, 300 MHz): δ = 12.74 (s, br, 1 H, OH), 8.50 (d, br, *J* = 6.8, 1 H, NH), 8.42 (s, 1 H, H-6), 7.34-7.20 (m, 6 H, Ph + OH), 5.07-4.98 (m, 1 H, C*H*NH), 4.81-4.68 (m, 1 H, C*H*CH_3), 3.40-3.15 (m, 3 H, CH_2Ph + C*H*HCHCH_3), 2.84 (dd, *J* = 17.6, 11.7, 1 H, CH*H*CHCH_3), 1.59 (d, *J* = 6.3, 3 H, Me).

^{13}C NMR ($CDCl_3$, 75 MHz). δ = 174.6, 169.7, 163.3, 159.1, 141.0, 139.0, 135.8, 129.3, 128.7, 127.3, 123.2, 120.2, 110.1, 75.9, 54.4, 37.3, 32.3, 20.7.

ESI-MS: *m*/*z* = 426 (68) [M + Na], 404 (100) $[M + H]^+$.

ESI-HRMS: *m*/*z* [M + Hr calcd for C2oli19CIN06: 404.0901; found: 404.0891.

MS/MS [M + Hr (ESI+, 20 eV): *m/z* = 404.08 (34.8) $[M + H]^+$, 386.07 (6.4) [M - H_2O + $H]^+$, 358.08 (79.1) [M - H_2O - CO + $H]^+$, 341.05 (17.6) [M - H_2O - CO - NH_3 + H]+, 239.00 (100.0) [M - $C_9$11$_{11}$1•10$_2]^+$, 220.99 (7.2) [M - $C_9H_{13}NO_3]^+$, 120.08 (6.2) $[C_8H_{io}N]^+$.

1b

Yield: 34 mg (34%); colorless solid; mp 182-183 °C; $[\alpha]^{25}_D$ ($CHCl_3$, c = 3 mg/mL) = + 66.7°.

IR (KBr): ν = 2924 (s), 1741 (s), 1673 (s),1615 (w) 1541 (m), 1427 (m), 1214 (s),1170 (m), 1139 (m), 809 (m), 742 (w), 705 (w).

^{1}H NMR ($CDCl_3$, 300 MHz): δ = 12.71 (s, br, 1 H, OH), 8.50 (d, br, *J* = 7.2, 1 H, NH), 8.40 (s, 1 H, H-6), 7.34-7.18 (m, 5 H, Ph), 7.08 (s, br, 1 H, OH), 5.05 (distorted dd, *J* = 12.6, 6.8, 1 H, C*H*NH), 4.80-4.66 (m, 1 H, C*H*CH_3), 3.39-3.15 (m, 3 H, C*H*$_2$Ph + C*H*HCHCH_3), 2.89-2.76 (m, 1 H, CH*H*CHCH_3), 1.58 (d, *J* = 6.3, 3 H, Me).

^{13}C NMR ($CDCl_3$, 75 MHz): δ = 174.8, 169.7, 163.1, 159.2, 141.0, 139.1, 136.0, 129.4, 128.7, 127.3, 123.3, 120.6, 110.2, 75.9, 54.5, 37.6, 32.4, 20.7

ESI-MS: *m/z* = 426 (100) [M + Na], 404 (87) $[M + H]^+$.

ESI-HRMS: *m/z* [M + Hr calcd for C20li19CIN06: 404.0901; found: 404.0886.

MS/MS [M + Hr (ESI+, 20 eV): *m/z* = 404.09 (33.7) $[M + H]^+$, 386.08 (5.9) [M - H_2O + H]+, 358.08 (81.4) [M - H_2O - CO + $H]^+$, 341.06 (17.9) [M - H_2O - CO - NH_3 + H]+, 239.01 (100.0) [M - $C_9$11$_{11}$1•10$_2]^+$, 221.00 (8.6) [M - $C_9H_{13}NO_3]^+$, 120.08 (8.1) $[C_8H_{io}N]^+$.

***tert-Butyl* L-d_5-Phenylalaninate (9)**

Coned $HClO_4$ (70%) (200 336 mg, 2.34 mmol) was added slowly to a suspension of L-d_5-phenylalanine (99% D; 255 mg, 1.5 mmol) in t-BuOAc (3.6 mL, 3.1 g, 26.7 mmol) under N_2 at 0 °C. After stirring of the mixture at r.t. for 12 h, H_2O (15 mL) followed by 1 N aq HC1 (10 mL) were added. The mixture was basified to pH 9 by the addition of 10% K_2CO_3, and then extracted with CH_2C1_2 (3 x 15 mL). The combined organic layers were dried (Na_2SO_4). After filtration, the solvent was removed by rotary evaporation, and the crude product was purified by column chromatography (silica gel, hexane-EtOAc, 1:1); this gave pure **9**.

Yield: 307.0 mg (90%); colorless oil.

IR (film): ν = 3381 (s, br), 1735 (m), 1621 (s), 1212 (m), 1154 (m) cm^{-1}.

^1H NMR ($CDCl_3$, 300 MHz): δ = 3.61 (distorted dd, *J* = 7.6, 5.8, 1 H, C*H*NH_2), 3.04 (distorted dd, *J* = 13.6, 5.8, 1 H, C*H*H), 2.84 (distorted dd, *J* = 13.6, 7.6, 1 H, CH*H*), 1.53 (s, br, 2 H, NH_2), 1.42 (s, 9 H, *t*-Bu).

GC-MS: *m/z* = 227 (M^+, absent), 125 (100), 107 (5), 106 (5), 96 (10), 74 (40). ESI-HRMS: *m/z* [M + Hr calcd for $C_{13}H_{15}D_5NO_2$: 227.1803; found: 227.1795.

MS/MS [M + Hr (ESI+, 20 eV): *m/z* = 171.11 (55.7) [M - t-Bu + H]+, 125.11 (100.0), 124.10 (16.0).

***tert-Butyl* N-{[(3R3S)-5-Chloro-8-hydroxy-3-methyl-1-oxoisochroman-7-yl]carbony1}-L-d_5-phenylalaninate (10a,b)**

EDC·HCl (208 mg, 1.08 mmol) was added to a stirred solution of **9** (227 mg, 1.0 mmol), 6 (269 mg, 1.05 mmol), and BtOH (142 mg, 1.05 mmol) in anhyd $CHC1_3$ (5 mL) under N_2 at 0 °C. After additional stirring of the mixture at 0 °C for

15 min and at r.t. for 20 h, $CHC1_3$ (10 mL) and H_2O (10 mL) were added. The organic layer was separated, washed sequentially with 1 N aq HC1, H_2O, 5% $NaHCO_3$, and brine (10 mL each), and then dried (Na_2SO_4). After filtration of the mixture, the solvent was removed by rotary evaporation; this gave a crude mixture of diastereomers **10a,b,** which was used as such for the next step.

(-)-N-{[(3R)-5-Chloro-8-hydroxy-3-methyl-l-oxoisochroman7-yl]carbony1}4,4$_5$-phenylalanine (d$_5$-Ochratoxin A, d$_5$-OTA, 2a) and (+)-N-{[(3S)-5-Chloro-8-hydroxy-3-methyl-1-oxoisochroman-7-yl]carbony1}-L-d$_5$-phenylalanine (2b)

The crude mixture of diastereomers **10a,b,** obtained as described above, was added to a soln of TFA (12.3 g, 108 mmol) in anhyd CH_2C1_2 (20 mL) at r.t. under N_2. The reaction mixture was stirred at r.t. and monitored by TLC. When the reaction was completed (ca. 6 h), the solvent and the excess TFA were removed by rotary evaporation. The resulting residual oil was dissolved in CH_2C1_2, washed with H_2O (3 x 10 mL), and dried (Na_2SO_4). After filtration and removal of the solvent by rotary evaporation, a colorless solid was obtained, which was crystallized from benzene; this gave a mixture of 2a and 2b.

Yield: 350 mg (82% from 6); colorless solid; mp 93-95 °C.

Separation of 2a and 2b

A mixture of the two diastereomers **2a** and **2b** (100 mg) dissolved in $CHC1_3$ was then subjected to preparative TLC on plates coated with silica gel (20 plates, 20 x 20 cm, 0.25 mm thickness, benzene-acetone-HCO_2H, 79:20:1); this gave pure **2a** (R_f= 0.47) and pure**2b** (R_f= 0.43)

2a

Yield: 47 mg (47%); colorless solid; mp 112-113 °C; $[\alpha]^{25}_{D}$ ($CHCl_3$, c = 5 mg/mL) = – 32.7°.

IR (KBr): ν = 2985 (m, br) 1728 (s), 1677 (s), 1611 (w) 1531 (m), 1426 (m), 1219 (m), 1138 (w), 810 (w), 770 (w).

^{1}H NMR ($CDCl_3$, 300 MHz): δ = 12.72 (s, br, 1 H, OH), 8.50 (d, br, *J* = 7.2, 1 H, NH), 8.41 (s, 1 H, H-6), 7.73 (s, br, 1 H, OH), 5.10-5.01 (m, 1 H, C*H*NH), 4.82-4.67 (m, 1 H, C*H*CH_3), 3.40-3.16 (m, 3 H, CH_2Ph + C*H*HCHCH_3), 2.84 (distorted dd, *J* = 17.6, 11.7, 1 H, CH*H*CHCH_3), 1.58 (d, *J* = 6.3, 3 H, Me).

^{13}C NMR ($CDCl_3$, 75 MHz): δ = 174.8, 169.7, 163.2, 159.2, 140.9, 139.1, 135.8, 129.0 (t, *J* = 23.8), 128.2 (t, *J* = 23.8), 127.3-126.3 (m), 123.3, 120.6, 110.2, 75.9, 54.4, 37.5, 32.4, 20.7

ESI-MS: *m*/*z* = 409 (100) [M + H]+.

ESI-HRMS: *m*/*z* [M Hr calcd for $C2_0{}^{11}14^{D}{}_5C^1N0_6$: 409.1210; found: 409.1199.

MS/MS [M + Hr (ESI+, 20 eV): *m*/*z* = 409.11 (43.03) [M + 391.10 (7.7) [M – H_2O + H]$^+$, 363.11 (86.8) [M – H_2O – CO + H]$^+$, 346.08 (17.6) [M – H_2O - CO – NH_3 + H]+, 239.00 (100.0) [M – $C_9H_6D_5NO_2$]$^+$, 220.99 (8.7) [M – $C_911_813_5NO_3$]$^\pm$, 125.11 (6.6) [$C_9H_5D_5N$]$^+$.

2b

Yield: 36 mg (36%); colorless solid; mp 183-185 °C; $[\alpha]^{25}_{D}$ ($CHCl_3$, c = 3 mg/mL) = + 60.2°.

IR (KBr): ν = 2927 (m, br), 1740 (s), 1672 (s),1626 (m), 1544 (s), 1427 (m), 1380 (w), 1214 (m), 1139 (w), 810 (w), 769 (w).

^{1}H NMR ($CDCl_3$, 300 MHz): δ = 12.70 (s, br, 1 H, OH), 8.49 (d, br, *J* = 7.2, 1 H, NH), 8.40 (s, 1 H, H-6), 7.16 (s, br, 1 H, OH), 5.05 (distorted dd, *J* = 12.2, 5.9, 1 H,

C*H*NH), 4.80-4.67 (m, 1 H, C*H*CH_3), 3.39-3.16 (m, 3 H, C*H*$_2$Ph + C*H*HCHCH_3), 2.89-2.76 (m, 1 H, CH*H*CHCH_3), 1.58 (d, *J* = 6.3, 3 H, Me).

^{13}C NMR ($CDCl_3$, 75 MHz): δ = 174.8, 169.7, 163.1, 159.2, 140.9, 139.1, 135.8, 129.0 (t, *J* = 23.8), 128.2 (t, *J* = 23.8), 127.2-126.3 (m), 123.3, 120.6, 110.2, 75,9, 54.5, 37.5, 32.4, 20.7

ESI-MS: *m/z* = 409 (100) [M + H]+.

ESI-HRMS: *m/z* [M + Hr calcd for $C_{20}11_{14}D_5C1N0_6$: 409.1210; found: 409.1192.

MS/MS [M + Hr (ESI+, 20 eV): *m/z* = 409.12 (41.20) $[M + H]^+$, 391.11 (7.4) [M – H_2O + $H]^+$, 363.11 (84.5) [M – H_2O – CO + $H]^+$, 346.08 (18.7) [M – H_2O - CO – NH_3 + H]+, 239.01 (100.0) [M – $C_9H_6D_5NO_2]^+$, 221.00 (9.2) [M – $C_9H_9D_5NO_3]^+$, 125.11 (8.0) $[C_9H_5D_5N]^+$.

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IV

Ochratoxin A Determination

4.1 Introduction

4.1.1 Background

After the discovery of ochratoxin in 1960's, the analytical methods for it, which typically occur in the mg/kg (ppb) range, have developed and expanded along with the general advances in analytical science. The earliest analytical methods were based on solvent extraction, crude clean-up on open-ended packed-silica columns and separation of the analytes of interest by thin layer chromatography (TLC). Such methods are still valid today and in the case of OTA, TLC with either visual assessment or instrumental densitometry is routinely applied in many laboratories in the developing world. The basic requirements of extraction, clean-up and separation for OTA determination in food matrices remain the same in current methods. Advances have come in the areas of sample purification techniques and in separation science with the development of high-performance liquid chromatography (HPLC) and associated detectors.

As in many other branches of analytical chemistry, the introduction of bench top mass spectrometers as detectors for GC or HPLC instruments has impacted on OTA analysis and has allowed detection at very low levels with simultaneous confirmation of the compounds of interest. Due to the range of chemical properties used for extract clean up and chromatographic detection. Another area of technological advance has been the introduction of solid phase extraction techniques using a range of chemistries (normal phase, reversed-phase, strong anion exchange). The development of immunoaffinity columns (IACs) containing antibodies specific to the analyte of interest has resulted in faster clean-ups and a greater degree of sample purification. This has been followed by the introduction of analytical methods which rely on direct fluorimetric measurement of the resulting purified extract (or a suitable derivatised product). When combined with HPLC separation, injected samples are

cleaner and the resulting chromatograms are less complex, with attendant advantages to HPLC column life and analytical reliability.

OTA analytical methods need to have low limits of detection (generally in the mg/kg range), be specific to avoid analytical interferences, be easily applied in routine laboratories, be economical for the laboratory involved and provide a confirmatory test. For official control and implemention of OTA regulations, a number of official methods, mostly based on HPLC, have been validated by interlaboratory collaborative studies conducted under the auspices of international bodies such as AOAC International. The validation process involves testing the within-laboratory repeatability, between-laboratory reproducibility, analytical recovery, and limits of detection and quantification. At the same time, the European Committee for Standardization (CEN) has published criteria for OTA analytical method performance.[1] However, aside from these official methods, a need exists for rapid screening methods which can be used for control purposes and in situations where rapid decisions are required, frequently in field situations at granaries, silos and factories. For this purpose, a number of screening methods and biosensors have been developed which are mostly based on immunological principles and use antibodies raised against OTA. These methods range from quantitative ELISA to qualitative tests based on obtaining a simple result of contamination above or below a set control level. Such tests, which are available in various formats such as lateral flow dipsticks, are evaluated by the level of false positive or false negative results. In terms of consumer protection, a level of false positives may be acceptable in that such batches will then be subject to more comprehensive testing. Thus in selecting a method for OTA analysis, it is necessary to consider the purpose for which the results are needed, the matrix to be analysed, the detection limit required and the expertise and infrastructure available.

In the next text, it will be reported the use of chromatographic techniques for OTA determination. Although the selectivity of MS is unchallenged if compared to common GC and LC detection methods and accuracy and precision are generally high, other factors must often be taken into account for evaluating the performances of MS detectors and the necessity of their use, such as the sensitivity, which may be different in different instruments and can be much lower than with fluorescence detectors, and the price of the instrument, which is generally higher than that of other detectors. The sensitivity issue may be a real problem especially in the case of LC-MS, in which the choice of the right interface is fundamental and the response can be very different for the different ionization techniques. The problem of sample preparation, which is of primary importance in OTA determination, will not be thoroughly approached, since it affects every chromatographic determination with any detector. When necessary, since it may affect recovery and sensitivity (due to matrix interference), a few comments on the pre-analysis preparation of the samples will be reported.

4.1.2 Ochratoxin A determination in food and feed by HPLC techniques and MASS spectrometry

LC is the technique of choice for OTA analysis, and MS detectors have long been used to confirm and quantitate ochratoxin in foods with high selectivity. However, as it will be discussed in the following text, LC using reversed phase columns and fluorescence detection appears to be fully adequate to the task, with lower LODs than MS/MS methods, being chromatograms usually clean enough to ensure a clear identification of the peak. Therefore, OTA detection and quantification is another case, such as for aflatoxins, where fluorescence is more satisfying than MS in terms of sensitivity, not to mention the easiness of operation and the lower cost of the

instrument. The higher selectivity displayed by MS detectors is not necessary for many food matrices, if a suitable sample preparation is performed. The best application for MS detectors is the clear-cut confirmation of ochratoxin in samples already turned out to be positive by LC/ FLD analysis. On the other hand, the use of MS detection, together with isotopically labeled standards, is fundamental to overcome problems encountered in the clean-up of the samples. It should be noted in any case that even with very specific MS/MS methods, problems due to matrix interference are often encountered in OTA determination in food matrices. One study discussing OTA determination, after derivatization, by GC/MS has also been reported.[2]

4.1.2.1 Gas Chromatography/Mass Spectrometry Determinations of ohratoxin A

A GC/MS method for the detection and quantification of OTA in wines was reported by Soleas, Yan, & Goldberg (2001).[2] The samples were extracted in dichloromethane, dried and derivatized with bis(trimethylsilyl)trifluoroacetamide (BSTFA) to yield the trimethylsilyl derivative of OTA (MW 1/4 619), which was analyzed by GC/MS in the SIM mode by monitoring eight specific ions. The LOD of the method was 0.1 ppb, higher than that obtained by an LC/PDA method presented in the same study (0.05 ppb). Moreover, also recovery and precision were less satisfactory in the GC/MS method, which is to be considered suitable for confirmation, due to its high selectivity, but not for routine detection of OTA in wines.

4.1.2.2 Liquid Chromatography/Mass Spectrometry Determination of Ochratoxin A

Earlier LC/MS methods made use of the first generation of interfaces, such as thermospray[3] or a direct liquid introduction,[4] with the known disadvantages in terms of robustness, sensitivity, and ease of operation. The first work with modern

instrumentation can be attributed to Becker et al. (1998),[5] who used an LC/ESI-MS/MS method for the quantification of OTA in wheat, coffee, and beer. The molecule was analyzed in the positive ion mode, where the fragmentation of the protonated molecular ion (m/z 404) yielded fragments corresponding to the loss of water (m/z 386), water/ carbon monoxide (m/z 358), phenylalanine (m/z 239), and water/ ammonia/carbon monoxide (m/z 341). The fragmentation of the deprotonated molecular ion (m/z 402) in the negative mode yielded only the fragment corresponding to the loss of carbon dioxide (m/z 358): being this loss not very characteristic, the positive ion mode was chosen for the selected reaction monitoring (SRM) analysis, by monitoring the fragments at m/z 358 and 239 generated by the protonated molecular ion. An LOD of 20 pg (as absolute amount injected) was obtained, almost comparable to that obtained with fluorescence detectors. In food samples, after an SPE and a concentration procedure with good recoveries, this meant an LOD of 0.01 ppb. Quantification was performed with standard solutions with external calibration (LOQ 60 pg or 0.03 ppb). Thus, the method can be a good confirmatory test for samples already turned out to be positive with fluorescence detection. The same procedure was applied by the same group[6] for the detection of OTA in beer. Again, the LC/MS/MS method was used mainly for confirmatory purposes, after a positive sample was detected by LC/FLD. In this case, the LODs were higher if compared to the previous study (0.1 ppb). Quite interestingly, dark beer could not be analyzed by LC/MS/MS due to matrix interferences.

A very interesting LC/MS/MS method for the analysis and confirmation of OTA in foods, after derivatization to the methyl ester and using the isotopically labeled OTA methyl(D_3) ester as internal standard, was reported by Jorgensen & Vahl (1999).[7] The deuterated methyl derivative was synthesized by the authors starting from the methanol(D_4)-boron trifluoride complex and a standard solution of OTA. The same

reaction with ''light'' methanol was then used to derivatize OTA in the food matrices, followed by mixing with the ''heavy'' internal standard OTA methyl(D_3) ester. The MS/MS detection for both isotopes was performed by ESI in the positive ion mode through a MRM experiment by monitoring three daughter ions for the ''light'' methyl ester derivative (m/z 239, 221, and 193) and one for the ''heavy'' internal standard methyl ester derivative (m/z 239). However, quantification was done by using only the daughter ion m/z 239 also for the ''light'' labeled analyte, and an LOD of 0.02 ppb was reported. The samples were extracted and concentrated before derivatization. The precision of the method was around 10% although the mean recovery was quite high (104– 121%). This could be due to the fact that the pseudomolecular ion at m/z 421 (the protonated molecular ion for the ''heavy'' standard) was present with discrete intensity also in the spectrum of the ''light'' analyte. As a consequence, the transition from 421 to 239 cannot be considered specific only for the standard, since it can be detected also for the analyte, thus interfering with an accurate determination of the real OTA content.

A comparison of two different LC/MS/MS methods, the former based on ESI interface and the latter on APCI interface, was reported by Lau et al.[8] In the positive ion mode, the ESI interface showed much higher sensitivity than the APCI interface. A sensitive LC/ESI-MS/MS method was extensively studied for the detection of OTA in human plasma and was also applied to the analysis of contaminated coffee. The fragmentation patterns were extensively discussed and the transitions to be monitored to achieve maximum sensitivity were carefully optimized: the monitored transitions were those from m/z 404 to 239, 404 to 358 (see above), and also the unusual 426 to 261, i.e., the transitions from the sodiated molecular ion to the sodiated 239 ion. Since a sodiated ion was also taken into account for quantification, the effect of the presence of alkali metal ions in the sample was also studied and found to be negligible. OTB was

used as internal standard in plasma, although its use in foods is not envisaged, since it may be present as contaminant. The LOD obtained with the optimized ESI-MS/MS detection was 5 pg (as absolute amount injected). Finally, by using LC/FLD to confirm the quantitative results, three different methods of quantification were compared: external standard, internal standard, and standard addition methods. The results were compared with those from the conventional LC/FLD method: all methods were in reasonable agreement (< ± 20% deviation from the average).

Two different columns were used by Zoellner et al.[9] to determine OTA in wines by LC/ESI-MS/MS in the MRM mode. The wines were extracted and concentrated by a SPE procedure. The monolithic column was found to give comparable results to the standard particle-based column, while enabling higher flow rates, thus reducing the analysis time. The usual transitions from 404 to 239 and to 358 and also the very specific transition from 406 (minor isotope of the protonated molecular ion) to 241 were monitored in the positive ion mode. Good linearity range, precision, and LOD (0.025 ppb) were achieved by using the mycotoxin ZEN as internal standard. Very interestingly, this method was compared in a subsequent study from the same group with widely used LC/FLD determinations of OTA in 18 naturally contaminated wine samples.[10] The simpler sample preparation by SPE allowed by the high selectivity of the MS method led to higher recoveries than liquid– liquid extraction and comparable with those obtained by IAC column purification. When the same sample preparation was used, the results of the MS method were highly consistent with those obtained by fluorescence detection. The major and evident drawback was in the LOD (and subsequently in the limit of quantification), which was five times higher (0.05 ppb against 0.01 ppb) by using MS detection. As a consequence, samples in which quantification was possible by LC/FLD were under the limit of quantification or even under the limit of detection when using LC/MS/MS. Although MS chromatograms

were usually cleaner, OTA in all wine samples could be confidently identified and quantified by LC/FLD.

Another recent study reported the use of LC/ESI-MS/MS (MRM mode), with procedures essentially analog to those previously reported, for the confirmation of OTA in South African wines previously turned out to be positive by LC/FLD analysis.[11] Recently, Dall' Asta et al.,[12] showed that changing the pH of the eluent (from 5.5 to 9.8) it is possible to detect OTA by directly injecting wine in HPLC/FLD with LOD of 0.05 ng/mL. Actually, the presence of OTA in wine is a widespread problem in the world and further studies are needed to investigate its natural occurrence in wines and for developing methods of prevention and decontamination.

An LC/FLD method with confirmation of positive samples by LC/ESI-MS (single quadrupole analyzer, positive ion mode, single ion recording of m/z 404) was also reported for the quantification of OTA in coffee.[13] The LOD of the fluorescence detector was reported to be 0.1 ppb, and the LOD of the MS detector was claimed to be the same, although data were not reported. The importance of the study mainly consists in the claim that a single quadrupole MS is sufficient for confirming by LC/MS the samples positive by LC/FLD.

A very interesting stable isotope dilution assay was recently reported by Lindenmeier, Schieberle, & Rychlik (2004),[14] who elegantly synthesized the ($^2H_5$)-OTA by hydrolyzing the phenylalanyl moiety from standard OTA and coupling the isocoumarin derivative with ($^2H_5$)-phenylalanine. The labeled internal standard was mixed with the matrix and OTA was extracted by an SPE technique or by immunoaffinity clean-up of wine and coffee samples.

Table 4.1 : Main recent LC/MS methods for ochratoxin A determination

Reference	Extraction / concentration	MS detection	Standard	LOD	LOQ
Becker et al., 1998; Degelmann et al. 1999	SPE	ESI positive ion mode MRM (triple quad.)	external	0.01 ppb[a]	0.03 ppb[a]
Jorgensen and Vahl, 1999	methylene chloride, then methanol/BF_3 (methyl ester)	ESI positive ion mode MRM (triple quad.)	(D_3)-OTA methyl ester	0.02 ppb	not given
Lau et al., 2000	SPE	ESI positive ion mode MRM (triple quad.)	variable	0.5 ppb[b]	not given
Zoellner et al., 2000a; Leitner et al., 2002	SPE	ESI positive ion mode MRM (triple quad.)	Zearalenone	0.05 ppb	0.15 ppb
Tuomi et al., 2001	SPE[c]	ESI positive ion mode MRM (ion trap)[c]	Reserpine	100 ng	200 ng
Lindenmeier et al., 2004	SPE + IAC	ESI positive ion mode SRM (triple quad.)	D_5-OTA	0.5 ppb	1.4 ppb

[a]These values are those found for wine. In beer, the LOD was higher than one order of magnitude (0.1 ppb).

[b]This value is somewhat higher than others because of the different matrix, since it has been calculated in human plasma.

[c]The method, although proposed for aflatoxins, OTA and other toxins, showed good recovery (99%) only for OTA. Moreover, the method also showed low precision and low accuracy. Again, OTA gave best results (5–27% RSD and 69–110% accuracy).

The ESI-MS/MS method was based on a SRM experiment by monitoring the specific transition from 404 to 358 for OTA and from 409 to 363 for labeled OTA. Both extraction procedures worked well for the wine samples, whereas for the coffee samples only the immunoaffinity clean-up yielded interferent-free MS chromatograms and was used as the preparative method of choice. The LOD was calculated at 0.5 ppb in wheat flour, with good recovery and precision. Finally, many different food samples were tested for their OTA content with the developed method. A summary of the main recent LC/MS methods for ochratoxin determination is reported in Table 4.1.

4.1.3. Conclusion

Several high-performance liquid chromatography (HPLC) methods with fluorometric detection (FLD) have been reported for the determination of ochratoxin A in wine or dried vine fruit, and two of them were successfully validated through collaborative studies, namely, for wine and beer [15] and for dried vine fruits.[16] The method of Visconti et al.[15] has been adopted as the official method by the Association of Official Chemists International (AOAC 2001.01), the European Committee for Standardization (CEN) (EN 14133), and the Organisation Internationale de la Vigne et du Vin (OIV 16/ 2001). The method of MacDonald et al.[16] is currently under discussion by CEN (working group TC/275/WG5) for approval as a European standard.. As it has been explained that LC using reversed phase columns and fluorescence detection appears to be fully adequate to the determination of OTA in food and feed. This is not only because the lower LODs than MS/MS methods , but also because the lower cost and the facility which make from it more convenient for the routine determination of OTA. Also the use of MS detection, together with isotopically labeled standards which is identically structure to OTA, is fundamental to overcome problems encountered in the clean-up of the samples. It should be noted in any case that even with very specific MS/MS methods,

problems due to matrix interference are often encountered in OTA determination in food matrices. The methods for determination of OTA in food and feed still need an improvement to overcome all the drawbacks and collect the advantages of the previous methods. The using of HPLC-FLD seems to be more sensitive than MS methods, and the using of an identically structure internal standard, which prevents the previously prevalent errors resulting from the using of a different structure internal standard, and provides a precision, accurate, and reliable results. If we can create a new methods can collect both of those factors, we can have the most reliable method, and this which will be explained in the next text.

4.2 New Method for the Determination of Ochratoxin A Based on High Performance Liquid Chromatography and Diastereomeric Dilution.

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Abstract

The development of sensitive and accurate analytical quantification of Ochratoxin A (OTA, a) in food products has recently received an increasing interest. In the presented work, we have developed a new and reliable method for OTA quantification, by using OTA 3-(S)- diastereoisomer (b) as internal standard by the means of Liquid Chromatography–Fluorescence Detection (LC-FLD), with immunoaffinity column cleanup. OTA 3-(S)-diastereoisomer is not naturally occurring but it was synthesized in our laboratory. OTA 3-(S)-diastereoisomer has nearly identical chemical and physical properties of the naturally occurring OTA; but HPLC-FLD allows discriminating between both of them by their retention time which is more than 1 minute difference. For The using of OTA 3-(S)-diastereoisomer as internal standard provides sensitive, accurate, reliable and simple method. The goodness of the methodology was confirmed by the data obtained from spiked samples in different concentration levels for wine, and wheat. The accuracy levels reached 98% and 99% for the two matrixes, while the RSD % was 1.1% and 1.5% in wine and wheat

respectively, a good correlation (r^2 = 0.9995), LOD(0.005 μg/L), and LOQ (0.007μg/L).in wine, and LOD(0.01 μg/Kg), and LOQ (0.015μg/Kg)in wheat.

Introduction

The quantification of organic substances has turned out to be of primary importance in the analytical chemistry field. Regulations and codex provides more and more severe limits in the amount of pollutants such as pesticides and toxins in various matrixes; furthermore, to ensure the quality of the foodstuff a deeper molecular investigation is required to finely characterize each product. In the two or three past decades the development of new analytical tools and devices has lead to a boost of performance in the assay of organic molecules in the low ppb or even in the low ppt range. The use of the chromatographic systems in conjunction with a variety of detection techniques has allowed sensitivity, reproducibility and in some cases good levels of specificity. Furthermore, the use of the internal standard method together with the latter techniques improved the repeatability and the accuracy of the measurements. The internal standard method is generally used to void some analytical errors in the measurement.

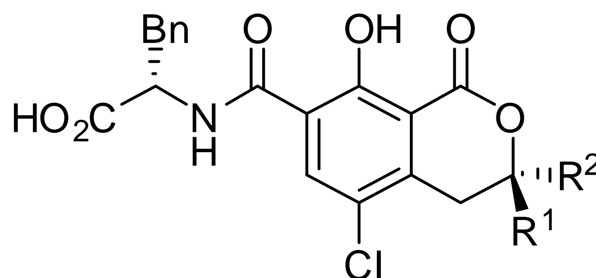
The internal standard is a compound that matches as closely, but not completely, the chemical species of interest in the samples, as the effects of sample preparation should, relative to the amount of each species, be the same for the signal from the internal standard as for the signal(s) from the species of interest in the ideal case. However, it can be difficult to find an appropriate substance that will elute in a position on the chromatogram that does not interfere or merge with any of the natural components of the mixture. The use of identically structure internal standard can not cause systematic errors and gives

a precision result. In other words, losses of the analyte are completely compensated for by identical losses of our IS, whereas in the using of a structurally different IS. is likely to show different losses and is, therefore, less suited. When structurally different internal standards are used, additional recovery and spiking experiments are necessary, which further increase the work-load. But often recoveries are not reproducible, which is an additional cause of imprecision.

This paper describes a new method of dilution analysis of ocrhratoxin A OTA, (**1)** based on the use of its *3-(S)-* diastereoisomer (**2**) as internal standard (Figure 4.1). The internal standard was easily synthesized in our laboratory;[17] the measurements were carried out using a HPLC system connected to a fluorescence detector after immunoaffinity clean up.

Ochratoxin A (R)-N-[(5-chloro-3,4-dihydro-8-hydroxy-3-(R)-methyl-1-oxo-1H-2-benzopyran-7-yl)carbonyl]-L-phenylalanine, **1**), a mycotoxin produced by some fungi of the Aspergillus and Penicillium species (such as Aspergillus ochraceus and Penicillium verrucosum), is usually detected and quantified by external standard methodology. It is found in raw and improperly stored food products.[18] OTA has been shown to be nephrotoxic, mutagenic, genotoxic, teratogenic, hepatotoxic, neurotoxic, immunotoxic, in both animals and humans,[19] and in 1993 was classified as a possible carcinogen to humans (Group 2B) by the International Agency for Research on Cancer (IARC).[20]

Figure 4.1 : structure of ochratoxin A and 3-(S)-ochratoxinA



Experimental section

Chemicals

Solvents were obtained commercially from Carlo Erba (Rodano, Italy); OTA (**1**) and other reagents were purchased from Sigma-Aldrich (St. Louis, MO); *3-(S)*-OTA (**2**), used as internal standard, was synthesized as reported.[17] OchraTest™ Immunoaffinity columns were purchased from Vicam (Milford, MA).

The PBS buffer was prepared by dissolving 8.0 g NaCl, 1.2 g Na_2HPO_4, 0.2 g KH_2PO_4 and 0.2 g KCl in 990 mL of purified water.

Sample Preparation.

a) **Wine**

50 µL of a 10 µg/Kg solution of internal standard (**2**) were mixed to 10 mL of wine, and were vigorously mixed with 10 mL of diluting solution (1% PEG + 5% $NaHCO_3$, pH 8.3) into a 100 mL flask, and then filtrated. 10 mL of the filtrate were passed onto the immunoaffinity column at a flow rate of 1 drop per second. The column was rinsed with 10 mL of washing solution (2.5% NaCl + 0.5% $NaHCO_3$) and then with 10mL of water at the same flow rate until dryness; compound **1** and **2** were eluted with 2 mL of CH_3OH: The solution was

evaporated to dryness at 50 °C under N_2. The residue was dissolved in 250 µL of HPLC mobile phase (see below), and the injected into HPLC.

b) **flour**:

500 µL of a 100 µg/Kg solution of internal standard (**2**) were mixed to 50 g of flour. The mixture was left to dry and then extracted at high speed with 100 mL of acetonitrile/water (60/40 v/v) for 5 minute. The residue was filtered onto a filter paper, and 10 mL of filtrate were added to 40 mL of PBS buffer. The resulting solution was filtered and 10 mL were poured into the immunoaffinity column at a flow rate of 1 drop/second. The column was then washed with 10 mL of PBS buffer and 10 mL of water at the same flow rate. The analytes were recovered from the column using 2 mL of CH_3OH. The solution was evaporated to dryness at 50 °C under N_2, dissolved in 1 mL of HPLC mobile phase (see below),and the injected into HPLC.

HPLC Conditions.

The analyses were performed using a HPLC 1100 from Agilent Technologies (Waldbronn, Germany) equipped with a fluorescence detector and a Luna C_{18} column, 25 cm × 4.6 mm (Supelco, Saint Louis, MO). The flow rate was fixed at 1 mL/min and the following isocratic eluents were used: 49% H_2O, 49% acetonitrile, 2% acetic acid. 60 µL of samples were injected into the loop, the run time was 20 min and the emission and absorption wavelength of the fluorescence detector were set to λ_{ex} = 333, and λ_{em} = 460, respectively

Analytical Parameters.

The limit of detection (LOD) and the limit of quantitation (LOQ) were calculated by applying equations 4.1 and 4.2, following the directives of IUPAC

and the American Chemical Society's Committee on Environmental Analytical Chemistry.

$$S_{LOD}=S_{RB} + 3\sigma_{RB} \quad \text{(eq. 4.1)}$$

$$S_{LOQ}=S_{RB} +10\sigma_{RB} \quad \text{(eq. 4.2)}$$

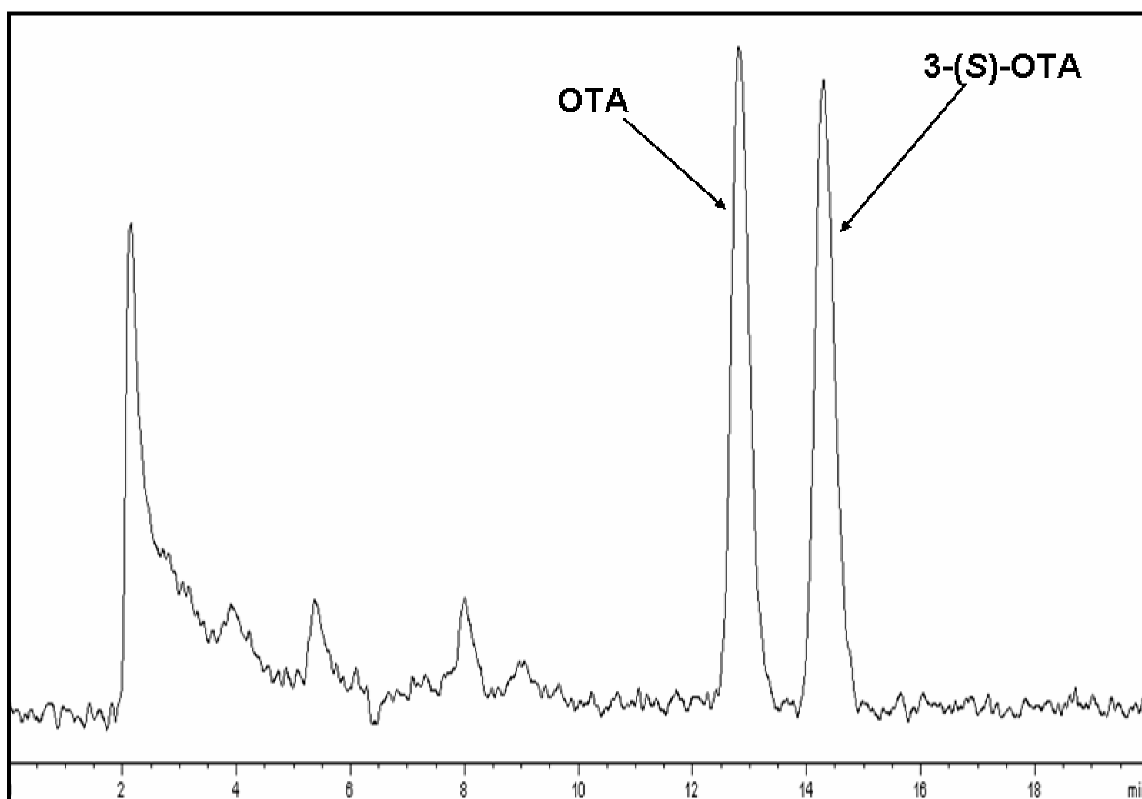
where S_{LOD} is the signal at the limit of detection, S_{LOQ} is the signal at the limit of quantitation, S_{RB} is the signal of white wine without OTA, and σ_{RB} is the standard deviation.

Result and discussion

OTA is generally and easily detected by fluorescence (FL) detection connected to a HPLC system, which is, in general, equipped with a C_{18} reversed phase column. This is made possible by the particular structure (figure 4.1) of the analyte that, absorbing the light at 333 nm, emits photons at 460 nm wavelength. To avoid interferences, the matrixes are in all cases subjected to previous steps of purification. The most common type of purification consists in the use of an immunoaffinity column which is based on the molecular recognition of the analyte.[21] Other methods of clean up relies on the exploitation of SPE cartridges packed with normal phase (silica),[22] reversed phase (C_{18} derivatized silica) or ion exchange.[23] The clean up procedures are useful also as pre-concentration step, and allow to lower the detection limit to a few ppt concentration detection limit. In addition to the FL detector, the assay of OTA has been performed with ultraviolet and mass spectrometry detection; in the latter case it is convenient to use an internal standard to avoid error on the determination of the analyte. In particular, ochratoxin B[24] and C[25] has been often added as internal standard, for their structural similarities to OTA; furthermore,

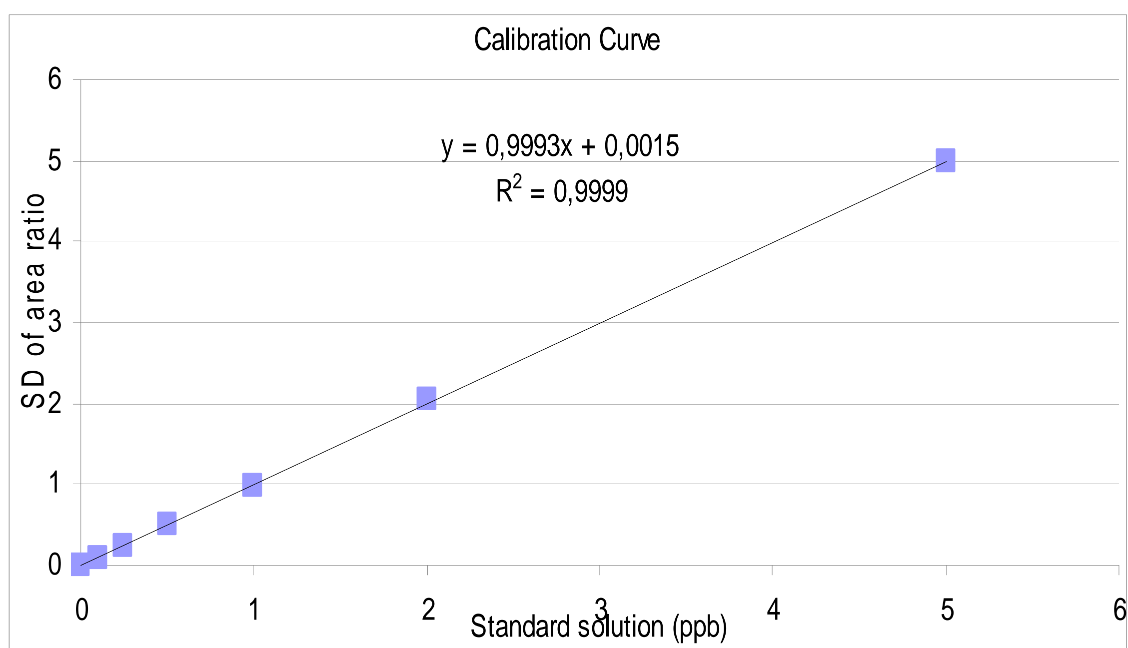
the mass spectrometric detection offers the advantage of measuring the signal due to the mass to charge values, so applications of isotope dilution may be found in literature.[26] To our knowledge there is only one paper regarding the use of a diastereomer as internal standard in a quantitative analysis, which, however, deals with the quantification of a peptide.[27] The internal standard used in the assay of OTA is the *3-(S)-* enantiomer (**2**); it should possess the same physical and chemical characteristic of the analyte; to check the latter assertion, a solution containing 1 μg/Kg of **1** and the same concentration of **2** has been submitted to the purification step on the immunoaffinity column. The recovered solution has been injected into the HPLC showing in the chromatogram two peaks at different retention times but with the same area (figure 4.2).

Figure 4.2 : The chromatogram of ochratoxin A and 3-(S)-ochratoxinA (1 μg/Kg each) after the submission to the immunoaffinity column



The calibration curve ($y = 0,9993x + 0,0015$, $R^2 = 0,9999$) has been obtained using standard solution of **1** in the range of concentration 0.1 to 5 ppb mixed with the internal standard (**2**) at the fixed concentration of 1 ppb. The correlation coefficient (R^2) shows that the linearity of the interpolated values is maintained in the range monitored (figure 4.3).

figure 4.3 : The calibration curve.



The methodology for the determination of OTA has been applied to wine and flour matrixes; to ensure the consistency of the method some accuracy tests were performed. A commercial wine and a packed commercial flour were submitted to classic quantitation of OTA using external standard solutions, and the addition standard method.[15] The latter experiments were conducted to know the exact amount of the analyte in those foods. The analyses revealed the presence of 254 ± 16 ppt and 31.5 ± 2 ppt of **OTA** in flour and wine respectively. The matrix samples were considered blanks, and, hence, spiked with a known amount of OTA and submitted to the analyses with the internal standard

methodology. The flour was spiked with **OTA** to reach a concentration of 0.4, 0.8, and 3.0 ppb. The results of the quantitation using **3-(S)-OTA** as internal standard gave 102.49%, 98.98% and 99.21% of accuracy for the spiked samples (Table 4.2). The replication of the measurements gave a precision value (RSD %) of about 1.7 to 4%.

Three samples of wine was altered by adding **OTA** to reach a mycotoxin concentration of 30, 45 and 150 ppt. Table 4.2 shows that the accuracy value, achieved from the analyses with the internal standard, were 101.2%, 98.99% and 102.1% respectively.

Table 4.2 : the accuracy and the RSD % values, for the spiked samples of wine and flour.

Sample type	OTA spiked Concentration (PPb)	Calculated concentration PPb	RSD (%) (average)	Accuracy (%) (average)
Wine	0.03	0,03036	1.52	101.20
	0.045	0,0445	1.81	98.98
	0.15	0,153	1.33	102.08
Flour	0.4	0,4099	1.71	102.50
	0.8	0,792	4.00	98.97
	3	2,977	1.76	99.22

Accordingly, The LOD and LOQ values, 0.010 μg/kg, 0.015 μg/kg for flour, respectively and 0.005 μg/kg, 0.007 μg/kg for wine, are well below than any other known method (table4.3). Finally, the use of identically structure internal standard shortens the time of analysis because the calibration curve is based on standard solutions, while the use of chemical analogue internal standards require calibration curves prepared in blank matrices.

Table 4.3: Reproducibility of the method (RSD %)*; Recovery, LOQ, and LOD measurements of analyzed matrices

matrix	LOD μg/kg	LOQ μg/kg	Recovery (%)	Reproducibility (RSD %) 0.04 μg/kg	Reproducibility (RSD %) 0.08 μg/kg
flour	0.01	0.015	97	3.5	4.1
matrix	**LOD μg/kg**	**LOQ μg/kg**	**Recovery (%)**	**Reproducibility (RSD %) 0.003 μg/kg**	**Reproducibility (RSD %) 0.015 μg/kg**
wine	0.005	0.007	98	3.2	3.9

*The reproducibility of the measurements was determined by extracting 3 times each foodstuff over a period of 1 week. The recovery was estimated by using an external calibration curve.

Our methodology also was applied for the determination of OTA in certificated wheat and was able to determine OTA in the limit of the reported concentration, with RDS% 2.47 for different 3 measurements. Also our methodology can be applied in the determination of OTA in different food matrixes, like corn milo, feeds, and raisins.

Conclusion

The aim of this work to develop a new mythology for the quantification of OTA by the means of HPLC-FLD using an identically structure internal standard 3-(S)OTA diastereoisomer. This new method prevents the previously Prevalent errors resulting from the using of a different structure internal standard, decreasing in the cost and the work load, and provides a precision, accurate, and reliable results. Due to the low limit of quantification and the best accuracy, this novel method is a potential tool for official OTA screening purposes in food and feed.

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V

Rotenone Properties, Toxicity, and Determination

5.1 Introduction

5.1.1 Background

Rotenone is a naturally occurring chemical with insecticidal and piscicidal properties obtained from the roots of several tropical and subtropical plant species belonging to genus Lonchocarpus or Derris. Rotenone is used for centuries as a selective fish poison and more recently as a commercial insecticide[1]. It is highly toxic to insects, fish and other aquatic life, but has low toxicity to birds and mammals. Rotenone is non-persistent in the environment, being quickly broken down by light and heat[2]. It does not accumulate in animals and is readily metabolised and excreted. For those reasons it has been used as a commercial insecticide for more than 150 years and for the management of fish populations since the 1930s. Nowadays rotenone is used in organic farming and for domestic gardening as a potent insecticide, sometimes in combination with pyrethrins and piperonyl butoxide.[3]

More than 2000 papers have been published on rotenone since 1990 and the literature is currently expanding at more than 100 papers per year. Recent research interest in rotenone stems mainly from biochemical interest in its highly specific action in selectively inhibiting mitochondrial activity and its possible anticancer properties. Most studies of rotenone have focused on the ability of this mitochondrial toxin to induce neuropathological effects reminiscent of those seen in Parkinson's disease.[4] A laboratory study suggests a possible link between high levels of exposure to rotenone and some forms of Parkinson's disease in animals; chronic subcutaneous exposure to low doses of rotenone (2.0–3.0 mg/kg/day) caused highly selective nigrostriatal dopaminergic lesions.[5] Many scientists now suspect that a vast majority of Parkinson's cases

are caused by interplay between an inherited genetic susceptibility and environmental toxins. However, rotenone and rotenoid-containing plants are reported to have anticancer activity in rats and mice.[6]

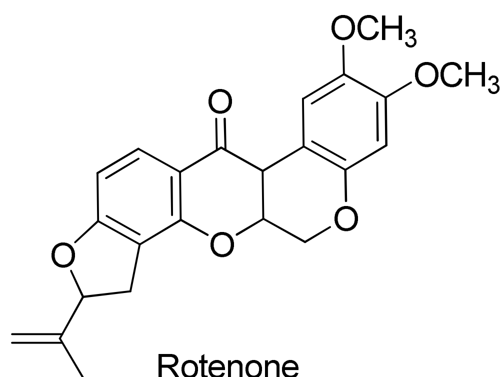
The widespread using of rotenone as an insecticide or in fishing management, and the recent studies which have related it to Parkinson's disease[5] gave alert to several national and international organizations and agencies to have another considerations about the human health risk assessment of the non synthetic pesticide, which was allowed by the USDA National Organic Program to be used to grow "organic" produce until 2005 when it was removed from the list of approved substances[7]. The use of rotenone in some country of EU has been prohibited, however, to clear their stock market has been extended until 2011 and its use is permitted for some crops, only[8]. A reregistration eligibility decision for Rotenone was issued quite recently by the US EPA, where the risks of exposure to this dangerous natural substance were revaluated.

5.1.2 Structure, properties, and stability

Rotenone, (6R,6aS,12aS)-1,2,6,6a,12,12a-hexahydro-2-isopropenyl-8,9-dimethoxychromenyl[3,4-b]furo(2,3-h)chromen-6-one, has an empirical formula $C_{23}H_{22}O_6$ (figure 5.1), is an isoflavonoid compound with a molecular weight 394.41. It consists of 70.04% carbon, 5.62% hydrogen, and 24.34% oxygen. It melts at 165-166°C, and it has high solubility in many organic solvents and is slightly soluble in water. It is derived from the roots of certain tropical species of the Leguminosae; in particular, cube or barbasco (Lonchocarpus utilis and L. urucu), derris (Derris elliptica), rosewood (Tephrosia spp.) and Rabbit's pea (Dalbergia paniculata). Most commercial

product comes from Central and South America. Dried derris roots contain an average of around 5% rotenone.

Figure 5.1 : the structure of rotenone



Rotenone is generally unstable and degrades rapidly in water. It has been shown to degrade as fast as within 2 weeks of application[9] but can also persist for periods up to 5 months.[10] The length of degradation time is dependent on many factors including light, temperature, turbidity, depth, presence of organic debris, and dose.[11] Rotenone is photochemically unstable and will readily breakdown in the presence of light into nontoxic dihydrorotenone and water.[9] This degradation process will occur at a quicker rate in the presence of higher water temperatures. Temperature appears to affect the breakdown of rotenone the most.[12]

6.1.3 Toxicity

5.1.3.1 general toxicity

Rotenone is a highly specific metabolic poison that affects cellular aerobic respiration, blocking mitochondrial electron transport by inhibiting NADH-ubiquinone reductase.[13] Rotenone is highly toxic to insects and aquatic life

including fish, temperature and contact time are the two main variables that significantly affect toxicity.[14] Adults amphibian and reptiles are less sensitive to rotenone; also it is slightly toxic to wildfowl, and birds. Rotenone is not easily absorbed in higher animals and does not accumulate in the body. Absorption of rotenone in the stomach and intestines is relatively slow and incomplete, although fats and oils in the diet promote its uptake due to solubility effects. Large oral doses (200 mg/kg in pigeons, 10 mg/kg in dogs) usually stimulate vomiting in animals.[15] The same is reportedly true for humans following suicidal ingestion of Derris root.[16] Once absorbed, rotenone is effectively broken down by the liver to produce less toxic excretable metabolites. Approximately 20% of the oral dose (and probably most of the absorbed dose) is excreted within 24 hours;[16] around 80% as water soluble products with the remainder as hydroxylated rotenoids,[17] the evidence for teratogenicity was inconclusive.[18] Recent research has revealed that rotenone may be an effective agent against certain types of cancers by its action in inhibiting cellular respiration.[6] Recently rotenone has been reported to cause effects in rats similar to those of Parkinson's disease. Lesions were observed in dopamine producing neurons of the substantia nigra of the brains of rats continuously exposed by IV. infusion for 5 weeks to 2–3 mg/kg body weight per day,[5] and it was investigated as a possible Parkinson-causing agent.

5.1.3.2 The proposed link between rotenone use and Parkinson's Disease

Following the publication of a scientific paper prepared by J. T. Greenamyre's group at Emory University titled "Chronic Systemic Pesticide Exposure Reproduces Features of Parkinson's Disease",[5] the news media picked up on this as there being a direct link between the development of Parkinson's Disease (PD) and rotenone and other pesticides. The stated goal of

Greenamyre's research was not to study health effects of pesticides, but rather "To develop a more accurate *in vivo* model of PD" to enable a method to induce Parkinsonism in lab animals to further the study of the onset and development of the disease. Because mitochondrial electron transport defects have been implicated in the onset of PD, and rotenone and a variety of other compounds are electron transport inhibitors, it was felt that rotenone might be useful in stimulating the onset of PD in laboratory animals under controlled conditions. To achieve the onset of PD in rats, Greenamyre' s group infused rotenone directly into the jugular veins of test animals at a dose of 2-3 mg/kg. Why did they choose to use direct injection rather than feed the animals rotenone, an experimentally more simple approach? Their paper states the answer, an answer that was well-known in the literature prior to their publication "Rotenone seems to have little toxicity when administered orally". Feeding 75 mg/kg of rotenone for two years (versus 3 mg/kg for 5 weeks in Greenamyre's study) resulted in no observed tissue or brain abnormalities. As discussed above, oral administration results in efficient metabolism of rotenone in the gut, and so little, if any, gets into the bloodstream and into the brain. These observations argue against the oral introduction of rotenone being a risk for the development of Parkinson's Disease, especially at piscicidal concentrations and the corresponding small dose to humans that might result from drinking a substantial quantity of rotenone-treated water. Also important to note, only a small fraction of people that die from PD have shown abnormal electron transport chemistries in their brain tissues, the majority have normal function. This observation argues against a direct link with rotenone in PD in humans.[19]

In piscicidal applications of rotenone, it is extremely unlikely that the route of ingestion would be anything other than ingestion through the drinking of rotenone-treated water. Under those conditions of use, it is highly unlikely, and indeed, not observed, that the Parkinsonism that Greenamyre detected from direct injection of rotenone into the bloodstream could occur under the conditions of the piscicidal use of rotenone. It is also important to note that there is not a documented link between rotenone use for agricultural or piscicidal applications and the development of PD. PD predates the onset of use of rotenone. There is no documented increased occurrence of PD among native Peruvians that were clearly exposed to significant amounts of rotenone through the pounding of cube roots to extract rotenone for their use in fishing, which included swimming in rotenone treated waters and consuming rotenone-killed fish. If ever there was a population that might exhibit rotenone-induced PD, the Peruvian natives would be one.

5.1.3.3 Dietary Risk

EPA estimated acute dietary exposure through food and water from the uses of rotenone. The acute dietary risk assessment considered only the population subgroup “females 13-49 years old” because an appropriate endpoint for this subgroup was available from a developmental toxicity study in rat. No acute dietary endpoint could be identified for the general population because other effects attributable dose were not observed in the available toxicity studies. The chronic dietary risk assessment considered drinking water for the general population and various population subgroups. The chronic assessment only considered drinking water because chronic exposure from food

(consumption of treated fish) is not expected based on rotenone's generally rapid degradation and low propensity to bioaccumulate in fish.

5.1.4. Rotenone and rotenone residue determination

The analytical studies of rotenone were started early since 1934 by using colorimetry,[20] and it was Paralleled to the progress in the analytical techniques. Since it started by colorimetry and passed through thin-layer chromatography,[21] gas chromatography,[22] Ultraviolet and infrared spectrometry,[23] and high-performance liquid chromatography (HPLC).[24-28] Mostly of those studies were established for the characterization, or for the determination of rotenone in water and fish tissue, and few were interesting in the determination of it in foods or studying its residue in crops. In 1980 Newsome and Shields[29] studied residues on lettuce and tomato, and it was the first study attracted the attention to the rotenone residue and the presence of rotenone in some dietary components. After Betarbet et al.[5] had reported real evidences were related strongly rotenone to Parkinson's disease, the interests about the determination of rotenone in dietary components has been increased. The first developed approach was involved by using Capillary Gas Chromatography,[30] but the GC method did not present the real approach that were presented by HPLC and MS methods which were reported later.

The determination of rotenone residues in raw honey was an important approach to direct the research recently to the residue of rotenone in foods.[31] Which was followed by many other studies were devoted to the development of more precious analytical methods that identify and quantify food contaminants and residue of rotenone. From 2004 until 2009 the development of the analytical

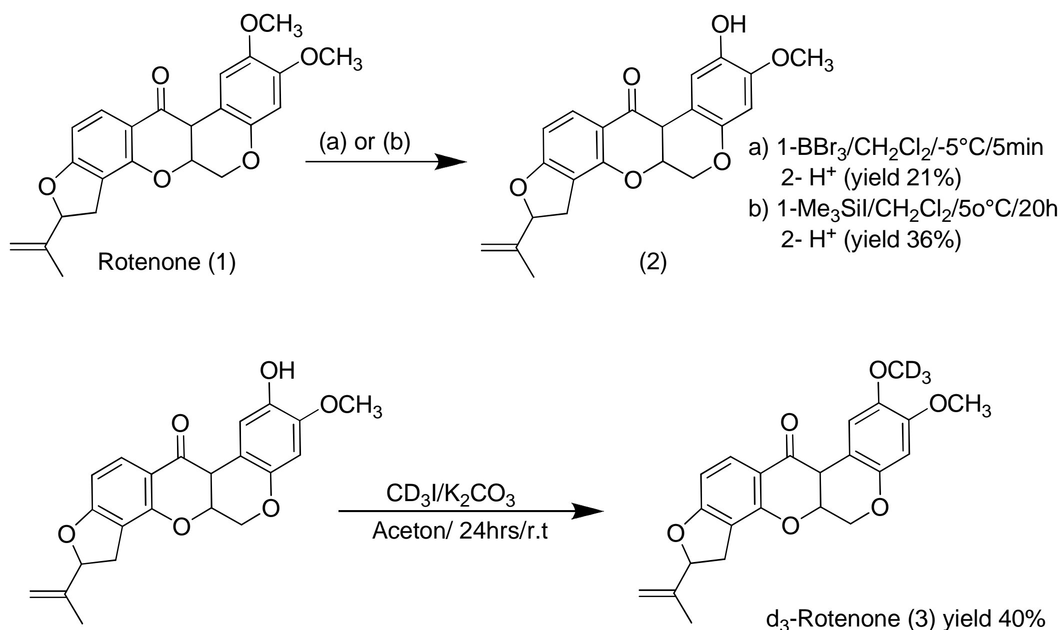
methods in the determination of rotenone in river water and olive oil have scored an increasing due to the high techniques methods have been used. High resolution electrospray tandem mass spectra, atmospheric pressure chemical ionization tandem mass spectrometry, high-throughput tandem mass spectrometry and multiple-reaction monitoring.[32-34] All those analytical methods were participated directly in the study of rotenone residue in olives and olive oil,[35-38] which gave new considerations about the rotenone residue in olive and olive oil. Those studies have corrected the considerations were taken about the decay rate of rotenone residue in olive oil, and they found the level of the rotenone residue was higher than the maximum residue legal limit.

5.1.5 The synthesis of d_3- rotenone

(-)-(6aS,12aS,5'R)-ROTENONE (l) contains ten ether C-O linkages, and fissions of the C(6a)-O(7) and 0(1')-C(5') bonds are involved in a number of its characteristic reactions. Specific ether cleavages open the way to new chemical transformations and offer potentialities for specific isotopic labeling necessary for our internal standard synthesis. With this in mind we have engaged in a study of rotenoid ether cleavages initiated by boron halides and other reagents capable of breaking C-0 bonds, and of allylic ether hydrogenolysis.

An earlier study [39] of the reaction between rotenone and boron tribromide has indicated that in the presence of 1 mol equiv. of reagent the 1',5'-seco-bromide is formed, whereas use of 2 mol equiv. causes additional demethylation of the C-2 methoxy-group giving (2); 3 mol equiv. of reagent de-O-methylates at C-2 and C-3, forming (2a) (scheme 5.1, 5.2). The n.m.r. data given in support of structure (2) seemed to us to be inconsistent with that formulation, and as the evidence for the position of initial de-O-methylation seemed slender, the reaction was re-examined.

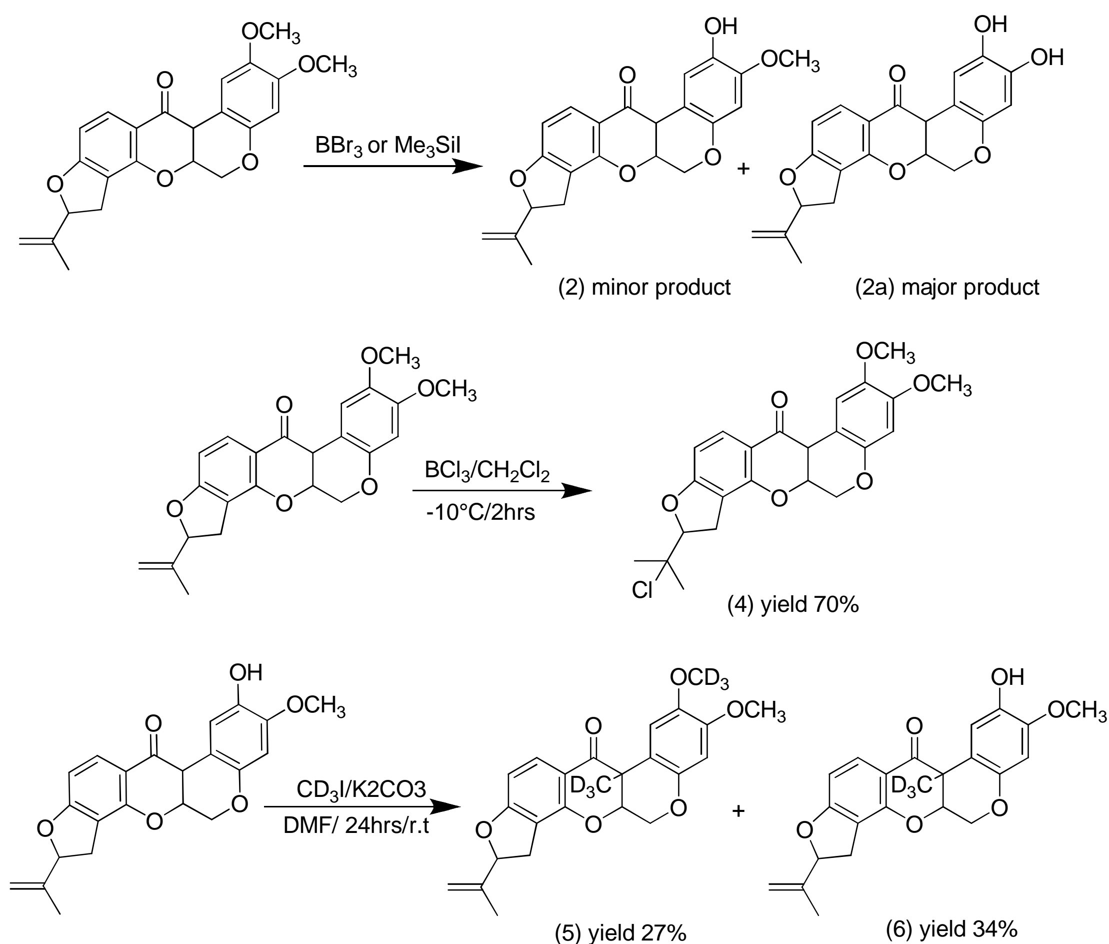
Scheme 5.2 : The best procedure for the synthesis of d3-rotenone.



*The products 2, 3 were characterized completely (HRMS, NMR, IR, and m.p),and all the yields were written are the separated yields.

The synthesis of 2-de-O-methylrotenon (2) can be done also using trimethylsilyl iodide as an O-demethylating agent, in which both boron tribromide or trimethylsilyl iodide can produce our desired product with yields of 21%, 36% respectively (scheme 1). But in case of using boron trichloride[40] which produced chlororotenone (4) as a major product (Scheme 5.2).

Scheme 5.2 : The byproduct can be exist with changing the concentration or the compounds.



Scheme (2)

*All the products (2a, 4, 5, 6) were characterized by only MS, and all the yields were written are the separated yields.

The methylation procedure was more easy, in which the 2-de-O-methylrotenon was dissolved in acetone in the presence of potassium carbonate and d_3-methyliodide, the d_3-rotenone (3) was obtained with a yield of 40%, this relatively low yield due to the isomeric changes and the corruption of the ring system of the rotenone. This can be very clear when it was used DMF as solvent in the same reaction condition,[41] it gives 6a-(d_3-methyl)-d_3-rotenon (5) and 2-de-O-methyl-6a-(d_3-methyl)-rotenon (6) which has the same molar mass and the same molecular formula of d_3-rotenone but it has not the same structure and it

can not be usable as internal standard because it has different MASS fragments (Scheme (2).Also deuterated diazomethane[42] can not be used as a deuterated methyl agent because of the isotopic purity of its product was less than 90%, and this isotopic purity can not qualified it to be used as internal standard. Because all of that the using of acetone as solvent in this reaction was the producible reaction for d_3-rotenone.

5.2 Isotope dilution method for the assay of rotenone in olive oil and river waters by liquid chromatography-multiple reactions monitoring tandem mass spectrometry

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Abstract

A new approach for the assay of rotenone (**1**) by isotope dilution mass spectrometry is presented. The extremely toxicity of rotenone, a natural phytodrug with insecticidal and piscicidal activity, recently banned by national and international environmental protection agencies, calls for the development of sensitive and accurate methods of analyses.
Accordingly, the proposed protocol is based on the availability of the labeled internal standard rotenone-d_3 (**3**) which can be conveniently prepared by consecutive and specific mono-O-demethylation, and remethylation with methyl iodide-d_3. The sensitivity of the method is confirmed by the very low

LOD and LOQ values achieved in the assay of (**1**) in two distinct fortified matrices, and is further supported by the observed accuracy values.

Keywords: Rotenone, Isotope dilution, APCI-MRM.

Running Title

Rotenone assay by isotope dilution.

Introduction

Rotenone ((2R,6aS,12aS)-1,2,6,6a,12,12a-hexahydro-2-isopropenyl-8,9-dimethoxychromeno[3,4-b]furo(2,3-h)chromen-6-one, **1** scheme 5.3) is a naturally occurring compound present in the roots of several tropical and subtropical plant species, belonging to genus Lonchocarpus or Derris,[43] characterised by insecticidal and piscicidal activity. It is used in powder or as emulsified liquid form in fisheries management to remove unwanted fish species, and in the eradication of exotic fish from non-native habitats.[44] Nowadays it is used in organic farming and for domestic gardening as a potent insecticide, sometimes in combination with pyrethrins and piperonyl butoxide.[3] Rotenone, once classified by the World Health Organisation as a moderately hazardous, class II, with LD_{50} for rats between 132 and 1500 mg per kilogram,[7] is now considered a risky natural molecules to be banned in agrochemical food chain. Most studies of rotenone have focused on the ability of this mitochondrial toxin to induce neuropathological effects reminiscent of those seen in Parkinson's disease.[45] A laboratory study suggests a possible link between high levels of exposure to rotenone and some forms of Parkinson's disease in animals; chronic subcutaneous exposure to low doses of rotenone

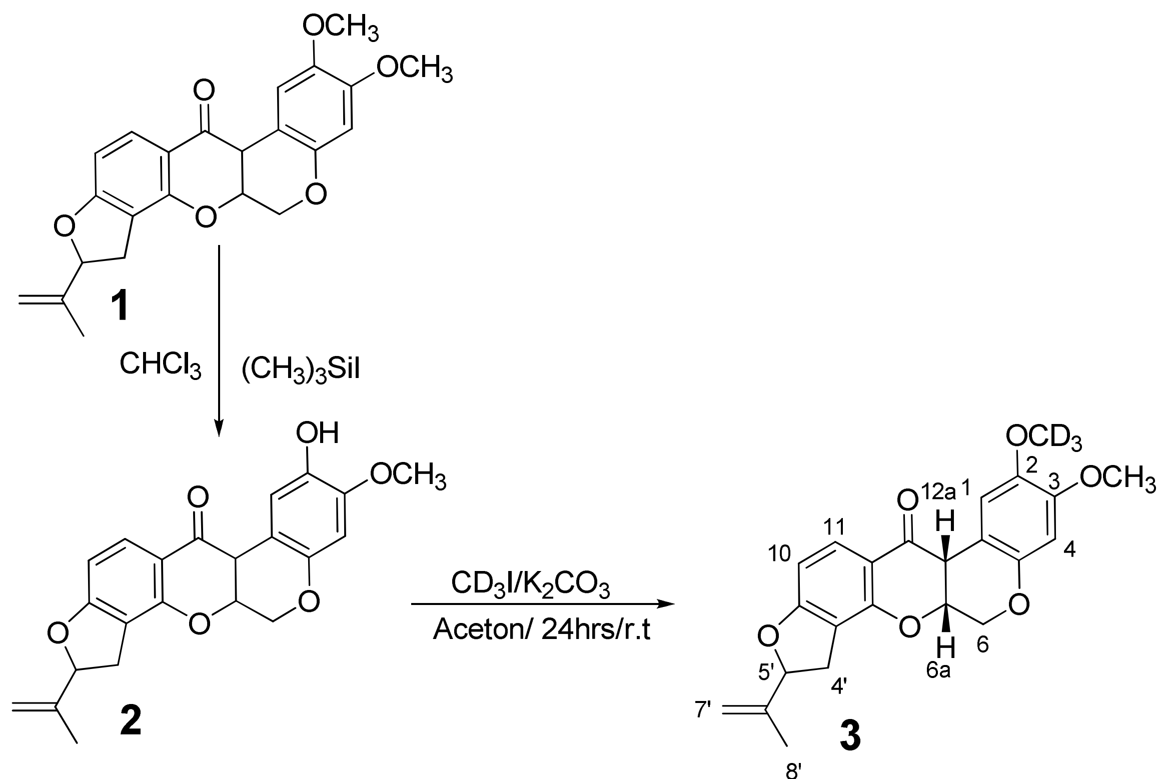
(2.0–3.0 mg/kg/day) caused highly selective nigrostriatal dopaminergic lesions.[5] Novel studies have shown that rats chronically treated with rotenone develop neuropathological and behavioural symptoms of Parkinsonism.[46] The use of rotenone in some country of EU has been prohibited; however, to clear the stocks, market has been extended until 2011 and its use is permitted for some crops, only.[8] A *reregistration eligibility decision* for rotenone was issued quite recently by the US EPA,[47] where the risks of exposure to this dangerous natural substance were revaluated.

The methods most frequently used to analyze rotenone in foods are based on liquid chromatography (LC) coupled to UV detection, [35,36] or hyphenated with mass spectrometers, as for the quantitation of this phytodrug in olive oil or in river water.[33,34] The assays of this analyte have been performed, up to now, by means of external standards or by rotenone derivatives used as internal standards.

The use of isotope dilution method, where the reference is represented by a labeled isotopomer should improve the reliability of the analytical procedure as recently shown in the quantification of micro-components and pollutants in foodstuffs.[48-51] By this approach, in fact, the sample loss risks in all the analytical steps are conveniently compensated.

The aim of the current study is, therefore, to develop an absolute isotope dilution method for rotenone assay, using the same matrices, previously analyzed in different experimental conditions, [33,34] to directly match the performances of the two approaches. The labeled internal standard rotenone-d_3 (**3**), was synthesized by demethylation of one of the methoxy groups followed by remethylation with methyl iodide-d_3 (scheme5.3).

Scheme 5.3 : Scheme of the synthesis of rotenone-d_3



Experimental section

Chemicals

Rotenone (95% purity) was used for the synthesis of **3**, while rotenone (PESTANAL® grade) was used for the assay. CD_3I, K_2CO_3, ACS grade acetone and chloroform were used for synthesis, while HPLC grade CH_3CN, H_2O and HCOOH were used for analyses. All the chemicals were purchased from Sigma-Aldrich, St. Louis, MO.

Synthesis of rotenone-d_3

2-de-O-methylrotenone (**2**, scheme 5.3) was obtained following literature methods.[52]

To a stirred solution of **2** (100 mg, 0.263 mmol) and K_2CO_3 (182 mg, 1.3mmol) in acetone (2 mL) CD_3I (381 mg, 164µL, 2.63 mmol) was added , under N_2 at 0°C; the mixture was stirred for 1 h at 0°C, and for further 24 h at room temperature. The reaction mixture was then partitioned between water and chloroform 10 ml each. The organic layers, washed with water and brine 10 ml each, and evaporated to dryness afforded a crude mixture which was further purified by preparative PTLC (chloroform-methanol, 99.50 : 0.50), to afford 41 mg of rotenone-d_3 (40% yield and 98% purity verified by HPLC). mp 145–148 °C.

IR (KBr): 2940 (s),1668 (m), 1611 (s), 1515 (s), 1466 (s), 1360(s), 1280 (m),1208 (s), 1100 (s), 837 (s), 822 (s), cm^{-1}.

^1H NMR (300 MHz, $CDCl_3$): δ = 7.81 (d, J = 8.5 Hz, 1 H, 11-H), 6.82 (s, 1 H, 1-H), 6.48 (d, J = 8.5 Hz, 1 H, 10-H), 6.42 (s, 1 H, 4-H), 5.21 (t, 1 H, J = 9.1 Hz, 1 H, 5'-H), 5.06 (br s, 1 H, 7'-H), 4.91 (br s, 1 H, 7'-H), 4.89 (br s, 1 H, 6a-H), 4.80(distorted dd, J = 2.9, 12 Hz, 1 H, 6-H),4.15 (d, J = 12.1 Hz, 1 H, 6-H), 3.81 (s, 1 H, 12a-H), 3.78 (s, 3 H, OCH_3) , 3.29 (dd, J = 9.9, 15.8 Hz, 1 H, 4 -H), 2.93 (distorted dd, J = 8.1, 15.7 Hz, 1 H, 4′-H), 1.75 (s, 3 H, 8′-CH_3)

^{13}C NMR: δ = 188.7, 167.3, 157.9, 147.1, 146.8, 143.2, 140.3, 130.1, 113.4, 113.3, 112.9, 112.4, 106.2, 104.8 100.3, 87.8, 72.3, 66.3, 56.6, 55.9, 44.7, 31.4, 17.2.

HRESI-MS: *m/z* 398.1680 $[M+H]^+$ calculated for $C_{23}H_{20}D_3O_6^+$ 398.1683. The isotopic distribution was d_3=96%, d_2=4%.

Sample preparation

Olive oil

A 20-μL portion of a stock solution (5 mg/kg) of internal standard was added to 1 g of olive oil spiked with rotenone and emulsified with 5 mL of CH_3CN. The mixture was placed in a sonic bath for 1 min and then loaded on to a C_{18} cartridge (5 g) previously washed with 10 mL of CH_3CN. The analyte was eluted with 30 mL of CH_3CN and the final solution was evaporated to dryness under reduced pressure. The residue was dissolved in 1 mL of CH_3CN and injected into LC-MS.

River water

A 20-μL portion of a stock solution (5 mg/kg) of internal standard was added to 50 mL of water taken from Emoli river in Rende and spiked with rotenone. The mixture was passed through a C_{18} cartridge (3 g) previously activated with 5 mL CH_3CN and 5 mL H_2O. The analyte was eluted with 30 mL CH_3CN and the final solution was evaporated to dryness under reduced pressure. The residue was dissolved in 1 mL CH_3CN and injected into LC-MS.

Mass spectrometry

The LC-MS analysis was carried out on a triple-quadrupole mass spectrometer LC 320 (Varian Inc., Palo Alto, CA), equipped with an APCI source interfaced with an HPLC Prostar 210 (Varian, Inc.). The chromatographic analysis was performed using a Pursuit, C_{18} column, 5.0 cm × 2.0 mm (Varian Inc.). The flow rate was fixed at 0.25 mL min^{-1} using the following eluants and

linear gradient: solvent A (H_2O, 0.1% formic acid), solvent B (CH_3CN); from 25% B to 95% B in 5 min; 3 min at 95% B isocratic; from 95% B to 25% B in 2 min; 2 min at 25% B isocratic. The corona needle current was fixed at 8 μA, the capillary was set at 85 V, the drying gas (N_2) pressure and temperature were 20 psi and 200 °C, respectively, while the nebulizing gas (N_2) pressure was 60 psi and the APCI gas (N_2) parameters were set to 20 psi and 350 °C; the housing temperature was fixed at 50 °C and the electron multiplier voltage was 1350 V. The dwell time was 0.200 s/scan, and the resolution was set using a mass peak width of 0.9 *m/z* units. The collision gas pressure (Ar) was fixed at 2 mTorr, and the collision energy was set to 19 eV for the transition *m/z* 395 → *m/z* 192 and *m/z* 398 → *m/z* 195, while for the transition *m/z* 395 → *m/z* 213 and *m/z* 398 →*m/z* 213 the collision energy was set to 18 eV.

High resolution experiments were carried out on a hybrid Q-Star Pulsar-i (MDS Sciex, Applied Biosystem, Toronto, Canada) mass spectrometer equipped with an ion spray ionization source. Samples were introduced by direct infusion (5 μL min^{-1}) of the sample containing the analyte (5 mg/kg), dissolved in a solution of 0.1% acetic acid, acetonitrile/water 50:50 at the optimum ion spray (IS) voltage of 4800 V. The source nitrogen (GS1) and the curtain gas (CUR) flows were set at pressures of 20 and 25 psi, respectively, whereas the first declustering potential (DP1), the focusing potential (FP), and the second declustering potential (DP2) were kept at 50, 220, and 10 V relative to ground, respectively.

The assay of rotenone was performed by MRM following the transitions *m/z* 395 → *m/z* 213 for **1** and *m/z* 398 → *m/z* 213 for the labeled internal standard (**3**);

Analytical parameters

The limit of detection (LOD) and the limit of quantitation (LOQ) were calculated following the directives of IUPAC and American Chemical Society's Committee on Environmental Analytical Chemistry, i.e. as follows:

$$S_{LOD} = S_{RB} + 3\sigma_{RB}$$

$$S_{LOQ} = S_{RB} + 10\sigma_{RB}$$

where S_{LOD} is the signal at the limit of detection, S_{LOQ} is the signal at the limit of quantitation, S_{RB} is the signal of each matrix without Rotenone, and σ_{RB} is the standard deviation. The concentrations were calculated using calibration curves. The recovery was calculated from the area of the signal obtained analyzing uncontaminated olive oil or river water added to a known amount of rotenone; the concentration of the latter samples was estimated by means of an external calibration curve.

Results and discussion

Isotope dilution is an analytical technique based on the addition of an isotopically labeled compound having the same structure of the analyte. Stable isotopes were particularly useful in the analysis of complex mixtures of organic compounds where the isolation of the desired analyte with satisfactory purity is difficult to obtain. The technique of isotope dilution improves precision and accuracy by reducing problems arising from matrix effects due to calibration and sample preparation. The main fragments of the MS/MS spectrum of the rotenone are the ions at *m/z* 192 (*m/z* 195 for the labeled species) and *m/z* 203.[53] These fragments are generated by two competitive fragmentation pathways. The ion at m/z 203 is obtained by a retro-Dies-Alder (RDA) of the protonated

molecular ion, while the radical cation at *m/z* 192 derives by RDA process of the transient species at *m/z* 394 obtained by hydrogen radical loss from the same precursor. Other fragments present in the MS/MS spectrum of the rotenone are the ions at *m/z* 367, *m/z* 241 and *m/z* 213, the exact mass of the latter is 213.0913 corresponding to elemental composition of $C_{14}H_{13}O_2^+$ and could likely be represented by the structure displayed in figure 5.2B.

Figure 5.2 : ESI MS/MS spectra of $[M + H]^+$ rotenone (**A**) and labeled rotenone (**B**).

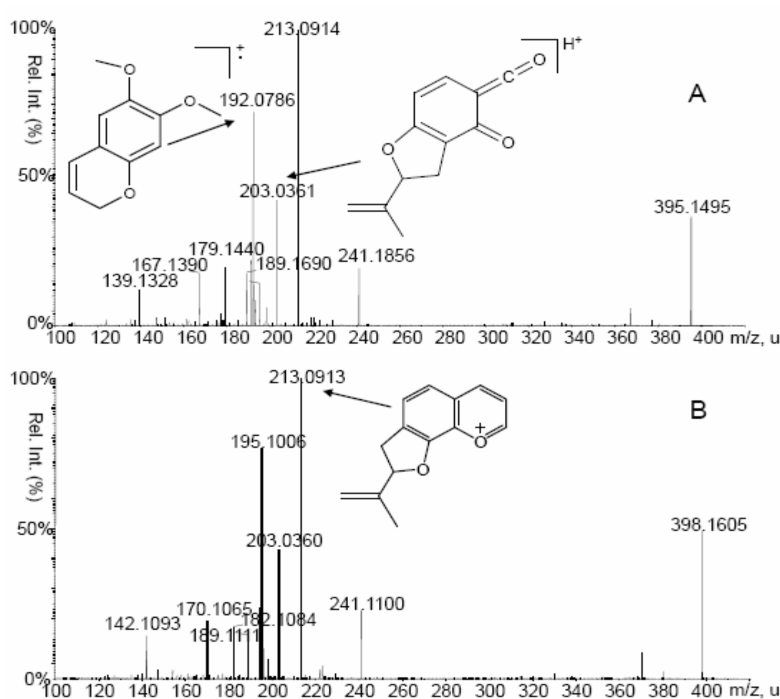


Figure 1

A calibration curve, built-up by triplicate injection of standard solutions of pure rotenone in the range 12.5 to 400 μg/kg containing the fixed amount of 100 μg/kg of **3** showed good linearity ($y = 2.6791x + 0.089$; $R^2 = 0.9995$). The sample preparation was performed using a SPE technique (see experimental), for both the oil and river water, in particular aqueous matrix were concentrated 50 times. A chromatographic step (twelve minutes) was essential when low amounts of contaminant were present in order to avoid unwanted interferences.

The new approach was developed by spiking with known amounts of analyte (**1**) and internal standard (**3**) two common matrices, such as olive oil and river water, in which rotenone is often employed.[12,13] The use of the labeled internal standard in the assay of **1** turns out to be more efficient if compared to any other known literature method. The accuracy values, in fact, were 102% and 104% in the two fortified olive oil samples, and 102 % for the river water (table 5.1). These values become 92% and 95% for olive oil and 92 % and 112% for river waters, when a chemical analogue is used as internal standard.[12, 13]

Table 5.1 : Analytical parameters of precision and accuracy

μg/kg	Calculated concentration	RSD % (average)	Accuracy % (average)
		Olive oil	
30	31.24 ± 0.35	1.10	104.12
300	307.47 ± 1.95	0.64	102.49
		River water	
1.2	1.22 ± 0.05	3.79	101.78
6.0	6.12 ± 0.03	0.53	102.03

Accordingly, The LOD and LOQ values, 2.0 μg/kg, 6.0 μg/kg for olive oil, respectively and 0.04 μg/kg, 0.11 μg/kg for river water, are well below than any other known method (table5.2). Finally, the use of the labeled internal standard shortens the time of analysis because the calibration curve is based on standard solutions, while the use of chemical analogue internal standards require calibration curves prepared in blank matrices.

Table 5.2 : Reproducibility of the method (RSD%)*; Recovery, LOQ, and LOD measurements of analyzed matrices

matrix	LOD µg/kg	LOQ µg/kg	Recovery (%)	Reproducibility (RSD %)	
				30 µg/kg	300 µg/kg
Olive oil	2.11	6.06	95	8.5	9.1

matrix	LOD µg/kg	LOQ µg/kg	Recovery (%)	Reproducibility (RSD %)	
				1.2 µg/kg	6.0 µg/kg
River water	0.04	0.11	97	9.2	6.1

*The reproducibility of the measurements was determined by extracting 3 times each foodstuff over a period of 1 week. The recovery was estimated by using an external calibration curve.

Conclusion

Among the different methods introduced for the assay of rotenone in natural matrices, mass spectrometry plays always a unique role for its specificity and sensitivity. The directives recently issued by international protection agencies on the rotenone disposal into the environment, call for the development of analytical methods suitable to determine traces of this extremely dangerous phytodrug in foods and in the environment. The isotope-dilution tandem-mass-spectrometric method here proposed, hyphenated with chromatographic devices allows an easy and accurate determination of this pollutant in complex mixtures.

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