ENHANCED DETECTION OF OCHRATOXIN A IN AGRICULTURAL COMMODITIES USING RABBIT ANTISERA AND LAYING-HEN EGG YOLK ANTIBODIES

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James Roger Clarke

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ENHANCED DETECTION OF OCHRATOXIN A IN AGRICULTURAL COMMODITIES USING RABBIT ANTISERA AND LAYING-HEN EGG YOLK ANTIBODIES

BY

JAMES ROGER CLARKE

A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

ABSTRACT

Ochratoxin A (OA) is a highly toxic and carcinogenic fungal metabolite. The wide-spread distribution of this toxin in nature and its incidence in the human food chain potentially poses a serious health threat to humans. These investigations were initiated to develop simple, specific, sensitive and economical immunobased assays for OA in selected agricultural commodities.

Laying-hen egg yolk was examined as an economical source of sensitive and specific antibody (IgY) for OA analysis. The OA directed IgY antibodies were found to cross-react with the ochratoxins A, B, C, α and the structurally related mycotoxin citrinin, 100, 100, 400, 33.3 and 1.9 %, respectively. Ochratoxin A could be detected in swine finisher diets using an indirect competitive enzyme-linked immunosorbent assay (ELISA) at concentrations greater than 50 ng/g. A simplified IgY extraction and purification procedure was developed based on aqueous buffer dilution, chloroform extraction and polyethylene-glycol 8000 induced IgY precipitation. Yields approached 70-80 % with corresponding purities of 86-92 %. These results demonstrated for the first time that laying-hen egg yolk antibodies specific for OA can be developed and applied to a quantitative ELISA.

Rabbit and laying-hen antisera specific for OA were compared in regard to sensitivity, specificity and production potential. The responses were examined using an ELISA. Rabbit produced OA-specific antisera faster and at greater concentrations in comparison to laying-hens. The two sources of antisera were

comparable in specificity but substantially different in sensitivity. The rabbit antisera could detect OA in spiked wheat samples at concentrations greater than 3 ng/g where as laying hens could only detect 50 ng/g or greater. These studies indicate rabbits may be a superior source of antisera for OA detection than laying-hens.

Ochratoxin A is known to accumulate at significant concentrations in the kidneys of swine fed contaminated diets, it was therefore necessary to develop a sensitive ELISA based on rabbit antisera. An improved sample preparation procedure was developed that was based on acidified ethyl acetate extraction. Extraction recoveries were determined by high-performance liquid chromatography (HPLC) and found to range from 91 to 110 % with an acceptable inter-assay coefficient of variation (CV) of < 12 % at OA concentrations greater than 4 ng/g. Rabbit antisera was developed and applied in an ELISA. Ochratoxin A could be detected reproducibly at concentrations greater than 8 ng/g with corresponding CV of 9 %. The ELISA of OA was highly correlated with conventional HPLC ($r = 0.94 \pm 0.07$). These studies resulted in the development of a simple, sensitive and specific immunoassay for OA that is sufficiently accurate for routine and regulatory analysis of swine kidneys.

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Finally, I wish to express my heartfelt gratitude and appreciation to my family. My parents, Maria and Roger and my sister Virginia have been an unlimited source of emotional, financial and nutritional support.

FOREWORD

This thesis has been written in the manuscript style. All three manuscripts have been accepted for publication. The first manuscript by the "Journal of Agricultural and Food Chemistry" the second by the "Journal of Food Protection" and the third in "Food and Agricultural Immunology".

The authors and titles of the manuscripts are:

I. Clarke, J. R., Marquardt, and Frohlich, A. A. 1993. Development of a quantitative and sensitive enzyme-linked immunosorbent assay for ochratoxin A using antibodies from the yolk of the laying-hen. J. Agric. Food. Chem. (accepted).

II. Clarke, J. R., Marquardt, R. R., and Frohlich, A. A. 1994. Comparative studies on the specificity and sensitivity of rabbit and laying-hen antisera to ochratoxin A. Food Agric. Immunol. (accepted)

III. Clarke, J. R., Marquardt, R. R., and Frohlich, A. A. 1994. Quantitation of ochratoxin A in swine kidneys by enzyme-linked immunosorbent assay using a simplified sample preparation procedure. J. Food. Prot. (accepted).

Three additional manuscripts which are not directly related to this thesis but are based on this research have been submitted and in one case published in a peer review journal.

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LIST OF ABBREVIATIONS

A_w.....water activity
BSA.....bovine serum albumin
CDI.....1,1'-carbonyldiimidazole
CFA.....complete Freund's adjuvant
$CHCl_3$.....chloroform
CV.....coefficient of variation
DCC.....dicyclohexylcarbodiimide
EIA.....enzyme-based immunoassay
ELISA.....enzyme-linked immunosorbent assay
h.....hour
H_3PO_4.....phosphoric acid
HPLC.....high-performance liquid chromatography
IFA.....incomplete Freund's adjuvant
IgY.....laying-hen egg yolk antibody
kg.....kilogram
M.....molarity
MA.....mixed anhydride
mg.....milligram
min.....minutes
ml.....millilitre
Na_2CO_3.....sodium carbonate
NaCl.....sodium chloride
NaOH.....sodium hydroxide
NHS.....N-hydroxysuccinimide
nm.....nanometres
OA.....ochratoxin A
Oα.....ochratoxin alpha
OB.....ochratoxin B
Oß.....ochratoxin beta
OC.....ochratoxin C
°C.....degrees Celsius
OD.....optical density units
ODS.....octyldecyl-silane
(4R,S)-OH-OA.....4-hydroxyochratoxin A
10-OH-OA.....10-hydroxyochratoxin A
OV.....hen egg ovalbumin
PEG.....polyethylene glycol-8000
PBS.....phosphate buffered saline
PBS-T.....phosphate buffered saline containing Tween-20

pKa.....dissociation rate constant
ppb.....parts per billion
ppm.....parts per million
r.....correlation coefficient
RIA.....radioimmunoassay
TLC.....thin-layer chromatography
μg.....microgram
μl.....microliters
UV.....ultraviolet-light
WSC.....water-soluble carbodiimide

GENERAL INTRODUCTION

Mycotoxins are a heterogenous group of low molecular weight compounds. These molecules are produced as a consequence of secondary metabolism in certain fungi and can in certain cases contaminate foods and feeds before and after harvest (Jelinek et al., 1989). Compounds such as these can cause deleterious effects in animals and have in certain cases been implicated as causal agents in human disease (CAST, 1989). Certain mycotoxins can accumulate and pass through the human food-chain. It is thus imperative that we minimize their presence in the feed-chain. By determining their incidences and concentrations in the food-chain, we can begin to understand their toxicity significance. The potential toxicities can therefore be alleviated by routine surveillance, sound management, and chemical or biological detoxification.

Ochratoxin A (OA) is a mycotoxin and is known to be produced by several fungi in the *Aspergillus* and *Penicillium* genera. Ochratoxin A is a phenylalanine substituted 7-carboxy-5-chloro-8-hydroxy-3-4-dihydro-methylisocoumarin (Cole and Cox, 1981) belonging to a class of molecules collectively referred to as ochratoxin. Several compounds belong to the ochratoxin group however OA is considered to be the most important member of this group (Marquardt and Frohlich, 1992). Many agricultural commodities in different areas of the world have been shown to contain OA. These include many different types of foods and

feedstuffs, for instance, corn, oats, wheat, barley, nuts, mixed grain feeds, and swine kidney (Kuiper-Goodman and Scott, 1989). Experimentally, it has been shown that exposure to OA can cause a wide variety of health problems in animals. These problems may include nephrotoxicity, carcinogenicity, hepatotoxicity and immunosuppression (Chu, 1974b; Steyn, 1984; Dwivedi and Burns, 1986 a,b). Ochratoxin A is known to accumulate in the kidneys and liver of the contaminated animals (Krogh, 1983) and has a relatively long half-life (89 h) in the blood because of the toxins high affinity for serum albumins (Galtier et al., 1981; Chu, 1974a,b), perhaps, increasing the potential toxicity to humans due to carry-over phenomena. Residues of OA have been routinely detected in the blood and tissues of livestock, notably swine (Marquardt et al., 1988; Scott, 1989) but more importantly in the blood of man (Hult et al., 1982; Gareis et al., 1988; Breitholtz et al., 1991; Frohlich et al., 1991). Ochratoxin A has been implicated in porcine nephropathy (Krogh, 1978), the symptoms of which include polydipsia, polyuria, interstitial fibrosis, tubular atropy and is likewise suspected to be a causal agent in Balkan endemic nephropathy, a fatal and chronic human renal disease found in parts of Bulgaria, Romania, and Yugoslavia (Krogh, 1977; Pavlovic et al., 1979). To date eleven or more countries have imposed or suggested strict regulatory limits for this toxin because of its widespread occurrence and potential toxicity to humans and livestock (van Egmond, 1991b). It was thus inevitable that

economic and sensitive monitoring techniques be developed for this toxin. The putative detection technique must be simple, fast, economical and above all specific and sensitive. Techniques such as thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) and more recently immunoassay have been developed for the routine detection of OA (van Egmond, 1991a). These techniques require that the samples be efficiently extracted. The most common solvent used in the extraction of OA from plant and animal tissues is chloroform, an extremely toxic and restricted chemical (van Egmond, 1991a). It would therefore be advisable to reduce the use of this solvent or substitute it with less toxic solvents. The conventional chromatographic approaches for OA are generally sensitive and specific, but are difficult, slow and relatively expensive to perform. The main draw-back being the need for extensive sample clean-up prior to analysis. The practical advantages of an immunoassay approach have been described for many mycotoxins (Pestka, 1988; Chu, 1990; Morgan, 1989; Wilkinson et al., 1992). These assays were found to be specific and sensitive and applicable to many sample matrixes. There are several reports of radio-immunoassay formats being used for the quantification of mycotoxins, however, the more commonly described technique is enzyme-linked immunosorbent assay (ELISA). The preference for ELISA can possibly be attributed to the hazardous and labile nature of radio-labels (Candlish, 1991). Most immunoassays for OA use

antisera from rabbits or monoclonal antibodies derived from mouse hybridoma (Chu et al., 1976; Pestka, 1981; Lee and Chu, 1984; Morgan et al., 1983, 1985, 1986; Rousseau et al., 1985, 1986, 1987; Chiba et al., 1985; Aizawa et al., 1987; Candlish et al., 1986, 1988; Fukal, 1987; Ruprich et al., 1988; Martlbauer and Terplan, 1988; Kawamura et al., 1989, 1990; Ramakrishna et al., 1990; Ueno et al., 1991; Breitholtz-Emanuelsson et al., 1992). These types of antibodies are sensitive and specific, however, they tend to be expensive to produce when large amounts are needed. Large amounts of antibody will need to be developed if immunochemical approaches are to become common-place in mycotoxin and more specifically for OA regulatory analysis. Laying-hen egg antibodies have been reported to be an excellent source of specific antibody. There is some evidence that suggests laying-hens can produce larger amounts of specific antibody when compared to rabbits. The antibodies can also be non-invasively collected from eggs, readily isolated and purified and applied to a wide variety of immunoassay applications (Gassmann et al., 1990). The recent works of Hsu and Chu (1992), Li et al. (1994) and Kierek-Jaszszuk et al. (1994) suggest that these antibodies can be used for the development of an ELISA for mycotoxins. Laying hen antibodies were developed against aflatoxin B1 and T-2 toxin, two highly toxic mycotoxins. The antibodies were readily produced by standard immunization techniques, the antibodies were easily isolated, and applied to a competitive-type ELISA. The

apparent antibody sensitivities seen with laying-hens for aflatoxin B1 are not as high as those previously reported with rabbits (Hsu and Chu, 1992; Candlish, 1991).

The present studies describe two polyclonal antibody sources, specifically, laying-hen egg-yolk antibody and rabbit antisera which are suitable for the development of an OA specific ELISA in selected agricultural commodities. A simple and efficient egg-yolk antibody isolation and purification procedure is described. Development of this procedure included a systematic investigation of the specificity and sensitivity differences of laying-hen and rabbit polyclonal antisera to OA using wheat as a test sample matrix. Two ELISA's are also described for the quantitation of OA in swine finisher diets (laying-hen egg antibody) and swine kidney (rabbit antisera). Two efficient and ELISA compatible solvent systems that avoid the use of chloroform are described for the extraction of OA from a swine finisher diet, a complex and mixed-grain matrix, and swine kidney.

LITERATURE REVIEW

1. Toxigenic Fungi.

Fungi are among the major causes of damage and spoilage in stored grains. Fungal contamination can result in decreased nutritional quality and hence reduced market value of the grain (CAST, 1989). Some species of fungi have the capacity to produce biologically active secondary metabolites, some of which are beneficial and can be used as antibiotics. The toxic metabolites produced by fungi are referred to as mycotoxins. The natural occurrence of fungi in stored grains and their potential for commodity destruction and or mycotoxin production is of economic concern and has far reaching implications in animal and human health.

1.1 Fungal Species Associated with Ochratoxin A Production and their Natural Occurrence

A large number of *Aspergillus* species have been reported to produce OA and include *A. ochraceus*, *A. ostianus, A. quercins, A. sulphureus* and potentially *A. niger* (Hesseltine et al., 1972; Abarca et al., 1994). However, *A. ochraceus* is the only member of this group which produces significant amounts of OA in cereals. The significance of *A. niger* contamination remains to be verified but in view of its high incidence in nature careful study should focus on this mold. Molds such as *A. ochraceus* are wide-spread pests in natures and have been found to contaminate a large-variety of agricultural commodities. Commodities infected

have included wheat, oats, barley, rice, animal feed, nuts, and animal products (Abramson et al., 1983; Yamazaki et al., 1970; Bacon et al., 1973; Doupnik and Bell, 1971; Doupnik and Peckman, 1970; Escher et al., 1973). Ochratoxin A has also been reported to be produced by *Eurotium herbariorum and Eurotium repens*, two species that occur widely in cereals (Chelkowski et al., 1987; Frisvad and Samson, 1991). Chemotypes I and II of *Penicillium verrucosum* are currently the only significant and confirmed OA producers in this genus (Pitt, 1987; Frisvad and Samson, 1991; Frisvad and Filtenborg, 1989). In earlier studies *P. viridicatum* was mistakenly identified as the OA producer (Pitt, 1987). Toxigenic strains other than *P viridicatum* have been isolated in stored cereals, corn, rapeseed and mixed-feed and include *P. cyclopium, P. chrysogenum, P. palitans* and *P. janthinellum* (Shotwell et al., 1969; Scott et al., 1972; Scott 1977; Mills and Abramson, 1982) however their identities may be suspect (El-banna et al., 1987; Frisvad, 1989).

1.2 Environmental Factors Consistent with Ochratoxin A Production by Fungi

P. verrucosum has a temperature and water-activity (aw) requirement lower than that of *A. ochraceus* for the production of OA in cereals (Northolt et al., 1979). *P. verrucosum* has a temperature and aw range of 16-24 °C and 0.90-0.93, respectively, for optimal growth and production of OA. The corresponding values for *A. ochraceus* are 31-37°C and 0.95-0.99 (Lillehoj and Elling, 1983). The minimum temperatures for OA production by *P. verrucosum* and *A. ochraceus* are

4 and 10°C in barley, respectively (Haggblom, 1982 and Domaglou et al., 1984). These findings agree well with the observations that *P. verrucosum* is the major OA producer in cereals in northern countries with cooler climates such as Scandinavia and Canada and *A. ochraceus* in warmer regions such as Yugoslavia and Australia (Krogh, 1987). Surveys of Canadian swine blood (Marquardt et al., 1988; Frohlich et al., 1991) have indicated that OA incidence is greatest in July with the greatest mean OA concentrations occurring in October. The higher levels and incidences associated with the months of July and October were attributed to higher temperatures in the stored grain mass during spring and fall. The higher temperatures and ambient moistures associated with spring and fall would be more conducive to the growth of the toxigenic fungi. Tholstrup and Rasmussen (1990) analyzed several cereals and observed a direct relationship between humidity at harvest time and fungal growth. Ochratoxin A levels were high when the cereals were harvested in wet years.

2. Chemical Characteristics of Ochratoxin

Several naturally ochratoxins have been observed and their structures have been elucidated. The dechlorinated form of OA is referred to as OB. The amide-bond hydrolyzed forms of OA and OB are referred to as Oα and Oß, respectively. The ethyl ester of OA is known as OC. Hydroxylated forms of OA have been

isolated and include (4R)-OH-OA, (4S)-OH-OA and 10-OH-OA (Cole and Cox, 1981; Marquardt and Frohlich, 1992) (Figure 1, Table 1).

2.1 Ochratoxin A

OchratoxinA,(R)-N-[(5-chloro-3,4-dihydro-8-hydroxy-3-methyl-1-oxo-1H-2-benzopyran-7yl)carbonyl]-L-phenylalanine, is a toxigenic secondary metabolite that is frequently produced by certain storage fungi in the *Aspergillus* and *Penicillium* genera. Ochratoxin A is a colourless and low molecular weight (403 MW) compound with a molecular formula of $C_{20}H_{18}NO_6Cl$. It is a heat stable and crystalline compound that is highly soluble in non-polar organic solvents such as chloroform and benzene, and in polar organic solvents such as methanol and acetonitrile (Roschenthaler et al., 1984). It is also highly soluble in aqueous solutions at pH values above its pKa, that is $NaHCO_3$, but only slightly soluble in distilled water (Steyn, 1984). Ochratoxin A has melting temperatures that depend on its solvent of crystallisation. It was found to melt at 90 and 169°C when crystallized from benzene and xylene, respectively. Ochratoxin A has a maximum ultraviolet adsorption spectrum at 213nm ($\in$ 36,800) and 332nm ($\in$ 6400) in absolute ethanol. In aqueous solutions OA has maximas at 333 and 380nm at pH 1.5 and 8.5, respectively, (Roschenthaler et al., 1984); this pH dependency was attributed to the dissociation in the phenolic hydroxyl-group (Steyn, 1984). Fluorescence emission of OA was found to be dependant on the solvent polarity

and composition (Hald et al., 1993). Fluorescence maximums are also known to increase with increasing pH (Scott, 1994). In aqueous solutions OA exists in both ionized and non-ionized forms. The dissociation constant of the phenolic hydrogen in the dihydroisocoumarin ring of OA is pKa 7.04 (Chu, 1974b). Infrared and ^{13}C nuclear magnetic resonance spectroscopy has indicated that OA exists in a ß-conformation where the NH group of the amide-functionality is hydrogen-bonded to the phenolic oxygen, and the phenolic hydrogen is hydrogen-bonded to the carbonyl group in the lactone-functionality (Steyn, 1984, Bredenkamp et al., 1989). Ochratoxin A is known to bind tightly but non-covalently with certain serum proteins of animals with a particularly high affinity for serum albumins (Chu, 1974a). Fluorescence maximums are also known to shift when OA is bound to the serum albumins. Oxidized iron is known to form a tight complex with OA. In the process of reduction free-radicals are formed which can potentially lead to lipid peroxidation (Rahmitula et al., 1988; Omar et al., 1990). Hasinoff et al. (1990) reported that the iron-complex of OA produced highly toxic hydroxyl radicals in the presence of NADPH and cytochrome P-450 reductase. The OA amide can be hydrolyzed by strong inorganic acids, action of the enzyme carboxypeptidase A, and microorganisms in the rumen, caecum, and large intestine to L-phenylalanine and 7-carboxy-5-chloro-8-hydroxy-3-4-dihydro-methylisocoumarin, Oα (Doster and Sinnhuber, 1972; Steyn, 1984).

2.2 Ochratoxin α

Ochratoxin α (Table 1) is a non-toxic, low molecular weight (256 MW), non-coloured, crystalline metabolite of OA which is primarily excreted in the urine and feces of animals consuming OA contaminated diets (Marquardt and Frohlich, 1992). It is more soluble than OA in distilled water and has a pKa of 11.0. It has been rarely identified in naturally contaminated grain samples but is known to occur in toxigenic *A. ochraceus* inoculated wheat (Xiao et al., 1994). There is some suggestion that Oα can cause free-radical damage at very high pH's since malondialdehyde was detectable (Rahimtula et al., 1988).

2.3 Ochratoxin B

Ochratoxin B (Table 1), the dechlorinated form of OA (369 MW) is considered to be substantially less toxic, than OA (Cole and Cox, 1981). It is similar to OA in solubility. It has a pKa of 8.0. Free-radical production is possible but less than that from OA (Rahimtula et al., 1988). It has been reported that OB is cleaved to L-phenylalanine and 7-carboxy-8-hydroxy-3-4-dihydro-methylisocoumarin, Oß, faster than OA is cleaved (Doster and Sinnhuber, 1972). The toxin is found in nature but at lower concentrations than OA. However, there are in vitro reports showing OB to be produced preferentially by *A. ochraceus* on oilseed crops such as soybeans and peanuts but not grains (Madhyastha et al., 1990).

FIGURE 1. The general structure of ochratoxin. See Table 1 for R-group structure assignment.

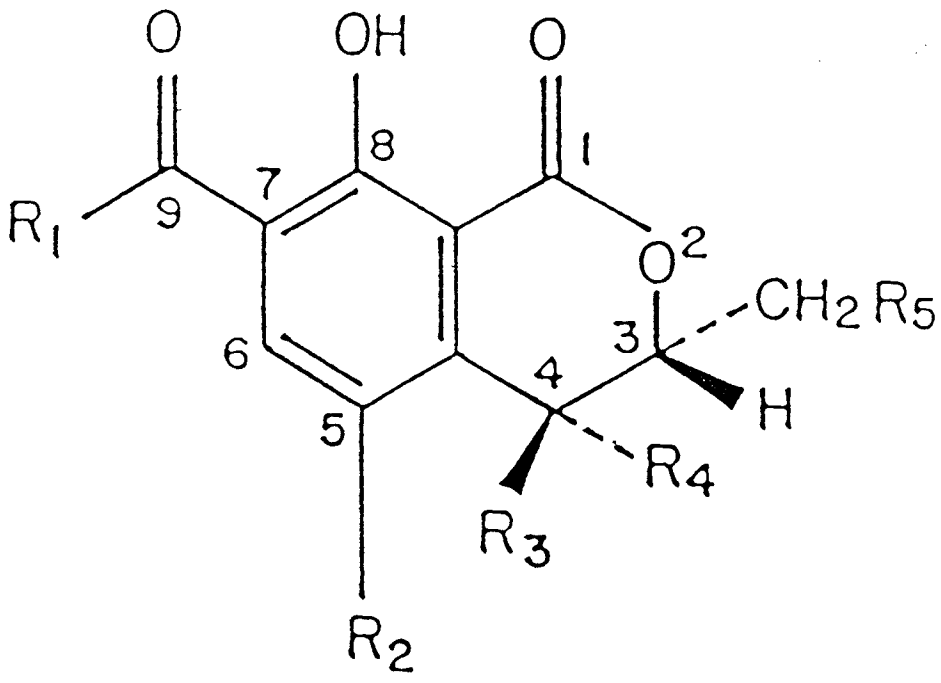
O
OH
O
8
1
7
9
R1
O2
CH2 R5
3
6
4
H
5
R4
R3
R2

TABLE 1. A Selection of Naturally Occurring Ochratoxins[a]

Common Name	Abbreviation	R1	R2	R3	R4	R5
Ochratoxin A	OA	Phenylalanyl	Cl	H	H	H
Ochratoxin α	Oα	OH	Cl	H	H	H
Ochratoxin B	OB	Phenylalanyl	H	H	H	H
Ochratoxin ß	Oß	OH	H	H	H	H
4(R)-Hydroxyochratoxin A	(4R)-OH-OA	Phenylalanyl	Cl	H	OH	H
4(S)-Hydroxyochratoxin A	(4S)-OH-OA	Phenylalanyl	Cl	OH	H	H
10-Hydroxyochratoxin A	(10)-OH-OA	Phenylalanyl	Cl	H	H	OH
Ochratoxin C	OC	Phenylalanyl-ethyl ester	Cl	H	H	H

[a] The location of the R-groups on the ochratoxin molecule are outlined in Figure 1. R-groups 1, 2, 3, 4, 5 are the functionalities bound to ochratoxins carbons 9, 5, 4, 4, and 3-methyl carbon, respectively. Adapted from Marquardt and Frohlich (1992).

2.4 Ochratoxin C

Ochratoxin C (Table 1), the ethyl ester of OA (431 MW), appears to be more toxic than OB but less than OA (Cole and Cox, 1981, Marquardt et al., 1990). When administered to animals it is rapidly hydrolysed to the parent form, OA (Fuchs et al., 1984). The toxins pKa was found to be 7.10 (Marquardt and Frohlich, 1992) and had been demonstrated to be able to cause lipid peroxidation, second only to OA (Rahimtula et al., 1988). This toxin, however, is rarely found in nature (Fuchs et al., 1984; Candlish et al., 1986). As a consequence of its rare occurrence, OA is sometimes chemically converted to OC by esterification as a confirmatory step in chromatographic analysis using boron trifluoride in methanol (Bauer and Gareis, 1987).

2.5 Hydroxylated Ochratoxin A

Ochratoxin A is hydroxylated by the action of certain cytochrome P-450 enzymes in the liver microsome (Stormer et al., 1983). Hydroxylated-OA includes (4R,S)- and 10-hydroxy OA [(4R)-OA-OH, (4S)-OA-OH, 10-OA-OH], with MW's of 419,419 and 419, respectively (Table 1). In *in-vivo* metabolism studies with rats it was shown that 1 to 1.5 % of the administered OA was excreted in the urine as (4R)-OA-OH and 25 to 27 % as Oα (Storen et al., 1982). 4R-hydroxyochratoxin A was the major hydroxylated metabolite formed in *A. ochraceus* inoculated wheat cultures (Xiao et al., 1994). These forms were found to be relatively non-toxic in rats (Hutchinson et

al., 1971) but toxic to yeast (Creppy et al., 1983). Adminstration of OC produced higher amounts of OH-OA in the urine when compared to OA in rats. The extent of hydroxylation could be further enhanced by the addition of phenobarbital, a known cytochrome-P450 enhancer (Frohlich et al., 1994).

3. Natural Occurrences of Ochratoxin A.

Ochratoxin A has been shown to occur in a wide variety of plant and animal tissues through-out the world (Kuiper-Goodman and Scott, 1989). It has been reported in at least 24 countries, 15 European and 9 non-European countries in the years leading up to 1992 (van Egmond and Speijers, in press; Kuiper-Goodman and Scott, 1989).

3.1 Ochratoxin A in Agricultural Commodities: A world-wide perspective

The toxin has been detected in maize, wheat, mixed feed, barley, coffee beans, beans, spices, rice, dry fruit, rye, oats, peanuts, kidneys of swine and chicken. The amounts of OA ranged from 10-500, 5-135, 10-200, 10-500, 2-100, 10, 19, and 1.5-2.5 μg/kg in maize, wheat, barley, beans, pig kidneys, calf kidneys, poultry kidneys, and poultry liver, respectively. Countries found to contain OA have included France, Yugoslavia, Germany, Netherlands, UK, Bulgaria, Poland, Italy, Austria, Denmark, Czechoslavia, Sweden, Belgium, Hungary, Norway, Egypt, Senegal, Sierra Leone, USA, Brazil and Canada (van Egmond and Speijers, in press).

3.2 Ochratoxin A in Agricultural Commodities: a Canadian Perspective

Canadian agricultural commodities such as swine blood, barley (Marquardt et al., 1988; Frohlich et al., 1991), mixed feeds (Prior, 1981), wheat (Abramson et al., 1990; Sinha et al., 1986) and peas and beans (Williams, 1985) have been shown to have significant levels of OA. Recent studies have ranked several Canadian grains in order of OA production potential with oats being the lowest risk crop, followed by Canadian Prairie Spring Wheat (HY320), hard red spring wheat, 2-row barley, 6-row barley, and corn, with amber durum having the highest potential to produce OA (Abramson et al., 1990). The presence of OA in Canadian plant products demonstrates that the Canadian human food-chain is contaminated with this mycotoxin.

3.3 Ochratoxin A in Swine Tissues: a world-wide problem

Swine kidney is a frequently consumed animal tissue, and very popular in European countries. Ochratoxin A is routinely detected in swine kidneys at various concentrations in most northern and central areas of Europe (van Egmond and Speijers, in press; Kuiper-Goodman and Scott, 1998). Countries such as Denmark have found OA in 20-100 % of all pigs kidneys tested at amounts ranging from 0.5 to 1955 μg/kg. In Germany, the incidence of contamination in kidneys has ranged from 14 to 21 % at levels ranging from 0.1 to 16.4 μg/kg. In the Netherlands, OA was detected in 7 to 100 % of the animals tested with the range of concentrations being from 0.2 to 240 μg/kg. Several country surveys have detected OA in swine

tissues including Hungary, Sweden, Poland, Belgium and Czechoslavia with incidences and concentrations ranging from 1.6 to 79.2 % and 0.2 to 104 μg/kg, respectively (Kuiper-Goodman and Scott, 1989; van Egmond and Speijers, in press).

3.4 Ochratoxin A in Swine Tissues: a potential Canadian problem

There are unfortunately few and no extensive studies on the natural occurrence of OA in swine kidneys in Canada (Kuiper-Goodman et al., 1993). Swine blood is known to be an excellent predictor of tissue and feed contamination (Krogh, 1976; Hult et al., 1980; Marquardt and Frohlich, 1992, Ominski, 1994). The amount of OA present in the swine kidney is about five times less than that seen in the serum. Due to the high incidence of OA in blood of Canadian swine (Marquardt et al., 1988; Frohlich et al., 1991, Ominski, 1994) it is quite likely that Canadian swine kidney tissues and their feeds are contaminated.

The ubiquitous nature of this toxin in foods and feeds suggests that humans and animal populations are at risk from this toxin. The presence of OA in the blood of Manitoban's (Ominski, 1994; Kuiper-Goodman et al., 1993) confirm that all levels of the Canadian and Manitoban human food chain are contaminated with this mycotoxin.

4 Experimental Ochratoxicosis in Animals.

The acute toxicity of OA has been extensively studied in rainbow trout, swine,

sheep, rats and chickens with the toxicities being found to be dependant on the species and the particular ochratoxin. The LD_{50} values (median lethal dose) for young chickens were as follows: OA, 150 μg; OC, 216 μg; OB, 1900 μg; Oα, >1900 μg per chick (Chu, 1974b). The LD_{50} values for rainbow trout was 4.65 mg/kg, intraperitoneally (Doster et al., 1972); 1-2 mg/kg for swine fed orally for 2 weeks (Chu, 1974b); 1 mg/kg in sheep, intravenously (Munro et al., 1973); 20-22 mg/kg in rats by gavage (Purchase et al., 1971), and 2.14 and 3.6 mg/kg for one day and three week old broiler chicks, respectively (Peckham et al., 1971). The toxin produces a dose dependant depression of growth, and a corresponding enlargement of the kidney and liver and a reduction in the size of the bursa of Fabricius and spleen in young chickens fed diets containing OA (Dwivedi and Burns, 1984). Histopathologically, rats administered a high dose of OA showed fibrin deposits in the spleen, brain choroid plexus, liver, kidney and heart. Renal tubular nephrosis, hepatic and lymphoid necrosis and necrotic enteritis were also observed (Albassam et al., 1987). Clinical symptoms of acute ochratoxicosis include anorexia, weight loss, emesis, retching, tenesmus, elevated rectal temperatures, bilateral purulent conjunctivitis, tonsillitis, polydipsia, polyuria, passing of blood stained mucus, dehydration, and prostration (Chu 1974b).

Ochratoxin A has been found to be carcinogenic in long term studies experimental animal models. Bendele et al. (1985) found increased incidences of renal adenomas

and carcinomas in mice fed OA. Long term carcinogenicity studies in rats have demonstrated a dose-dependant increase in the incidence of tubular adenomas and carcinomas. These types of studies also demonstrated that male rats were more susceptible in comparison to females (Boorman, 1989).

Ochratoxin A is known to be immunosuppressive. In turkeys it caused extensive depletion of lymphocytes in the thymus and significant diminishment of the delayed hypersensitivity response. This response strongly suggests an overall reduction in cell mediated immunity (Dwivedi and Burns, 1985; 1986b). A strong suppression of the delayed hypersensitivity responses were observed in gilts (Harvey et al. 1992). The cell-mediated immune response of swine was inhibited in a dose dependant manner. Lymphocyte response could be diminished 10 % at a concentration of 60 ng per ml of serum (Holmberg et al., 1988). Natural killer cells, T and B lymphocytes, are all reported to be inhibited by this toxin (Lea et al., 1989).

5 Natural Ochratoxicosis in Livestock and Man

Natural occurring ochratoxicosis in poultry resulted in increased mortality, poor feed conversions, reduced growth rates, and feed refusals. Fifty eight percent of 16,000 young turkeys that were affected by this toxin, died by marketing age. There have been five independently reported cases of ochratoxicosis in poultry, one episode involving 970,000 turkeys, two episodes in 70,000 laying-hens and two cases with

12,000,000 broiler chickens (Hamilton et al., 1982). Ochratoxin A was found in the diets and ranged from 0.3 to 16 mg/kg OA. Ruminants appear to be more resistant to ochratoxicosis owing to the fact that OA is rapidly cleaved in the rumen to the non-toxic form Oα (Xiao et al., 1991a; 1991b). Nevertheless, there are some reports that suggest naturally occurring OA produces renal uremia, hepatoses, gastrointestinal ulcers and pneumonia in cattle (Lloyd, 1980); the effects however, were transient (Ribelin et al., 1978). Experimental porcine nephropathy, including acute nephritis, hepatic degeneration, enteritis and kidney lesions, has been induced in pigs fed diets containing 0.2-4 mg/kg OA (Krogh et al., 1973; Krogh et al., 1974; Steyn, 1984). The kidney lesions had similar morphologies and were identical to those seen with naturally occurring mold induced porcine nephropathy. They subsequently found high levels of OA in the kidneys of the afflicted swine (Krogh et al., 1973; Krogh, 1977; Peterson and Ciegler, 1978).

Ochratoxin A has been speculated to be a causal agent of the irreversible and fatal kidney disease, Balkan endemic nephropathy. The symptoms displayed were identical to those observed in OA induced porcine nephropathy. Two Yugoslavian studies showed a higher upper range of OA and incidence of contamination in human serum in endemic areas (Hult et al., 1982). In Bulgaria, a significantly greater number of human serum samples contained OA in areas of high endemic nephropathy and urinary system tumours (Petkova-Bocharova et al., 1988). Higher levels of OA were

found in the blood and tissues of swine and stored cereals in endemic areas in Yugoslavia (Pepeljinjak and Cvetnic, 1985). Ochratoxin A levels in bean samples and maize were also found to be higher in endemic areas when compared to control areas (Petkova-Bocharova and Castegnaro, 1985). These results demonstrate the potential risk of OA contamination in human populations.

6. Avoidance Strategy.

Many different strategies have been employed in the reduction of OA contamination and consequent toxicities. Techniques have included, regulation, screening and subsequent diversion of toxin contaminated grains and or mold growth minimization by improved stored grain management practices and chemical control. Alternatively, the toxins have been destroyed *in situ* by chemical and biological treatment (kuiper-Goodman and Scott, 1989).

6.1 Regulatory Levels for Ochratoxin A in Agricultural Commodities.

Eleven countries as of 1991 have proposed or imposed regulatory limits for OA. These tolerance limits are mostly restricted to European countries. The acceptable limits have ranged from 1-50 and 100-1000 μg/kg for foods and feeds, respectively (van Egmond, 1991b). These levels are apparently based on a number of factors and include toxicity, distribution, dietary exposure, sensitivity of available analytical techniques, and potential impact on the economy. Denmark, regulates the level of OA

in swine kidneys. Swine kidneys are routinely examined for the presence of macroscopic lesions. The presence of a lesion will automatically render the animal suspect. Kidneys of the suspected animal are then subjected to OA analysis. Swine kidneys are condemned if any trace of OA is present, at concentrations above 10 ng/g but below 25 ng/g all visceral organs are destroyed and at levels above 25 ng/g the whole carcass is condemned. There are problems associated with the macroscopic examination protocols as recently contaminated animals may not have had enough time to form the characteristic lesions; that is, there is a high likelihood of false negatives (Buchmann and Hald, 1985). An alternate and better screening approach would be to directly analyze the concentration of OA in a representative herd blood or kidney sample. The concentration of OA in the blood could then be used to predict, with a fair degree of accuracy, the OA concentrations in tissues (Marquardt et al. 1988, Ominski, 1994). This type of approach would minimize the likelihood of false negatives. A fast analytical approach for swine serum and kidneys would facilitate routine regulatory analysis.

6.2 Control of Toxigenic Fungal Growth *in-situ*.

Production of OA by toxigenic strains of *Aspergillus* and *Penicillia* are known to be affected by the temperature and a_w (Northolt et al., 1979). Other factors include seed coat damage, gas composition, microbe and fungal composition, extent of insect infestation and type of substrate (Hesseltine, 1974). Alteration of any one of these

factors can minimize the growth of these fungi.

Frohlich et al. (1991) has demonstrated that 35 % of the harvested barley in Manitoba could produce OA in storage when moistened. It would therefore be prudent to minimize moisture levels in the grain. Naturally contaminated corn and wheat stored for 52 and 60 weeks at 21 and 19 % moisture produced high levels of OA. This production of OA could be minimized by decreasing the moisture contents of the grains to 16 % (Abramson et al., 1985; 1990). Naturally contaminated barley, wheat and corn sample stored for 20 weeks at a moisture content of 19 % contained OA. These grains when stored at 15 % moisture produced no OA over a 20 week period (Abramson et al., 1992). The relationship between the average swine blood OA concentrations in a herd and the average moisture content in the barley has been studied in Sweden. The percentage of OA positive animals increased from 12 to 35 % when the moisture content of the stored barley increased from 18 to 25 % (Holmberg et al., 1991). This data suggests that moisture content is one of the most important factors dictating growth of the toxigenic mold. The suggested practice is to ensure the grain is harvested in a relatively dry state, alternatively, grain drying equipment can be used to reduce the moisture content. Drying with heated air was found to be more efficient at stopping mold growth and subsequent OA production than drying with ambient temperature air (Holmberg et al., 1990). The grain storage facilities can be additionally weather-proofed to minimize water-leakage and the grain

mass turned to prevent hot-spots from forming. Irradiation is an effective means of controlling mold growth. The recent works of Chelack et al. (1991a, 1991b) have demonstrated efficient killing of mold spores, specifically, *A. ochraceus*. The major draw-back of this technique is that opportunistic and potentially mutated toxigenic molds may re-infect the grain. *A. ochraceus* when inoculated post-irradiation produced more OA than the corresponding control treatment. This may have been a consequence of reduced environmental competition or potentially increased nutrient availability to the mold. Bullerman et al. (1984), has reported competition to be a valid technique in the control of mold growth. The use of chemicals to control growth of the mold has been extensively examined. The addition of methyl paraben, potassium sorbate, and sodium propionate as anti-microbials inhibited the growth and production of *Aspergilli* and *Penicillium* in foods and feedstuffs (Tong and Draughan, 1985). The OA producing ability of fungi were found to be inhibited by high levels of CO_2, specifically, concentrations greater than 30 %. At higher concentrations of CO_2 the mold growth could be prevented. The general difficulties of maintaining a controlled atmosphere and the resumption of growth and OA production following treatment suggests this approach has no practical application with existing grain storage facilities (Paster et al., 1983).

6.3 Destruction of Ochratoxin A *in-situ*.

Chemical, physical and biological detoxification of OA is another means by which

toxicity can be avoided. An ideal scenario would be were OA is completely in-activated, by-products are minimized and nutritional quality of the food or feeds are conserved.

In modern feed manufacturing techniques, raw materials are subjected to heat for improved quality of the processed products. These treatments include steam flaking, explosion cooking, dry heat roasting, micronizing and popping. These heating regimes are commercially viable and potentially applicable for OA detoxification. Ochratoxin A in spiked coffee beans could be reduced to 0 % by roasting at 200 °C, for 5 min. In contrast, naturally contaminated coffee beans when roasted at 200 °C for 20 min showed only slight degradation as 88 to 100 % of the OA was recovered after heat treatment. This indicates the importance of the use of naturally contaminated samples in decomposition studies. Naturally contaminated cereal products could be decontaminated (30 % OA remaining) by autoclaving at 120 °C for 3 h (Samarajeewa, 1991). Trivedi et al. (1992) has reported that heat destruction was facilitated by NaOH addition. Heating under dry or moist conditions in the absence of NaOH had no effect on OA as this procedure did not change the toxicity in a mammalian cell culture assay. Heating the toxin under dry and moist conditions in the presence of NaOH resulted in the destruction of OA and removal of toxicity. The data suggests that modern heating techniques must be aided by strong-base chemical treatment to ensure destruction of OA.

Ammoniation has been shown to decompose OA in common grains with the degree of decomposition being dependent on the temperature of the reaction. The use of 25 % ammonia did not reduce OA or the viability of the sclerotia of *A. ochraceus* at 28 °C (Paster et al., 1985). The use of 2 % (W/W) ammonia at 45 °C for 6 weeks seemingly destroyed all the OA in corn, however, it was less effective when applied to wheat. The toxicity values for both grains were basically unchanged when fed to chickens. There was some evidence that the ammoniated corn was less palatable and has a lower protein utilization potential in rats (Golinski and Kubiak, 1983; CheŁkowski et al., 1982). Madsen et al. (1983) found ammoniation of barley using 5 % ammonia for 96 h at 70 °C to be more efficient than heating at 105 °C for less than 10 min with 0.5 % NaOH or autoclaving at 132 °C for 30 min. The OA content was seemingly reduced by 95 % using ammoniation whereas a 16-18 % reduction was observed with the two other techniques. A corresponding reduction in OA toxicity was not observed in the feeding trial with pigs. The authors concluded that the ammoniation approach was not practical due to the lack of observable detoxification.

Several chemisorbents can be added to diets to sequester OA and therefore reduce bioavailabilty. Reagents that have been used to remove OA have included hydrated sodium calcium aluminosilicates (HSCAS), bentonite, charcoal and cholestyramine (Marquardt and Frohlich, 1992). Charcoal appears to be the most efficient chemisorbent as it could reduce up to 80 % of OA in the blood and tissues of swine

when compared to control animals. The HSCAS salts and bentonite had no effect on OA levels in the tissues and blood of swine. This was surprising as *in-vivo* studies have demonstrated that HSCAS, when incorporated into poultry diets at a level of 0.5 %, significantly protected the chicks from aflatoxins, a ubiquitously occurring family of highly toxic mycotoxins (CAST, 1989). Cholestyramine is a commercial anion exchange resin that effectively binds bile acids in the gastrointestinal tracts of non-ruminants. Madhyastha et al., (1992) demonstrated that a 50 % reduction of OA in the blood could be accomplished by the addition of 0.5 % cholestyramine to the diet of rats. There was a decrease of OA excretion in the urine and a corresponding increase in the faeces. The major drawback of the technique was the high cost of the binding agent.

The addition of chemical additives such as anti-oxidants may reduce toxicity in animals. Ochratoxin A is known to produce free-radicals (Hasinoff et al., 1990) and thus lipid-peroxidation. A free-radical scavenger may ameliorate this effect. Ascorbic acid has been reported to be effective in prevention of ochratoxicosis in laying-hens (Haazele, 1992). Vitamin E is also known to be an efficient scavenger of free-radicals therefore one might speculated similar protective effects. The addition of phenylalanine has been shown to prevent protein inhibition by OA in cell cultures and mice. Phenylalanine also reduced renal tubular injury in rats, and mortality in chicks (Creppy et al., 1984; Kane et al., 1986; Bailey et al., 1990). The recent work of

Rotter et al. (1989) has, however, demonstrated that this approach was impractical due to the high mortalities of chickens fed diets containing this supplement.

Ruminant animals such as sheep can effectively hydrolyse OA to Oα and thus reduce bioavailability (Xiao et al., 1991a,b). Mature sheep fed diets containing hay could hydrolyse OA to Oα more effectively than when fed grain. The bioavailability of OA in sheep fed hay was 4.3 times less than that seen with grain fed animals. The reduced bioavailability was attributed to a lowering of the pH in the rumen and alteration of the microbial population compositions. The noticeable absence of cattle and sheep ochratoxicosis tends to confirm these observations. This biological approach would be an economical and viable means of utilizing contaminated feeds, however, the grains must be fed infrequently or at diluted concentrations to prevent excessive accumulation of the toxin in the kidney. It has been reported that OA will accumulate in the kidneys of cows fed diets containing OA at levels of 1 mg/kg for extended periods of time (Raisbeck et al., 1991). This detoxification process might also be facilitated by the incorporation of routine hay-feeding.

Sound prevention practices such as drying, the addition of feed additives such as vitamins or feed diversion to ruminants will probably be the most easily applied and economical approaches to detoxification. These types of techniques can be easily implemented at the farm level.

7 Conventional Analytical Procedures for Ochratoxin A in Agricultural Commodities

Trace analytical methodologies are required to determine the concentrations of mycotoxins such as OA. These compounds are usually present in agricultural commodities and biological materials in concentrations ranging from low ppb to high ppm. Several factors are known to affect the success of an analytical procedure for a given mycotoxin and include the complexity of the sample matrix, the reliability of the initial sampling, extent of sample comminution, sub-sampling size, extraction efficiency and sensitivity and specificity of the detection technique (van Egmond, 1991a).

7.1 Sampling at Trace Levels

Mycotoxin contamination in grain is not homogeneous as only a small population in the seed sample may be contaminated. Even the amounts of toxin from seed to seed within the sample can be highly variable. This is very evident in aflatoxin sampling procedures for peanuts and corn (CAST, 1989). Certain similarities exist for all mycotoxin analysis procedures. All techniques must include appropriate and valid sampling procedures which give an accurate measure of the real situation. Original test samples must be large and may range as high as 2-10 kg for reproducible and accurate aflatoxin analysis (CAST, 1989). The variability in the original sample can be reduced by extensive comminution. In the process of comminution, the sample

contamination becomes more homogeneous, in addition, the surface area of the ground particles increases thus facilitating greater solvent accessability and consequently extraction efficiencies. Variability in the sub-sample can then be reduced by the use of a larger sub-sample (CAST, 1989). Sub-samples sizes that are normally collected for OA analysis vary from 25-100g for grains and tissue samples or a few millilitres for biological fluids such as blood, milk and urine (van Egmond, 1991a).

7.2 Sample Extraction

One of the most crucial steps in mycotoxin analysis is extraction of the sub-sample. This basically involves the separation of the test analyte from the bulk of the matrix. This will also render the sample smaller and more manageable. The choice of extraction solvent depends on the matrix, the mycotoxin, and the type of analysis. The most common technique for OA extraction by far, is to extract plant or animal commodities with chloroform, following acidification with aqueous H_3PO_4 (van Egmond, 1991a; Candlish, 1991; Nesheim et al., 1992; Hald et al., 1993). This procedure will normally require the extract to be dried and reconstituted with a system compatible solvent. Aqueous organic solvents such a methanol, acetonitrile in combination with water or acidified water have been found to be effective for the quantitative extraction of OA from grains (Ramakrishna et al., 1990; Lee and Chu, 1984). These types of solvents are compatible with most chromatographic methods and immunoassays. Immunoassays usually require the sample to be introduced into

the assay system in an aqueous solvent as the antibodies are susceptible to denaturation by organic solvents. Several other combined solvents systems have been described for the extraction of OA and include chloroform-methanol (1:1), methanol-water-hexane (55:45:40) and 1 % sulphuric acid (20 %), chloroform-methanol-hexane (8:2:1), chloroform, chloroform-0.1M phosphoric acid (10:1), chloroform-water (10:1), acetonitrile-4 % aqueous potassium chloride (9:1), acetonitrile-4 % aqueous potassium chloride and 2 % sulphuric acid (9:1), chloroform at pH 1.5, chloroform-methanol (7:3), chloroform-methanol (4:1); chloroform-methanol at pH 1, ethyl acetate at pH <3, acetone-water (85:15), and acetonitrile-water-hexane (9:1:5) (Steyn, 1984). Extraction solvents are brought into contact with the sample matrix by a variety of techniques which might include soxhlet extraction, blending, shaking or homogenization (Steyn, 1984; Hald et al., 1993). Shaking times are generally longer in comparison to blending, however, blending and homogenization can be more labour intensive especially in cases were large numbers of samples are to be analyzed. Tissues, unavoidably, must be homogenized, a time consuming and laborious technique, especially when large numbers of samples are tested. Liquid samples may be directly extracted in separatory funnels or by using solid-phase extraction column (van Egmond, 1991a). Certain animal tissue and biological fluid extraction procedures have been facilitated by digestion with enzymes (Hunt et al., 1979; Orti et al., 1986).

7.3 Sample Clean-up

The usual step following extraction is limited purification, more commonly referred to as sample clean-up. This was found to be essential for most chromatographic based analytical procedures and in certain cases, immunoassays (van Egmond, 1991a; Candlish, 1991). It has normally included the passage of the sample through columns packed with either chemical or immunoaffinity based adsorbents. The use of normal phase and reversed-phase chemical adsorbents have become increasingly popular for the clean-up of mycotoxin extracts. The extracts are passed through the column where the mycotoxin is selectively retained. The column or cartridge may be then washed with a solvent to remove non-bound contaminants followed by selective elution of the mycotoxin. Both silica gel and reversed-phase stationary phases have been successfully employed for OA clean-up (Neisham et al., 1992; Hald et al., 1993; van Egmond 1991a). Immunoaffinity-based column clean-up cartridges which have unique specificities for the analytes of interest are now gaining interest (Katz and Brady, 1990). The unique specificity of the antibody for the analyte allows for selective retention and thus enhanced clean-up. Immunoaffinity columns specific for OA have been applied in several studies for the clean-up of coffee, cereals and animal tissues. Interfering compounds were washed-off with an aqueous buffer and the bound OA was eluted using an organic solvent (Nakajima et al., 1990; Ueno et al., 1991; Sharman et al., 1992; Hald et al., 1993). Ochratoxin A containing extracts have also been conveniently cleaned using a column containing diatomaceous earth impregnated

with sodium bicarbonate solution. The principle is similar to liquid partitioning. The interfering compounds are subsequently removed using a mixture of hexane and chloroform. The remaining OA is eluted with a mixture of benzene and acetic acid (Krogh and Nesheim, 1982). Partitioning of chloroform and or ethyl acetate extracts with sodium bicarbonate in separatory funnels (Hald et al., 1993) has yielded excellent recoveries but tends to be time-consuming especially in cases were large numbers of samples are analyzed. TLC clean-up of sample extracts have also been successfully employed prior to HPLC analysis. The technique results in good recoveries and clean-up, however, it requires custom made recovery equipment (Frohlich et al., 1988).

7.4 Bioassay Detection of Ochratoxin A

A number of different separation and detection approaches have been developed for OA in agricultural commodities. Biological assays which do not require separation for detection have included the use of chick embryos (Vesley et al., 1982), zebra fish larvae (Abedi and Scott, 1969), human girardi heart (Lompe and Milczewski, 1979), *Bacillus thuringiensis* (Boutibonnes et al., 1985), *Photobacterium phosphoseum* (Yates and Porter, 1982), and *Bacillus brevis* (Madhyastha et al., 1994). These biological methods have been found to be useful for broad screening of toxicity and do not normally require clean-up of the sample extract, however, they tend to lack specificity, sensitivity and speed (Candlish, 1991; Chu, 1992). The recent work of Madhyastha et al. (1994) has indicated that the pH of the medium is a factor that

significantly influences the sensitivity of the bacteria to OA, however, the sensitivity of the approach is still many times less than that seen with modern physicochemical approaches. Physicochemical type assays in many respects are superior and are consequently found to be the procedure of choice for the routine analysis of OA. Thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC) in conjunction with fluorescence detection are by far the most important and useful OA analytical tools.

7.5 Thin-layer Chromatographic Detection of Ochratoxin A

Thin-layer chromatography has found wide-spread use for the separation of mycotoxins. The use of silica and/or reversed-phase stationary phases have enabled adequate separation of OA from agricultural commodity extracts (van Egmond, 1991a). This technique has been found to be suitable for complex matrixes where the OA concentrations are relatively low. Detection of OA after TLC is easily achieved as it produces a distinct blue-green fluorescence when the plate is subjected to longwave U.V. light (365 nm). The fluorescence can be further enhanced by treating the plate with ammonia. One and two dimensional thin-layer chromatographic techniques have been developed for OA in a wide variety of agricultural commodities using fluorescence, or ammonia enhanced fluorescence for detection and include matrixes such as barley, green-coffee, oats, wheat, corn, feeds, beans, liver, swine kidney and muscle tissues (Stoloff and Scott, 1984). Additional matrixes include rye,

peanuts, rice, cassava beans, coconuts, peas, olive oil, dried milk, whey, beer and eggs (Asensio et al., 1982; Czerwiecki, 1982; Jizdny, 1983, Frohlich et al., 1986; LeTutour et al., 1983; Majerus and Woller, 1983; Piskorska-Pliszczynska, 1985; Soares and Rodriguez-Amaya, 1985). The limits of detection by TLC are, 0.2-0.5, 0.5, 3, 2.4-4, and 10 ng/g in swine kidney, eggs and swine tissues, cereals, rice, and feeds, respectively (Asensio et al., 1982; Czerwiecki, 1982; Paulsch et al., 1982; Piskorska-Pliszczynska, 1984; Pogrebnyak and Volkov, 1985; Scheuer and Leistner, 1986; Soares and Rodriguez-Amaya, 1985; Wilken et al., 1985).

7.6 High-performance liquid chromatographic Detection of Ochratoxin A

High-performance liquid chromatographic techniques are in general more precise than TLC. Increased precision, accuracy, resolution, sensitivity and reproducibility are obtained with HPLC approaches (van Egmond, 1991a; Lepom, 1986). This approach is more readily adapted for large-scale analysis as auto-injectors are commercially available.

The sensitivities of the HPLC method are dependant on the choice of detector. The most common detector with the greatest selectivity and sensitivity for OA is fluorescence based (Hald et al., 1993). Ochratoxin A has been readily separated and quantified on HPLC, using a wide variety of stationary phases; the most common being the bonded C^{18} reversed-phase packing (Hald et al., 1993). Advanced detection techniques such as thermo-spray quadrapole mass spectrometry have also been

described but are still less common than fluorescence detection due to the prohibitively high costs of instrumentation, and skill-sets required to operate the equipment (Abramson, 1987; Chu, 1992). HPLC techniques for OA in agricultural commodities have included matrixes such as animal feeds, cereals, wheat, barley, corn, coffee and cocoa beans, swine kidneys, bread, and flour (Osborne, 1979; Hunt et al., 1980; Nesheim, 1982; Cantafora et al., 1983; Hurst and Martin, 1983; Visconti and Bottalico, 1983; Cohen and Lapointe, 1986; Lepom, 1986; Terada et al., 1986; Abramson, 1987; Bauer and Gareis, 1987; Chamkasen et al., 1989; Scott et al., 1991; Nesheim et al., 1992; Dunne et al., 1993). The sensitivity of HPLC using fluorescence detection is approximately ten times higher than that seen with TLC and superior in regards to precision and accuracy. The detection limits were generally found to be greater than TLC with limits as low as 0.1 ng/g in swine kidney and feedstuffs (Bauer and Gareis, 1987).

7.7 Direct Spectrophotometric Detection of Ochratoxin A

A direct spectrophotometric method for OA in blood has been described and offers several potential advantages over TLC and HPLC. Ochratoxin A is cleaved into Oα and L-phenylalanine using the proteolytic enzyme carboxypeptidase A. The technique is based on the loss in fluorescence at the wavelength were OA has its excitation maximum. This technique has been successfully employed in the case of barley and pig kidneys. Limits of detection where in the range of 1-4 ng/g (Hult and Gatenbeck,

1976). The sensitivity is slightly better than that described for TLC but less than that seen normally with HPLC. There is however a question of specificity as other fluorescent compounds with similar emission spectrums may interfere with the quantitation of OA.

Many different procedures can be utilized to prepare plant and animal tissues for OA analysis. Acidified chloroform appears to be the most common solvent for the quantitative extraction of OA from plant and animal tissues. However, this solvent is toxic and its use is often restricted. It therefore would be beneficial to replace this solvent with solvents that are less toxic and easier to dispose of. Solid-phase clean-up columns using C18 or silica appear to be the methods of choice for routine analysis of OA, and commercial companies supply pre-packed columns. There is also the possibility of automation which would greatly facilitate routine analysis. Despite extraction and clean-up, the final sample may still contain compounds that could interfere with the direct detection and analysis of OA, thus suitable separation and detection procedures sometimes have to be developed. Thin-layer chromatography is generally the most economical choice of separation, especially in cases were low sample through-put is necessary, however, HPLC offers increased speed, resolution and increased sensitivity.

8 General Strategies for the Development of Ochratoxin A Specific

Immunoassays.

Immunoassays offer a unique advantage over conventional chromatographic procedures because they are normally highly specific, sensitive, rapid, relatively inexpensive to perform and can be readily adapted to large-scale and routine analysis (Pestka, 1988; Chu, 1990; 1992). The speed of the immunoassay approach is related to the less stringent requirement for sample clean-up and the possibility of performing several assays simultaneously with appropriate standards and controls.

8.1 Preparation of an Immunogenic Ochratoxin A-Protein Conjugate

Mycotoxins such as OA are by themselves incapable of eliciting immune responses due to their size, but can be rendered immunogenic by covalent attachment to large molecular weight carrier proteins (Candlish, 1991; Harlow and Lane, 1988). It is generally believed that animals respond against the toxin moiety most distal to the region of linkage (Pestka, 1988). It is thus prudent to select an appropriate linkage-site on the mycotoxin that induce and maximize the immune response. Alteration of linkage-sites of aflatoxin B1 dramatically varied the specificities of the antibodies to this toxin (Candlish, 1991).

Ochratoxin A possesses a free carboxyl-group which can be readily coupled to protein amines to form a stable amide bond. Several amide-bond formation reactions have been utilized for OA conjugation and have included those that make use of the N-hydroxysuccinimide activated esters (NHS), water-soluble carbodiimides (WSC),

and mixed anhydrides (MA) (Wilkinson et al., 1992). The polyclonal and monoclonal antibodies for OA raised using these conventional amide formation chemistries are generally similar in specificities and sensitivities (Table 2; Table 3). These result suggest the linkage between the carboxyl group of OA and the amines of proteins are sufficiently similar for the production of antibodies with similar specificity and sensitivity.

Several problems have, however, been encountered with the use of MA and WSC coupling chemistries including the formation of cross-reacting immunogenic side-reaction products (Gendloff et al., 1986). These side-reaction products manifested themselves as high background absorbances and reduced sensitivities in the subsequent mycotoxin immunoassays. These interferences could only be reduced by cross-absorption of non-specific antibodies with protein-conjugates formed in the absence of the mycotoxin, affinity purification of the antibody using the unwanted determinant as a ligand or alteration of the amide-bond formation chemistries (Gendloff et al., 1986; Albro et al., 1979; Wilkinson et al., 1992; Xiao et al., 1994).

Recently Xiao et al. (1994) has proposed the use of 1,1'-carbonyldiimidazole (CDI), a multi-functional coupling reagent which can form non-charged and zero-length amide bonds between carboxyl functionalities of target haptens and amine groups of proteins. The reaction was found suitable for the conjugation of OA to proteins and has additionally been found useful for many other carboxyl containing

mycotoxins. The reaction is efficient, controllable and produces conjugates that are recognizable by OA-specific antibodies produced using different amide-bond formation chemistries. The carboxyl groups of OA are suspected to be activated by CDI to form a highly reactive acylimidazole intermediate. This intermediate presumably is replaced with nucleophiles such as amines to form stable, covalent amide-linkages. The un-reacted intermediates are decomposed by water thus minimizing non-toxin determinant formation. This reaction was deemed to be faster than any existing conjugation method including product work-up. The CDI reaction should therefore complement other OA coupling chemistries as it offers an alternative reaction mechanism that minimizes the formation of unwanted determinants.

8.2 Selection of Antibody Source.

The choice of antibody source must fit the intended application. An immunoassay which is intended for large-scale screening and regulatory analysis must be sensitive, specific and economically available.

The most convenient source of mycotoxin specific antibody is polyclonal antibody from antisera. Animals found useful for the production of polyclonal antibodies have included rabbits, sheep, goats, donkeys, and horses. Rabbits are however, the most commonly described species for the production of OA specific polyclonal antibodies (Chu, et al., 1976; Fukal, 1987; Pestka et al., 1981; Morgan et al., 1983;1986; Lee and Chu, 1984; Martlebauer and Terplan, 1988; Breitholtz-Emanuelson et al., 1992).

These types of antibodies have been found to be sensitive and specific enough for the quantitative detection of OA. These high sensitivities and specificities are a reflection of the antibody development mechanism. Prolonged immunization with low doses can "fine-tune" due to affinity maturation the immune response and minimize heterogeneity of the immune response (Harlow and Lane, 1988, Morgan, 1989). Any contributions made by the low affinity and non-specific antibodies to the assay performance will normally be minimized by prudent choices of immunogens, competing antigens, and assay formats. A disadvantage of using polyclonal antibodies is that there performance characteristics are very hard to reproduce from animal to animal and within animals, however, a pooled sample from a high titre rabbit can produce sufficient amount of antibody for thousands of assays.

Murine monoclonal antibodies (Mabs) as the name suggests are the products of a single antibody producing cell-line. Many of the immunoassays for OA use this type of antibody (Rousseau et al., 1987; Chiba et al., 1985; Candlish et al., 1986, 1988; Kawamura et al., 1989, 1990; Ramakrishna et al., 1990; Ueno et al., 1991). The techniques for the production of Mabs are well described (Harlow and Lane, 1988). In comparison to polyclonal antibodies, Mabs have an unlimited production potential which ensures consistent specificities and sensitivities from batch to batch, and thus reduced assay variabilities. Immunoaffinity-based clean-up column development can also be facilitated by Mabs usage while polyclonal antibodies are not normally

recommended to be immobilized (Harlow and Lane, 1988). A major disadvantage of Mabs, is that they can be expensive to produce, and can also be too specific. It might be desirable in certain cases to have a assay for the mycotoxin group, especially when other toxic members can occur. The singular and generally lower affinities of Mabs can also limit its applications in ELISA's due to reduced sensitivities (Chu, 1990; Morgan, 1989).

It is a well known phenomena that laying-hens protect their offspring by transferring antibodies from serum to the egg yolk, a process referred to as passive immunity. These yolk antibodies, IgY, have been used in several analytical techniques, including ELISA (Ricke et al., 1988), RIA (Fertel et al., 1981) and rocket immunoelectrophoresis (Altschuh et al., 1984). Recently laying-hen antibodies have been found useful for the development of immunoassays for aflatoxin B1 and T-2 (Hsu and Chu, 1992; Li et al., 1994; and Kierek-Jaszczuk et al., 1994). The antibodies could be used in a quantitative ELISA format. The authors reported good specificities, however, in comparison to conventional rabbit antisera the laying hen antibody was less sensitive (Hsu and Chu, 1992). Regardless of the lower sensitivities, Li et al. (1994), described an assay for the quantitation of aflatoxin B1 in peanuts that could readily detect this toxin in the low ppb. Other applications may include in the future passive immunity therapy for humans and mice against rotoviral infection (Yolken et al., 1988; Lösch et al., 1986). Although the chicken has not been widely

used as a source of mycotoxin specific polyclonal antibody it offers several important advantages over conventional mammalian polyclonal antibody preparations and murine Mabs. The collection of eggs is convenient and non-invasive, as compared to the repeated bleeding of mammals (Gassman et al., 1990). The technical expertise required for the collection of eggs and the requirement for specialized housing facilities is minimal; furthermore, their is some evidence that indicates egg yolk antibodies are produced in larger quantities than that seen with rabbits (Gassman et al., 1990; Polson et al., 1980a,b; Gottestein and Hemmelar, 1985). The high sensitivities and ease of production associated with mammalian polyclonal antibodies and the hen egg-yolk antibody should facilitate the development of a readily available, sensitive and specific assay that is suitable for the routine screening of OA.

8.3 Purification of Antibodies

There are a wide variety of techniques for the isolation and purification of antibodies. The techniques selected are dependant on many variables and may, in certain cases, be influenced by the particular animal species and antibody source including serum from rabbits, ascites or tissue culture supernatant from mice and egg-yolk from the laying-hen; the particular class or subclass of the antibody and most importantly its application. Some researchers even avoid purification and just dilute the antibody prior to application in the assay (Newsome, 1986). Antibodies from typical mammalian sources can be purified using conventional methods such as

TABLE 2. Quantitative Immunoassays for Ochratoxin A Based on Rabbit Antibodies

Assay Format	Sample Type	Extraction Solvent	Clean-up Procedure	Standard Curve Range (ng/ml)	Limits of Detection (ng/g)	Reference
RIA	Barley	Acidified $CHCl_3$	Normal-phase column clean-up	2.0-800	2.5	Rousseau et al., 1985
RIA	Swine Serum	Acidified $CHCl_3$	Normal-phase column clean-up	2.0-10000	0.4	Rousseau et al., 1981
RIA	Swine Kidney	Acidified $CHCl_3$	Reversed-phase column clean-up, partioning into $NaHCO_3$	0.2-1.6	1	Fukal, 1987
RIA	-	-	-	2.5-200	-	Chu et al., 1976
ELISA	-	-	-	0.5-25	-	Pestka et al., 1981
ELISA	Human Serum	Acetone	CHCl3:buffer partitioning	-	0.02	Breitholtz-Emanuelsson et al., 1992
ELISA	Swine Serum	Acidified $CHCl_3$	Partioning into $NaHCO_3$	0.07-4.5	0.5	Martlbauer and Terplan, 1988
ELISA	Wheat	Methanol	C18 column clean-up, partioning into $NaHCO_3$	1.0-100	1	Lee and Chu, 1984
ELISA	Barley	Acidified $CHCl_3$	-	0.1-100	0.06	Morgan et al., 1983
ELISA	Kidney	Acidified $CHCl_3$	Partioning into $NaHCO_3$ and methanol	0.33-333	0.5	Morgan et al., 1986

TABLE 3. Quantitative Immunoassays for Ochratoxin A Based on Mouse Monoclonal Antibodies

Assay Format	Sample Type	Extraction Solvent	Clean-up Procedure	Standard Curve Range (ng/ml)	Limits of Detection (ng/g)	Reference
RIA	Swine Kidney	Acidified $CHCl_3$	Reversed-phase column clean-up, partioning into $NaHCO_3$	0.75-50	0.2	Rousseau et al., 1987
ELISA	Swine Serum	Acidified $CHCl_3$	-	0.05-10	0.1	Kawamura et al., 1989
ELISA	-	-	-	1000-100000	-	Porter, 1990
ELISA	Barley	Acidified $CHCl_3$	$NaHCO_3$ partioning	0.5-250	2.5	Candlish, 1991
ELISA	Barley	Acetonitrile: Water	-	1.0-250	0.5	Ramakrishna et al., 1990
ELISA	Barley	Acidified $CHCl_3$	$NaHCO_3$ partioning	5-250	5.0	Candlish et al., 1988
ELISA	-	-	-	5-10000	-	Candlish et al., 1986

precipitation and column chromatographies. Techniques are numerous and include, precipitation with reagents such as ammonium sulphate and column chromatographic procedures that depend on size exclusion or absorption. Absorption chromatographies have included the use of ion-exchange resins such as diethylaminoethyl resin, specific absorbents such as caprylic acid and hydroxyapatite, and affinity-based absorption materials such as proteins A and G, and specifically immobilized-ligands (Harlow and Lane, 1988). The egg-yolk of a laying-hen is a complex matrix, containing lipids, lipoproteins, and a mixture of water-soluble proteins. Isolation and purification of antigen-specific IgY requires the initial removal of bulk lipids and lipoproteins (bulk lipid), followed by the purification of the IgY protein fraction. Several procedures have been developed to remove the bulk lipid from egg yolk, including precipitation with PEG (Polson et al., 1980a,b;1985), dextran sulphate (Jensenius et al., 1981) or more recently, natural gums (Hatta et al., 1990). Organic solvents have also been found to be useful; for example, chloroform (Aulisio and Shelokov, 1976; Polson, 1990) and isopropyl alcohol (Bade and Stegemann, 1984) were used with acceptable recoveries. Purification of the IgY has been successfully accomplished with a variety of techniques. Selective protein precipitation agents have been used to purify and concentrate IgY; including ammonium sulphate (Vieira et al., 1984), sodium sulphate (Hatta et al., 1990) and PEG (Polson et al., 1980ab; 1985). Other

more advanced IgY purification techniques have been examined which include ion exchange chromatography (Gassmann et al., 1990; Hatta et al., 1990; McCannel et al., 1990), copper-loaded metal chelate interaction chromatography (McCannel et al., 1989), hydrophobic and size exclusion chromatography (Hassl and Aspöck, 1988). Antigen specific IgY can be further purified by the use of affinity chromatography. Specific IgY antibodies for proliferating cell nuclear antigen and calf thymus RNA polymerase II have been isolated using such techniques (Gassmann et al., 1990; Carroll and Stollar, 1983). The procedure suggested by Polson (1990) utilized several conventional techniques for the extraction and purification of this antibody; chloroform-lipid extraction coupled with PEG protein precipitation. The procedure was reported to be high yielding in comparison to other existing extraction procedures, convenient and produced pure and active antibody.

8.4 Conventional Immunoassay Formats.

Immunoanalysis of mycotoxins utilizes the unique abilities of antibodies to recognize and strongly bind the toxin at extremely low concentrations. The amounts of antibody-hapten complex formed can be determined by the use of a label. The labels must possess a unique characteristic that can be monitored visually or by instrumental techniques (Wilkinson et al., 1992; Harlow and Lane, 1988).

Radioimmunoassays are based on the competition between an isotopically labelled toxin and an unlabelled toxin for a specific antibody in limited concentrations. They are also considered to be homogeneous as all reactants are in the same phase, that is, all reactants are in liquid-phase. As the amount of unlabelled toxin increases there is a corresponding decrease in the amount of labelled toxin-antibody complex formed if labelled toxin and antibody concentrations are kept constant. A systematic comparison of the ratios of bound to unbound label with that obtained from a series of toxin standards will allow quantitation of unknowns. Once equilibrium has occurred, the antibody free fraction can be removed by the addition of activated charcoal, alternatively, the toxin-antibody complex can be precipitated with ammonium sulphate or by the use of an anti-antibody (Candlish, 1991). The use of an anti-antibody is less disruptive of the complex, however, it can take a considerably longer time to perform (Candlish, 1991; Wilkinson et al., 1992). The amount of labelled toxin in the supernatant or precipitate can then be instrumentally measured. Radio-labels are usually short-lived, expensive, highly restricted, and hazardous. In addition the standard and competing toxin must be radio-labelled, thus, availability of the assay reagents may be restricted (Candlish, 1991; Wilkinson et al., 1992).

Competitive and enzyme-based immunoassays (EIA) are similar in principle to RIA for mycotoxins, however, the radioisotopic label has been replaced with an

enzyme. These enzymes catalyze the conversion of a non-coloured substrate to a coloured form. Important criteria for the selection of an appropriate enzyme have been recently described (Candlish, 1992). The enzymes must have a high turnover rate, produce a readily discernable signal, be capable of being conjugated to other molecules, operate under normal assay conditions, be capable of being stored, and should be readily available as well as inexpensive. Several enzymes have been used for this type of application and normally include horseradish peroxidase and alkaline phosphatase. The substrates are varied but normally produce a colour which is highly visible and readily quantified. The chromogenic substrates used in the reactions must ideally be stable, non-toxic and water soluble. Chromogenic substrates found useful for alkaline phosphatase have included p-nitrophenol phosphate and for horseradish peroxidase, 2,2'-azinodi[ethylbenzthiazoline]sulfonate, o-phenylenediamine and 3,3',5,5'-tetramethylbenzidine (Harlow and lane, 1988). The ELISA formats are considered to be heterogeneous. They normally involve the separation of unbound toxin in one phase, usually liquid, from bound toxin in another phase, a solid. In this type of assay, it is routine to bind the antibodies or antigens to the solid surfaces such as polystyrene microtiter wells, polystyrene tubes or nylon beads. Methods of attaching the antibodies and antigens include air drying, passive absorption in high pH carbonate or Tris buffers and covalent attachment with gluteraldehyde

(Candlish, 1991).

Three competitive ELISA formats are used for mycotoxin detection, specifically, direct antigen-capture, direct antibody-capture and indirect antibody-capture. In the direct antigen capture ELISA format, mycotoxin specific antibodies are immobilized on the surface of the solid phase. A fixed amount of enzyme-labelled toxin is mixed with the unlabelled toxin (standard or unknown) and the two forms of the toxin are allowed to compete for binding to the immobilized antibody. The presence of an excess amount of unlabelled toxin will normally completely inhibit the binding of an enzyme-labelled toxin to the antibody. The unbound enzyme-labelled toxin is removed by washing and the reaction is monitored by the addition of the substrate. In the direct antibody capture method the antigen is initially bound to the surface of the plate. Competition is allowed to take place between the free toxin and the free but limited amount of toxin specific antibody which has been previously labelled with an enzyme. The unbound enzyme-labelled antibody is removed by washing and the reaction is monitored directly by the addition of the substrate. The indirect antibody capture format is identical to the direct antibody capture method with the exception that the free antibody is unlabelled. The amount of antibody bound to the coated antigen is determined by the addition of an anti-antibody labelled with a colour producing enzyme. The amount of enzyme signal produced are inversely proportional to the

amount of unlabelled toxin added.

Radioimmunoassays have been gradually replaced with the ELISA due to ease of label separation and preparation of the labelled toxins. Chu (1990) and Candlish (1991) have reported that the ELISA format is faster than RIA as they do not normally need extended equilibration times for antibody antigen complexing. There are also reports of the use of solid-phase RIA, however, they are uncommon; this may be due, in-part, to the high costs of scintillation counters designed for microtiter plate analysis and the restricted nature of the radiolabels. There is some indication that the ELISA formats are more sensitive than the RIA and that RIA requires more sample clean-up than ELISA.

8.5 Performance Criteria for Mycotoxin Immunoassay

The criteria for good mycotoxin immunoassays have been previously described (Chu, 1990; Morgan, 1989; Candlish 1991; Wilkinson et al., 1992). The assays must be sensitive, specific, simple, fast, quantitative and reproducible.

The direct format ELISA's are considerably faster than the conventional indirect techniques due to the removal of two washings and the enzyme-labelled anti-antibody incubation step. Several authors have modified the indirect antibody capture approach by pre-incubation of the anti-antibody enzyme conjugate with the toxin specific antibody prior to carrying out the ELISA. This complex, when used in an indirect competitive assay, reduced the time of the assay because the second

incubation and washing step were not required. An additional advantage of this type of approach is that the anti-antibody enzyme conjugate can be obtained commercially, while toxin-enzymes conjugates must generally be prepared in-house (Candlish, 1991). The direct antigen capture methods have been methods of choice because of speed and simplicity even though they appears to be less sensitive and requires 100 times more antibody per assay than the antibody capture methods.

The sensitivity of an assay is normally defined as the lowest concentration of toxin required to give a significant inhibition in the standard competitive immunoassay. This is usually twice the standard deviation of maximal signal. Sensitivity can also be assessed by the slope of the standard curve and the concentration of toxin required to give 50 % of maximal signal (IC50). A steep slope and low IC50 are indicative of a sensitive immunoassay and high affinity antibody (Candlish, 1988). A relatively small change in concentration will manifest its self as a large change in the assay signal. This signal response would be ideal for rapid and semi-quantitative assay where the lack of inhibition indicates that the sample falls below the legal toxin limit and inhibition indicates that the sample is in violation of the tolerance limits. The sensitivity of the assay can be further improved by the prudent choice of antibody, antigen concentrations (Li et al., 1994; Van de Water and Haagsma, 1990). ELISA sensitivities were increased by the systematic reduction in the amount of coating antigen and antibody. These

reductions altered the competition reaction in favour of the unlabelled antigen because the unlabelled antigen competes with fewer binding sites on the surface of the microtiter plate or antibody. Alteration of the coating antigen for a less recognized form, that is, the antibody has less affinity for this form, may also favour competition for the free ligand, however this remains to be tested with mycotoxins. The polyclonal antibody preparation can also be affinity purified with the goal of isolating a more sensitive population of antibodies (Assil et al., 1992).

Relative cross-reactions of the antibody are usually expressed as the ratios, given as a percentages, of the amount of standard toxin to unknown that give 50 % of the maximal signal. The assay must be specific enough to match the application. Regulatory agencies usually require an assay to be highly specific for a particular mycotoxin especially when other closely related forms are non-toxic; for example, OA is the naturally occurring and most toxic member of this group of toxins (Marquardt and Frohlich, 1992). Group specific assays should also be developed were closely related and co-occurring family members are toxic and regulated. A group specific antibody for aflatoxin B1 (CAST, 1989) and its closely related and toxic family members would facilitate routine regulatory analysis. The cross-reactivities of antibodies can be controlled before or after immunization. The immunogens linkage site can be selected to maximize or minimize specificities of the ensuing antibody. Antibodies, for example, can be specifically generated to the

dihydrofuran-ring of aflatoxin B1 when this toxin is conjugated via the cyclopentanone groups. Likewise, antibodies can be directed against the cyclopentanone ring structure by preparing the conjugates through the dihydrofurn moiety (Candlish, 1991). Mycotoxin specific monoclonal antibodies can be additionally selected during the cloning process which react specifically or non-specifically with the toxins of interest. The antisera can also be affinity purified to maximize and ensure defined specificities (Assil et al., 1992).

Toxin standards diluted in blank matrix when compared to standard in assay buffer must have superimposable inhibition curves in the assay. Interferences will be manifested as a deviated standard curve. In certain cases sample matrix can decrease maximal signal, which may suggest that competing compounds could be present. An increased maximal signal suggests that sample extraction solvent is interfering with the assay. These effects can be minimized by extensive sample dilution, sample extract clean-up or incorporation of blank matrix extract into standards solutions (Wilkinson et al., 1992; Chu, 1990).

Recovery and reproducibility of all steps in the assay must be determined. Recoveries can be conveniently determined by spiking toxin at different concentrations in the sample matrix prior to extraction. The analytical technique can be evaluated if extraction recoveries are high and reproducible at all concentrations tested. A consistent but lowered recovery can be mathematically

compensated for in normal analysis (van Egmond and Wagstaffe, 1989).

8.6 Quantitative Immunoassays for Ochratoxin A in Agricultural Commodities

Several ELISA and RIA methods have been described for the determination of OA in agricultural commodities. These methods have been used on commodities such as wheat, barley, pig kidney, pig serum, feeds (Lee and Chu, 1984; Morgan et al., 1983, 1985, 1986; Rousseau et al., 1985, 1986, 1987; Chiba et al., 1985; Fukal, 1987; Candlish et al., 1988; Ruprich et al., 1988; Ramakrishna et al., 1990). ELISA methods are generally preferred for OA. The limits of detection of some ELISA's for OA are comparable to those of chromatographic procedures (Table 2; Table 3). The assay sensitivities ranged from 0.06 to 5 ng/g for plant tissues, 0.2 to 1 ng/g for swine kidney and 0.1 to 0.5 ng/mL for swine serum. The polyclonal antibodies are generally 2 to 3 times more sensitive than the Mabs for OA (Table 2; Table 3). The RIA based on polyclonal antibodies appears to be generally less sensitive than that obtained with the ELISA's. The polyclonal antibodies were as specific for OA and the other ochratoxins as the Mabs (Table 4). The majority of antibody preparations showed significant cross-reactivities with OC, the ethyl-ester of OA, but little cross-reaction with any other naturally occurring forms. The lack of any cross-reaction with phenylalanine and the small response seen against Oα suggests the antibodies are specific for the whole molecule (Chu et al., 1976; Morgan et al., 1983; Candlish et al., 1988). Solvents

TABLE 4. Comparison of Antibody Specificites

Antibody Type*	Ochratoxin cross-reactivity**					
	OB	Oα	OC	(4)-OH-OA	Phenylalanine	Ref.
Mab	>1	>1	-	-	>1	Rousseau et al., 1987
Mab	1-100	0.1	63.1-99.5	<0.1	<0.01	Kawamura et al., 1989
Mab	-	0.6	8.2	-	<0.1	Ramakrishna et al., 1990
Mab	-	3.9	150	-	<0.1	Candlish et al., 1986
Mab	19	31	4	-	3	Porter, 1990
Pab	4	-	27	-	<0.01	Fukal, 1987
Pab	14	0.1	44	-	-	Pestka et al., 1981
Pab	0.75	<0.01	37.5	-	<0.01	Chu et al., 1976
Pab	0.5	2.4	-	1.3-7.2	<0.01	Morgan et al., 1986
Pab	0	0	100	-	-	Martlebauer and Terplan, 1988
Pab	0.08-0.2	1.5-3.5	15-20	3-5	<0.01	Breitholtz-Emanuelsson et al., 1992

* Mab and Pab refer to mouse monoclonal antibody and rabbit antisera, respectively. ** The percent relative cross-reactivity. Relative cross-reactivities were normally determined by comparison of the concentrations of toxin required to give 50 % maximal signal in the immunoassay.

used in the extraction of grains and swine tissues are varied and include acidified-chloroform, methanol and acetonitrile. The methanol and acetonitrile solvents were recommended for grain extraction as they could be easily diluted in water for direct analysis and gave comparable results to those obtained with acidified-chloroform (Ramakrishna et al., 1990; Lee and Chu, 1983). The two aqueous solvents are generally regarded as being less toxic, more easily disposed and economical for routine grain analysis. The swine kidney tissue extractions procedures were based on acidified-chloroform and required sample clean-up by partitioning against sodium bicarbonate and or passing through reversed-phase packed columns (Morgan et al., 1986; Rousseau et al., 1987; Fukal, 1987). A sensitive ELISA which is fast, economical and specific, and avoids the use of chloroform and extensive sample clean-up should facilitate future large-scale regulatory screening programs for grains and swine tissues.

9 Summary and Conclusions

Ochratoxin A has been detected in human blood and milk and is known to contaminate the human food-chain because the mycotoxin can occur in both edible plants and livestock tissues. The human food-chain appears to be contaminated throughout the world and is not restricted to under-developed countries. Ochratoxin A is primarily a nephrotoxin which has been identified as the causal

agent of porcine nephropathy, and a suspected agent in Balkan endemic nephropathy. Several techniques have been proposed for minimization of potential ochratoxicosis and include, large-scale regulatory screening followed by subsequent condemnation or diversion of contaminated products, improved commodity management practices, amelioration of toxic effects by chemical supplementation, prevention of toxigenic mold growth, and *in-situ* destruction of toxin by physical, biological and chemical techniques. The most viable control approaches are the maintenance of low moistures in the harvested grains, routine surveillance at the lower levels of the human food chain, condemnation and diversion of heavily contaminated commodities, supplementation of diets with free-radical scavenging vitamins and commingling and feeding of slightly contaminated grains to adult ruminant-livestock. Several trace-level chromatographic techniques have been developed for the surveillance of mycotoxins. These analytical techniques are sensitive and specific but require extensive sample clean-up. Immunoassays, specifically ELISA, offer many potential advantages in that they can be highly sensitive and specific without the need for extensive clean-up. Immunoassays in comparison to conventional chromatographic approaches are more easily adapted to large-scale screening applications because multiple samples, including controls and standards, can be run simultaneously. Polyclonal antibodies are the most practical and economical source of antibody for ELISA development as sufficient

amounts can be obtained in relatively short periods of time. Rabbit antibodies have proved to be sufficiently sensitive and specific for the development of ELISA's for mycotoxins. Further research, however, must be carried out to establish the usefulness of chicken IgY for such assays. The development of a simplified sample extraction procedure which is compatible with the ELISA and avoids the use of chloroform will facilitate future regulatory analysis. The research presented in this thesis addressed these concerns.

MANUSCRIPT I

Development of a Quantitative and Sensitive Enzyme-Linked Immunosorbent Assay for Ochratoxin A using Antibodies from the Yolk of the Laying-Hen

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ABSTRACT

Antibodies directed against ochratoxin A (OA) were obtained from laying-hens egg yolk using an optimized purification procedure and applied in an ELISA for OA in swine finisher diets. The egg yolk antibody could be recovered at levels as high as 70-80 % with purities of 86-92 % using a mixture of aqueous buffer and chloroform for lipid extraction and polyethylene glycol for antibody precipitation. Ochratoxins C, B, α and the structurally related mycotoxin citrinin where found to cross-react with the antibody, 400, 100, 33.3 % and 2 %, respectively in an indirect competitive ELISA. Ochratoxin A could be detected in swine finisher diets at levels greater than 50 ng/g using a simplified sample preparation procedure and a indirect competitive ELISA. Recoveries of OA from the diets were validated by conventional HPLC analysis using a proven sample extraction protocol. ELISA determined OA values correlated highly with those obtained using HPLC analysis ($r = 0.98$). Assay sensitivity was found to be dependant on background absorbance. The mixed anhydride (MA) coupling chemistry used to prepare the immunogens promoted high background absorbances in the quantitative ELISA. The background was overcome by using N-hydroxysuccinimide based coupling chemistry for the preparation of plate coating antigen and or incubation of the antibody with bovine serum albumin that had been subjected to the MA reaction. This study demonstrates that antibodies from hen egg yolk can be readily obtained in good yield and purity and used to develop a highly sensitive ELISA for OA.

INTRODUCTION

Ochratoxin A, a secondary fungal metabolite belonging to the group of compounds known as mycotoxins, is known to be hepatotoxic, nephrotoxic, teratogenic, and mutagenic to a wide variety of animals (Roschenthaler et al., 1984; Steyn, 1984; Marquardt et al., 1990). The wide-spread and natural occurrence of this mycotoxin in animal feeds and tissues and its potential hazard to humans has lead to much research in its detection. Techniques such as TLC, HPLC and more recently ELISA's have been described for the determination of OA (van Egmond, 1991a). Conventional chromatographic techniques often lack sensitivity as is the case for TLC and usually require extensive sample clean-up prior to analysis. Alternatively, ELISA's offer several potential advantages, less stringent requirement for sample clean-up, the possibility of large sample through-put, and improved sensitivity and specificity (Pestka, 1989 ; Chu, 1990). Mycotoxin specific antibodies are primarily obtained from rabbits or mice but interestingly no previous report of the use of laying hen egg yolk polyclonal antibodies, IgY. Although the chicken has not been widely used as a source of polyclonal antibodies it offers several important advantages over conventional mammalian antisera. Collection of the eggs is convenient and noninvasive, as compared to the repeated bleeding of mammals and there appears to be a quantitative advantage in the production of antigen specific antibody (Gassman et al., 1990). Several IgY purification protocols exist with high yields and purities.

Techniques of purification have included simple precipitation with reagents such as polyethylene glycol, PEG (Polson et al., 1980), organic solvent extraction of the lipid component (Auliso and Shelokov, 1976; Bade and Stegemann, 1984) and advanced column chromatographic protocols (McCannel et al., 1990; Hassl and Aspock, 1988). The procedure of Polson (1990) was selected for anti-OA IgY purification as the technique proved to be relatively simple with the potential for further yield improvement and the yields were better than earlier published protocols. This paper reports methodology for the production and purification of OA specific polyclonal antibodies from the yolk of a laying hen, and the development of a sensitive and quantitative ELISA for OA in swine finisher diets.

MATERIAL AND METHODS

Materials. Pure OA was obtained via surface liquid fermentation with *Aspergillus ochraceus* Whilhelm, NRRL 3174) as previously described (Sreemannarayana et al., 1988). Ochratoxin alpha was prepared by hydrolysis in strong acid according to the procedure of Doster and Sinnhuber (1972). Ochratoxin C was prepared by esterification with ethanol (Fuchs et al., 1984). Ochratoxin B was isolated in the same manner as that reported by Madhyastha et al. (1990) while both citrinin, L-phenylalanine, bovine serum albumin (BSA), dicyclohexylcarbodiimide (DCC), N-hydroxysuccinimide, complete Freund's adjuvant (CFA), polyethylene glycol-8000 (PEG) were obtained from Sigma Chemical Co., St. Louis, MO. Microtiter plates (Falcon, 3911, Microtest III,) were obtained from Becton Dickinson Labware, Oxnard, CA. All remaining solvents and reagents were of reagent grade quality or better.

Immunization of the laying hens. White Leghorn laying hens (Shavers SX 288) that were approximately 20 weeks of age were immunized and used for egg production in accordance with standard animal care regulations. The immunogen consisted of 2 mg of bovine serum albumin-OA conjugate (BSA-OA) prepared according to the MA procedure of Chu et al. (1982). The immunogen was dissolved in 0.5 mL of 0.1 M saline and emulsified with an equal volume of CFA prior to injection. One millilitre of the mixture was injected intramuscularly

(pectoral muscles) at two different sites on the hen. Two booster injections using the same preparation were given subsequently at days 21 and 70. Eggs were collected every day following the final boost for two months, pooled, purified and stored at 4 °C.

ELISA used for optimization of IgY purification and comparison analysis. The amount of IgY recovered during an extraction was indirectly quantified on the basis of the final recoverable anti-ochratoxin A IgY (anti-OA) activity in a manner similar to that previously described (Kühlmann et al., 1988). In this procedure, the total amount of IgY in the sample as determined by the indirect non-competitive ELISA was estimated on the basis of the dilution of the unknown sample that was required to produce the same absorbance as a standard anti-OA IgY preparation. A reference absorbance was selected at 25 % of the maximal absorbance and was found to yield acceptable accuracy and reproducibility when compared to the originally proposed value of 10 %.

Ochratoxin A was conjugated to ovalbumin using the mixed anhydride reaction previously described (Chu et al., 1982). The ELISA plates were coated with 100 μL of 0.1 μg/ml ovalbumin-OA conjugate (OV-OA) in 0.05 M carbonate buffer (pH 9.6) overnight at 4 °C. After washing the plates twice with 0.1 M, pH 7.2 phosphate buffer containing 0.1 M saline (PBS), the plates were "blocked" using PBS containing 2 % instant skim milk powder. Following the "blocking" step the

plates where washed twice with PBS and 50 μL of the whole yolk or partially purified IgY possessing anti-OA activity that was diluted initially 1:100 and then subsequently diluted by doubling serial dilutions. The plates were again incubated at 37 °C for 1 hr. Following 1 hr incubation the plates were washed twice again with PBS containing 0.05 %(v/v) Tween 20 (PBS-T). Apply 50 μL of the alkaline phosphatase labelled rabbit anti-chicken IgG diluted 1:2500 in PBS-T containing 0.2 % skim milk powder. Incubate the plate for 1 hr at 37 °C. Plates at this last step where washed extensively using PBS-T (at least six washing cycles) and then developed by the addition of 50 μl of the substrate solution (1 mg/ml p-nitrophenyl phosphate, diethanolamine buffer, pH 9.8. The plates were incubated at room temperature for 1 hr, after which the absorbence was measured using a microplate reader ((Bio-Rad Laboratories Ltd., Mississauga, Ontario, Canada, model 450) fitted with a 405 nm filter. The reference yolk sample was found to be stable for a period of at least 3 months at 4 °C. Recoveries of anti-OA IgY activity and total protein were determined for the procedures of Polson et al. (1980, 1985) and Polson (1990). The total proteins following the extraction were adjusted to the same concentration and the activity recoveries determined for comparison purposes.

Optimal conditions for IgY purification. Egg yolk antibody, IgY, was purified as described by Polson (1990) with the following yield optimizing modifications.

In general, one volume of yolk was diluted with four volumes of 0.1 M pH 7.2 PBS, and one volume of chloroform. The mixture was shaken well and centrifuged for 30 min at 10,000 x g. The top aqueous layer was recovered and IgY was precipitated twice with 14 % (W/W) polyethylene glycol-8000. The IgY precipitate was reconstituted in PBS containing sodium azide (0.005 %) and 5 % glycerol and was stored at -20 °C. IgY preparations for routine usage were kept at 4 °C after washing and concentration of the IgY preparation by ultrafiltration (Amicon, Centriprep-10 concentrator, Beverly, MA, USA) to approx. 5-10 mg ml^{-1}. This preparation appeared stable for more than six months even though some precipitation was observed.

Total protein determination. The protein content of the extracted yolk samples was determined using the bicinchoninic acid method for determining total protein concentration (Smith et al., 1985). Bovine serum albumin was selected as a standard. The final sample absorbence was measured using a microplate reader (Bio-Rad Laboratories Ltd., Mississauga, Ontario, Canada, model 450) fitted with a 570 nm filter.

Electrophoresis. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions was performed according to Laemmli (1970) using a Mini-Protein II electrophoresis apparatus from (Bio-Rad

Laboratories Ltd., Mississauga, Ontario, Canada). A commercially prepared gradient polyacrylamide gel with a concentrations range of 4-20 % (Bio-Rad Laboratories Ltd., Mississauga, Ontario, Canada, 161-0900) was selected for separating protein bands. The resulting protein bands were stained according to manufacturers instructions (Bio-Rad Laboratories Ltd., Mississauga, Ontario, Canada, 161-0900 product manual) using CCB R250. Purity of final protein preparation was estimated using a Beckman DU-8 spectrophotometer fitted with a gel scanning apparatus set to read proteins peaks at 590nm.

Preparation of indirect competitive ELISA plate coating antigen. Ochratoxin A was conjugated to ovalbumin (OA-OV) using the MA reaction described by Chu et al. (1982). The alternate plate coating antigen was prepared in a manner similar to that previously reported by Gendloff et al. (1986). Ten milligrams of pure OA, 40 mg of NHS and 40 mg of DCC was added to 1 ml of dry tetrahydrofuran. The mixture was allowed to react in the dark for at least 30 min at 25 °C with constant stirring. The white precipitate (oxidized DCC) that was formed during the reaction was removed by centrifuging at 10,000 x g for 15 min. Residual reactant was removed from the precipitate by several re-extractions with tetrahydrofuran followed by filtration through a pasteur pipet plugged with a glass wool frit. Supernatant and washings were pooled and dried under a stream of nitrogen gas. The dried product was reconstituted in 2 mL, final volume, of N,N-

dimethylformamide. Two millilitres of the activated OA-NHS ester was added slowly and dropwise to a continuously stirred solution containing ovalbumin (100 mg in 50 mL of 0.1 M sodium bicarbonate). This mixture was allowed to react for 30 min at 25 °C with constant stirring in the dark and was subsequently dialysed against 6 L of distilled water (at least three changes). The conjugate following dialysis was collected, diluted with PBS to a concentration of approximately 1 mg mL^{-1} and stored at 4 °C until coated onto ELISA plates.

Preparation of mixed anhydride modified bovine serum albumin. A bovine serum albumin conjugate, termed "mock conjugate" that contained only MA determinants was prepared by a modified version of the MA reaction of Chu et al. (1982). In this procedure OA was removed from the reaction and substituted with a mole equivalent of L-glycine while BSA was selected as protein for conjugation. L-Glycine was selected as a substitute carboxyl containing molecule. The conjugate denoted BSA-MA was exhaustively dialysed against double distilled water to remove all organic solvents and un-reacted L-glycine. The protein was subsequently diluted with PBS (1-5 mg mL^{-1}) and stored at 4 °C with sodium azide (0.005 %). Storage periods of greater than 3 months did not affect its ability to counteract the non-specific background binding.

Preparation of OA contaminated swine finisher diet. Crushed and moistened

(32 % water by weight) soybeans were inoculated under sterile conditions with a preparation of *A. ochraceus* spores and incubated at 28 °C for 30 days. Inoculated soybean samples were analyzed for OA production by HPLC (data not shown) and were added to an OA-free swine finisher diet. The diet samples were blended and re-ground to give the desired concentrations of OA. The mixed diets were composed of 89 % barley, 7.8 % soybean meal, 2.5 % vitamin pre-mix and an additional 0.7 % of OA contaminated crushed soybeans.

Simplified swine finisher diet OA extraction procedure. A "simplified" methanol-based extraction procedure was evaluated and involved the extraction at 25 °C with constant shaking of a 5 g sample of diet with 30 mL of methanol-water (80 : 20) in a closed capped Nalgene centrifuge tube for 30 min. The mixture was centrifuged for 30 min at 5000 x g and the cleared supernatant was recovered by decanting. Aliquots of supernatant were removed, diluted with HPLC grade methanol, and filtered through a 0.5 μ filter prior to HPLC and ELISA analysis.

Ochratoxin A extraction procedure used for comparison of recoveries. The methanol-based extraction and reversed-phase cartridge clean-up procedure, that was used for comparison was a slight modification of that originally described by Lee et al. (1984) for the determination of OA in wheat by ELISA. In brief, 5 g of diet sample was mixed with 15 mL of pure methanol and shaken for 30 min.

The mixture was filtered through a Whatman (Number 4) paper filter and the filtrate was passed through a C18 Sep-Pak ™ (Waters Associates, Milford, Ma). The samples, following clean-up, were dried using a rotary evaporator (Buchi, Brinkman, Rotavapor R110), reconstituted in a small volume of methanol and passed through 0.5 μ filters prior to ELISA or HPLC analysis.

High performance liquid chromatography for OA recovery analysis and ELISA correlation. Analytes were separated on a Beckmann 5 μm Ultrasphere ODS reversed-phase C18 25 cm x 4.6 mm analytical column using an isocratic mobile phase containing 70 % methanol and 30 % distilled water adjusted to pH 2.1 using H_3PO_4 with a system similar to that described by Sreemannarayana et al., (1988). Ten to 50 μL of diet extract was injected and chromatographed using a solvent flow rate of 1.6 mL min^{-1} at a column temperature of 40 °C. OA peaks were detected using fluorescence emission with a Shimadzu model RF-535 detector set at 333nm for excitation and 450nm for emission.

Indirect competitive ELISA for quantitation of OA in swine finisher diets and determination of antibody cross-reactivity. Coat ELISA plates with 2.5 μg per well of the activated ester prepared coating antigen (OA-OV) overnight at 37 °C. The coating antigen was dissolved in 0.05 M carbonate buffer (pH 9.6). Wash plates two times with PBS and block plate with PBS containing 1 % skim milk

(200 μL/well) for 1hr at 37 °C. Coated and blocked plates can be stored empty and dry at 4 °C for several months. Dilute anti-OA antibody and mock conjugate in PBS to achieve dilution of antibody and a final protein concentration of the mock conjugate of 1:1500 and 3 mg/mL, respectively. Incubate the mixture for 1 hour at 37 °C prior to adding to ELISA plates. Apply onto the coated and blocked ELISA plates, consecutively; 65 μL PBS then 10 μL of the sample which contains either unknown or OA standards in methanol and then 75 μL of the diluted and pre-incubated with the mock conjugate, laying hen anti-OA IgY. Mix wells carefully and incubate for 1 hour at 37 °C. Wash plates with PBS-T three times and dry plates. Apply 150 μL of the alkaline phosphatase labelled rabbit anti-chicken IgG diluted at 1:2000 in PBS-T containing 0.2 % skim milk powder. Incubate the plate for 1 hr at 37 °C. Wash plates six times with PBS-T and dry plates thoroughly. Apply 150 μL of alkaline phosphatase substrate solution and incubate the plate for 30 min at 37 °C or until absorbence of the sample with no free OA was greater than 1.5 OD units. Read plates directly on microplate reader at 405 nm.

RESULTS AND DISCUSSION

Selection and Optimization of a Procedure for the Purification of Anti-OA IgY.

Several procedures for the purification of antigen specific IgY were examined for recovery and purity and the values obtained were compared with those reported in the literature. The amounts of IgY recovered in the comparison experiment, as determined by the indirect non-competitive ELISA, compared favourably with those values originally reported in the literature (Table 5). The results obtained for the Polson (1990) technique were unexpected because they were different from the 1990 report which suggested higher yields than Polson et al. (1980); yet in comparison to the other isolation procedures tested the procedure of Polson (1990) was the most readily improved. The systematic investigation of factors affecting the yield and purity of IgY demonstrated that the most practical ratio of solvents for the extraction of yolk was 1(10 mL):1(10 mL):4(40 mL), respectively for yolk, chloroform and PBS (Table 6). The anti-OA activity appeared to be quantitatively precipitated with polyethylene glycol-8000 at a concentration of greater than 14 % (W/W) with maximal purity being obtained after two successive precipitations, figure 2 and table 7, respectively. Gradient gel (4-20 %) SDS-PAGE carried out according to Laemmli (1970) followed by scanning densitometry demonstrated the final IgY preparation was reasonably pure as it contained between 8-14 % non-IgY

protein (Figure 3). The final IgY recovery and purity for the optimized procedure is shown in comparison to the earlier suggested purification procedures (Table 5). The optimized procedure is consequently an excellent means of preparing near pure and immunologically active IgY in relatively short periods of time and avoids the use of cumbersome column chromatographies.

Relative cross-reactivities of the laying hen ochratoxin A specific antibody preparation.

The anti-OA IgY preparation used for the development of the OA indirect competitive ELISA appeared to have antibodies with noticeable specificity differences, that is, the presence of non-toxin specific antibodies. In a normal indirect non-competitive ELISA using a mixed anhydride prepared coating antigen (OA-OV), and OA as the competing antigen, full inhibition of antibody response by the free ochratoxin A was not possible (Figure 4). This observation is consistent with the findings of Gendloff et al. (1986) who also reported the presence of high backgrounds in their mycotoxin specific ELISA's. Their experiments confirmed the presence of new antigenic determinants and attributed their presence to the mixed anhydride coupling reaction which was used to prepare the toxin-protein immunogen. One solution proposed by this group was to change the plate coating antigen coupling chemistry. They selected NHS activated ester

TABLE 5. Percent Recovery of Anti-Ochratoxin A(OA) IgY Activity and the Relative Fold Purification of IgY.

Procedure	Percent anti-OA IgY activity recovered[a]	Relative fold purification	Yield of IgY (mg/egg)[b]	(mg/egg)[b]
Polson et al., (1980)	61.2	11.5	77.5	80, 65, 83, 50[c]
Polson et al., (1985)	29.6	5.6	37.5	45, 45[d]
Polson (1990)	57.1	10.8	72.3	-
Current procedure	75.0	14	95	-

[a] Values shown are the means of at least four replicates, standard errors ($\leq \pm 5$) [b] Calculation of yields were made on the assumption that the volume of an average egg yolk is 15 mL and its total protein content is 160 mg/mL. Recovery assay was the same as that described in Material and Methods. [c] Values reported by Altschuh et al., (1984), Gassmann et al., (1990), Gottstein and Hemmeler (1985) and Hassl et al., (1987). [d] Values reported by Hassl and Aspöck (1988) and Hassl et al., (1987).

TABLE 6. The Percent Recovery of Total Protein, Anti-Ochratoxin A (OA) IgY Activity and the Relative Fold Purification of IgY in the Aqueous Supernatant Following Lipid Extraction.[a]

Volume of $CHCL_3$ (ml)	Volume of PBS dilutant (ml)			
	0	10	20	40
Percent recovery of total protein in supernatant.[b]				
0	100.0 ± 3.1	51.8 ± 1.5	59.4 ± 1.9	71.4 ± 2.0
10	10.6 ± 0.6	23.1 ± 1.1	24.9 ± 1.4	32.2 ± 1.8
40	4.8 ± 0.2	13.7 ± 0.8	22.9 ± 1.3	31.0 ± 1.7
Percent recovery of anti-OA IgY activity in supernatant.[b]				
0	100.0 ± 11.1	100.4 ± 9.6	90.0 ± 10.6	95.2 ± 8.5
10	23.7 ± 4.2	69.2 ± 6.8	72.2 ± 5.6	86.0 ± 6.4
40	16.3 ± 2.9	49.4 ± 3.9	67.0 ± 7.4	85.8 ± 8.0
Relative fold purification of IgY [b c]				
0	1.0 ± 0.1	1.9 ± 0.2	1.5 ± 0.2	1.3 ± 0.1
10	2.2 ± 0.5	3.0 ± 0.4	2.9 ± 0.4	2.7 ± 0.3
40	3.4 ± 0.7	3.5 ± 0.5	2.9 ± 0.5	2.8 ± 0.4

[a] The volume of yolk extracted for all tests was 10 mL. The concentration of protein in the egg yolk was 160 mg/ml. [b] Values represent the mean and standard error of at least 8 replicas. [c] Relative fold purification =(recovered anti-OA IgY activity in sample / recovered total protein in sample).

FIGURE 2. Percent recovery of IgY anti-ochratoxin A (-●-●-) activity and relative fold purification of the protein precipitate (-○-○-) following addition of different concentrations of PEG-8000 to aqueous extract of yolk. Percent total protein recovery and IgY activity values are the mean of eight replicates and the vertical bars represent the standard error of mean.

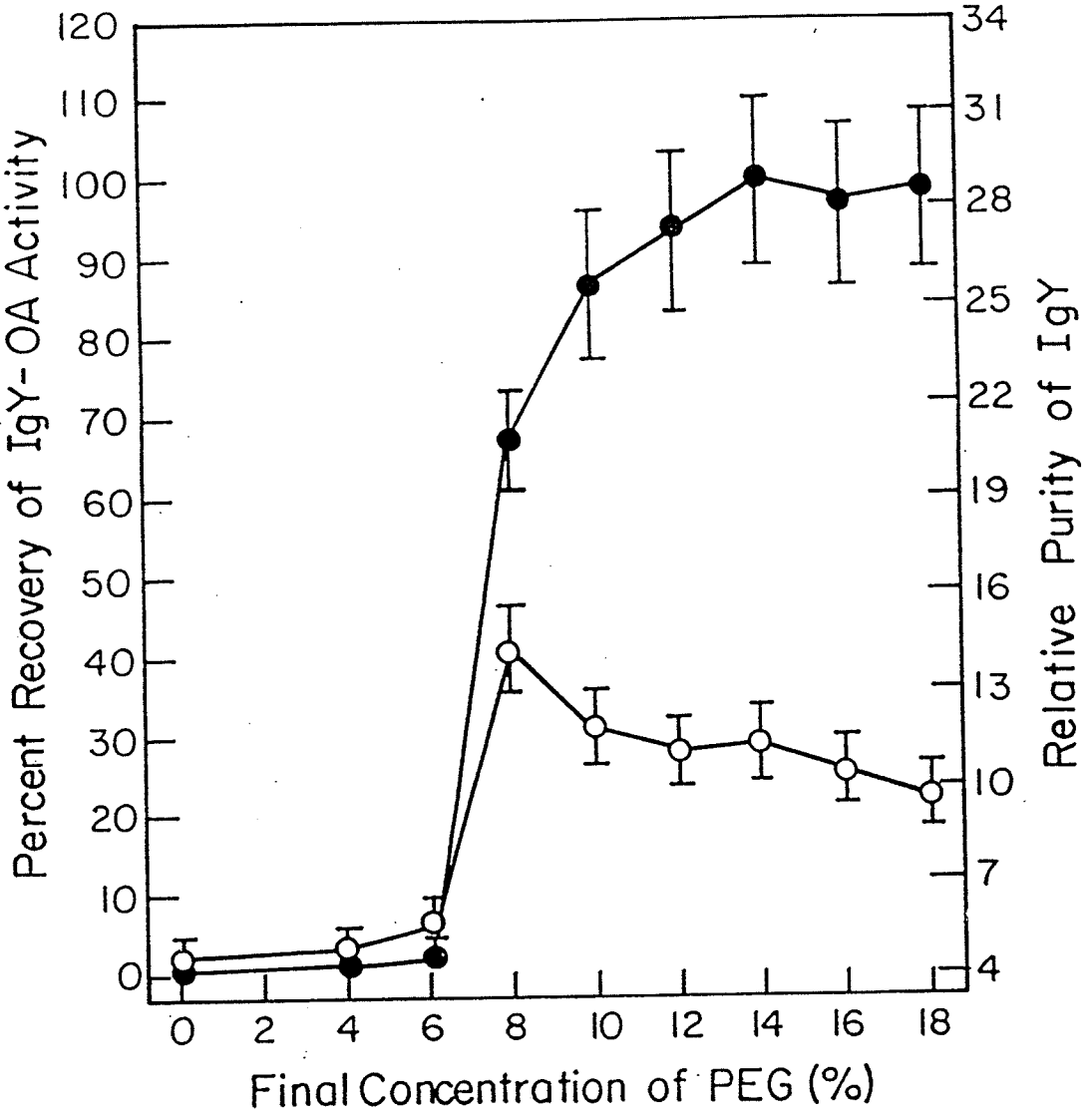

Percent Recovery of IgY-OA Activity
Relative Purity of IgY
Final Concentration of PEG (%)
120
110
100
90
80
70
60
50
40
30
20
10
0
34
31
28
25
22
19
16
13
10
7
4
0
2
4
6
8
10
12
14
16
18

TABLE 7. Percent Recovery of Total Protein and Anti-Ochratoxin A IgY Activity and Corresponding Relative Fold Purification Following Successive Precipitation with PEG-8000.[a]

	Percent total protein recovered[b] (x)	Percent anti-OA IgY activity recovered[b] (y)	Relative fold purification (x/y)
Whole yolk	100.0 ± 7.0	100.0 ± 3.9	1.0 ± 0.1
Aqueous supernatant	21.5 ± 1.5	85.7 ± 3.4	4.0 ± 0.4
Protein precipitate 1[c]	7.5 ± 0.6	82.5 ± 3.3	11.0 ± 1.2
precipitate 2[c]	5.4 ± 0.5	75.0 ± 5.0	14.0 ± 1.8
precipitate 3[c]	5.2 ± 0.6	44.2 ± 4.4	8.5 ± 1.6
precipitate 4[c]	4.8 ± 0.6	31.2 ± 3.1	6.5 ± 1.3

[a] The procedure used for total activity determination was as outlined in Figure 2 and described in Materials and Methods. [b] Values shown are the mean and standard error of at least four replicates. [c] Protein sample collected following repetitive precipitations.

FIGURE 3. Reducing SDS-PAGE electrophoretic evaluation of extracted proteins following different steps of antibody purification. Lanes A and G, pre-stained, low molecular protein standards (Bio-Rad Laboratories Ltd., Mississauga, Ontario, Canada); lane B, diluted whole egg yolk; lane C, aqueous supernatant following lipid solvent extraction; lane D and E, aqueous supernatant following one and two 14 % PEG-6000 precipitations; lane F, commercially prepared IgY standard H and L, denote heavy and light chains of IgY, respectively.

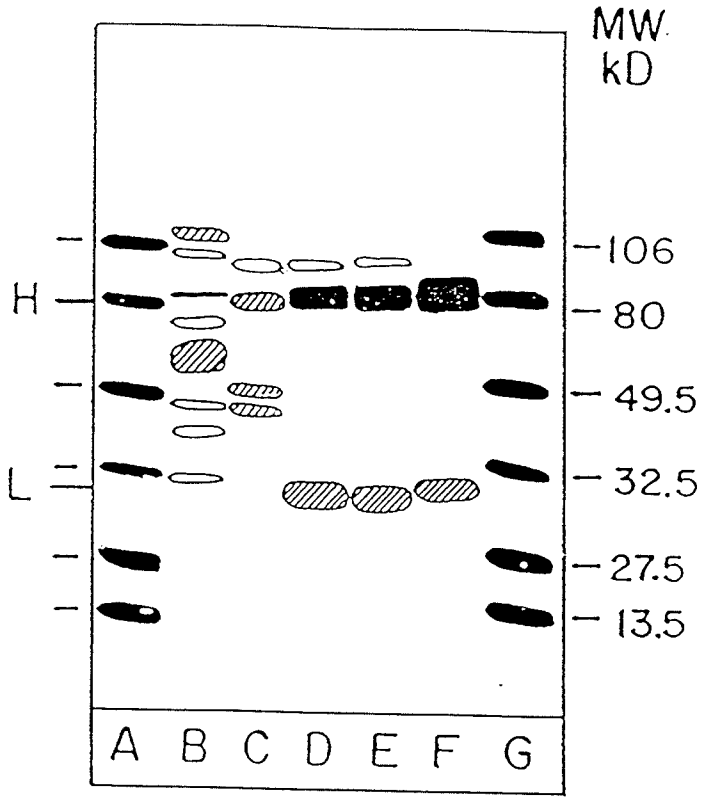
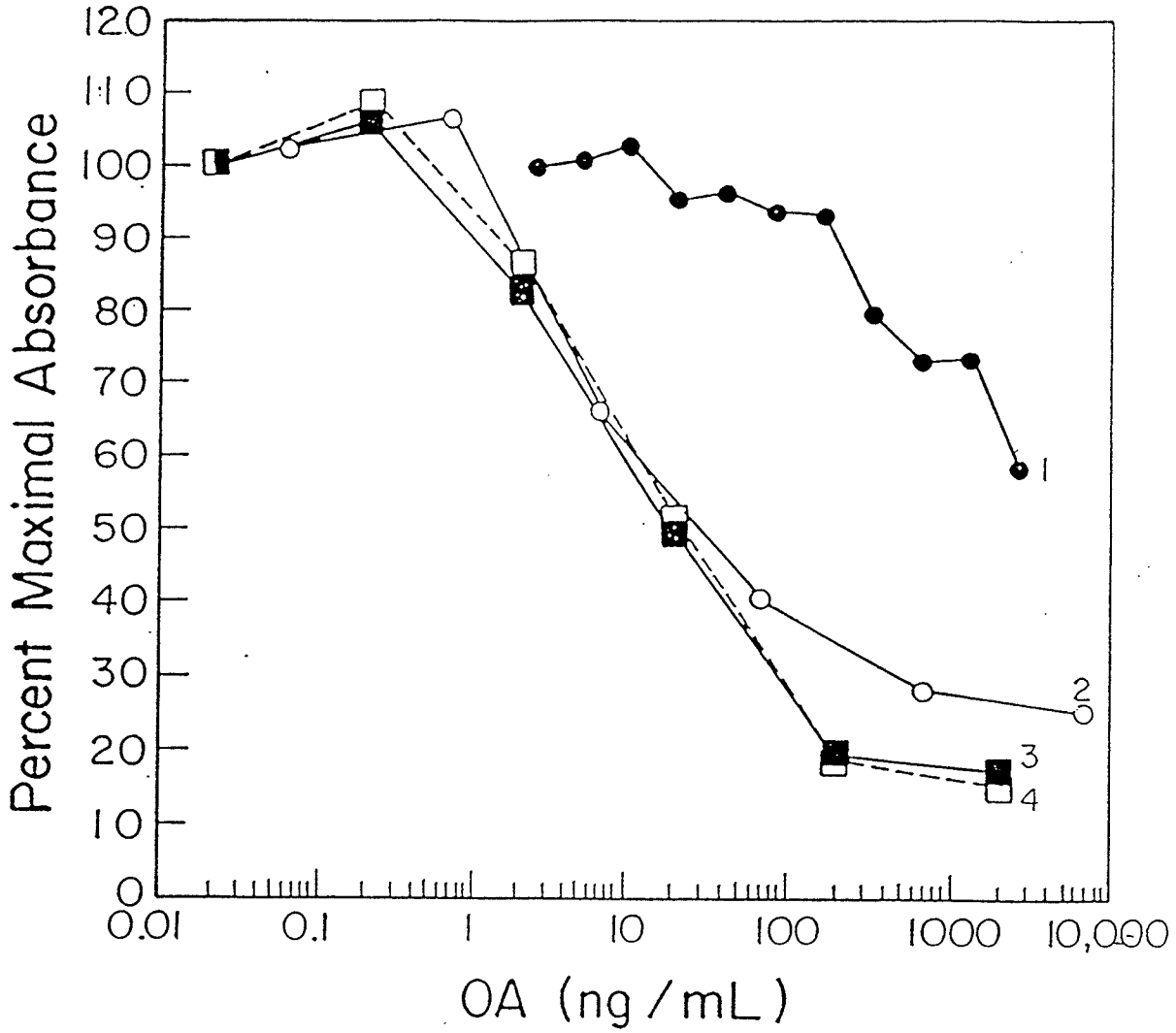

MW
kD
106
80
49.5
32.5
27.5
13.5
H
L
A B C D E F G

FIGURE 4. The reduction in background binding and concurrent increase in sensitivity due to the substitution of coating antigen (OV-OA) and addition of BSA-MA mock protein conjugate: (1) OV-OA conjugated with MA (●-●); (2) OV-OA conjugated with NHS(○-○); (3) OV-OA conjugated by MA and 3 mg mL^{-1} MA derivitized BSA(■-■); (4) OV-OA conjugated by NHS and 3 mg mL^{-1} MA derivitized BSA (□-□). Standard errors (n=6) in all cases were less than 10 %.


Percent Maximal Absorbance
120
110
100
90
80
70
60
50
40
30
20
10
0
0.01
0.1
1
10
100
1000
10,000
OA (ng/mL)
1
2
3
4

procedures instead of MA coupling chemistries to prepare the plate coating antigen. Although they found the technique to be generally useful the approach was shown to not work in all cases, therefore alternative techniques needed to be developed. In the current study, the MA coupling chemistry used to prepared the OA-OV plate coating antigen was replaced with the NHS activated ester coupling reaction. This technique as demonstrated in figure 4 reduced backgrounds significantly. Alternatively, the high background binding observed could be reduced by the pre-incubation of anti-OA IgY preparation with BSA which had been treated with the mixed anhydride coupling reagents (BSA-MA). This course of action could prove useful when alteration of plate coating antigen chemistries fail. The combination of both techniques yielded low backgrounds sufficient for routine analysis and was therefore included in the final ELISA for quantitation of OA in swine finisher diets.

The antibody was found to be ochratoxin group specific as it exhibited high relative cross-reactivities with other ochratoxin A like molecules in an indirect competitive ELISA (Table 8). The relative cross-reactivities are of interest as OB and OC are toxic whereas Oα and L-phenylalanine are not (Marquardt et al., 1990). The general specificity of this antibody preparation is not expected to interfere with quantitation of OA by ELISA in grain-based diets as OA is the most predominant member of the group to occur naturally, but may depend on the

substrate and fungi infecting the grain. In grains such as corn and barley, OB is reduced in comparison to OA, but in the presence of peanuts, soybean, and rapeseed, OB production can be enhanced (Roth et al., 1989; Madhyastha et al., 1990). Ochratoxin C, which competes extensively in this ELISA is a rare molecule and is unlikely to interfere with OA quantitation under normal conditions (Candlish et al., 1986; Fuchs et al., 1984). Ochratoxin alpha has not been reported in grains but is an excellent indicator of past OA contamination (Screemannarayana et al., 1988; Xiao et al., 1991). Identification of OA and citrinin toxicosis is possible as the antibody showed little recognition. Citrinin often co-occurs with OA and has similar modes of toxicity (Damoglou, 1984; Krogh, 1973b). Although their exists the possibility of erroneous OA analysis, the antibody developed can be considered sufficiently specific for determination of OA in grain.

Development of a quantitative indirect competitive ELISA for determination of OA in Swine finisher diets.

The development of a quantitative ELISA for OA in swine finisher diets could be practical and useful application of this antibody since swine finisher diets are composed of a myriad of complex and potentially interfering matrixes which could conceivably be encountered in routine grain testing.

The indirect competitive ELISA that was developed used a simple sample

TABLE 8. Relative Cross-Reactivity of Laying-Hen Anti-Ochratoxin A IgY with Various Ochratoxin A like Molecules in an Indirect Competitive ELISA.

Structurally related molecules	Cross reactivity[a](%)
Ochratoxin A	100
Ochratoxin B	100
Ochratoxin α	33
Ochratoxin C	400
L-Phenylalanine	0
Citrinin	2

[a] Relative cross-reactivity was determined by comparing the concentrations of toxin required to give 50 % maximal absorbency in the indirect competitive ELISA. All analysis was performed in at least triplicate.

TABLE 9. Effect of Sample Preparation on Recovery (Percent) of Ochratoxin A (OA) from Swine Finisher Diet by HPLC[a]

Toxin added to diet ng/g (spiked level)	Simplified sample preparation procedure	Comparison sample preparation procedure
50	100.0 ± 4.0	100 ± 4.0
100	100.0 ± 10.0	100.0 ± 10.0
200	110.0 ± 4.5	n.a.[b]
1000	85.0 ± 23.0	96.0 ± 2.1
2000	93.0 ± 5.9	100 ± 3.5
5000	97.2 ± 5.9	n.a.[b]

[a] Mean and standard deviation. Each sample analyzed by HPLC was done in at least triplicate. The correlation between OA recoveries for the two extraction procedures was ($r=0.98$, $P<0.05$) as determined by HPLC. [b] n.a. = not analyzed.

TABLE 10. Recovery of Ochratoxin A from Swine Finisher Diet Samples as Determined by the Quantitative Indirect Competitive ELISA using the Simplified Sample Preparation Procedure[a].

Toxin added to diet ng/g (spiked level)	Toxin detected by ELISA ng/g	Recovery, %
50	55 ± 5	110.0 ± 9.1
100	115 ± 20	115.0 ± 17.4
200	215 ± 30	107.5 ± 14.0
1000	815 ± 80	81.5 ± 9.8
2000	1945 ± 150	97.2 ± 7.7
5000	4565 ± 400	91.3 ± 8.7

[a] Mean and standard deviation. Each sample analyzed by the ELISA was done in at least triplicate. Recoveries by ELISA and HPLC (Table 9) using the simplified extraction gave a correlation of ($r=0.99$, $P<0.05$).

preparation procedure which avoided extensive sample clean-up. The proposed sample extraction procedure was validated by conventional HPLC recovery analysis using a published comparison procedure involving the use of a more elaborate OA extraction protocol (Table 9). An important feature of the simplified procedure for sample preparation is its use of methanol. This solvent has many advantages over chloroform, a commonly used OA extraction solvent, in that it is less toxic, more easily disposed, has comparable OA extraction recoveries, and is readily diluted with aqueous buffers necessary for the ELISA. It is also known that acetonitrile can be used effectively for the extraction of OA (Ramakrishna et al., 1990), however, it also has considerable toxicity and is in general more expensive for routine analysis. The recovery and quantitation of OA by the ELISA method was confirmed by correlation of observed OA contamination in OA spiked diets (Table 10). Ochratoxin A was reproducibly detectable in swine finisher diets between 50 and 5000 ng/g. The high sensitivity of the ELISA for OA coupled with the simplified sample preparation procedure and the ease of antibody purification makes this assay attractive for routine OA analysis.

MANUSCRIPT II

Comparative Studies on the Specificity and Sensitivity of Rabbit and Laying-Hen Antisera to Ochratoxin A

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ABSTRACT

Polyclonal antisera specific for ochratoxin A (OA) were developed in laying-hens and rabbits immunized with OA conjugated to bovine serum albumin. The rabbits and laying-hens were immunized and tested under parallel conditions. Ochratoxin A specific antisera activity was monitored by the use of an indirect competitive ELISA. Rabbits and hens produced OA specific antisera within the first 51 days of the immunization schedule. The rabbits consistently produced antisera to OA that was ten fold higher in concentration than that observed with laying-hens. Rabbit and laying-hen antisera were found to be equally specific at most times in the immunization schedule for ochratoxins B and C, but not α. Antisera from either source could be used to quantify OA in spiked wheat. The rabbit antisera reproducibly detected OA at 3 ng/g or higher whereas the hens antisera could only detect 50 ng/g or greater. The optimal assay pH for the specific and sensitive detection of OA in wheat was 7.0 using either source of antisera. The antisera from both sources were similar in their cross-reactions with structurally related compounds.

INTRODUCTION

Ochratoxin A (OA) is a highly toxic and frequently encountered secondary fungal metabolite produced by toxigenic strains of *Penicillium* and *Aspergillus*. This mycotoxin is known to be hepatotoxic, nephrotoxic, teratogenic, carcinogenic and mutagenic to a wide variety of animals (Roschenthaler et al., 1984; Steyn, 1984; Marquardt et al., 1990, Marquardt et al., 1992). Economical and large scale monitoring techniques should be developed in view of the widespread occurrence of OA and potential toxicity to humans and animals. Ochratoxin A occurs in relatively low concentrations in grain and frequently occurs along with other co-occurring mycotoxins such as citrinin (Kuiper-Goodman and Scott, 1989; Krogh et al., 1973). Several countries have imposed strict regulatory limits for this mycotoxin at low concentrations (van Egmond, 1991b). The putative detection technique must be therefore very sensitive and specific. The method must also be simple and inexpensive to be of practical benefit in routine analysis. Techniques such as TLC, HPLC, and more recently immunoassays have been described for OA detection (van Egmond, 1991a). The conventional chromatographic approaches are sensitive and specific, however, they tend to be slow and lack sensitivity, are not simple and are in general expensive. The many practical advantages of immunoassays for mycotoxins, specifically competitive ELISA methods, have been described (Pestka, 1989; Chu, 1990). The widespread application of

immunochemical methods for OA detection requires a readily available source of OA specific antibody. In general, mammalian sources of polyclonal and monoclonal antibody have been used for mycotoxin detection (Wilkinson et al., 1992). Recently, due to the many advantages of raising and collecting laying-hen antibodies (Gassmann et al., 1990), OA specific antibodies from the yolk of a laying-hen have been developed (Clarke et al., 1993). The apparent specificity and sensitivity of the laying-hen antibody preparation was not as high as those previously reported for OA in mammals (Chu, 1989; Candlish, 1991). The recent works of Otani et al. (1991); Hassl et al. (1987); Hsu and Chu (1992) and Bauwens et al. (1987) have also suggested differences in specificities of the two sources of antibody. Hsu and Chu (1992) have also suggested that laying-hen egg yolk antibody specific for the mycotoxin aflatoxin B1 had lower sensitivity than that obtained with rabbit polyclonal antisera.

This study systematically investigated the changes in specificity and sensitivity of laying-hen and rabbit polyclonal antisera to OA over time and the influence of pH on the ELISA performance.

MATERIALS AND METHODS

Materials. Ochratoxin A was isolated from liquid culture of *Aspergillus ochraceus* (Sreemannarayana et al., 1988). Ochratoxin α (Oα) was prepared by acid hydrolysis of OA (Doster and Sinnhuber, 1972), ochratoxin B (OB) by catalytic de-chlorination of OA with palladium on activated carbon (Bredenkamp et al., 1989) and ochratoxin C (OC) by esterifying OA with ethanol (Fuchs et al., 1984). Bovine serum albumin (BSA), ovalbumin (OV), 1,1'-carbonyldiimidazole (CDI), N-hydroxysuccimide, complete and incomplete adjuvants (CFA and IFA), p-nitrophenyl phosphate, diethanolamine, goat anti-rabbit IgG coupled to alkaline phosphatase (A3812) with specific activity of 3700 units per ml, were obtained from Sigma Chemical Co., St. Louis, MO, USA. Rabbit anti-chicken IgG coupled to alkaline phosphatase (303-055-003), with comparable specific activity was obtained from Jackson Laboratories, USA. Microtiter plates (Falcon 3911, Microtest III) were obtained from Becton Dickson Labware, Oxnard, CA., USA. All solvents and reagents used were of analytical grade.

Preparation of Immunogen and Plate Coating Antigen. Ochratoxin A was covalently conjugated to BSA (BSA-OA) using a novel coupling reagent. The OA carboxyl functional group was activated with 1,1'-carbonylimidazole and allowed to react with the ϵ-amino groups of lysine in the proteins (Xiao et al., 1994). In

brief 1 mg of OA and 1.5 mg of CDI were dissolved and mixed in 0.1 ml acetone. The mixture was allowed to react for 10 min at 25 °C in the dark with constant stirring. The activated OA mixture was added slowly and dropwise to a continuously stirred solution of BSA (10 mg) in 0.1 M Na_2CO_3 (1 ml). The solution was allowed to react for 2 h at 25 °C in the dark with continuous stirring followed by exhaustive dialysis with 0.05 M carbonate buffer (pH 9.6), to remove any uncoupled OA and subsequently distilled water to remove carbonate salts (18 h). The molar binding ratio was determined with a Beckman DU-8 UV-VIS spectrophotometer. OA was measured at 333 nm and total protein was quantified by a dye binding method (Bradford, 1976). The observed extent of coupling was 6 mole OA per mole BSA. The conjugate following clean-up was collected, diluted to a concentration of 1 mg mL^{-1} and stored at -20 °C. The plate coating antigen, ovalbumin-OA (OV-OA), was prepared by the N-hydroxysuccinimide activated-ester procedure previously described by Clarke et al. (1993). The observed molar binding ratio was 3-4 mol OA per mol OV. The plate coating-antigen was also diluted to a concentration of 1 mg mL^{-1} and stored at -20 °C.

Immunization. Two dutch-belted type rabbits and two white-leghorn laying-hens (Shavers SX 288), 12 and 20 wk of age, were each immunized with 500 μg of the BSA-OA immunogen (prepared as described above) in 0.5 mL of 0.1 M saline,

emulsified with an equal volume of CFA. The mixtures were injected intramuscularly at multiple sites into the thigh muscles of rabbits and the pectoral muscles of laying-hens. Four boosters were given subsequently at days 51, 102, 132, 166 and consisted of 250 μg BSA-OA in 0.5 mL saline emulsified with an equal volume of IFA. The booster injections were administered in the same manner as the initial injections. Blood was collected from the marginal ear-vein of the rabbits and from the wing-veins of the laying-hens on days 0, 30, 51, 81, 102, 132, 166. The serum was allowed to separate from the blood at 25°C for 3 h, followed by centrifugation at 5000xg for 30 min and collection of the serum. The rabbit and laying-hens antisera were used directly in the ELISA after dilution with assay buffer. The sera was stored frozen in aliquots at -20°C.

Indirect Competitive ELISA for Quantitation of OA in Wheat and Determination of Antisera Cross-Reactivities. The microtiter plate wells were sensitized with the antigen by coating with OV-OA (200 μL) at a concentration of 12.5 μg mL^{-1}, overnight at 4 °C, in 0.1 M phosphate buffered saline (pH 7.2). The plates wells were washed twice with 0.1 M phosphate buffered saline (pH 7.2) containing 0.01 % TWEEN-20 (WB-T), and then air dried at 25 °C. Coated and air-dried plates could be stored at 4 °C for several months without any significant decrease in activity. Prior to analysis the microtiter plates were washed once with

WB-T. The following were sequentially added to the appropriate microtiter plate wells; 65 μL of pH 6.0, 7.0 or 8.0 (0.1 M) phosphate buffered saline containing 0.01% TWEEN-20 (PBS-T), 10 μL of toxin standard or wheat extract in methanol and 75 μL of rabbit or laying-hen antisera diluted 1:2000 or 1:200 in pH 6.0, 7.0 or 8.0 PBS-T. The plates wells were mixed carefully and incubated for 60 min at 37 °C. The plates following incubation were carefully emptied, washed three times with WB-T, and then dried. Commercial alkaline phosphatase conjugated anti-rabbit IgG or anti-chicken IgG as the second antibody (150 μL of 1:5000, 1:2000 in WB-T, respectively) was added to all wells, followed by incubation for 1 h at 37 °C. The wells were emptied carefully and the wells were washed six times with WB-T. Alkaline phosphatase substrate solution [150 μL of 1 mg mL^{-1} p-nitrophenyl phosphate in diethanolamine buffer (pH 9.8)] was added to all wells followed by incubation of the plate for 30 min at 37 °C or until the absorbency of the sample with no free OA was greater than 1.5 absorbence units. The plates were directly read in a microtiter plate reader at 405 nm (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada, Model 450).

Preparation of OA-Spiked Wheat Samples. Clean and intact wheat was ground in a Willey Mill fitted with a 1 mm screen. Pure OA (1 mg) in chloroform (2 mL) was mixed with 100 g of clean, ground wheat followed by evaporation of the

chloroform at 25 °C. The spiked wheat was blended with 900 g of ground wheat to give a stock concentration of 1000 μg OA per kg of ground wheat. The stock sample was diluted with clean and ground wheat to give a contamination range of 0 to 100 μg OA per kg of ground wheat. The concentration of toxin in the spiked wheat samples were verified by HPLC analysis using conditions previously described by Clarke et al. (1993).

Extraction Procedure for Ochratoxin A from Wheat. The methanol-based wheat extraction technique, which was used to test sensitivities of the two antisera sources, was a slight modification of that originally described by Clarke et al. (1993) for the determination of OA in swine finisher diets by ELISA. Methanol-H_3PO_4 acidified water (15 ml, 80:20, pH 2.1) was added to 5 g samples of ground wheat in capped centrifuge tubes (Nalgene). The samples were extracted by shaking for 30 min at 25 °C and then centrifugation for 15 min at 10000xg. The supernatants were recovered by decanting and used directly in the ELISA.

RESULTS AND DISCUSSION

There are many reports in the literature of highly specific and sensitive antibody responses being produced to mycotoxins in mammals (Wilkinson et al., 1992) but few reports in laying-hens (Clarke et al., 1993; Hsu and Chu 1992). This is unfortunate as hyperimmunized laying-hen are a potentially valuable source of antigen specific antibody. The laying-hen can efficiently transfer serum antibodies to the developing egg yolk. These transferred egg yolk antibodies (IgY) are equivalent in specificity and sensitivity to those found in the serum (Kühlmann et al., 1988; Vieira et al., 1984), are maintained for long periods of time and can be easily isolated and purified using non-invasive collection techniques (Gassmann et al., 1990). Thus, the hen egg yolk appears to be an ideal, alternative source of readily purifiable mycotoxin specific antibody. Few comparison studies have been performed with mammalian antisera in regards to specificity and sensitivity to immunogens in general (Otani et al., 1991; Hassl et al., 1987; and Bauwens et al., 1987) and no systematic studies for small molecular weight mycotoxins. Hen antiserum was therefore used in this current study for comparison of sensitivities and specificities rather than egg yolk as it was more comparable in nature with the rabbit antisera. Hen antiserum had the added advantage that no purification was necessary prior to testing in the immunoassay. In addition, protein purification reagents such as the commonly used protein precipitating agent, PEG, could cause

laying-hen antibody aggregation which may alter the activity of the antibody (Hassl et al., 1987). Perhaps purification removes specific classes of antibody which might otherwise be present in sera (McCannel and Nakai 1990) and certain classes of antibody may be excluded from the egg yolk during the transfer of antibody into the egg. Immunoglobulin M is such a class of antibody (Rose et al., 1974). In addition, serum samples can be readily and consistently collected from the hens wing-veins thus ensuring comparability and continuity of the sampling.

Anti-OA activities were detected in both rabbits and laying-hens after one injection with BSA-OA. After four injections the activities were found to be substantially different. Rabbit anti-OA immune responses as determined by the ELISA were ten fold higher than that seen in laying-hen antisera even after 166 days of repetitive boosting (Figure 5). The recent work of Otani et al. (1991) also demonstrated large differences in total antibody activity. They observed a 100-fold difference in activity when rabbits were compared to laying-hens. Polson et al. (1980) has suggested that laying-hens respond poorly to antigens with molecular weights less than 30000. The lowered response may be partially attributed to the continuous transfer of immunoglobulin from the blood of the laying-hen to the developing egg yolks resulting in a depletion of anti-OA antibody activity in the blood. A more meaningful comparison of normal titers might therefore be obtained by comparison of rabbits with male chickens or non-laying hens.

The immune responses of rabbits and laying-hens to OA appear to be similar in regards to specificity as both sources of antisera had consistently similar relative cross-reactivities to OC and OB at all time points in the immunization schedule (Table 11). Ochratoxin alpha, interestingly showed significant relative cross-reactivities in one laying-hen at all points in the immunization schedule. The specificity of this antisera improved with time as the relative cross-reactivity with Oα decreased from 100 % to 60 % between the 51 st and 166 th day of immunization. The reason for this unexpected cross-reactivity is unknown but would not greatly interfere with an assay for OA as Oα is unlikely to occur in matrixes such as wheat since it is formed as a consequence of intestinal microflora and proteolytic enzyme metabolism (Xiao et al., 1991). The consistently low relative cross-reactivities with OB seen in both rabbits and hens will aid the development of a specific immunoassay for OA in oil rich matrixes such as soybean, rape-seed (Madhyastha et al., 1990). The high cross-reactivities with OC are not unexpected as mammals immunized with OA routinely show high cross-reactivities with the ester form of this molecule (Candlish, 1991). An earlier work with laying-hens also showed high relative cross-reactivities with OC as the response was greater than 100 % (Clarke et al., 1993). The high responses to OC currently observed in both rabbits and laying-hens will not pose any problems in the development of a quantitative ELISA in grain or animal tissues as this mycotoxin is very rare in nature and is rapidly converted to OA in-vivo (Fuchs et

FIGURE 5. The changes in anti-OA antisera activity over the course of the immunization schedule in rabbits and laying-hens. Rabbits (■-■) and laying-hen (□-□) antisera diluted 1:2000, 1:200 were allowed to develop for 15 and 60 min, respectively. The ELISA was the same as that described in Material and Methods with the exception that no free OA was present.

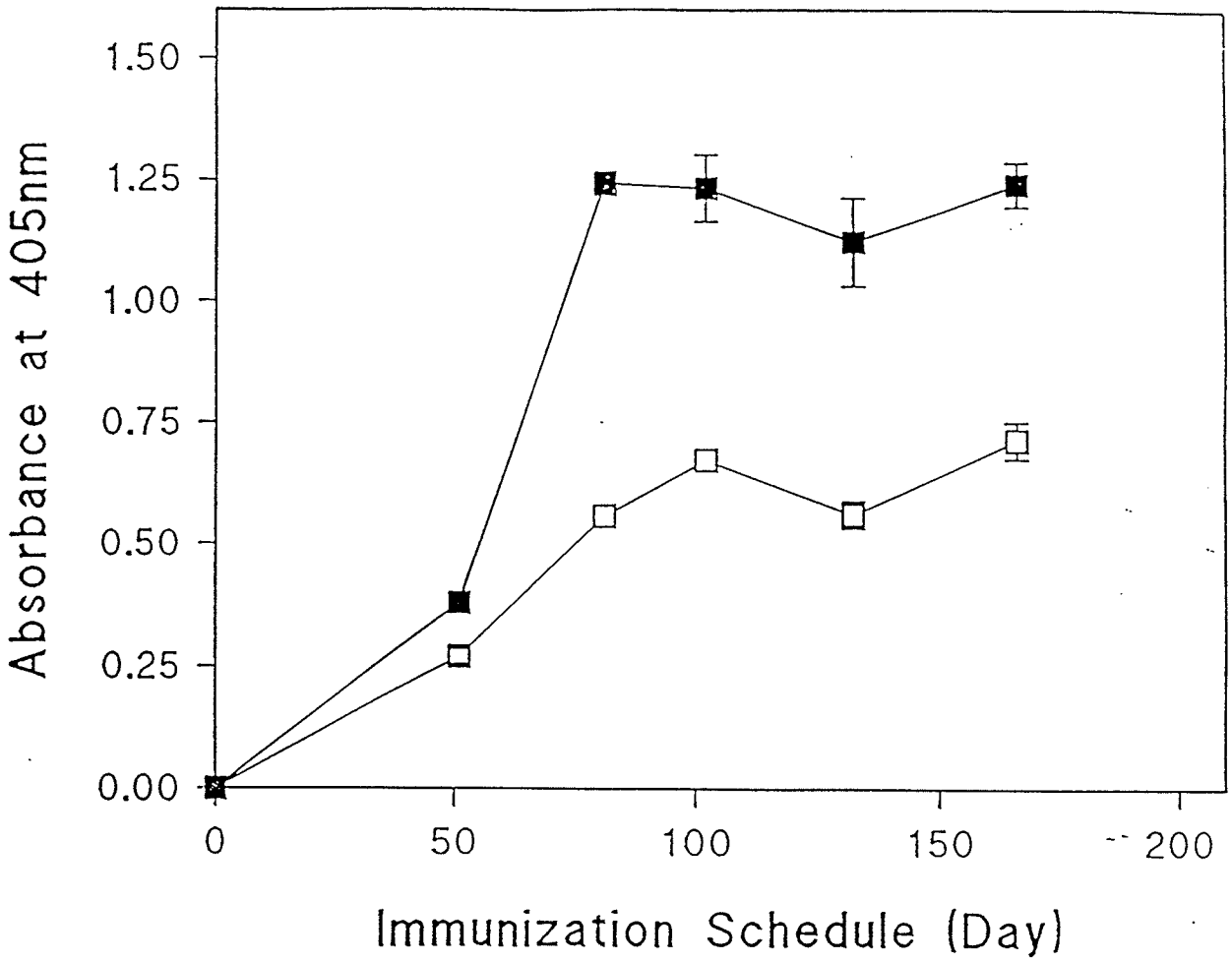
1.50
1.25
1.00
0.75
0.50
0.25
0.00
Absorbance at 405nm
0
50
100
150
200
Immunization Schedule (Day)

TABLE 11. Relative Cross-Reactivity[1] of Rabbit and Laying-Hen Anti-Ochratoxin A Antisera's with Various Ochratoxin A (OA) Like Molecules in an ELISA at Different Time Points in the Immunization Schedule.

Antiserum source	Sampling day	Structurally Related Ochratoxin Molecules[2]							
		OA		Oα		OC		OB	
		1	2	1	2	1	2	1	2
Rabbit	51	100	100	20	1	88	100	10	3
	81	100	100	1	2	95	88	2	3
	102	100	100	3	1	100	60	7	4
	132	100	100	1	2	100	67	3	2
	166	100	100	1	1	90	71	11	3
Laying-hen	51	100	100	0.05	100	150	75	5	8
	81	100	100	0.1	134	55	94	20	9
	102	100	100	0.4	100	40	76	17	9
	132	100	100	0.5	67	60	71	17	8
	166	100	100	1.0	60	50	83	12	7

[1] The ELISA is as described in Material and Methods. A pH 7.0 PBS-T was used for this study. [2] The values shown are the relative cross-reaction percentages seen in two hens and two rabbits (#1 and 2). The analysis was performed in at least triplicate. The inter-assay coefficient of variation for all assays was less than 15 %.

al., 1984). The work of Hsu and Chu (1992) also suggests that laying-hen egg-yolk antibodies and rabbit antisera are similar but not identical in specificity to small molecules such as aflatoxin B_1.

In an earlier study were rabbit antibodies specific for OA were applied to swine kidney samples in a ELISA, a lowered pH was found to be optimal. Ochratoxin A could be detected reproducibly and quantitatively at pH 6.0 and to a lesser extent at two higher pH's, 7.0 and 8.0 (Clarke et al., 1994; Manuscript III). These observations were unexpected but useful for the development of a highly sensitive assay. The specificities of the two antisera were tested systematically and found in the current study to be equally influenced by the pH used for the ELISA. At pH 6.0 the specificities of the two antisera were reduced compared to pH 7.0 and 8.0 and, as a consequence, the relative cross-reactivities with the other structurally related ochratoxins increased. The specificities of both antisera were the highest at the highest pH (pH 8.0) and was most noticeable for OC (Table 12). These observations suggest that a broad specificity screening assay could be developed at a low pH while a more specific assay may be obtained by increasing the pH of the assay. The sensitivities of the two antisera sources were also compared by ELISA at a conventional assay pH of 7.0 and additionally at pH 6.0 and 8.0. Clean wheat samples were spiked with 0 to 100 ng/g OA in wheat to simulate concentrations found in nature (Kuiper-Goodman and Scott, 1989). The two

TABLE 12. Relative Cross-Reactivity of Rabbit and Laying-Hen Antisera in an ELISA[1] with Various Ochratoxin A (OA) Like Molecules when Assayed at Three Different pH's.

Antiserum Source	OA-like molecules	pH 6.0		pH 7.0		pH 8.0	
		ng/mL[2]	%[3]	ng/mL[2]	%[3]	ng/mL[1]	%[2]
Rabbits							
	OA	4	100	0.7	100	0.8	100
	OB	15	26	3.5	20	10	8
	Oα	20	20	7	10	20	4
	OC	2	200	0.8	88	2	40
Hen							
	OA	10	100	15	100	15	100
	OB	600	1.6	30000	0.05	2000	0.7
	Oα	40	2.5	300	5	150	10
	OC	3	330	10	150	15	100

[1] The analysis was performed in at least triplicate as described in Material and Methods. The inter-assay coefficient of variation for all assay was less than 15 %. [2] The concentration of toxin required to give 50 % inhibition of the competitive inhibition standard curve for antisera from rabbit #2 and hen #1 at day 51 of the immunization schedule. [3] The values shown are the relative cross-reaction percentages.

TABLE 13. Estimation of the Amount of Ochratoxin A (OA) Present in Spiked Wheat as Determined by ELISA using Rabbit and Laying-Hen Antisera[1] at an Assay pH of 6.0, 7.0 and 8.0.

Antiserum Source	OA added (ng/g)[2]	Ochratoxin A detected by the ELISA (ng/g)[3]		
		pH 6.0	pH 7.0	pH 8.0
Rabbit				
	3	2 ± 2	4 ± 1	2 ± 2
	6	3 ± 3	8 ± 2	4 ± 2
	12	6 ± 4	12 ± 2	8 ± 3
	23	8 ± 4	21 ± 4	16 ± 1
	58	41 ± 22	53 ± 7	51 ± 11
	116	100 ± 45	121 ± 9	125 ± 25
Laying-hen				
	3	0	2 ± 2	0
	6	0	3 ± 4	0
	12	0	5 ± 7	0
	23	0	19 ± 16	34 ± 11
	58	12 ± 13	49 ± 15	62 ± 20
	116	25 ± 19	145 ± 23	139 ± 55

[1] Antisera came from rabbit #2 and laying-hen #1 at day 166 of the immunization schedule. [2] Ochratoxin A was determined by HPLC analysis according to Material and Methods. [3] The values are the means and S.D. of at least four independent assays with each assay being comprised of three replicas.

sources of antibody could detect OA in wheat but with differing levels of sensitivities. The optimal conditions for quantitation of OA in wheat by rabbits and laying-hens was pH 7.0 (Table 13). Quantitation of OA by rabbit antisera appears to be less dependant on the pH of the assay than that of the laying-hen. Rabbit antisera could detect OA at all concentrations and at all pH's whereas the laying-hen antisera could only detect the higher concentrations at pH 7.0. The reasons for this observation are unknown but may be related to subtle changes in the antibody and or antigen. Ochratoxins have generally two ionizable groups, the 8-hydroxy group in the 7-carboxy-5-chloro-8-hydroxy-3,4-dihydro-3R-methylisocoumarin type moiety and the 7-carboxyl group from the attached phenylalanine group. The dissociation constant (pKa) for the 8-hydroxy group are dependant on the structures of ochratoxin; the pKa values being 7.05, 7.10, 8.0, 11.0 for OA, OC, OB, Oα, respectively (Marquardt et al., 1992). The pKa of the carboxy group are far below the assay pH's used in this study. Subtle changes in the specific analyte are thus possible but unlikely to cause such large changes in assay performance at the chosen pH's. Antibodies understandably perform better at pH's approaching physiological conditions, and any deviation in pH will affect performance of the antibody. It is thus conceivable that the changes in specificity and sensitivity of this assay are more dependent on the antibodies than the analyte. Subtle changes in sample matrix could also be a factor leading to increased or decreased performance

of the two assays. The optimal pH for quantitation of OA in swine kidneys by rabbit antisera was 6.0 (data not shown), however, the optimal pH in this study for the wheat matrix was 7.0 (Table 13). It is therefore difficult to determine which side of the antibody-antigen interaction is most dependant on pH, however, it is relatively easy to ascertain which conditions are optimal for assay development.

The rabbit antisera were found to be 16 fold more sensitive for the detection of OA than laying-hen antisera at the neutral pH. Rabbit antisera could reproducibly detect OA at 3 ng/g or higher whereas laying-hen antisera could only detect OA at 50 ng/g or greater (Table 13). These differences in sensitivity agree with the observations of Hsu and Chu (1992). They reported that the sera from laying-hens were 25 to 50 fold less sensitive to aflatoxin B_1 compared to that obtained with a previously prepared rabbit antisera. These results are also in agreement with Clarke et al. (1993) who also observed a less sensitive response to OA in swine finisher diets. Although this source of antibody appears to be less sensitive than that from rabbits it can be more economically produced and uses non-invasive collection procedures. The rabbit antisera compared to that of the laying-hen also appears to be less dependant on pH for quantitation of OA in wheat although the concentration of toxin required to give 50 % inhibition in the ELISA is higher at pH 6.0 (Table 12). This is probably a reflection of the lower sensitivity of the

laying-hen antisera.

In summary, antisera responses to OA in the laying-hen when compared to those obtained with the rabbit appeared to have similar specificities but laying hen antisera was inferior in respect to sensitivity and total activity. This novel source of mycotoxin specific antibody, although less sensitive, can be potentially used to screen OA in wheat due to its relatively high specificity and potential ease of collection. Further studies are needed to fully evaluate the usefulness of OA specific antibodies in the laying-hen.

MANUSCRIPT III

Quantitation of Ochratoxin A in Swine Kidneys by Enzyme-linked Immunosorbent Assay using a Simplified Sample Preparation Procedure.

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ABSTRACT

An improved procedure for sample preparation and quantitation of ochratoxin A (OA) in swine kidneys was developed. Kidney samples were homogenized in acidified ethyl acetate, centrifuged, sub-sampled, dried, reconstituted with methanol and directly assayed using an indirect competitive ELISA. The rabbit antisera used in the development of this assay was found to have a high degree of relative cross-reaction with ochratoxins A and C but not with ochratoxins B, α, 4-OH-OA, and two structurally similar molecules L-phenylalanine and citrinin with the values being 100, 80, 3.33, 10, 1.4, 0 and 0.04 %, respectively. Extraction recoveries as determined by high performance liquid chromatography in kidneys spiked with 0.97 to 15.62 ppb OA were determined. The recovery values ranged from 91 to 110 % with acceptable inter-assay CV being obtained at the 3.9 ng/g spiking concentration or higher. The lowest reproducible OA detection limit for the ELISA in the spiked swine kidney samples was 7.81 ng/g with inter-assay CV of 8.85 %. The ELISA analysis of the spiked samples correlated highly with conventional HPLC analysis but was dependant on the conditions of the assay. Standards prepared in methanol or extract prepared from a kidney had correlation coefficients (r) of 0.91 $\pm$ 0.09 and 0.94 $\pm$ 0.07, respectively. The assay is sensitive, specific, simple and sufficiently accurate for routine analysis of swine kidneys.

INTRODUCTION

Ochratoxin A is a highly toxic and commonly observed secondary fungal metabolite produced by certain species of *Penicillium* and *Aspergillus*. This mycotoxin has been found to contaminate a wide variety of agricultural commodities in many areas of the world. Ochratoxin A is a known nephrotoxin and potent carcinogen which accumulates in the kidneys of animals fed contaminated diets (Steyn, 1984; Roschenthaler et al., 1984; Marquardt et al., 1990; Marquardt and Frohlich, 1992; Jelinek et al., 1989). This mycotoxin has been implicated in porcine nephropathy and perhaps contributes to human Balkan endemic nephropathy, both diseases are characterised by extensive changes in the renal interstitium (Austwick, 1975; Barnes et al., 1977; Krogh, 1978; Krogh, 1980; Krogh et al., 1974; Krogh et al., 1977). The prevalence of OA in mouldy foods and feeds and the subsequent contamination of edible animal tissues poses a serious health risk to humans and thus requires extensive surveillance. Swine kidneys in Denmark are routinely examined for OA and the contamination amounts are all strictly regulated. The kidneys are condemned if OA is detected at any level. At levels above 10 ng/g but less than 25 ng/g, kidneys, liver and other visceral organs are condemned and at concentrations greater than 25 ng/g the whole carcass is destroyed (van Egmond, 1991b; Jelinek et al., 1989). It is therefore essential that the determination method perform accurately under such

stringent regulations. Several analytical methods including TLC and HPLC have been developed for OA analysis in agricultural commodities including swine kidneys (van Egmond, 1991a). These techniques were found to be sensitive and reproducible but generally required extensive sample preparation. Mycotoxin assays based on immunological reactions have also been extensively examined and applied to a wide range of agricultural commodities. These immuno-based assays were found to be specific, sensitive, fast, economical, versatile, and have a minimal requirement for sample clean-up (Pestka, 1988; Chu, 1990; Candlish, 1991; Wilkinson et al., 1992). In view of these advantages, immunologically based assays such as radioimmunoassay (RIA) and enzyme-linked immunosorbent assays (ELISA) have been developed for OA in swine kidneys but they still require the toxic and carcinogenic solvent chloroform for sample preparation and in the case of the RIA, hazardous and restricted radio-labels (Morgan et al., 1986; Rousseau et al., 1987). The ELISA with a high sample through-put, high specificity and sensitivity coupled with a simplified and less hazardous sample preparation procedure will make routine analysis of OA more feasible in swine kidneys. The current study describes the development of such a simplified ELISA technique for the analysis of OA in swine kidneys which avoids the use of hazardous reagents and solvents and is well suited for routine and large scale surveillance.

MATERIAL AND METHODS

Materials. Ochratoxin A was isolated from liquid culture of *Aspergillus ochraceus* (Sreemannarayana et al., 1988). Ochratoxin α (Oα) was prepared by acid hydrolysis of OA (Doster and Sinnhuber, 1972). Ochratoxin B (OB) was obtained by catalytic de-chlorination of OA with palladium on activated carbon (Bredenkamp et al., 1989). Ochratoxin C (OC) was prepared by esterifying OA with ethanol (Fuchs et al., 1984). 4-Hydroxy-ochratoxin A (4-OH-OA) was donated by Dr. M. Castegnaro, IARC, Lyon, France. Citrinin, L-phenylalanine, bovine serum albumin (BSA), ovalbumin (OV), 1,1'-carbonyldiimidazole (CDI), N-hydroxysuccinimide (NHS), complete and incomplete Freund's adjuvant (CFA and IFA) p-nitrophenyl phosphate, diethanolamine, goat anti-rabbit IgG coupled to alkaline phosphatase were obtained from Sigma Chemical Co., St. Louis, MO. Microtiter plates (Falcon 3911, Microtest III) were purchased from Becton Dickson labware, Oxnard, CA. All solvents and reagents used were of analytical grade.

Preparation of immunogen and plate coating antigen. Ochratoxin A was chemically conjugated to bovine serum albumin (BSA-OA) using a novel zero-length coupling agent; that is, CDI activation of carboxyl functional groups in mycotoxins. In brief, 1 mg of OA and 1.5 mg of CDI were dissolved in 100 μL

acetone, mixed, and allowed to react for 10 min at 25°C in the dark with constant stirring. The 1,1'-carbonylimidazole activated OA mixture was added slowly and dropwise to a continuously stirred solution of BSA (10 mg) in 0.1 M Na_2CO_3 (1000 μL). The solution was allowed to react for 2 h at 25°C in the dark with continuous stirring followed by dialysis with 0.05 M carbonate buffer (pH 9.6), to remove any un-bound OA and subsequently distilled water to remove the carbonate salts. The conjugate following clean-up was collected, diluted to a concentration of 1 mg mL^{-1} and stored at -20°C. The plate coating antigen, ovalbumin-ochratoxin A (OV-OA), was prepared by the N-hydroxysuccinimide activated-ester procedure previously described (Clarke et al., 1993). The plate coating antigen was also stored at -20°C at a concentration of 1 mg mL^{-1} until coated onto microtiter plates.

Immunization of the rabbits. Two Dutch belted type rabbits, approximately 12 weeks of age, were immunized, boosted and anti-OA antisera harvested in accordance with current Canadian Council on Animal Care guidelines. The initial immunogen consisted of 500 μg of BSA-OA prepared as described in 0.5 mL 0.1 M saline, emulsified with an equal volume of CFA. The mixture was administered intradermally to 15 sites on the rabbits backs. Three boosters were given subsequently at 8 week intervals and consisted of 250 μg BSA-OA in 0.5 mL

saline emulsified with an equal volume of IFA. The boosters were given intramuscularly in both thigh muscles of the rabbits. Blood was collected from the marginal ear vein following final boosting, the serum was harvested and following centrifugation the rabbit antisera was purified by ammonium sulphate precipitation and stored frozen in aliquots at -20°C (Harlow and Lane, 1988).

Preparation of OA spiked swine kidney samples. Five grams of store purchased swine kidney was placed into a capped Nalgene centrifuge tube. Fifty microliters of methanol containing OA was added to the kidney and allowed to evaporate for 30 min at room-temperature to give a working concentration range of 0 to 16 ng/g. Samples were stored frozen at -20°C prior to extraction.

Simplified extraction procedure for OA in swine kidney. Five millilitres of double distilled water was added to 5 g of thawed swine kidney cortex in a capped Nalgene centrifuge tube. The mixture of kidney and water was shaken and then homogenized (Janke and Kunkel, Ultra-Turrax T25, Homogenizer) for 30 seconds. Ethyl acetate (10 mL) was added to the kidney slurry followed by homogenization for 30 seconds. A further 10 mL of ethyl acetate and 250 μL of concentrated H_3PO_4 was added and the mixture was shaken and re-homogenized. The homogenate turned from a red to brown colour after homogenization with the acid.

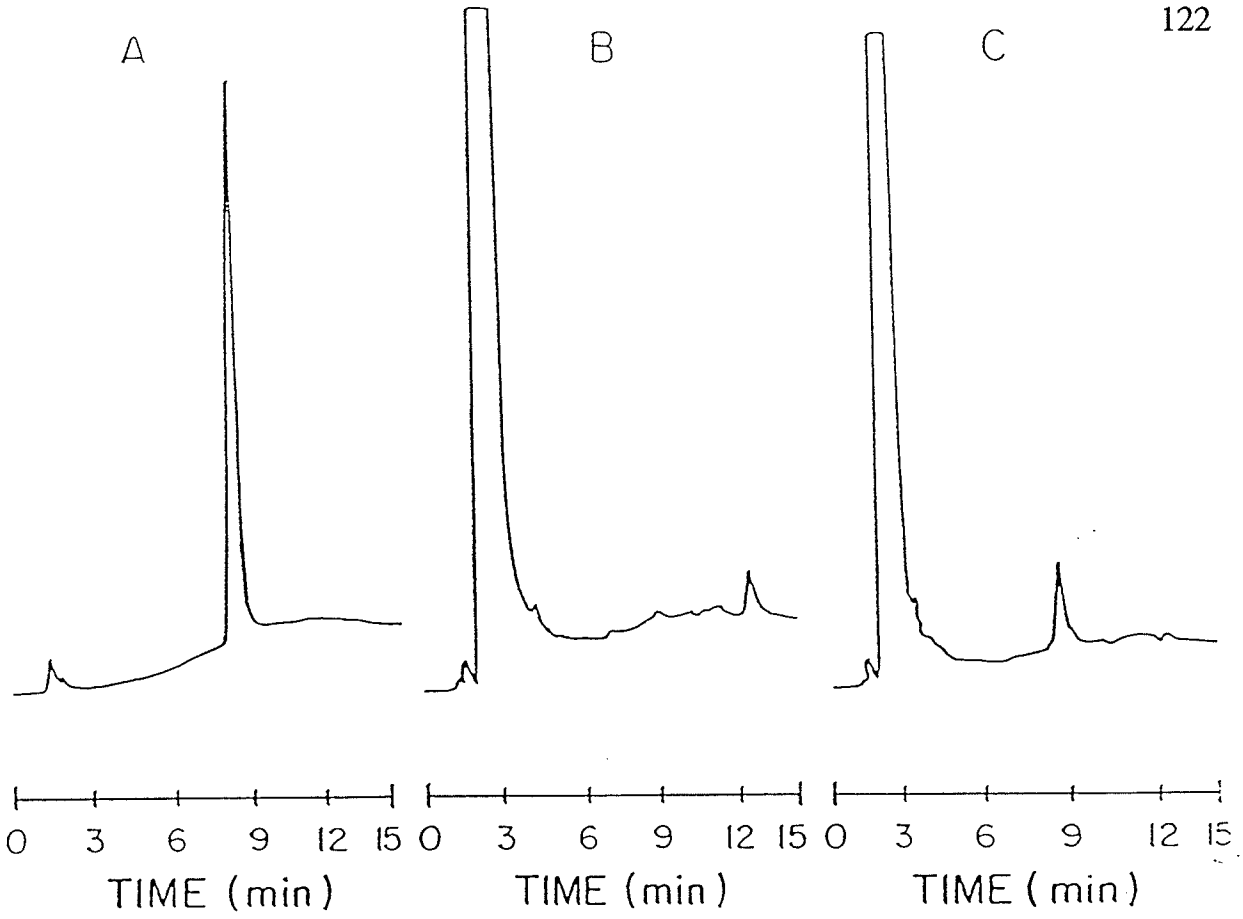
The extract was centrifuged for 30 min at 10000xg and a 1.5 mL aliquot of the supernatant was removed, dried under nitrogen and reconstituted with 1.2 mL methanol. This reconstituted sample was stored at -20°C until analyzed by the ELISA or HPLC.

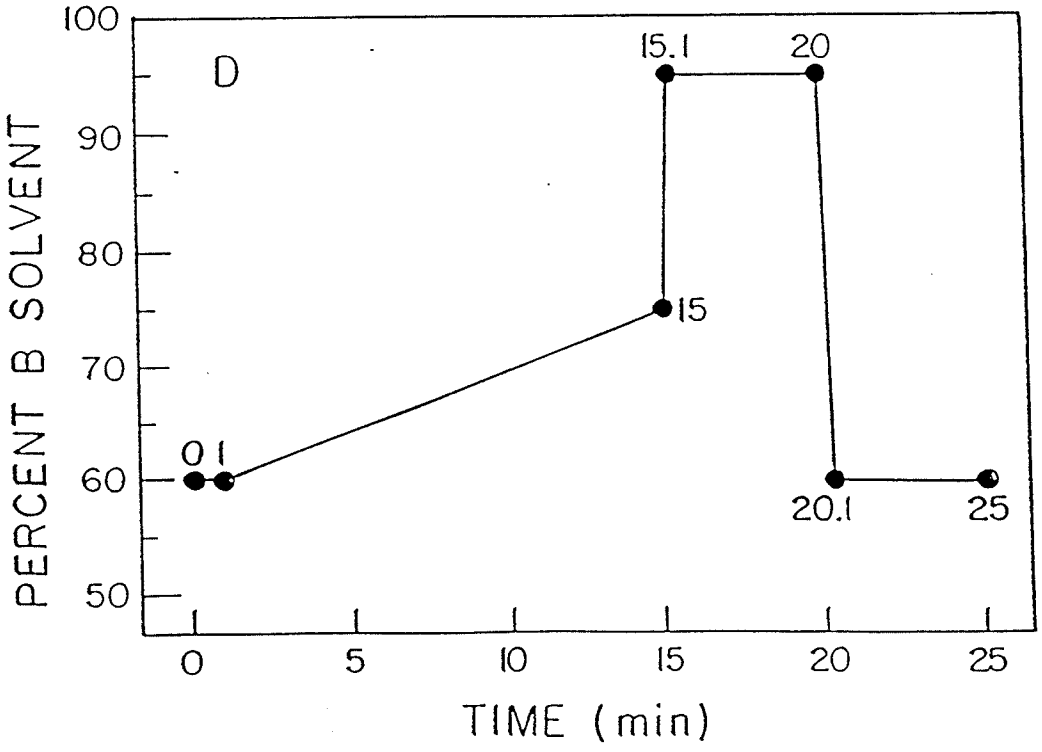
High-performance liquid chromatography for OA. Analytes were separated on a Beckmann 5-μm ODS reversed-phase C18 25 cm X 4.6 mm analytical column using a gradient mobile phase (Figure 6). The HPLC system was similar to that previously described (Frohlich et al., 1988). Fifty microliters of the kidney extract was injected and chromatographed using a solvent flow rate of 1.6 mL min^{-1} at a column temperature of 40°C. Fluorescence of OA peaks were detected using with a Shimadzu Model RF535 fluorescence detector set at 333 nm for excitation and at 450 nm for emission.

Indirect competitive ELISA for quantitation of OA in swine kidneys. The microtiter plates were coated with 2.5 μg per well of the OV-OA dissolved in 0.1 M phosphate buffered saline (pH 7.2) and incubated overnight at 4°C. The plates were washed twice with pH 7.2 phosphate buffered saline containing 0.01 % Tween-20 (pH 7.2 PBS-T), and then air dried at 25°C. Coated and dried plates could be stored at 4°C for several months without any significant loss in activity.

Fixed amounts of kidney extract (10 μl) or OA standards diluted in methanol or control kidney extract (10 μL) and rabbit anti-OA antisera (140 μL) diluted with pH 6.0 phosphate buffered saline containing 0.1 M NaCl and 0.01 % Tween-20 were added to each appropriate well in the OV-OA coated microtiter plate. The microtiter plates were incubated at 37°C for 60 min, the contents of the plate emptied and the individual wells washed three times with pH 7.2 PBS-T. Goat anti-rabbit IgG alkaline phosphatase conjugate (150μL) diluted 1:5000 in pH 7.2 PBS-T was added to each well and the microtiter plate incubated for 60 min at 37°C. The plates were washed six times with pH 7.2 PBS-T and dried thoroughly. Alkaline phosphatase substrate solution [1 mg mL^{-1} p-nitrophenyl phosphate in diethanolamine buffer (pH 9.8)] was added to each well (150 μL) and the microtiter plates incubated for 30 min at 37°C or until absorbency of the sample with no free OA was greater than 1.5 absorbency units. Read plates directly on microtiter plate reader at 405 nm (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada, Model 450).

Figure 6. HPLC separation profiles and solvent gradient used for resolving ochratoxin A from swine finisher kidney extracts. Tracings represent typical separation profiles for A = ochratoxin A standards, B = control swine kidney extracts and C = swine kidney extract with ochratoxin A contamination. The solvent gradient in figure 6D, consisted of two components, distilled water adjusted to pH 2.1 with H_3PO_4 (solvent A) and 90 % methanol with 10 % isopropanol (solvent B).


A
B
C
0 3 6 9 12 15
TIME (min)
0 3 6 9 12 15
TIME (min)
0 3 6 9 12 15
TIME (min)


D
100
90
80
70
60
50
PERCENT B SOLVENT
0.1
15
15.1
20
20.1
25
0 5 10 15 20 25
TIME (min)

RESULTS AND DISCUSSION

The current indirect competitive ELISA used a simplified sample preparation procedure which avoids the extensive sample clean-up normally required for analysis by conventional HPLC and TLC methods. The preparation procedure was adapted from a published HPLC sample extraction procedure for OA (Krogh and Nesheim, 1982). The extraction procedure was tested for efficiency and reproducibility of recovery by measuring OA recoveries from spiked kidneys as determined by HPLC (Table 14). The extraction procedure yielded acceptable recoveries above the 3.9 ng/g spiking level and decreasing variations with increasing OA concentrations. The CV became unacceptable for routine analysis at concentrations less than 3.9 ng/g. An important feature of this assay is its use of ethyl acetate as the OA extraction solvent. This solvent in comparison to chloroform is less toxic, non-carcinogenic more easily disposed of and gives comparable OA recoveries. The current extraction procedure also reduces the time of sample preparation because it does not have the sodium carbonate buffer partitioning step required by the two existing kidney immunoassay procedures (Morgan et al., 1986; Rousseau et al., 1987). These advantages make this extraction method an ideal alternative for routine and large scale swine kidney analysis.

The rabbit anti-OA antisera was found to be highly specific for OA and showed

TABLE 14. The Recoveries of Ochratoxin A (OA) from Spiked Kidney Samples using the Simplified Extraction Procedure as Determined by HPLC.

OA added to Kidney (ng/g)*	Observed OA (ng/g)**	Observed OA recovery (%)	Inter-assay coefficient of variation
15.62	15.44	98.85	5.73
7.81	7.08	90.66	8.51
3.9	4.30	110.3	12.00
1.95	1.90	97.45	20.38
0.97	1.0	103.0	106.84
0	n.d.***	n.d.	n.d.

* OA Spiked kidneys were prepared as described in Material and Methods. **The values shown are the means of at least three replicas. ***n.d. indicates not detected.

little or no relative cross-reactivity with the other and important ochratoxins. The antisera was found to have a high degree of relative cross-reaction with ochratoxins A and C but not with ochratoxins B, α, 4-OH-OA, and two structurally similar molecules L-phenylalanine and citrinin with the values being 100, 80, 3.33, 10, 1.4, 0 and 0.04 %, respectively. The high cross-reactivity with OC is of interest as this form of the toxin is considered equally toxic, yet, unlikely to cause false positives in the assay due to its very low incidence of occurrence (Fuchs et al., 1984; Candlish et al., 1986). The low cross-reactivity with citrinin is important as this mycotoxin can co-occur with OA and cause similar destruction of the kidney interstitium, potentially leading to nephropathy (Hald, 1991). The low cross-reactivity for the other ochratoxins, Oα, OB, 4-OH-OA makes this assay sufficiently specific for routine regulatory analysis.

Quantitation of OA in kidneys by the ELISA method was validated by correlation of observed OA contamination in OA spiked kidneys with calculated and HPLC chromatographic analysis (Tables 15 and 16). The ability of the ELISA to quantitate OA in swine kidneys depended on the manner in which the standards were analyzed. Ochratoxin A standards prepared in the extract from the control kidney gave higher absorbances in the ELISA as compared to the standards prepared in methanol (Figure 7). It was therefore necessary to run the unknowns and standards in the same milieu; that is, prepare the standards in the extracts

obtained from the control-kidney instead of methanol (Chu, 1990; Wilkinson et al., 1992). The correlations of ELISA with HPLC were found to improve slightly when the standard and sample solvent was switched from methanol ($r = 0.91 \pm 0.09$) to the control-kidney extract ($r = 0.94 \pm 0.07$) however the two approaches were not significantly different (Figure 8). There was however no observable increase in the sensitivity of the assay (Table 15, 16). Ochratoxin A in the spiked kidneys could only be quantified reproducibly with the current ELISA when the levels exceeded 7.8 ng/g. At levels below 7.8 ng/g the assay could only be considered qualitative. The lower reproducible limits of OA detection in this current assay is not as high as those previously reported and is due in part to the reduction in sample load, 2.5 mg kidney per assay. The two previously reported immunoassay techniques used 400 mg and 28 mg per assay to achieve 0.2 ng/g and 0.5 ng/g sensitivity, respectively (Rosseau et al., 1987; Morgan et al., 1986). Additionally, the current immunoassay in comparison to the two earlier reported techniques did not use extensive partitioning of the kidney extract or solid phase clean-ups. There is therefore the possibility of further improvement in sensitivity of the current ELISA. An increase in sensitivity is possible as extraction and concentration steps can be modified to further increase sample load but this will require further testing and validation. The use of sample clean-up is another possibility to improve performance of this assay. Techniques such as

TABLE 15. Recovery of Ochratoxin A (OA) from Spiked Swine Kidneys as Determined by the Quantitative ELISA using Methanol to Dilute OA Standards and Unknowns.

OA added to Kidney (ng/g)*	OA detected by ELISA (ng/g)**	Recovery of OA (%)	Inter-assay coefficient of variation
15.62	15.13	96.89	7.48
7.81	6.93	88.73	10.35
3.90	3.17	81.30	45.28
1.95	0.55	28.29	100.89
0.97	0.12	12.29	173.20
0	n.d.***	n.d.	n.d.

* OA Spiked kidneys were prepared as described in Material and Methods. **The values shown are the means of at least four replicas. ***n.d. indicates not detected.

TABLE 16. Recovery of Ochratoxin A (OA) from Spiked Swine Kidneys as Determined by the Quantitative ELISA using the Extract of Control Kidney to Dilute Ochratoxin A Standards and Unknowns.

OA added to Kidney (ng/g)*	OA detected by ELISA (ng/g)**	Recovery of OA (%)	Inter-assay Coefficient of Variation
15.62	14.42	92.35	11.99
7.81	6.97	89.31	8.85
3.90	3.22	82.65	29.90
1.95	0.67	34.57	101.22
0.97	0.20	20.48	173.20
0	n.d.***	n.d.	n.d.

* OA Spiked kidneys were prepared as described in Material and Methods. **The values shown are the mean of at least four replicas. ***n.d. indicates not detected.

FIGURE 7. The effects of control swine kidney extract on the ochratoxin A ELISA standard competitive inhibition curves. Ochratoxin A standards diluted with methanol (▲-▲) and ochratoxin A standards prepared in control swine kidney extract (■-■). Values are the mean of at least 3 replicas and corresponding standard deviations.

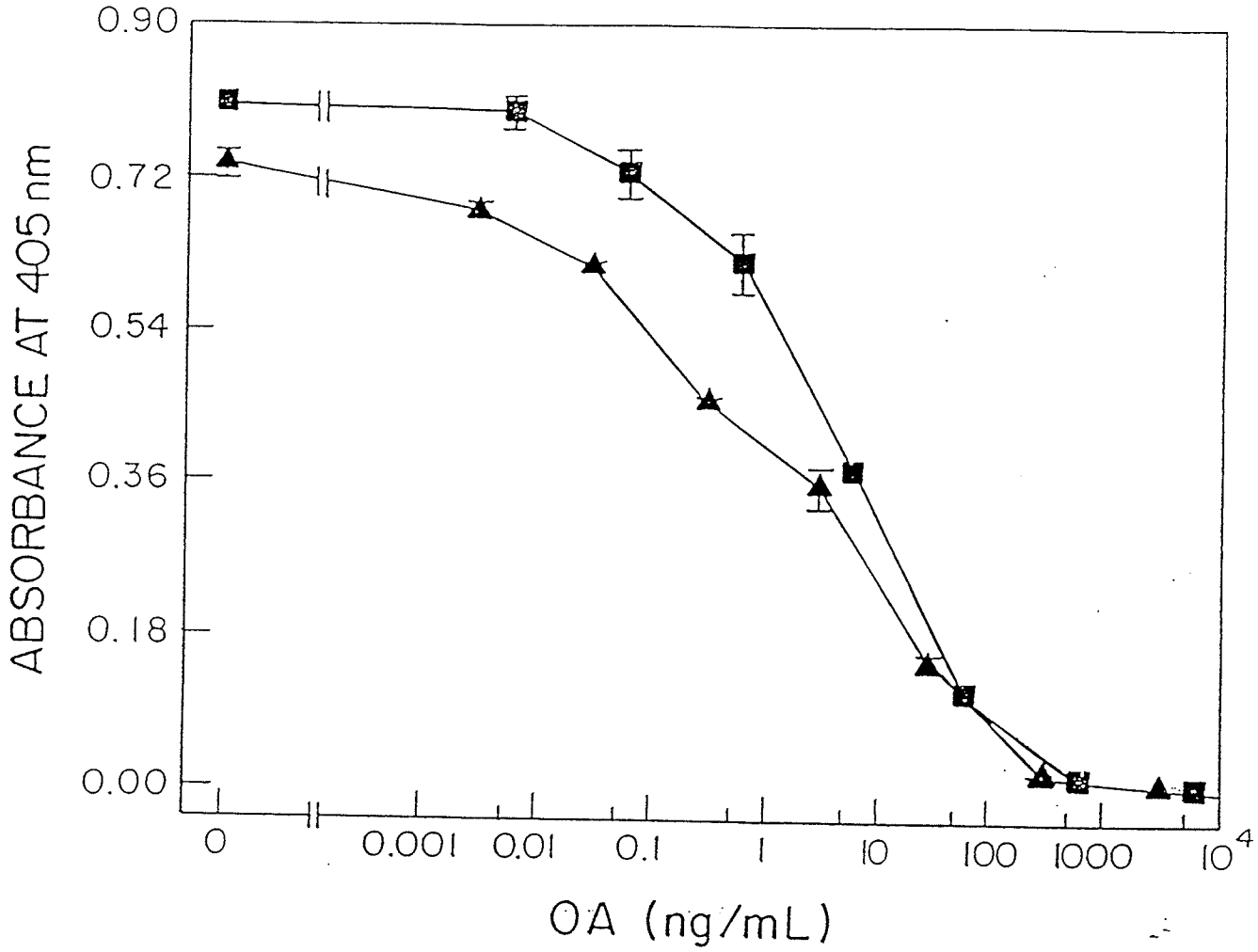
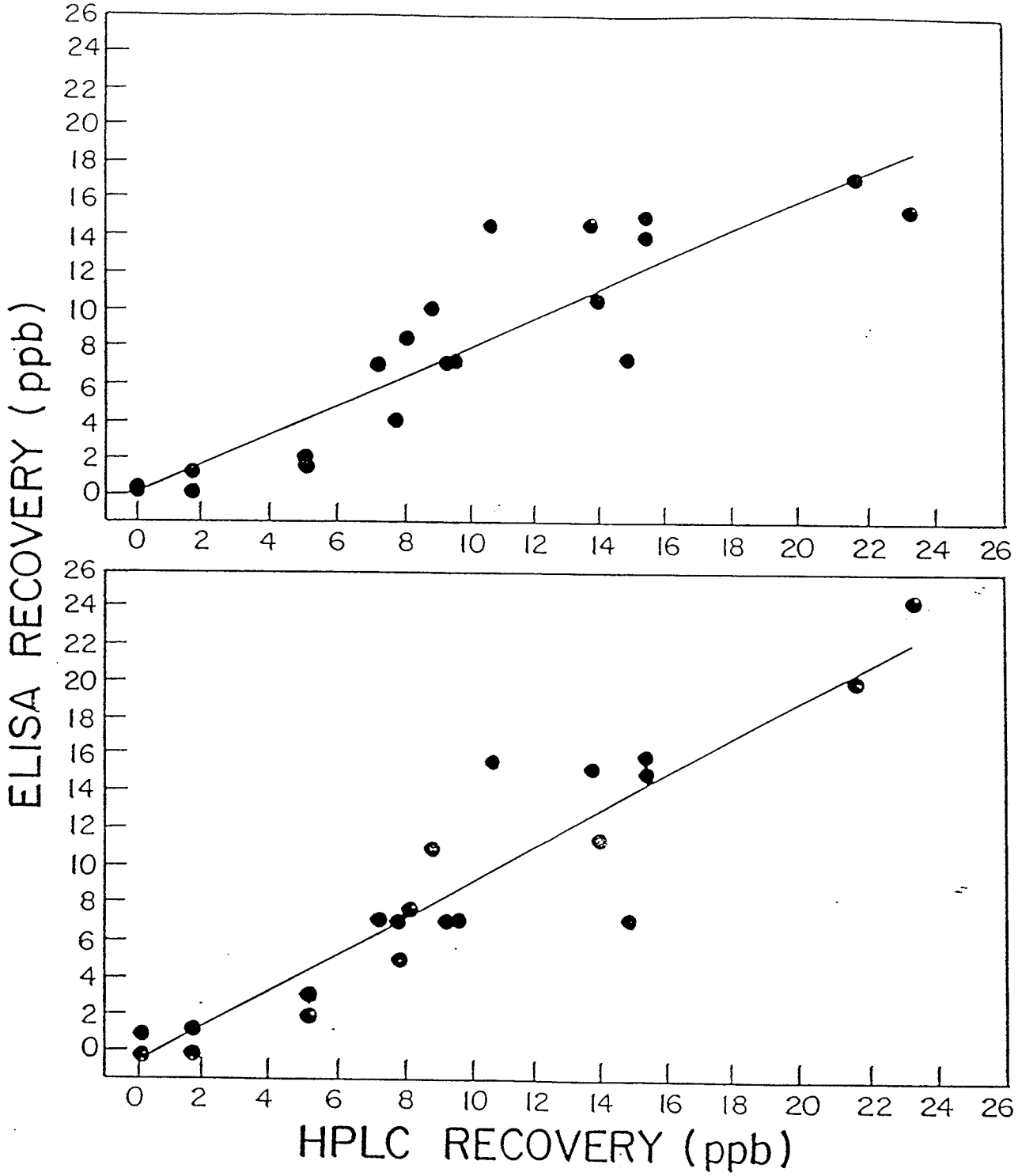

0.90
0.72
0.54
0.36
0.18
0.00
ABSORBANCE AT 405 nm
0
0.001
0.01
0.1
1
10
100
1000
10^4
OA (ng/mL)

FIGURE 8. Comparison of ochratoxin A recoveries in spiked swine kidney samples by HPLC and indirect competitive ELISA. Figures (ABOVE) indicate the effects of diluting ochratoxin A standards in methanol ($r = 0.91 \pm 0.09$) and (BELOW) extract prepared from swine kidney ($r = 0.94 \pm 0.07$) on ochratoxin A analysis.


ELISA RECOVERY (ppb)
HPLC RECOVERY (ppb)
0
2
4
6
8
10
12
14
16
18
20
22
24
26

immunoaffinity (Sharman et al., 1992), solvent partitioning (Morgan et al., 1986; Rousseau et al., 1987), solid-phase clean-ups are possible and have been shown in many cases to improve precision of the analysis (van Egmond, 1991b), however, they tend to lessen the simplicity of the assay and increase the cost of the analysis. Alternatively the assays sensitivity can be improved by further optimization of reagent concentrations, specifically, by the reduction of the amount of coating antigen used in the assay (Van der Water and Haagsma, 1990). This reduction in the coating antigen lessens the competition between the antibody and the solid phase antigen in favour of the free antigen, thus making the unknown antigens more competitive. It should be noted that without any modification to the current ELISA protocol the assay can detect OA at the required regulatory levels without the need for extensive sample preparation, increased sample loading and clean-up. The current immunoassay is similar in format to the previously reported swine kidney ELISA (Morgan et al., 1986) however the assay times have been substantially improved from 7 hours to 3 hours. The indirect competitive ELISA format eliminates the needs for hazardous and restricted radio-labels and the need for large amounts of anti-OA specific antisera (Chu, 1990). In view of all these findings the current assay developed is sufficiently sensitive, specific, simple and accurate for the routine analysis of swine kidneys.

GENERAL DISCUSSION

The wide-spread occurrence of this carcinogenic and nephrotoxic mycotoxin in agricultural commodities and its significant incidence in the human food-chain (Kuiper-Goodman and Scott, 1989; Kuiper-Goodman et al., 1993; Ominski, 1994) suggests that there is a potentially serious health risk to humans and animals. The presence of OA in Canadian grains, animal feedstuffs, and swine blood (Marquardt and Frohlich, 1992) demonstrates that Canadians are at risk. The high incidence of OA in Canadian swine blood strongly suggests that the swine kidneys are also tainted. The disposition of OA-contaminated grains is a problem as it is not readily destroyed by chemical, physical or most biological treatments. Ruminants, however, are able to neutralize the toxic effects of OA by hydrolysis to Oα in the rumen. Routine surveillance of the human food-chain are therefore necessary to minimize further contamination. All raw agricultural commodities such as the grains should be monitored at the farm level or at the elevators prior to commercial distribution. Tainted grain commodities can be diverted and commingled with clean grain and used as a feed for ruminant type animals. It would also be prudent to screen swine finisher-diets for OA prior to slaughter as this may identify a possible contamination problems in the herd. The herd could conceivably be diverted from slaughtering and fed an OA free diet until the concentrations of this toxin in the tissue reaches acceptable limits. This approach

is economically viable and would minimize contamination of the upper levels of the human food-chain.

Assays for the monitoring of OA in agricultural commodities must be inexpensive, sensitive, specific and available. Immunoassays, specifically ELISA procedures, are well suited for this application as they meet all the above mentioned criteria. ELISA determination offer added advantages in that they require minimal sample clean-up and can accommodate large sample through-put. Chromatographic techniques although highly sensitive and specific require extensive sample clean-up and long run-times. Rabbit and potentially laying-hen are the most readily adapted sources of antisera or IgY as they produce fairly sensitive and specific antibodies to the toxin. Both the rabbit antisera and the IgY can be readily and economically purified. In the current studies, the rabbit antisera were found to be very effective at quantifying OA in wheat and swine kidney (Manuscript II, III). The laying-hen antibodies offered the unique advantage that they could be more economically produced (Manuscript I) even though the sensitivities of the assays from this source are reduced in comparison to rabbits (Manuscript II). The level of sensitivity obtained with the laying-hen antibody, nevertheless, should be sufficient for the routine surveillance of swine finisher diets prior to animal slaughter. Rabbit antisera should be used for the regulatory based surveillance of OA in swine kidneys as the levels present are usually below

the limits of detection of the assay when IgY is the source of the antibody.

Routine surveillance requires that the procedure for sample extraction be simple and safe. Conventional approaches have normally included the use of chloroform, however, its toxic properties limits its use. The development of a methanol-based diet extraction procedure and an ethyl-acetate based swine kidney extraction technique, both of which use relatively non-toxic solvents, will facilitate the routine surveillance of plant and tissue commodities at the field and regulatory laboratory level.

The two ELISA methods developed in this study should facilitate the analysis of OA in foods and feeds. This would help ensure that our foods do not exceed the maximal acceptable levels of OA and would contribute to the continued safety of the Canadian food-chain.

CONCLUSIONS

Based on the research conducted, it can be concluded that:

1) Laying-hen egg yolk antibody was generated with specificity for OA. The OA directed IgY antibodies were found to cross-react with some of the common forms of ochratoxin. The relative cross-reactivities of OA, OB, OC, Oα and the structurally related mycotoxin, citrinin, were 100, 100, 400, 33, and 2 %, respectively. The toxins having the greatest cross-reactivities were those that are significantly toxic, with the exception of citrinin.

2) Ochratoxin A could be detected reproducibly in swine finisher diets and wheat using an indirect antibody capture type ELISA based on laying-hen antibody. Ochratoxin A could be detected at concentrations greater than 50 μg/kg in both commodities. There was a high correlation between ($r > 0.99$) between the ELISA values and those obtained using a conventional HPLC approach.

3) The methanol-based plant tissue sample extraction procedure gave comparable recoveries with the published literature cited chloroform-based techniques. The extraction recoveries as determined by HPLC ranged from 85 to 110 % with the average inter-assay CV being 9 %. The proposed methanol-based extraction

technique was faster, cheaper and less hazardous than extractions using chloroform.

4) A simplified IgY extraction and purification procedure was developed using an aqueous buffer for yolk dilution, chloroform extraction for the partitioning of the aqueous and non-aqueous phases and polyethylene-glycol 8000 for the selective precipitation of IgY from the aqueous phase. The yields of IgY approached 80% with the corresponding purity being 86-92 %.

5) Rabbit and laying-hen antisera were tested in a parallel study. The two antisera sources were found to have similar specificities but were notably different in regards to sensitivity and possibly production potential. The rabbit and laying-hen antisera relative cross-reactions with the common toxins OA, OB, OC, Oα, were 100, 20, 88, 10 and 100, 0.05, 5, 150 %, respectively, at an assay pH of 7.0. The relative cross-reactivities of both antisera could be easily modified by a slight change in assay pH. The specificities were both optimal at pH 8.0. The rabbit antisera could detect OA in spiked wheat samples at concentrations greater than 3 ng/g whereas laying-hens could only detect 50 ng/g or greater. Rabbits produced OA-specific antisera faster and at greater concentrations in the blood in comparison to laying-hens. Further egg yolk

antibody production studies are needed for valid conclusions about yield advantages. These studies indicate rabbits may be a superior source of antisera for OA detection at lower concentrations (<50 ng/g) when compared to laying-hens IgY, due to its greater sensitivity.

6) Ochratoxin A was detected reproducibly in swine kidneys and wheat at concentrations of greater than 7.8 and 3 ng/g, respectively, using an indirect antibody capture type ELISA based on rabbit antisera. The ELISA results were highly correlated with those obtained using conventional HPLC analysis ($r > 0.91$).

7) An improved kidney sample preparation procedure was developed that was based on acidified ethyl-acetate extraction. Extraction recoveries as determined by HPLC, ranged from 91 to 110 % with the inter-assay CV being less than 12 % at OA concentrations greater than 3 ng/g.

8) The two sources of antibody and the ELISA procedures developed could facilitate the routine and widespread surveillance of OA in animal and plant tissues destined for human consumption.

Further research and recommendations:

1) Additional research is under-way to confirm the general applicability of IgY to mycotoxin analysis. The toxins that are to be examined are T-2 toxin and deoxynivalenol. Further studies are needed to ascertain the economic advantage of the use of egg-yolk IgY.

2) Additional optimization of the OA ELISA procedure is necessary. The sensitivity of the swine finisher diet and swine kidney ELISA might be improved by affinity purification of the antibody preparation. The use of a direct antigen-capture format should facilitate routine usage of these assays as they are faster and easier to perform that the conventional indirect antibody capture methods used in these studies.

3) Alternate OA extraction procedures that avoid the use of chloroform must be employed if this toxin is to be analyzed on a routine basis. The procedures reported in this thesis would be suitable alternatives.

4) A large-scale survey of Canadian swine-finisher diets and swine kidneys using the current ELISA would help identify problem areas and could stimulate

interest in the use of a routine surveillance program by regulatory agents for this highly toxic and nephropathic mycotoxin.

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January 25, 1995

Mrs. Helen Agar
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Dear Mrs. Agar

Please accept this letter as verification of our consent to be listed as a co-author of the three manuscripts included in James R. Clarke's Ph. D. thesis entitled "Enhanced Detection of Ochratoxin A in Agricultural Commodities using Rabbit Antisera and Laying-hen Egg yolk Antibody".

Sincerely,

Dr. R. R. Marquardt.

Dr. A. A. Frohlich

al., 1984). The work of Hsu and Chu (1992) also suggests that laying-hen egg-yolk antibodies and rabbit antisera are similar but not identical in specificity to small molecules such as aflatoxin B_1.

In an earlier study were rabbit antibodies specific for OA were applied to swine kidney samples in a ELISA, a lowered pH was found to be optimal. Ochratoxin A could be detected reproducibly and quantitatively at pH 6.0 and to a lesser extent at two higher pH's, 7.0 and 8.0 (Clarke et al., 1994; Manuscript III). These observations were unexpected but useful for the development of a highly sensitive assay. The specificities of the two antisera were tested systematically and found in the current study to be equally influenced by the pH used for the ELISA. At pH 6.0 the specificities of the two antisera were reduced compared to pH 7.0 and 8.0 and, as a consequence, the relative cross-reactivities with the other structurally related ochratoxins increased. The specificities of both antisera were the highest at the highest pH (pH 8.0) and was most noticeable for OC (Table 12). These observations suggest that a broad specificity screening assay could be developed at a low pH while a more specific assay may be obtained by increasing the pH of the assay. The sensitivities of the two antisera sources were also compared by ELISA at a conventional assay pH of 7.0 and additionally at pH 6.0 and 8.0. Clean wheat samples were spiked with 0 to 100 ng/g OA in wheat to simulate concentrations found in nature (Kuiper-Goodman and Scott, 1989). The two

TABLE 12. Relative Cross-Reactivity of Rabbit and Laying-Hen Antisera in an ELISA[1] with Various Ochratoxin A (OA) Like Molecules when Assayed at Three Different pH's.

Antiserum Source	OA-like molecules	pH 6.0		pH 7.0		pH 8.0	
		ng/mL[2]	%[3]	ng/mL[2]	%[3]	ng/mL[1]	%[2]
Rabbits							
	OA	4	100	0.7	100	0.8	100
	OB	15	26	3.5	20	10	8
	Oα	20	20	7	10	20	4
	OC	2	200	0.8	88	2	40
Hen							
	OA	10	100	15	100	15	100
	OB	600	1.6	30000	0.05	2000	0.7
	Oα	40	2.5	300	5	150	10
	OC	3	330	10	150	15	100

[1] The analysis was performed in at least triplicate as described in Material and Methods. The inter-assay coefficient of variation for all assay was less than 15 %. [2] The concentration of toxin required to give 50 % inhibition of the competitive inhibition standard curve for antisera from rabbit #2 and hen #1 at day 51 of the immunization schedule. [3] The values shown are the relative cross-reaction percentages.

TABLE 13. Estimation of the Amount of Ochratoxin A (OA) Present in Spiked Wheat as Determined by ELISA using Rabbit and Laying-Hen Antisera[1] at an Assay pH of 6.0, 7.0 and 8.0.

Antiserum Source	OA added (ng/g)[2]	Ochratoxin A detected by the ELISA (ng/g)[3]		
		pH 6.0	pH 7.0	pH 8.0
Rabbit				
	3	2 ± 2	4 ± 1	2 ± 2
	6	3 ± 3	8 ± 2	4 ± 2
	12	6 ± 4	12 ± 2	8 ± 3
	23	8 ± 4	21 ± 4	16 ± 1
	58	41 ± 22	53 ± 7	51 ± 11
	116	100 ± 45	121 ± 9	125 ± 25
Laying-hen				
	3	0	2 ± 2	0
	6	0	3 ± 4	0
	12	0	5 ± 7	0
	23	0	19 ± 16	34 ± 11
	58	12 ± 13	49 ± 15	62 ± 20
	116	25 ± 19	145 ± 23	139 ± 55

[1] Antisera came from rabbit #2 and laying-hen #1 at day 166 of the immunization schedule. [2] Ochratoxin A was determined by HPLC analysis according to Material and Methods. [3] The values are the means and S.D. of at least four independent assays with each assay being comprised of three replicas.

sources of antibody could detect OA in wheat but with differing levels of sensitivities. The optimal conditions for quantitation of OA in wheat by rabbits and laying-hens was pH 7.0 (Table 13). Quantitation of OA by rabbit antisera appears to be less dependant on the pH of the assay than that of the laying-hen. Rabbit antisera could detect OA at all concentrations and at all pH's whereas the laying-hen antisera could only detect the higher concentrations at pH 7.0. The reasons for this observation are unknown but may be related to subtle changes in the antibody and or antigen. Ochratoxins have generally two ionizable groups, the 8-hydroxy group in the 7-carboxy-5-chloro-8-hydroxy-3,4-dihydro-3R-methylisocoumarin type moiety and the 7-carboxyl group from the attached phenylalanine group. The dissociation constant (pKa) for the 8-hydroxy group are dependant on the structures of ochratoxin; the pKa values being 7.05, 7.10, 8.0, 11.0 for OA, OC, OB, Oα, respectively (Marquardt et al., 1992). The pKa of the carboxy group are far below the assay pH's used in this study. Subtle changes in the specific analyte are thus possible but unlikely to cause such large changes in assay performance at the chosen pH's. Antibodies understandably perform better at pH's approaching physiological conditions, and any deviation in pH will affect performance of the antibody. It is thus conceivable that the changes in specificity and sensitivity of this assay are more dependent on the antibodies than the analyte. Subtle changes in sample matrix could also be a factor leading to increased or decreased performance

of the two assays. The optimal pH for quantitation of OA in swine kidneys by rabbit antisera was 6.0 (data not shown), however, the optimal pH in this study for the wheat matrix was 7.0 (Table 13). It is therefore difficult to determine which side of the antibody-antigen interaction is most dependant on pH, however, it is relatively easy to ascertain which conditions are optimal for assay development.

The rabbit antisera were found to be 16 fold more sensitive for the detection of OA than laying-hen antisera at the neutral pH. Rabbit antisera could reproducibly detect OA at 3 ng/g or higher whereas laying-hen antisera could only detect OA at 50 ng/g or greater (Table 13). These differences in sensitivity agree with the observations of Hsu and Chu (1992). They reported that the sera from laying-hens were 25 to 50 fold less sensitive to aflatoxin B_1 compared to that obtained with a previously prepared rabbit antisera. These results are also in agreement with Clarke et al. (1993) who also observed a less sensitive response to OA in swine finisher diets. Although this source of antibody appears to be less sensitive than that from rabbits it can be more economically produced and uses non-invasive collection procedures. The rabbit antisera compared to that of the laying-hen also appears to be less dependant on pH for quantitation of OA in wheat although the concentration of toxin required to give 50 % inhibition in the ELISA is higher at pH 6.0 (Table 12). This is probably a reflection of the lower sensitivity of the

laying-hen antisera.

In summary, antisera responses to OA in the laying-hen when compared to those obtained with the rabbit appeared to have similar specificities but laying hen antisera was inferior in respect to sensitivity and total activity. This novel source of mycotoxin specific antibody, although less sensitive, can be potentially used to screen OA in wheat due to its relatively high specificity and potential ease of collection. Further studies are needed to fully evaluate the usefulness of OA specific antibodies in the laying-hen.

MANUSCRIPT III

Quantitation of Ochratoxin A in Swine Kidneys by Enzyme-linked Immunosorbent Assay using a Simplified Sample Preparation Procedure.

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ABSTRACT

An improved procedure for sample preparation and quantitation of ochratoxin A (OA) in swine kidneys was developed. Kidney samples were homogenized in acidified ethyl acetate, centrifuged, sub-sampled, dried, reconstituted with methanol and directly assayed using an indirect competitive ELISA. The rabbit antisera used in the development of this assay was found to have a high degree of relative cross-reaction with ochratoxins A and C but not with ochratoxins B, α, 4-OH-OA, and two structurally similar molecules L-phenylalanine and citrinin with the values being 100, 80, 3.33, 10, 1.4, 0 and 0.04 %, respectively. Extraction recoveries as determined by high performance liquid chromatography in kidneys spiked with 0.97 to 15.62 ppb OA were determined. The recovery values ranged from 91 to 110 % with acceptable inter-assay CV being obtained at the 3.9 ng/g spiking concentration or higher. The lowest reproducible OA detection limit for the ELISA in the spiked swine kidney samples was 7.81 ng/g with inter-assay CV of 8.85 %. The ELISA analysis of the spiked samples correlated highly with conventional HPLC analysis but was dependant on the conditions of the assay. Standards prepared in methanol or extract prepared from a kidney had correlation coefficients (r) of 0.91 $\pm$ 0.09 and 0.94 $\pm$ 0.07, respectively. The assay is sensitive, specific, simple and sufficiently accurate for routine analysis of swine kidneys.

INTRODUCTION

Ochratoxin A is a highly toxic and commonly observed secondary fungal metabolite produced by certain species of *Penicillium* and *Aspergillus*. This mycotoxin has been found to contaminate a wide variety of agricultural commodities in many areas of the world. Ochratoxin A is a known nephrotoxin and potent carcinogen which accumulates in the kidneys of animals fed contaminated diets (Steyn, 1984; Roschenthaler et al., 1984; Marquardt et al., 1990; Marquardt and Frohlich, 1992; Jelinek et al., 1989). This mycotoxin has been implicated in porcine nephropathy and perhaps contributes to human Balkan endemic nephropathy, both diseases are characterised by extensive changes in the renal interstitium (Austwick, 1975; Barnes et al., 1977; Krogh, 1978; Krogh, 1980; Krogh et al., 1974; Krogh et al., 1977). The prevalence of OA in mouldy foods and feeds and the subsequent contamination of edible animal tissues poses a serious health risk to humans and thus requires extensive surveillance. Swine kidneys in Denmark are routinely examined for OA and the contamination amounts are all strictly regulated. The kidneys are condemned if OA is detected at any level. At levels above 10 ng/g but less than 25 ng/g, kidneys, liver and other visceral organs are condemned and at concentrations greater than 25 ng/g the whole carcass is destroyed (van Egmond, 1991b; Jelinek et al., 1989). It is therefore essential that the determination method perform accurately under such

stringent regulations. Several analytical methods including TLC and HPLC have been developed for OA analysis in agricultural commodities including swine kidneys (van Egmond, 1991a). These techniques were found to be sensitive and reproducible but generally required extensive sample preparation. Mycotoxin assays based on immunological reactions have also been extensively examined and applied to a wide range of agricultural commodities. These immuno-based assays were found to be specific, sensitive, fast, economical, versatile, and have a minimal requirement for sample clean-up (Pestka, 1988; Chu, 1990; Candlish, 1991; Wilkinson et al., 1992). In view of these advantages, immunologically based assays such as radioimmunoassay (RIA) and enzyme-linked immunosorbent assays (ELISA) have been developed for OA in swine kidneys but they still require the toxic and carcinogenic solvent chloroform for sample preparation and in the case of the RIA, hazardous and restricted radio-labels (Morgan et al., 1986; Rousseau et al., 1987). The ELISA with a high sample through-put, high specificity and sensitivity coupled with a simplified and less hazardous sample preparation procedure will make routine analysis of OA more feasible in swine kidneys. The current study describes the development of such a simplified ELISA technique for the analysis of OA in swine kidneys which avoids the use of hazardous reagents and solvents and is well suited for routine and large scale surveillance.

MATERIAL AND METHODS

Materials. Ochratoxin A was isolated from liquid culture of *Aspergillus ochraceus* (Sreemannarayana et al., 1988). Ochratoxin α (Oα) was prepared by acid hydrolysis of OA (Doster and Sinnhuber, 1972). Ochratoxin B (OB) was obtained by catalytic de-chlorination of OA with palladium on activated carbon (Bredenkamp et al., 1989). Ochratoxin C (OC) was prepared by esterifying OA with ethanol (Fuchs et al., 1984). 4-Hydroxy-ochratoxin A (4-OH-OA) was donated by Dr. M. Castegnaro, IARC, Lyon, France. Citrinin, L-phenylalanine, bovine serum albumin (BSA), ovalbumin (OV), 1,1'-carbonyldiimidazole (CDI), N-hydroxysuccinimide (NHS), complete and incomplete Freund's adjuvant (CFA and IFA) p-nitrophenyl phosphate, diethanolamine, goat anti-rabbit IgG coupled to alkaline phosphatase were obtained from Sigma Chemical Co., St. Louis, MO. Microtiter plates (Falcon 3911, Microtest III) were purchased from Becton Dickson labware, Oxnard, CA. All solvents and reagents used were of analytical grade.

Preparation of immunogen and plate coating antigen. Ochratoxin A was chemically conjugated to bovine serum albumin (BSA-OA) using a novel zero-length coupling agent; that is, CDI activation of carboxyl functional groups in mycotoxins. In brief, 1 mg of OA and 1.5 mg of CDI were dissolved in 100 μL

acetone, mixed, and allowed to react for 10 min at 25°C in the dark with constant stirring. The 1,1'-carbonylimidazole activated OA mixture was added slowly and dropwise to a continuously stirred solution of BSA (10 mg) in 0.1 M Na_2CO_3 (1000 μL). The solution was allowed to react for 2 h at 25°C in the dark with continuous stirring followed by dialysis with 0.05 M carbonate buffer (pH 9.6), to remove any un-bound OA and subsequently distilled water to remove the carbonate salts. The conjugate following clean-up was collected, diluted to a concentration of 1 mg mL^{-1} and stored at -20°C. The plate coating antigen, ovalbumin-ochratoxin A (OV-OA), was prepared by the N-hydroxysuccinimide activated-ester procedure previously described (Clarke et al., 1993). The plate coating antigen was also stored at -20°C at a concentration of 1 mg mL^{-1} until coated onto microtiter plates.

Immunization of the rabbits. Two Dutch belted type rabbits, approximately 12 weeks of age, were immunized, boosted and anti-OA antisera harvested in accordance with current Canadian Council on Animal Care guidelines. The initial immunogen consisted of 500 μg of BSA-OA prepared as described in 0.5 mL 0.1 M saline, emulsified with an equal volume of CFA. The mixture was administered intradermally to 15 sites on the rabbits backs. Three boosters were given subsequently at 8 week intervals and consisted of 250 μg BSA-OA in 0.5 mL

saline emulsified with an equal volume of IFA. The boosters were given intramuscularly in both thigh muscles of the rabbits. Blood was collected from the marginal ear vein following final boosting, the serum was harvested and following centrifugation the rabbit antisera was purified by ammonium sulphate precipitation and stored frozen in aliquots at -20°C (Harlow and Lane, 1988).

Preparation of OA spiked swine kidney samples. Five grams of store purchased swine kidney was placed into a capped Nalgene centrifuge tube. Fifty microliters of methanol containing OA was added to the kidney and allowed to evaporate for 30 min at room-temperature to give a working concentration range of 0 to 16 ng/g. Samples were stored frozen at -20°C prior to extraction.

Simplified extraction procedure for OA in swine kidney. Five millilitres of double distilled water was added to 5 g of thawed swine kidney cortex in a capped Nalgene centrifuge tube. The mixture of kidney and water was shaken and then homogenized (Janke and Kunkel, Ultra-Turrax T25, Homogenizer) for 30 seconds. Ethyl acetate (10 mL) was added to the kidney slurry followed by homogenization for 30 seconds. A further 10 mL of ethyl acetate and 250 μL of concentrated H_3PO_4 was added and the mixture was shaken and re-homogenized. The homogenate turned from a red to brown colour after homogenization with the acid.

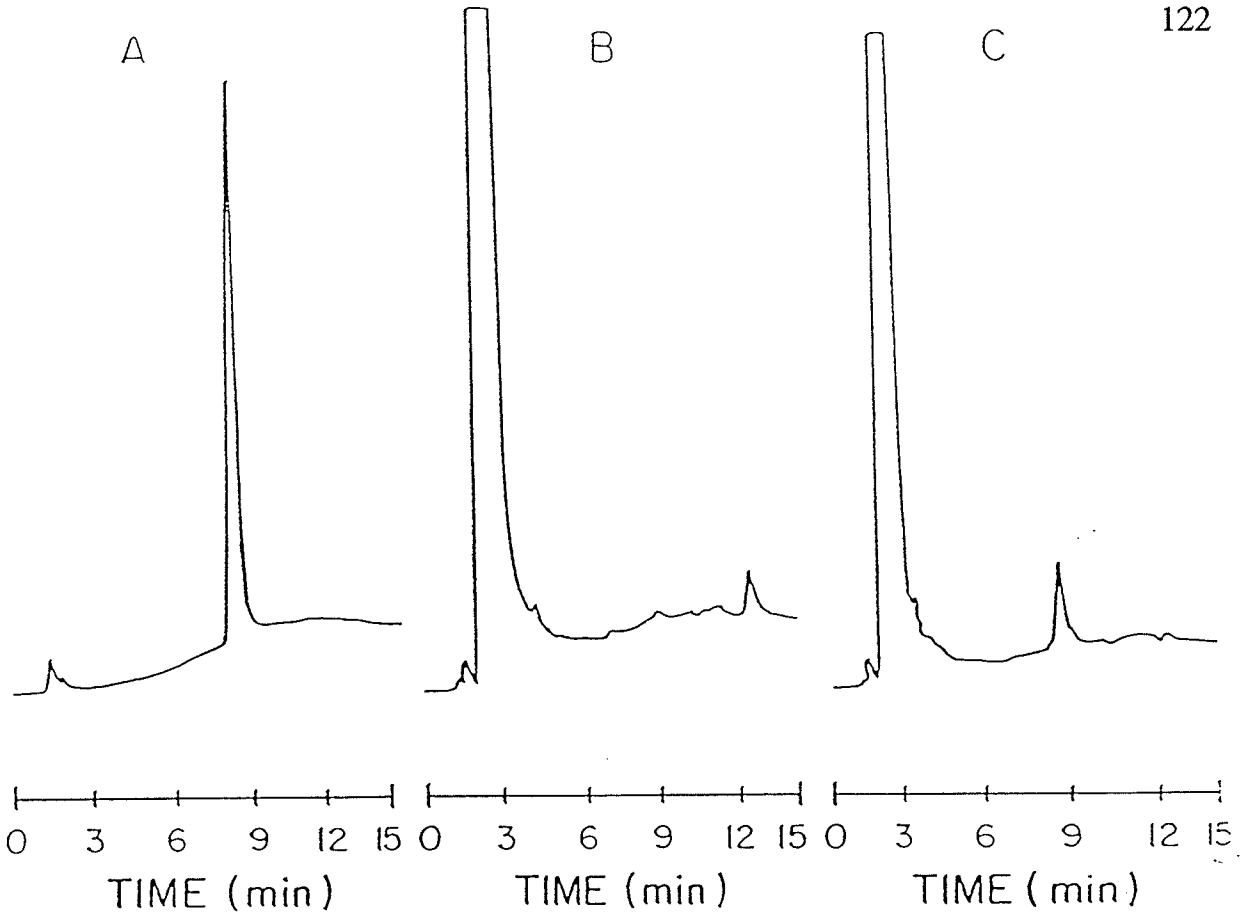
The extract was centrifuged for 30 min at 10000xg and a 1.5 mL aliquot of the supernatant was removed, dried under nitrogen and reconstituted with 1.2 mL methanol. This reconstituted sample was stored at -20°C until analyzed by the ELISA or HPLC.

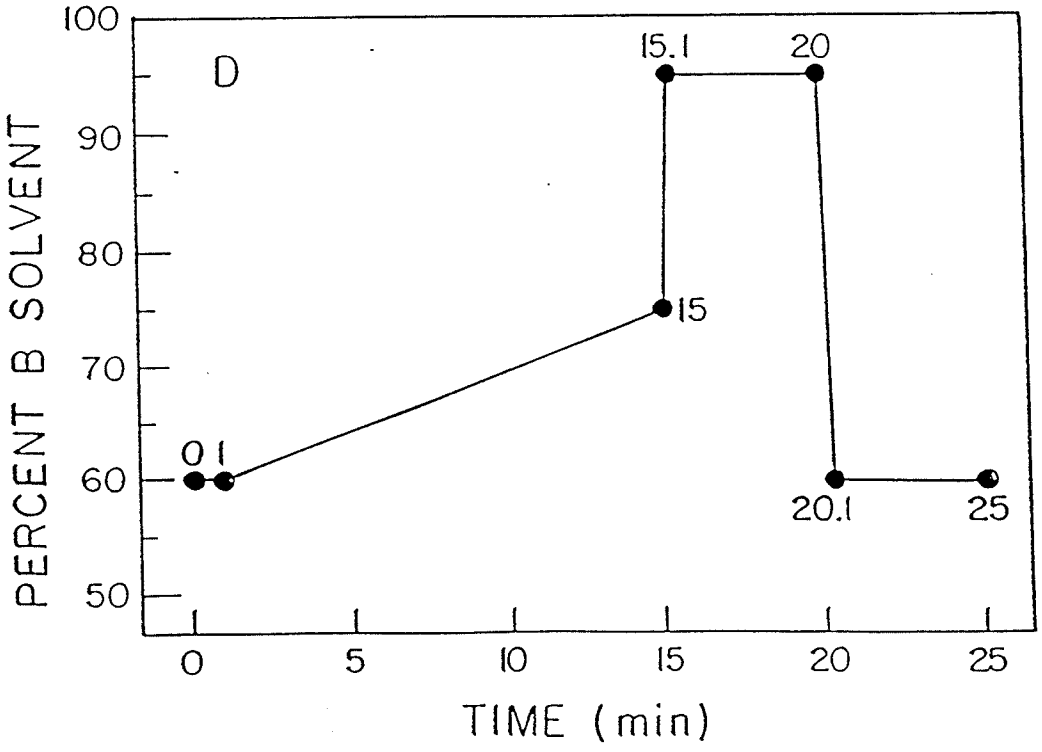
High-performance liquid chromatography for OA. Analytes were separated on a Beckmann 5-μm ODS reversed-phase C18 25 cm X 4.6 mm analytical column using a gradient mobile phase (Figure 6). The HPLC system was similar to that previously described (Frohlich et al., 1988). Fifty microliters of the kidney extract was injected and chromatographed using a solvent flow rate of 1.6 mL min^{-1} at a column temperature of 40°C. Fluorescence of OA peaks were detected using with a Shimadzu Model RF535 fluorescence detector set at 333 nm for excitation and at 450 nm for emission.

Indirect competitive ELISA for quantitation of OA in swine kidneys. The microtiter plates were coated with 2.5 μg per well of the OV-OA dissolved in 0.1 M phosphate buffered saline (pH 7.2) and incubated overnight at 4°C. The plates were washed twice with pH 7.2 phosphate buffered saline containing 0.01 % Tween-20 (pH 7.2 PBS-T), and then air dried at 25°C. Coated and dried plates could be stored at 4°C for several months without any significant loss in activity.

Fixed amounts of kidney extract (10 μl) or OA standards diluted in methanol or control kidney extract (10 μL) and rabbit anti-OA antisera (140 μL) diluted with pH 6.0 phosphate buffered saline containing 0.1 M NaCl and 0.01 % Tween-20 were added to each appropriate well in the OV-OA coated microtiter plate. The microtiter plates were incubated at 37°C for 60 min, the contents of the plate emptied and the individual wells washed three times with pH 7.2 PBS-T. Goat anti-rabbit IgG alkaline phosphatase conjugate (150μL) diluted 1:5000 in pH 7.2 PBS-T was added to each well and the microtiter plate incubated for 60 min at 37°C. The plates were washed six times with pH 7.2 PBS-T and dried thoroughly. Alkaline phosphatase substrate solution [1 mg mL^{-1} p-nitrophenyl phosphate in diethanolamine buffer (pH 9.8)] was added to each well (150 μL) and the microtiter plates incubated for 30 min at 37°C or until absorbency of the sample with no free OA was greater than 1.5 absorbency units. Read plates directly on microtiter plate reader at 405 nm (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada, Model 450).

Figure 6. HPLC separation profiles and solvent gradient used for resolving ochratoxin A from swine finisher kidney extracts. Tracings represent typical separation profiles for A = ochratoxin A standards, B = control swine kidney extracts and C = swine kidney extract with ochratoxin A contamination. The solvent gradient in figure 6D, consisted of two components, distilled water adjusted to pH 2.1 with H_3PO_4 (solvent A) and 90 % methanol with 10 % isopropanol (solvent B).


A
B
C
0 3 6 9 12 15
TIME (min)
0 3 6 9 12 15
TIME (min)
0 3 6 9 12 15
TIME (min)


D
100
90
80
70
60
50
PERCENT B SOLVENT
0.1
15
15.1
20
20.1
25
0 5 10 15 20 25
TIME (min)

RESULTS AND DISCUSSION

The current indirect competitive ELISA used a simplified sample preparation procedure which avoids the extensive sample clean-up normally required for analysis by conventional HPLC and TLC methods. The preparation procedure was adapted from a published HPLC sample extraction procedure for OA (Krogh and Nesheim, 1982). The extraction procedure was tested for efficiency and reproducibility of recovery by measuring OA recoveries from spiked kidneys as determined by HPLC (Table 14). The extraction procedure yielded acceptable recoveries above the 3.9 ng/g spiking level and decreasing variations with increasing OA concentrations. The CV became unacceptable for routine analysis at concentrations less than 3.9 ng/g. An important feature of this assay is its use of ethyl acetate as the OA extraction solvent. This solvent in comparison to chloroform is less toxic, non-carcinogenic more easily disposed of and gives comparable OA recoveries. The current extraction procedure also reduces the time of sample preparation because it does not have the sodium carbonate buffer partitioning step required by the two existing kidney immunoassay procedures (Morgan et al., 1986; Rousseau et al., 1987). These advantages make this extraction method an ideal alternative for routine and large scale swine kidney analysis.

The rabbit anti-OA antisera was found to be highly specific for OA and showed

TABLE 14. The Recoveries of Ochratoxin A (OA) from Spiked Kidney Samples using the Simplified Extraction Procedure as Determined by HPLC.

OA added to Kidney (ng/g)*	Observed OA (ng/g)**	Observed OA recovery (%)	Inter-assay coefficient of variation
15.62	15.44	98.85	5.73
7.81	7.08	90.66	8.51
3.9	4.30	110.3	12.00
1.95	1.90	97.45	20.38
0.97	1.0	103.0	106.84
0	n.d.***	n.d.	n.d.

* OA Spiked kidneys were prepared as described in Material and Methods. **The values shown are the means of at least three replicas. ***n.d. indicates not detected.

little or no relative cross-reactivity with the other and important ochratoxins. The antisera was found to have a high degree of relative cross-reaction with ochratoxins A and C but not with ochratoxins B, α, 4-OH-OA, and two structurally similar molecules L-phenylalanine and citrinin with the values being 100, 80, 3.33, 10, 1.4, 0 and 0.04 %, respectively. The high cross-reactivity with OC is of interest as this form of the toxin is considered equally toxic, yet, unlikely to cause false positives in the assay due to its very low incidence of occurrence (Fuchs et al., 1984; Candlish et al., 1986). The low cross-reactivity with citrinin is important as this mycotoxin can co-occur with OA and cause similar destruction of the kidney interstitium, potentially leading to nephropathy (Hald, 1991). The low cross-reactivity for the other ochratoxins, Oα, OB, 4-OH-OA makes this assay sufficiently specific for routine regulatory analysis.

Quantitation of OA in kidneys by the ELISA method was validated by correlation of observed OA contamination in OA spiked kidneys with calculated and HPLC chromatographic analysis (Tables 15 and 16). The ability of the ELISA to quantitate OA in swine kidneys depended on the manner in which the standards were analyzed. Ochratoxin A standards prepared in the extract from the control kidney gave higher absorbances in the ELISA as compared to the standards prepared in methanol (Figure 7). It was therefore necessary to run the unknowns and standards in the same milieu; that is, prepare the standards in the extracts

obtained from the control-kidney instead of methanol (Chu, 1990; Wilkinson et al., 1992). The correlations of ELISA with HPLC were found to improve slightly when the standard and sample solvent was switched from methanol ($r = 0.91 \pm 0.09$) to the control-kidney extract ($r = 0.94 \pm 0.07$) however the two approaches were not significantly different (Figure 8). There was however no observable increase in the sensitivity of the assay (Table 15, 16). Ochratoxin A in the spiked kidneys could only be quantified reproducibly with the current ELISA when the levels exceeded 7.8 ng/g. At levels below 7.8 ng/g the assay could only be considered qualitative. The lower reproducible limits of OA detection in this current assay is not as high as those previously reported and is due in part to the reduction in sample load, 2.5 mg kidney per assay. The two previously reported immunoassay techniques used 400 mg and 28 mg per assay to achieve 0.2 ng/g and 0.5 ng/g sensitivity, respectively (Rosseau et al., 1987; Morgan et al., 1986). Additionally, the current immunoassay in comparison to the two earlier reported techniques did not use extensive partitioning of the kidney extract or solid phase clean-ups. There is therefore the possibility of further improvement in sensitivity of the current ELISA. An increase in sensitivity is possible as extraction and concentration steps can be modified to further increase sample load but this will require further testing and validation. The use of sample clean-up is another possibility to improve performance of this assay. Techniques such as

TABLE 15. Recovery of Ochratoxin A (OA) from Spiked Swine Kidneys as Determined by the Quantitative ELISA using Methanol to Dilute OA Standards and Unknowns.

OA added to Kidney (ng/g)*	OA detected by ELISA (ng/g)**	Recovery of OA (%)	Inter-assay coefficient of variation
15.62	15.13	96.89	7.48
7.81	6.93	88.73	10.35
3.90	3.17	81.30	45.28
1.95	0.55	28.29	100.89
0.97	0.12	12.29	173.20
0	n.d.***	n.d.	n.d.

* OA Spiked kidneys were prepared as described in Material and Methods. **The values shown are the means of at least four replicas. ***n.d. indicates not detected.

TABLE 16. Recovery of Ochratoxin A (OA) from Spiked Swine Kidneys as Determined by the Quantitative ELISA using the Extract of Control Kidney to Dilute Ochratoxin A Standards and Unknowns.

OA added to Kidney (ng/g)*	OA detected by ELISA (ng/g)**	Recovery of OA (%)	Inter-assay Coefficient of Variation
15.62	14.42	92.35	11.99
7.81	6.97	89.31	8.85
3.90	3.22	82.65	29.90
1.95	0.67	34.57	101.22
0.97	0.20	20.48	173.20
0	n.d.***	n.d.	n.d.

* OA Spiked kidneys were prepared as described in Material and Methods. **The values shown are the mean of at least four replicas. ***n.d. indicates not detected.

FIGURE 7. The effects of control swine kidney extract on the ochratoxin A ELISA standard competitive inhibition curves. Ochratoxin A standards diluted with methanol (▲-▲) and ochratoxin A standards prepared in control swine kidney extract (■-■). Values are the mean of at least 3 replicas and corresponding standard deviations.

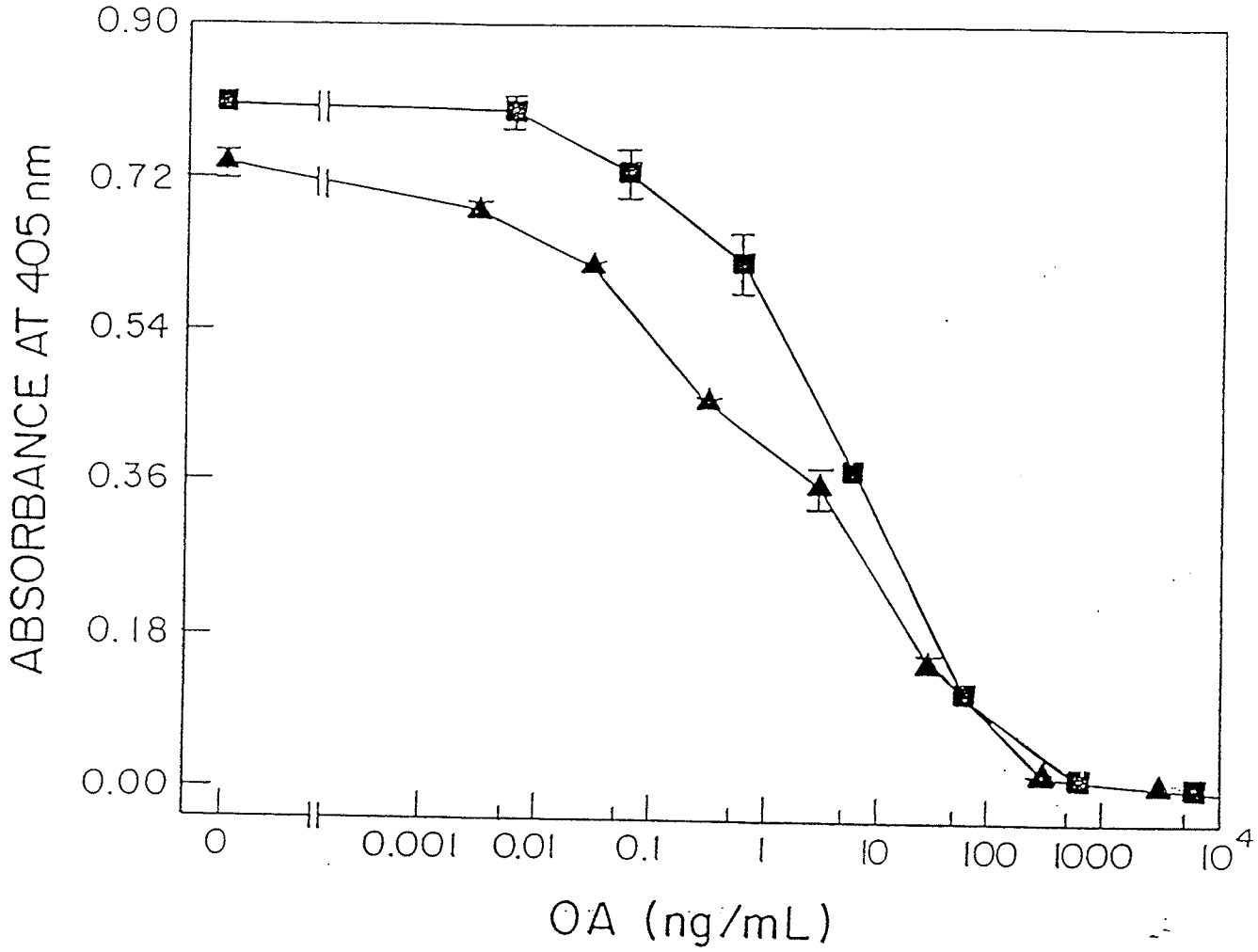
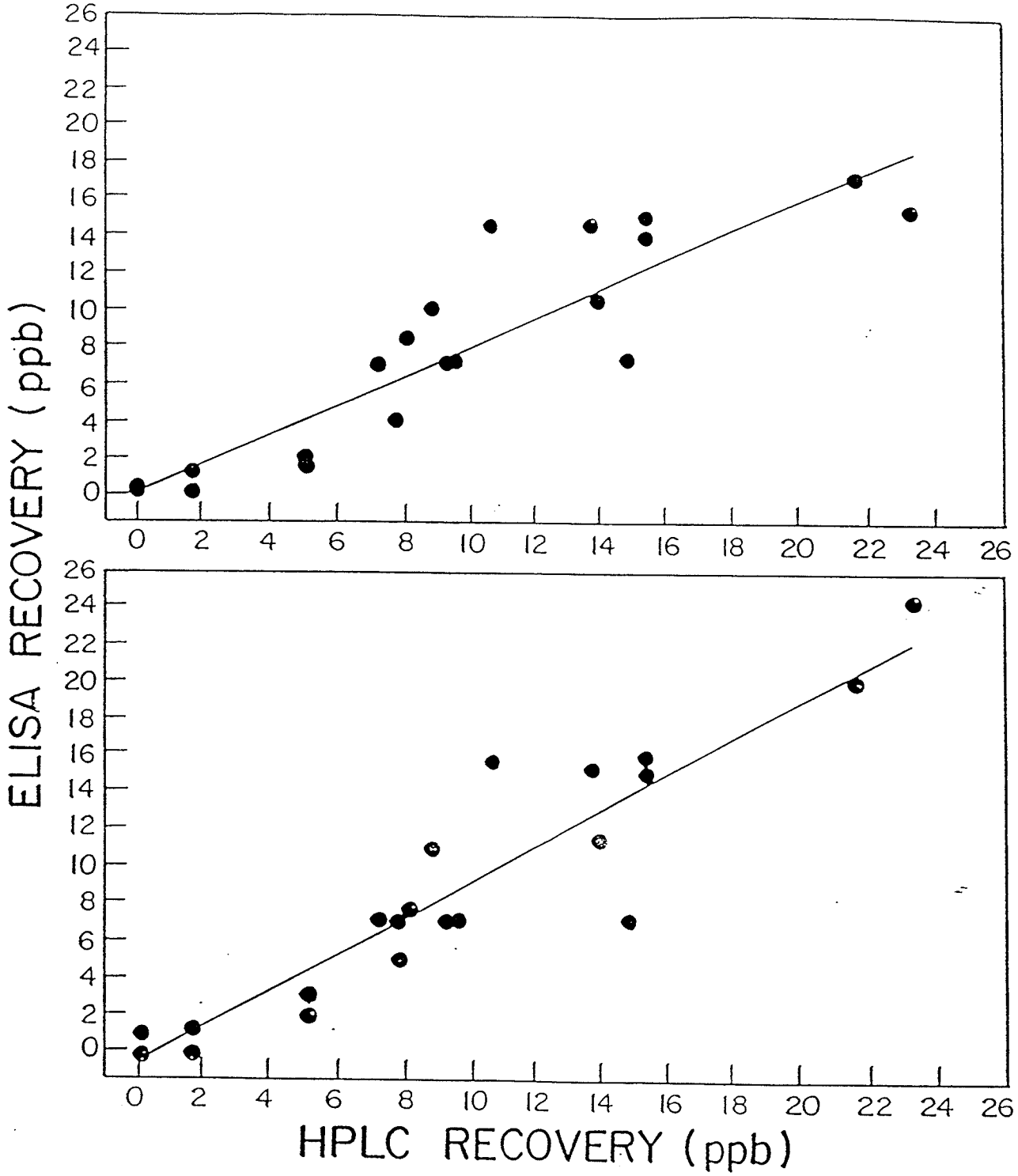

0.90
0.72
0.54
0.36
0.18
0.00
ABSORBANCE AT 405 nm
0
0.001
0.01
0.1
1
10
100
1000
10^4
OA (ng/mL)

FIGURE 8. Comparison of ochratoxin A recoveries in spiked swine kidney samples by HPLC and indirect competitive ELISA. Figures (ABOVE) indicate the effects of diluting ochratoxin A standards in methanol ($r = 0.91 \pm 0.09$) and (BELOW) extract prepared from swine kidney ($r = 0.94 \pm 0.07$) on ochratoxin A analysis.


ELISA RECOVERY (ppb)
HPLC RECOVERY (ppb)
0
2
4
6
8
10
12
14
16
18
20
22
24
26

immunoaffinity (Sharman et al., 1992), solvent partitioning (Morgan et al., 1986; Rousseau et al., 1987), solid-phase clean-ups are possible and have been shown in many cases to improve precision of the analysis (van Egmond, 1991b), however, they tend to lessen the simplicity of the assay and increase the cost of the analysis. Alternatively the assays sensitivity can be improved by further optimization of reagent concentrations, specifically, by the reduction of the amount of coating antigen used in the assay (Van der Water and Haagsma, 1990). This reduction in the coating antigen lessens the competition between the antibody and the solid phase antigen in favour of the free antigen, thus making the unknown antigens more competitive. It should be noted that without any modification to the current ELISA protocol the assay can detect OA at the required regulatory levels without the need for extensive sample preparation, increased sample loading and clean-up. The current immunoassay is similar in format to the previously reported swine kidney ELISA (Morgan et al., 1986) however the assay times have been substantially improved from 7 hours to 3 hours. The indirect competitive ELISA format eliminates the needs for hazardous and restricted radio-labels and the need for large amounts of anti-OA specific antisera (Chu, 1990). In view of all these findings the current assay developed is sufficiently sensitive, specific, simple and accurate for the routine analysis of swine kidneys.

GENERAL DISCUSSION

The wide-spread occurrence of this carcinogenic and nephrotoxic mycotoxin in agricultural commodities and its significant incidence in the human food-chain (Kuiper-Goodman and Scott, 1989; Kuiper-Goodman et al., 1993; Ominski, 1994) suggests that there is a potentially serious health risk to humans and animals. The presence of OA in Canadian grains, animal feedstuffs, and swine blood (Marquardt and Frohlich, 1992) demonstrates that Canadians are at risk. The high incidence of OA in Canadian swine blood strongly suggests that the swine kidneys are also tainted. The disposition of OA-contaminated grains is a problem as it is not readily destroyed by chemical, physical or most biological treatments. Ruminants, however, are able to neutralize the toxic effects of OA by hydrolysis to Oα in the rumen. Routine surveillance of the human food-chain are therefore necessary to minimize further contamination. All raw agricultural commodities such as the grains should be monitored at the farm level or at the elevators prior to commercial distribution. Tainted grain commodities can be diverted and commingled with clean grain and used as a feed for ruminant type animals. It would also be prudent to screen swine finisher-diets for OA prior to slaughter as this may identify a possible contamination problems in the herd. The herd could conceivably be diverted from slaughtering and fed an OA free diet until the concentrations of this toxin in the tissue reaches acceptable limits. This approach

is economically viable and would minimize contamination of the upper levels of the human food-chain.

Assays for the monitoring of OA in agricultural commodities must be inexpensive, sensitive, specific and available. Immunoassays, specifically ELISA procedures, are well suited for this application as they meet all the above mentioned criteria. ELISA determination offer added advantages in that they require minimal sample clean-up and can accommodate large sample through-put. Chromatographic techniques although highly sensitive and specific require extensive sample clean-up and long run-times. Rabbit and potentially laying-hen are the most readily adapted sources of antisera or IgY as they produce fairly sensitive and specific antibodies to the toxin. Both the rabbit antisera and the IgY can be readily and economically purified. In the current studies, the rabbit antisera were found to be very effective at quantifying OA in wheat and swine kidney (Manuscript II, III). The laying-hen antibodies offered the unique advantage that they could be more economically produced (Manuscript I) even though the sensitivities of the assays from this source are reduced in comparison to rabbits (Manuscript II). The level of sensitivity obtained with the laying-hen antibody, nevertheless, should be sufficient for the routine surveillance of swine finisher diets prior to animal slaughter. Rabbit antisera should be used for the regulatory based surveillance of OA in swine kidneys as the levels present are usually below

the limits of detection of the assay when IgY is the source of the antibody.

Routine surveillance requires that the procedure for sample extraction be simple and safe. Conventional approaches have normally included the use of chloroform, however, its toxic properties limits its use. The development of a methanol-based diet extraction procedure and an ethyl-acetate based swine kidney extraction technique, both of which use relatively non-toxic solvents, will facilitate the routine surveillance of plant and tissue commodities at the field and regulatory laboratory level.

The two ELISA methods developed in this study should facilitate the analysis of OA in foods and feeds. This would help ensure that our foods do not exceed the maximal acceptable levels of OA and would contribute to the continued safety of the Canadian food-chain.

CONCLUSIONS

Based on the research conducted, it can be concluded that:

1) Laying-hen egg yolk antibody was generated with specificity for OA. The OA directed IgY antibodies were found to cross-react with some of the common forms of ochratoxin. The relative cross-reactivities of OA, OB, OC, Oα and the structurally related mycotoxin, citrinin, were 100, 100, 400, 33, and 2 %, respectively. The toxins having the greatest cross-reactivities were those that are significantly toxic, with the exception of citrinin.

2) Ochratoxin A could be detected reproducibly in swine finisher diets and wheat using an indirect antibody capture type ELISA based on laying-hen antibody. Ochratoxin A could be detected at concentrations greater than 50 μg/kg in both commodities. There was a high correlation between ($r > 0.99$) between the ELISA values and those obtained using a conventional HPLC approach.

3) The methanol-based plant tissue sample extraction procedure gave comparable recoveries with the published literature cited chloroform-based techniques. The extraction recoveries as determined by HPLC ranged from 85 to 110 % with the average inter-assay CV being 9 %. The proposed methanol-based extraction

technique was faster, cheaper and less hazardous than extractions using chloroform.

4) A simplified IgY extraction and purification procedure was developed using an aqueous buffer for yolk dilution, chloroform extraction for the partitioning of the aqueous and non-aqueous phases and polyethylene-glycol 8000 for the selective precipitation of IgY from the aqueous phase. The yields of IgY approached 80% with the corresponding purity being 86-92 %.

5) Rabbit and laying-hen antisera were tested in a parallel study. The two antisera sources were found to have similar specificities but were notably different in regards to sensitivity and possibly production potential. The rabbit and laying-hen antisera relative cross-reactions with the common toxins OA, OB, OC, Oα, were 100, 20, 88, 10 and 100, 0.05, 5, 150 %, respectively, at an assay pH of 7.0. The relative cross-reactivities of both antisera could be easily modified by a slight change in assay pH. The specificities were both optimal at pH 8.0. The rabbit antisera could detect OA in spiked wheat samples at concentrations greater than 3 ng/g whereas laying-hens could only detect 50 ng/g or greater. Rabbits produced OA-specific antisera faster and at greater concentrations in the blood in comparison to laying-hens. Further egg yolk

antibody production studies are needed for valid conclusions about yield advantages. These studies indicate rabbits may be a superior source of antisera for OA detection at lower concentrations (<50 ng/g) when compared to laying-hens IgY, due to its greater sensitivity.

6) Ochratoxin A was detected reproducibly in swine kidneys and wheat at concentrations of greater than 7.8 and 3 ng/g, respectively, using an indirect antibody capture type ELISA based on rabbit antisera. The ELISA results were highly correlated with those obtained using conventional HPLC analysis ($r > 0.91$).

7) An improved kidney sample preparation procedure was developed that was based on acidified ethyl-acetate extraction. Extraction recoveries as determined by HPLC, ranged from 91 to 110 % with the inter-assay CV being less than 12 % at OA concentrations greater than 3 ng/g.

8) The two sources of antibody and the ELISA procedures developed could facilitate the routine and widespread surveillance of OA in animal and plant tissues destined for human consumption.

Further research and recommendations:

1) Additional research is under-way to confirm the general applicability of IgY to mycotoxin analysis. The toxins that are to be examined are T-2 toxin and deoxynivalenol. Further studies are needed to ascertain the economic advantage of the use of egg-yolk IgY.

2) Additional optimization of the OA ELISA procedure is necessary. The sensitivity of the swine finisher diet and swine kidney ELISA might be improved by affinity purification of the antibody preparation. The use of a direct antigen-capture format should facilitate routine usage of these assays as they are faster and easier to perform that the conventional indirect antibody capture methods used in these studies.

3) Alternate OA extraction procedures that avoid the use of chloroform must be employed if this toxin is to be analyzed on a routine basis. The procedures reported in this thesis would be suitable alternatives.

4) A large-scale survey of Canadian swine-finisher diets and swine kidneys using the current ELISA would help identify problem areas and could stimulate

interest in the use of a routine surveillance program by regulatory agents for this highly toxic and nephropathic mycotoxin.

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January 25, 1995

Mrs. Helen Agar
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Dear Mrs. Agar

Please accept this letter as verification of our consent to be listed as a co-author of the three manuscripts included in James R. Clarke's Ph. D. thesis entitled "Enhanced Detection of Ochratoxin A in Agricultural Commodities using Rabbit Antisera and Laying-hen Egg yolk Antibody".

Sincerely,

Dr. R. R. Marquardt.

Dr. A. A. Frohlich