

**CONCURRENT ANALYSIS OF THE MYCOTOXINS,  
CYCLOPIAZONIC ACID, MONILIFORMIN  
AND OCHRATOXIN A  
USING CAPILLARY ZONE ELECTROPHORESIS**

by

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## DECLARATION

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I, Urishanie Govender, declare that unless otherwise indicated, this dissertation is my own work and that it has not been submitted for a degree at another University or Institution.

U. Govender (19819)

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## ABSTRACT

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Mycotoxins are a group of natural poisons produced by certain strains of fungal species when they grow under favourable conditions on a wide variety of different substrates. These toxins have been implicated in a wide range of acute diseases in man and animals. Their toxic effects include oesophageal cancer and liver diseases in humans, and carcinogenic effects in experimental rats and poultry. Hence, there is a need to monitor toxin levels in food commodities.

Many fungi occurring in food and feeds are capable of producing more than one mycotoxin. The co-production of the mycotoxins has additive toxic effects and increases the toxigenic potential of the fungus. Hence, in a contaminated medium, there could be several fungi, each producing a wide range of mycotoxins. Therefore, there is a need to develop multitoxin assays to establish co-occurrence of mycotoxins.

Cyclopiazonic acid has been reported to co-occur with aflatoxin, zearalenone and ochratoxin A. The natural co-occurrence of moniliformin, deoxynivalenol and zearalenone in agricultural commodities has also been documented. Therefore, cyclopiazonic acid, moniliformin and ochratoxin A were chosen for this study in developing a multitoxin assay. Cyclopiazonic acid, ochratoxin A and moniliformin are acidic toxins, the latter being a small polar compound, with each having different biochemical properties. Furthermore, very little is known with respect to their occurrence in South African commodities.



The project focuses on the development of an analytical technique for the multitoxin analysis of cyclopiazonic acid, moniliformin and ochratoxin A. Three analytical systems, viz., thin layer chromatography, high performance liquid chromatography and capillary electrophoresis were chosen for the study. High performance liquid chromatography techniques used were reversed phase, paired ion chromatography, normal phase and ion chromatography. Capillary electrophoresis techniques used were capillary zone electrophoresis and micellar electrokinetic chromatography.

Thin layer chromatography and high performance liquid chromatography methods were successfully implemented for the analysis of individual toxins, with high performance liquid chromatography systems having higher sensitivities.

The technique of high performance liquid chromatography was explored for the simultaneous analysis of cyclopiazonic acid, moniliformin and ochratoxin A, by manipulating the mobile phase composition, buffer concentration, pH and temperature with reference to various optimisation strategies. Multitoxin analysis using various high performance liquid chromatography techniques were unsuccessful at separating the three toxins. This may be due to the toxins having very different chemical structures and varying polarities. Alternatively, it may be that the toxins have separated, but not in a chromatographically useful way.

A capillary electrophoresis method was developed using the Beckman P/ACE 5510 system equipped with a photodiode array detector, and an uncoated capillary column. Buffer conditions viz., concentration, pH and micelle concentration were optimised using the overlapping resolution mapping scheme. The optimum conditions for applied voltage and injection time were obtained from a plot of the required parameters as a function of column efficiency. Optimum conditions were 10 mM phosphate buffer (pH 7.5), UV - 226 nm working wavelength, 15 kV applied voltage and 3 seconds injection time.

The repeatability, reproducibility and linearity of the developed multitoxin assay by capillary electrophoresis compare favourably with individual high performance liquid chromatographic systems, with the relative standard deviation below 2 %.

The detection limit for the capillary electrophoresis method was less sensitive than the high performance liquid chromatography methods. However, the on-column sensitivity of capillary electrophoresis was significantly higher than the high performance liquid chromatography systems.

The developed multitoxin capillary electrophoresis assay was evaluated using established extraction methods on spiked samples and naturally contaminated maize and coffee samples. Recovery values obtained for the spiked maize and coffee samples, using individual high performance liquid chromatography methods compared favourably with the developed capillary electrophoresis method. High performance liquid chromatography and capillary electrophoresis quantification results for the naturally contaminated samples compare favourably for the maize and coffee extracts, with the 'F' values all below the critical value of 6.39.

Rural maize samples showed relatively high levels of cyclopiazonic acid contamination (74.1 – 1181.0 ng/g). The five coffee samples that were obtained from a West African source were contaminated with ochratoxin A (30.0 – 44.0 ng/g). These results are a cause for concern, and highlight the need for the monitoring of mycotoxin levels in food commodities.

This is the first successful report to concurrently separate cyclopiazonic acid, moniliformin and ochratoxin A using capillary electrophoresis.

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## INTRODUCTION AND LITERATURE REVIEW

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The aim of this chapter is to briefly introduce mycotoxins, their biochemical properties and the incidence of co-occurrence of mycotoxins in food samples. The chapter focuses on a review of the analytical techniques available for the analysis of mycotoxins.

### 1.1 Characteristics of mycotoxins

Mycotoxins are defined as “fungal metabolites which when ingested, inhaled, or absorbed through the skin cause lowered performance, sickness or death in man, animal or bird” (Moss, 1992). Thousands of fungi are capable of growing on agricultural commodities. However, only a proportion of fungal species produce mycotoxins, and even this is strain dependent. They are chemically diverse, extremely potent and several are carcinogenic (Betina, 1993).

The diseases that occur as a result of the poisoning of the host, which follows ingestion of mycotoxin-contaminated food or feed are regarded as “mycotoxicoses”. Mycotoxins occur world wide in a wide variety of food and feed and have been implicated in a range of human and animal diseases (Smith *et al.*, 1994). Ingestion of large amounts of toxin in a short period of time will cause acute toxicity leading to death while small doses in a prolonged length of time will result in chronic effects to the consumer.

In Africa, reports on mycotoxicoses have not been as widespread as those of the rest of the world, and this has been due to the limited knowledge in disease diagnosis, occurrence, and the acute and chronic effects of mycotoxins. Mycotoxins are reported to be the causative agent of diseases, for example marasmic kwashiorkor, hepatocellular carcinoma in humans, and hepatocarcinomas, encephalopathy and other acute diseases in animals, in African countries (Sibanda *et al.*, 1997).

## INTRODUCTION AND LITERATURE REVIEW

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By 1984, over 240 fungal metabolites were reported to have toxic properties towards animals and animal cell cultures (Cole and Cox, 1981). Pathological effects vary between different mycotoxins and different animals. The major mycotoxins of concern in South Africa are aflatoxins, cyclopiazonic acid, fumonisins, moniliformin, ochratoxin A, sterigmatocystin and zearalenone. A summary of the characteristics of these toxins is presented in Table 1.1.

### 1.2 Implication of co-occurrence of mycotoxins

It is widely known that many fungi occurring in food and feeds are capable of producing more than one mycotoxin (Gqaleni, 1996). Most fungal species can produce two to four mycotoxins. However, some species produce as much as seven or twelve toxins, e.g., *A. flavus* produces nine different toxins. Table 1.2 shows a list of important mycotoxigenic fungal species and the mycotoxins they are capable of producing (Lillehoj, 1991). Some species use a single biochemical pathway to produce related toxins, for example aflatoxins and sterigmatocystin are synthesised in the polyketide pathway. Other fungi, such as *A. flavus*, use two or more different biochemical pathways to produce chemically diverse mycotoxins, such as polyketides and cyclopiazonic acid (Lillehoj, 1991).

The ability of fungi to produce more than one mycotoxin has been extensively researched and documented. The co-production of the mycotoxins has additive toxic effects and increases the toxigenic potential of the fungus. Further, the contamination of several mycotoxins in food or feed sample results in "synergism". Mycotoxin-mycotoxin synergism is defined as the combined effects of multiple mycotoxin ingestion in an animal such that the summation of these effects is more severe than would be predicted based upon the known effects of the individual mycotoxin (Wyatt, 1991). In addition, mycotoxin-mycotoxin synergism can be regarded as the combined action of multiple mycotoxin exposure such that the net result is a unique condition in affected animals. Since signs of intoxication resulting from simultaneous feeding of mycotoxins are different, in many cases the presence of certain mycotoxins are unreported (Wyatt, 1991).

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**Table 1.1: Characteristics of commonly – occurring mycotoxins<sup>#</sup>**

<b>Mycotoxin</b>	<b>Producing fungi</b>	<b>Natural occurrence (Moss, 1998)</b>	<b>Toxic effects</b>	<b>Chemical structural information (Moss, 1998)</b>	<b>Toxicity data</b>
Aflatoxin	<i>Apergillus flavus</i> <i>Aspergillus parasiticus</i>	Maize, peanuts, rice sorghum, oats, and peas.	Carcinogenic. Liver disease in humans. Death in poultry. (Cole and Cox, 1981).	Highly fluorescent, highly oxygenated, heterocyclic compounds characterised by dihydrodifurano or tetrahydrodifurano moieties fused to a substituted coumarin moiety.	The FDA* established action levels of 20 µg/g for grain and feed products, and 0.5 µg/g for milk (Charles, 1997).
Cyclopiazonic acid	<i>A. flavus</i> <i>Penicillium cyclopium</i>	Maize, sorghum, cereal, rye.	Acutely toxic to rats. Lesions produced include necrosis in the liver, spleen, kidney and skeletal muscles.	Indole tetramic acid.	The LD <sub>50</sub> value is 36 mg/kg for male and 63 mg/kg for female rats. (Smith and Henderson, 1991).

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**Table 1.1 Characteristics of commonly – occurring mycotoxins<sup>#</sup>**

<b>Mycotoxin</b>	<b>Producing fungi</b>	<b>Natural occurrence (Moss, 1998)</b>	<b>Toxic effects</b>	<b>Chemical structural information (Moss, 1998)</b>	<b>Toxicity data</b>
Fumonisin	<i>Fusarium moniliforme</i>	Maize, rice.	Oesophageal cancer in human Leukoencephalomalacia in horses. Pulmonary edema in pigs. Carcinogenic to rats.	Group of closely related polar metabolites.	Recommended to be not higher than 5 mg/g for horses, 10 mg/g for swine and 50 mg/g for cattle. (Charles, 1997).
Moniliformin	<i>Fusarium subglutinans</i> <i>Fusarium fusaroides</i>	Maize, sorghum, cereals, rye.	Death in experimental animals. Phytotoxic effects on plant systems.	The toxin occurs naturally as a sodium or potassium salt of 1-hydroxycyclobut-1-ene-3, 4-dione. It is an ionic, low molecular weight, water-soluble cyclobutenedione.	Toxic effects are observed in concentrations as low as 5 µM in experimental rats. (Charles, 1997).



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 Table 1.1 : Characteristics of commonly – occurring mycotoxins<sup>#</sup>

Mycotoxin	Producing fungi	Natural occurrence (Moss, 1998)	Toxic effects	Chemical structural information (Moss, 1998)	Toxicity data
Ochratoxin A	<i>Aspergillus ochraceus</i> <i>Penicillium viridicatum</i>	Maize, coffee, beer, grain, sorghum, cereal, rye, citrus fruit.	Teratogenic and carcinogenic. Causes acute hepatic injury, fatty infiltration and focal necrosis in liver.	family of dihydroisocoumarins.	Mice exhibited gross abnormalities to the kidneys at 1 mg/kg dose. (Hayes, 1974).
Sterigmatocystin	<i>Bipolaris versicolor</i> <i>Bipolaris sorokiniana</i>	Maize, grains, fruit juices, green coffee beans and cheese.	Hepatocarcinogenic. Inhibits Deoxyribonucleic acid synthesis.	Related difuroxanthones, that represents a family of mycotoxins whose structure is very similar to aflatoxins.	The LD <sub>50</sub> value for CPA is 32 mgkg <sup>-1</sup> in vervet monkeys (Smith and Henderson, 1991).
Zearalenone	<i>Fusarium roseum</i> <i>Fusarium tricinctum</i>	Maize, barley.	Pregnant swine abort. Implicated in reproductive problems in horses.	Class of resorcylic acid lactones. It is an estrogenic mycotoxin.	Toxic effects to horses at 0.1 – 5 µg/ml level. (Smith and Henderson, 1991).

\*Food and Drug Administration # Compiled from references as indicated in Table 1

## INTRODUCTION AND LITERATURE REVIEW

**Table 1. 2 : Mycotoxigenic fungi and the mycotoxins they are capable of producing (Smith and Ross, 1991)**

Fungal species	Mycotoxin produced
<i>A. flavus</i>	Aflatoxin B <sub>1</sub> and B <sub>2</sub> , aflatrem, aflavinins, aspergillic acids, cyclopiazonic acid, kojic acid, 3-nitropropionic acid, paspalinins
<i>F. moniliforme</i>	Moniliformin, fumonisins, bikaverins, cyclonerodoil, fusarin C, fusariocins, gibberellins, naphthoquinones
<i>P. chrysogenum</i>	Emodic acid, meleagrin, penicillin, roquefortine C
<i>P. verrucosum</i>	Citrinin and ochratoxin A
<i>Claviceps paspali</i>	Ergot alkaloids, paspalicin, paspaline, paspalinine

In a contaminated medium, there could be several fungi, each producing a wide range of mycotoxins. Hence, in an extract from contaminated food, a mixture of mycotoxins will be present. This, together with the interactive toxicity of mycotoxins prompted much multitoxin research.

Cyclopiazonic acid has been reported to co-occur with aflatoxins. The study by Gallagher *et al.* (1978) revealed that cyclopiazonic acid occurred with a frequency similar to or greater than that of aflatoxins. Cyclopiazonic acid has also reported to co-occur with aflatoxin, zearalenone and ochratoxin A in corn (Widiastuti *et al.*, 1988). Cole (1986) reported, in retrospect, certain distinguishing clinical signs that were more consistent with cyclopiazonic acid poisoning rather than aflatoxin alone, hence the severity of the Turkey 'X' disease, that occurred in the 1960's, in which thousands of turkeys and other poultry lost their lives, after being fed contaminated feed.

Thiel *et al.* (1982) reported the natural co-occurrence of moniliformin, deoxynivalenol and zearalenone in agricultural commodities. Leon *et al.* (1996) investigated the additive toxic effects of moniliformin and aflatoxins.



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These studies revealed that the combination of both toxins were more toxic than the individual toxins, and may pose a potentially greater problem to the poultry industry than either of the toxins individually. In combination with other mycotoxins, ochratoxin A showed varied effects. When ochratoxin A (2 mg/kg) and T2-toxin (0.5 mg/kg), were injected together in experimental mice, ochratoxin A exacerbated the incidence of T-2 induced gross malfunctions. Further, when ochratoxin A (1 mg/kg) and citrinin (30 mg/kg) were administered to a pregnant rat, gross malfunctions and skeletal defects were observed, as compared to the individual toxin treatment. These experiments suggested that when ochratoxin A and another toxin are administered concurrently, they interact to enhance the toxic effects (Smith and Henderson, 1991).

This interactive toxic effect of the various co-occurring mycotoxins in a sample is an important phenomenon that demands monitoring. In order to rapidly identify and accurately quantify the levels of the various mycotoxins in a food sample, multitoxin assay systems based on analytical techniques need to be developed. Cyclopiazonic acid, moniliformin and ochratoxin A were targeted for this study of developing a multitoxin assay, since the reports on co-occurrence with other toxins are increasing (Smith and Henderson, 1991; Thiel *et al.*, 1982; and Widiastuti *et al.*, 1988).

Cyclopiazonic acid, ochratoxin A and moniliformin are acidic toxins, the latter being a small polar compound, with each having different biochemical properties (Table 1.3). Furthermore, very little is known with respect to their occurrence in South African commodities.

Sensitive analytical methods are required to separate and quantify the various mycotoxins present in a sample. The co-occurrence of mycotoxins having different biochemical properties, and structurally dissimilar, renders multitoxin analysis a challenge to the analytical chemist.

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**Table 1.3 : Biochemical properties of cyclopiazonic acid, moniliformin and ochratoxin A**

Mycotoxin	Biochemical properties
Cyclopiazonic acid	Monobasic, indole acid (Holzapfel, 1968). Molar absorptivity coefficient in methanol was $1972 \pm 15$ , for $21.19 \mu\text{mol/l}$ solution in methanol at $\lambda_{\text{max}}$ 281 nm (Pohland <i>et al.</i> , 1982). Toxicity has been demonstrated in a variety of animal species, and a variety of symptoms, including weight loss, depression, convulsions, and death were observed in rats and turkeys (Norred and Lansden, 1987).
Moniliformin	$\text{pKa} = 1.70$ (Steyn <i>et al.</i> , 1978). Molar absorptivity coefficient in methanol was 18 000 for $21.19 \mu\text{mol/l}$ solution in methanol at $\lambda_{\text{max}}$ 229 nm (Steyn <i>et al.</i> , 1978). Appears to have stability when heated, i.e., 40% survival when heated at $150^\circ\text{C}$ for fifteen minutes as the salt, and 30 – 40% survival when added to the maize and heated at high temperature (Gilbert, 1989). Exhibits its inhibitory effects in a manner similar to arsenite. (Smith and Henderson, 1991).
Ochratoxin A	Most important toxin produced by the <i>Penicillium</i> species, and the most widely studied (Smith and Henderson, 1991). The $\text{pKa}$ value for the carboxyl group of phenylalanine part is 4.4, and the $\text{pKa}$ of the phenolic hydroxyl group is 7.3 (Valenta, 1998). The molar absorptivity coefficient in methanol was $3720 \pm 55$ , for $21.8 \mu\text{mol/l}$ solution in methanol at $\lambda_{\text{max}}$ 214 nm (Pohland <i>et al.</i> , 1982). Specific kidney toxin in outbreaks of porcine nephropathy and implicated in human Balkan endemic nephropathy. The toxin is a potent inhibitor of protein biosynthesis, carcinogenic and also immunosuppressive (Moss, 1998). Ochratoxin A is heat stable and was found to survive the roasting process involved in the manufacture of coffee (Cosic, 1996).

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### 1.3 Analysis of mycotoxins

Extensive research efforts have been devoted over the last 30 years to developing methods for detection and quantification of mycotoxins in foods and agricultural commodities. Standard methods for mycotoxin analysis are required for compliance with legislation, monitoring and survey work, and for research. The methods available are thin layer chromatography (Rao and Hussain, 1985 and Rabie *et al.*, 1987), and high performance liquid chromatography (Scott *et al.*, 1998 and Skaug *et al.*, 1998). More recently (Holland and Sepaniak, 1993) capillary electrophoresis has been successfully used for mycotoxin analysis.

#### 1.3.1 Thin layer chromatography

Since the discovery of aflatoxins in 1961, thin layer chromatography has been the procedure of choice for separating and identifying these and other mycotoxins from concentrated extracts. Thin layer chromatography methods continue to be used routinely in most mycotoxin analytical laboratories, but are only published for new applications and improvements to the standard methods (Scott, 1995).

The Official Methods of the Association of Official Analytical Chemists (AOAC) aptly lists a vast combination of solvent mixtures for the different mycotoxin separation. Betina (1991) also details the standard solvent systems for the different mycotoxins. Table 1.4 lists the various conditions used to analyse selected mycotoxins.

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**Table 1.4 : Standard stationary phase, developing solvents and visualising agents for cyclopiazonic acid, moniliformin and ochratoxin A**

<b>Mycotoxin</b>	<b>Stationary phase</b>	<b>Developing solvent</b>	<b>Visualising agent</b>	<b>R<sub>f</sub> value</b>	<b>Reference</b>
Cyclopiazonic acid	Silica gel treated with oxalic acid	Toluene:ethyl acetate:formic acid (5:4:1)	Ehrlich's reagent	0.63	Rao and Hussain (1985)
Moniliformin	Silica gel	Methanol:chloroform (2:3)	2,4 Dinitrophenol	0.53	Rabie <i>et al.</i> (1978)
Ochratoxin A	Silica gel	Toluene:ethyl acetate:formic acid (6:3:1)	*None	0.55	Abarca <i>et al.</i> (1994)

\*Naturally fluorescent under visible light

Quantitative assessment of mycotoxin spots on the thin layer chromatography plate can be achieved by visual estimation in which the colour and intensity of the sample spot is compared with that of different concentrations of mycotoxins. Visual assessment can give excellent results, provided the spot is small enough to fall within the human optically linear range (Smith and Henderson, 1991). However, photoelectric densitometers are more reliable and less subjective than visual assessment.

Multitoxin thin layer chromatography systems for various toxins have been investigated (Gorst-Allman and Steyn, 1979); however, they do not give clear separation of the toxins due to overlapping and tailing of some of the mycotoxins. Further, Smith and Henderson (1991), concluded that multitoxin thin layer chromatography systems gave more false positive and false negative results than the thin layer chromatography procedure dedicated to one mycotoxin, especially at low concentrations, and when analysing complex matrices such as cereals and

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animal feeds. Hence, multitoxin assays are used primarily for screening purposes and cannot be used for confirmation of identity.

### 1.3.2 Gas chromatography

Gas chromatography (GC) is a relatively simple technique that is widely used for a variety of purposes in food chemistry. However, the analysis of mycotoxins by gas chromatography has not gained widespread popularity owing to the fact that the toxins lack volatility. Consequently, the toxins must be derivatized to increase their volatility. Generally, gas chromatography is performed when the toxin does not possess fluorescent properties and does not yield sharp absorption peaks when exposed to ultraviolet-visible radiation. One class of mycotoxins that have been successfully analysed by gas chromatography is the trichothecenes. Smith and Henderson (1991) reviewed the analysis of deoxynivalenol by gas chromatography and the application to complex matrices. Generally, packed columns were successful at separating derivatized toxin, although trends were towards capillary columns, which have higher resolving power and greater efficiency. All procedures use relatively non-polar chromatographic stationary phases. Flame ionisation and electron capture detectors were commonly employed, with the electron capture detector being the most sensitive for the heptafluorobutyryl derivative of deoxynivalenol. The technique has been successful at detecting low levels of deoxynivalenol (2 to 100 ng/g) in wheat, cereal grain, maize and mixed feeds.

### 1.3.3 High performance liquid chromatography

High performance liquid chromatography (HPLC) is rapidly replacing thin layer chromatography, as the most commonly used analytical technique for the quantitative determination of mycotoxins. It is a form of liquid chromatography in which the mobile phase is placed under pressure generated by pumps, and is forced through a column containing the stationary phase. The column is either a finely divided solid or liquid coated on a solid support, or a solid support which has been chemically modified by the introduction of organic groups. The size of

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the particles of the column packing and the type of surface modification influence the separation mechanism in high performance liquid chromatography.

- **Cyclopiazonic acid**

Lansden (1984) described a reversed phase high performance liquid chromatography method for the analysis of cyclopiazonic acid in peanuts, using a Partisil -10 ODS column. The mobile phase consisted of 40% acetonitrile: 30% isopropyl alcohol: 1% ammonium acetate: 0.025% 4-dodecyldiethylenetriamine: 0.001 M zinc acetate in water. The paper addressed two shortfalls observed in the analysis of cyclopiazonic acid by high performance liquid chromatography: severe peak tailing and the dependence of the peak retention time on the amount of cyclopiazonic acid injected. The method was linear up to 1 µg cyclopiazonic acid, with a detection limit of 4 ng (two times baseline noise).

Goto *et al.* (1987) described a normal phase high performance liquid chromatography method for the analysis of cyclopiazonic acid. The normal phase system with a stainless steel (10 cm x 4 mm i.d.) column packed with silica was used. The optimum mobile phase used was ethyl acetate: 2-propanol: 25% ammonia (55:20:5). The paper reports that from preliminary investigation of reversed phase systems, none of the systems were successful for the analysis of cyclopiazonic acid, because the reversed phase methods were lacking in reproducibility, accuracy and sensitivity. The detection limit for the normal phase system was 0.20 ng at 284 nm. This detection limit was reported to be twenty times greater than the reversed phase system reported by Lansden (1984). A linear calibration curve was obtained in the range 0.50 ng to 3 µg. Later Rao (1993) used this normal phase method (silica column 150 x 250 mm, and ethyl acetate, isopropyl alcohol and 25% ammonia as mobile phase) for the separation of cyclopiazonic acid by high performance liquid chromatography. The detection limit was reported to be 20 ng.

Matsudo and Sasaki (1995) separated cyclopiazonic acid by high performance liquid chromatography using a 1 mM zinc sulphate solution in a 45: 55 phosphoric

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acid : acetonitrile mixture as the mobile phase, with a Tosoh ODS – 80TM (4.6 i.d. x 250 mm) column. The relative standard deviation for the repeatability studies for a 10 µg/ml solution was 3.13%, with a detection limit of 0.3 ng. An internal standard (indomethacin) had to be used to improve the repeatability of the method. The linearity range was 1 µg/ml to 50 µg/ml with an R-squared value of 0.999.

Prasongsidh *et al.* (1997) performed gradient elution separation of cyclopiazonic acid from milk with zinc sulphate solution: methanol: water mixtures, with a Nova-pak (150 x 3.9 mm) C18 column. The analytical response for the high performance liquid chromatography method was linear from 500 ng/ml to 100 µg/ml, with R-squared value of 0.9999937. The detection limit for the method was 50 ng/ml in spiked milk samples.

Sobolev *et al.* (1998) described the high performance liquid chromatography determination of major secondary metabolites (cyclopiazonic acid, o-methylsterigmatocystin, and versicolorins) produced by *A. flavus*, *A. parasiticus*, *A. tamarii* and *A. caelatus* using normal phase ion pair partition chromatography. The mobile phase used was n-heptane: 2-propanol: n-butanol: water: tetrabutyl ammonium hydroxide (2560: 900: 230: 32: 8), with a Zorbax (250 x 4.6 mm i.d.) silica column. The limit of detection for cyclopiazonic acid, at 280 nm was 0.25 µg/ml. The ultraviolet-visible response to cyclopiazonic acid was linear to 0.50 µg per injection.

Cyclopiazonic acid was analysed by normal phase and reversed phase chromatography. Reversed phase chromatography methods were not investigated in this study, as normal phase chromatography was described to produce the best peak shape, while the reversed phase methods showed poor reproducibility, accuracy and sensitivity.

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- **Moniliformin**

Steyn *et al.* (1978) described an ion exchange chromatography method for the isolation and purification of moniliformin. The column (2.5 x 40 cm) was packed with a Bio-Rad anion exchange resin. The chloride form of the resin was converted to the hydroxyl form.

The column was then washed with water until the eluate was neutral. The aqueous extract of the fungal material was applied to the column at a flow rate of 8 ml/min. The moniliformin was eluted with 6 litres of sodium chloride concentration gradient (0 to 2 M). The absorbance of the eluate was observed with an ultraviolet-visible detector at 254 nm. Moniliformin yields of 2 to 16 g/kg were reported.

Thiel (1979) described a method for the separation of moniliformin with the addition of ion pairing reagent to the buffer (tetrabutylammonium bromide salt), and a Partisil 10 ODS (4.6 mm id x 25 cm) column, with 0.1 M sodium phosphate buffer, pH 7 and 8% methanol as the mobile phase. The paper also described a successful anion exchange chromatographic method using a Partisil 10 SAX (4.6 mm id X 25 cm) column and 0.01 M sodium phosphate buffer, pH 5. In both procedures, moniliformin eluted as a sharp, reproducible peak. The detection limit for both techniques was 1 µg/g when monitored at 229 nm.

Rabie *et al.* (1982) reported on moniliformin incidence in South Africa. Two techniques were described, viz., ion exchange chromatography and paired ion chromatography. For the former, a Partisil 10 SAX column (4 mm i.d. x 30 cm) with a mobile phase of 0.01 M sodium phosphate (pH 5), at a flow rate of 1 ml/min was used. Under these conditions, the moniliformin peak eluted at 9.7 minutes. The second procedure was done with a Bondapak C18 column (4 mm i.d. x 30 cm), with 0.1 M sodium phosphate buffer (pH 5) with 5 mM tetrabutylammonium hydroxide sulphate and 8% methanol as the mobile phase, at a flow rate of 1 ml/min. The retention time for the moniliformin peak was



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9.4 minutes. The detection limit for both methods were 1 ng of pure moniliformin, and the lower limit of detection of the sample extract was 1 mg/kg.

Filek and Lindner (1996) reported on the determination of moniliformin in cereals by high performance liquid chromatography. The analysis was performed using a diode array detector, and fluorescence detector in series, an ODS (15 cm x 4.6 mm i.d.) column, and acetonitrile:ammonium acetate (35:65), pH 7 as the mobile phase, with post column derivitisation using 1,2-diamino-4,5-dichlorobenzene (DDB). The detection limit in diverse cereal samples and maize, was 0.05 mg/kg for ultraviolet-visible detection and 0.02 mg/kg for fluorescence detection.

The general trend for moniliformin determination was ion exchange chromatography and paired ion chromatography. Both the detection limits compare favourably. However, ion exchange chromatography is not a very popular technique, largely due to the problems encountered (poor reproducibility and peak shape) when using these systems. The column variability tends to be more of a problem when using bonded-phase ion-exchange packing, and the columns are less stable than the other chromatographic columns (Poole and Poole, 1991). Hence, a paired ion chromatography method was investigated for the high performance analysis of moniliformin in this study.

- **Ochratoxin A**

Abarca *et al.* (1994) used a S5 octadecyl silane 2 column (4.6 mm id x 25 cm) and 57% acetonitrile, 41% water and 2% acetic acid as the mobile phase, to successfully analyse for ochratoxin A produced from strains of *Aspergillus niger* var. *niger*.

Hurst *et al.* (1998) described a high performance liquid chromatography method for the analysis of ochratoxin A in artificially contaminated cocoa beans. The high performance liquid chromatography column used was 5 µm Spherisorb ODS-2 column (150 x 4.6 mm) with a mobile phase of acetonitrile: water: trifluoroacetic

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acid (40:59.7:0.3). Further, 0.2 mM  $\beta$ -cyclodextrin was added to the mobile phase to enhance separation of ochratoxin A from interfering components in the matrix. A fluorescence detector set at an excitation wavelength of 330 nm and emission wavelength of 460 nm was used. The detection limit (at signal-to-noise ratio at 3) was calculated to be 2  $\mu\text{g/g}$ . Relative standard deviation of 1.10% was obtained from data collected for ten injections.

Jorgensen (1998) reported the incidence of ochratoxin A in pork, coffee, beer and pulses in Denmark, using high performance liquid chromatography analysis. The mobile phase employed was acetonitrile: water: acetic acid (50:49:1), post column addition of 6% ammonia for enhanced fluorescence detection, reversed phase, C18, 5  $\mu\text{m}$  column, and fluorescence detection with excitation wavelength at 440 nm, and emission wavelength at 385 nm. The limit of detection (signal to noise - 3:1), was 0.02 to 0.03  $\mu\text{g/kg}$  for pork and poultry, 0.001  $\mu\text{g/kg}$  for beer, and 0.1  $\mu\text{g/kg}$  for roasted coffee and pulses.

Zimmerli and Dick (1995) used post column addition of ammonia to enhance the fluorescence signal, to detect ochratoxin A in serum, blood, milk and other foodstuff. An acidic aqueous mobile phase (methanol: 9% acetic acid, (18:7), and an ODS-2 spherisorb (30 x 4.6 mm) column was used. A detection limit (signal to noise ratio at 3:1) of 2 pg of ochratoxin A was achieved. The quantification limit for ochratoxin A in blood or plasma was estimated to be 5 pg/g.

Scott *et al.* (1998) surveyed Canadian human blood plasma for the presence of ochratoxin A using high performance liquid chromatography. The mobile phase was acetonitrile: methanol: 0.15 M phosphoric acid (1:1:1). An ultrasphere C18 (25 cm x 3.2 mm) column was used, with fluorescence detection ( $\lambda_{\text{excitation}} = 330 \text{ nm}$  and  $\lambda_{\text{emission}} = 470 \text{ nm}$ ). The detection limit for the method was 0.12 ng/ml (signal to noise ratio at 3:1).

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Skaug *et al.* (1998) described the analysis of naturally occurring ochratoxin A from human milk samples in Norway using high performance liquid chromatography. The samples were analysed by the high performance liquid chromatography ion pairing technique, at an alkaline pH, with fluorescence detection ( $\lambda_{\text{excitation}} = 380 \text{ nm}$  and  $\lambda_{\text{emission}} = 450 \text{ nm}$ ). Separation was carried out on a Spherisorb S3ODS2, C18, 4.6 x 150 mm column. The mobile phase consisted of 10 mM tetrabutylammonium bromide in a methanol - potassium phosphate buffer, pH 7.5 mixture - (51: 49). The detection limit for spiked milk samples was 10 ng/ml.

The calibration curve was developed in the concentration range 70 to 1750 pg/ml. The coefficient of variation of the peak heights of ochratoxin A at different injections of the same standard solution was 3% (within day variation), and that from the spiked milk samples was 12% (between days).

The general trend for the analysis of ochratoxin A by high performance liquid chromatography has been by reversed phase chromatography with a C-18 column with acetonitrile: methanol: acetic acid (1:1:1) as the mobile phase.

### 1.3.4 Multitoxin high performance liquid chromatography

Schweighardt *et al.* (1980) analyzed zearalenone and deoxynivalenol in food and animal feeds using two separate high performance liquid chromatography systems. Zearalenone was separated on a polar stationary phase (25 cm x 4.6 mm i.d.) column, and eluted with chloroform: isooctane (75:25 v/v): methanol (1.5%) mixture, and detected fluorometrically. Deoxynivalenol was analysed with a C18 column (25 cm x 4 mm i.d.) with methanol:water (60:40) as the mobile phase. Additional clean up was obtained by combination of gas liquid chromatography and thin layer chromatography with ultraviolet-visible detection. The detection limit for zearalenone was 2  $\mu\text{g/kg}$ , and 25  $\mu\text{g/kg}$  for vomitoxin in cereal products.

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Howell and Taylor (1981) determined the presence of aflatoxins, ochratoxin A and zearalenone in mixed feeds. Their paper describes high performance liquid chromatography methods for each of the toxins with either a stainless steel (250 x 4.6 mm i.d.) 5  $\mu$ m silica column, or a 5  $\mu$ m ODS column. The aflatoxins were analysed using the silica column and water-saturated toluene: ethyl acetate: formic acid, (85: 25: 5) as the mobile phase. Zearalenone and ochratoxin A was individually analysed using a reversed column, and a mobile phase of acetonitrile: orthophosphoric acid (55: 45) and (50: 50) for ochratoxin A and zearalenone respectively. The limit of detection was 1  $\mu$ g/kg for each of the toxins.

Schweighardt (1980) and Howell and Taylor (1981), investigations reported on the multitoxin contamination of a sample by evaluating several high performance liquid chromatography systems. These investigations however, do not report on a single high performance liquid chromatography method for the separation and detection of several toxins.

### 1.3.5 Capillary electrophoresis

Electrophoresis has been defined as the differential movement of charged species (ions) by attraction or repulsion in an electric field. Tiselius (1937) first introduced electrophoresis as a separation technique. Placing protein solutions between buffer solutions in a tube, and applying an electric field, he found that sample components migrate in a direction and at a rate determined by their charge and mobility. Thermal diffusion and convection limited the separation efficiency in this set up. Therefore, electrophoresis was traditionally carried out in an anti-convective media, such as agarose gel. However, gel slab electrophoresis technique suffers from long analysis time, low efficiency, and difficulties in detection and automation. In order to improve separation efficiency and efficiently dissipate the heat produced, electrophoresis was attempted in a narrow bore capillary (Heiger, 1992).

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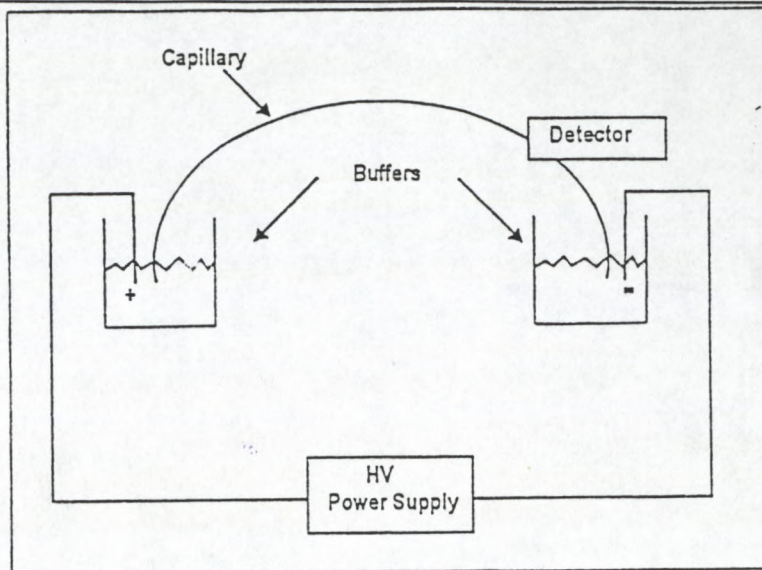
Capillary electrophoresis was born of the marriage of the powerful separation technique of electrophoresis and the instrumentation and automation concepts of chromatography. In capillary electrophoresis, electrophoresis is performed using narrow-bore capillaries, typically 25 to 75  $\mu\text{m}$  (i.d.). The capillaries have numerous advantages, particularly in respect to the detrimental effects of Joule heating. The high electrical resistance of the capillaries enables the application of very high electric fields (100 to 500 V/cm) with only minimal heat generation. Moreover, the large area to volume ratio of the capillary efficiently dissipates heat that is generated. The use of the high electric fields results in short analysis times and high efficiency and resolution. Peak efficiency (N), generally greater than  $10^5$ , is due to the plug profile of the electro-osmotic flow. This flow also enables the simultaneous analysis of all solutes, regardless of charge.

In addition, there are numerous separation modes that offer different separation mechanisms and selectivities. Capillary electrophoresis is fast becoming a premier separation technique, due to the minimal sample volume requirements (1 to 10 nL), on column detection and the potential for quantitative analysis and automation (Heiger, 1992).

A schematic diagram of a capillary electrophoresis system is shown in Figure 1.1. Briefly, the ends of a narrow bore, fused silica capillary are placed in buffer reservoirs. The contents of the reservoirs are identical to that within the capillary. The reservoirs also contain the electrodes used to make electric contact between the high voltage power supply and capillary. Sample is loaded onto the capillary by replacing one of the reservoirs (usually at the anode) with a sample reservoir and applying either an electric current or an external pressure. After replacing the buffer reservoirs, the electric field is applied and the separation performed. Optical detection occurs at the opposite end, directly through the capillary wall (on-line detection), (Heiger, 1992).



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**Figure 1.1: Schematic diagram of the basic components of a capillary electrophoresis system (Heiger,1992).**

Separation by electrophoresis is based on differences in solute velocities in an electric field. The velocity of an ion is given by Equation 1.

$$V = \mu_e E \quad \text{Equation 1}$$

Where,  $V$  = ion velocity  
 $\mu_e$  = electrophoretic mobility  
 $E$  = applied electric field.

During electrophoresis a steady state, defined by the balancing of the electric force (Equation 2) and the frictional force (Equation 3) of the ion under investigation, is attained. At this point these forces are equal, but opposite.

$$F_e = qE \quad \text{Equation 2}$$

Where,  $F_e$  = electric force  
 $q$  = ion charge  
 $E$  = applied electric field.

$$F_f = -6\pi\eta rv \quad \text{Equation 3}$$

Where,  $\eta$  = solution viscosity  
 $r$  = ion radius  
 $v$  = ion velocity

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Substituting this information in Equation 1 yields an equation (Equation 4) that describes the mobility of an ion in terms of its physical parameters.

$$\mu_e = \frac{q}{6\pi\eta r} \quad \text{Equation 4}$$

Hence, from Equation 4, small, highly charged species have high mobilities whereas large, minimally charged species have low mobilities.

A fundamental constituent of capillary electrophoresis operation is the electroosmotic flow (EOF). Electroosmotic flow is the bulk flow of liquid in the capillary and is a consequence of the surface charge on the interior capillary wall. The surface of the silicate capillary contains negatively-charged functional groups (Si-OH) that attract positively charged counterions. The positively-charged ions migrate towards the negatively-charged electrode or cathode and carry solvent molecules in the same direction. This overall solvent movement is called electroosmotic flow. During a separation, uncharged molecules move at the same velocity as the electroosmotic flow (with very little separation). Positively charged ions move faster and negatively charged ions move slower (Heiger, 1992).

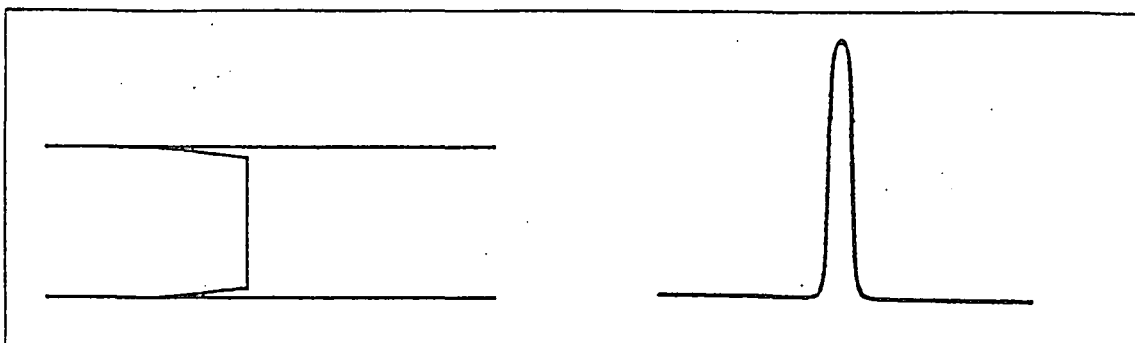
A unique feature of the electroosmotic flow in the capillary is the flat profile of the flow, as depicted in Figure 1.2 (i). Since the driving force of the flow is uniformly distributed along the capillary there is no pressure drop within the capillary, and the flow is nearly uniform throughout. The flat flow profile is beneficial since it does not directly contribute to the dispersion of the solute zones. This is in contrast to that generated by an external pump, which yields a laminar flow due to the shear force at the wall (Heiger, 1992).

Another benefit of EOF is that it causes movement of nearly all species, regardless of charge, in the same direction. Under normal conditions, the flow is from the anode to the cathode. Anions will be flushed towards the cathode since the magnitude of flow can be more than an order of magnitude greater than their

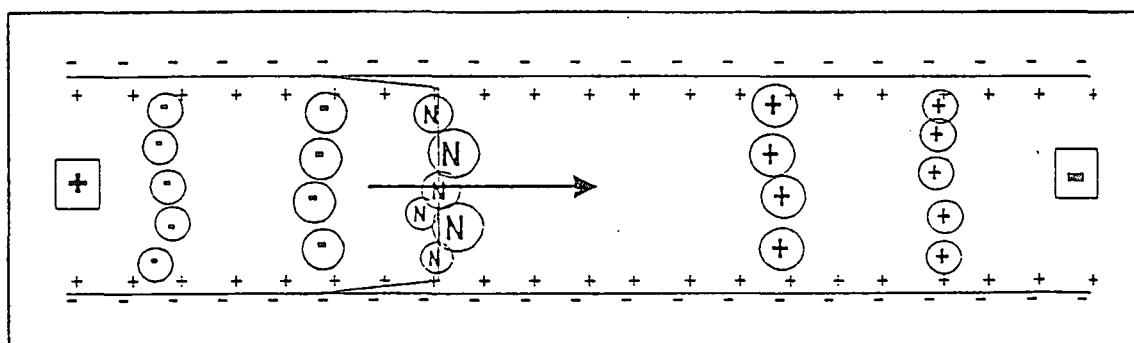
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electrophoretic mobility. Thus, cations, neutrals and ions can be electrophoresed in a single run since they all migrate in the same direction. Cations migrate the fastest, since the electrophoretic attraction towards the cathode and the EOF is in the same direction. Neutrals are carried at the velocity of the EOF, but are not separated from each other. Anions migrate the slowest since they are attracted to the anode, but are still carried by the EOF towards the cathode, as shown in Figure 1.2 (ii), (Heiger, 1992).

(i)



(ii)



**Figure 1.2 (i): Flat profile and corresponding solute zone and (ii) Differential solvent migration superimposed on electroosmotic flow in capillary electrophoresis (Heiger, 1992).**



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By incorporating micelles in the capillary system, neutral species can be separated on the basis of their differential distribution between the mobile and micellar phase, and is termed micellar electrokinetic capillary chromatography (MEKC).

Capillary electrophoresis has been compared very favourably with high performance liquid chromatography for the analysis of ionic compounds (John, 1997 and Moodley, 1995). Capillary electrophoresis is a versatile analytical technique that has gained much attention, particularly in the biological field.

Holland and Sepaniak (1993) described a multitoxin method, with high-resolution separation of citrinin, zearalenone, penicillic acid, aflatoxin B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, G<sub>2</sub>, ochratoxin A, roridin A, and sterigmatocystin. The separation was performed on an uncoated capillary column, with 50 mM sodium dodecylsulphate (SDS) in 10 mM phosphate – 6 mM borate buffer, pH 9.3, at 20 kV applied voltage and 20 seconds injection time. The article focused on trends in the retention of these toxins, resulting from change in mobile phase composition and pH. The data was generated from mycotoxin standards ( $1 \times 10^{-4}$  M). In addition, the qualitative aspects of the analysis were evaluated on randomly generated mycotoxin-interferent mixtures.

Bohs *et al.* (1995) analysed ochratoxin A, ochratoxin B, zearalenone and moniliformin by capillary electrophoresis. Various buffer systems were evaluated for the capillary electrophoresis separation. The analysis was performed using an uncoated capillary column (75  $\mu$ m X 75 cm). Peak detection was accomplished by diode array detection, and off line determination of fluorescence spectra with a fluorescence spectrophotometer. The detection limit for the standard sample at 220 nm detection and 20 mM phosphate buffer, pH 7, and 10 kV applied voltage, was 1  $\mu$ g/ml. The absolute amount injected at a pressure of 50 mbar for 3 second was 15 pg (total volume = 16.6 nl). The R-squared value measuring the concentration range from 10 to 120  $\mu$ g/ml was 0.998.

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Prasongsidh *et al.* (1997) performed micellar electrokinetic chromatography (MEKC) analysis of cyclopiazonic acid using the buffer system described by Holland and Sepaniak (1993), with the absorbance of cyclopiazonic acid monitored at 225 nm wavelength. The analytical response for the capillary electrophoresis was linear from 40 ng/ml to 100 µg/ml, with R-squared value of 0.99995. The detection limit for the method was 20 ng/ml in spiked milk samples. Prasongsidh *et al.* (1998) described a MEKC method to analyse for cyclopiazonic acid in milk. The buffer system was as described by Holland and Sepaniak (1993). The capillary electrophoresis used (HP<sup>3D</sup> system) was equipped with a capillary extended light path feature, that permitted the accumulation of the three times the sample at the bubble cell, where light throughput occurs. This increased the signal and thus improved the lower detection limit. The analytical response was linear from 40 ng/ml to 100 µg/ml (R-squared value = 0.99995). The detectable limit of cyclopiazonic acid by MEKC was  $0.27 \times 10^{-7}$  pg/ml. In addition, capillary electrophoresis analysis was reported to be a better and quicker method for cyclopiazonic acid detection in milk as compared to high performance liquid chromatography. Capillary electrophoresis was capable of isolating the cyclopiazonic acid peak at lower concentration, although the sample injection volume (8.3 nl) was several times lower than that of high performance liquid chromatography (20 µl).

Corneli and Maragos (1998) described a capillary electrophoresis method with laser-induced fluorescence detection for ochratoxin A. The capillary electrophoresis unit was modified by replacing the emission filter with a 399 nm long-pass filter and 465 nm band pass filter. This combination permitted the collection of light between 400 and 539 nm. The fluorescence of ochratoxin A was measured with a helium – cadmium laser unit, at 325 nm. The detection limit of the method, using an untreated fused silica capillary, 20 mM phosphate buffer at pH 7.00 as the running buffer, 5 seconds sample injection time and 20 kV applied voltage, was 10 ng/ml for spiked coffee samples. The method was linear with an R-squared value of 0.999.

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The literature indicates that both modes of capillary electrophoresis have been used for the analysis of mycotoxins, that is capillary zone electrophoresis and micellar electrokinetic capillary chromatography. However, there are more publications on MEKC, with the MEKC buffer used by Holland and Sepaniak (1993), being the most common for the separation of toxins (including ochratoxin A).

### 1.3.6 Extraction techniques

The detection of trace quantities of toxin dictates that the toxin be isolated from interfering compounds present in the sample, while retaining a high recovery of toxin. Mycotoxins vary greatly in their structural properties and, thus, also in their physical properties such as solubilities and partition coefficients (Betina, 1993). In addition, food and feedstuff present a complex matrix with many interfering substances. Hence, extraction techniques vary for different classes of toxins.

Gorst-Allman and Steyn (1979) described a general procedure for the extraction of thirteen mycotoxins (aflatoxin B<sub>1</sub>, sterigmatocystin, zearalenone, patulin, T2 - toxin, roquefortine, penitrem A, fumitremorgen B, roridin A, ochratoxin A, citrinin, cyclopiazonic acid and penicillic acid) from spiked maize, that could be separated as neutral and acidic metabolites. The spiked maize samples were extracted with methanol: chloroform (1:1), filtered and evaporated to dryness. The resultant residue was partitioned between hexane and methanol; the hexane layer was discarded while the methanol layer was evaporated to dryness. The residue was then partitioned between chloroform and water, and the chloroform layer was extracted with saturated sodium bicarbonate solution and contained all the neutral toxins. The aqueous layer was acidified and extracted with chloroform. The chloroform extracts were concentrated and contained the neutral toxins.

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- **Cyclopiazonic acid**

Holzapfel (1968) described the isolation cyclopiazonic acid from *P. cyclopium*, which were grown on sterilised wet maize meal. The dried mouldy meal was extracted with chloroform: methanol and the solvent removed *in vacuo*.

The extract was distributed between chloroform and methanol (5:8). The chloroform extract was evaporated and the residue distributed methanol and hexane (3:4). The methanol was evaporated and the residue was chromatographed on a cellulose column, pregated with  $\text{HCONH}_2$ :  $(\text{COOH})_2$  (50:3). The lipids were eluted with hexane. The column was developed with hexane: benzene (20:1) to (3:1) and (70 x 150 ml) fractions collected. The cyclopiazonic acid fractions (30 – 48) were purified by column chromatography with Dowex 1 packed column. The column was developed by gradient elution with formic acid solutions, and (50 x 25 ml) fractions collected. Fractions (28 to 38) gave pure cyclopiazonic acid (395 mg).

Gorst-Allman and Steyn (1979) described a method to purify cyclopiazonic acid in the presence of other co-metabolites using a DEAE-cellulose packed column. The activated Whatman cellulose, previously washed with 0.02 M ammonium hydrogen carbonate solution, was packed into a glass column and allowed to settle under gravity. A crude extract of *P. griseofulvum*, a known precursor of cyclopiazonic acid (Jesus *et al.*, 1981), was injected into the column, and fractions were collected while monitoring the ultraviolet-visible absorption of the eluate at 280 nm. Initially 0.02 M ammonium hydrogen carbonate solution was pumped through the column for two hours, and then a linear gradient from 0.02 M to 0.2 M was started, the final molarity was reached after 26 hours. Elution with the final molarity ammonium hydrogen carbonate solution was maintained until cyclopiazonic acid was eluted. The method was described to be ideal for preparative separation and accumulation of substantial amounts of the two metabolites.

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Lansden and Davidson (1983) described a liquid-liquid extraction method for the extraction of cyclopiazonic acid from peanuts. The sample (100 g each) was defatted with petroleum ether, vacuum filtered and the sample allowed to dry overnight. Samples were then extracted with 250 ml of 20% methanol in chloroform, with 0.5 ml phosphoric acid. The extract was filtered, flash evaporated and extracted with 50 ml of sodium bicarbonate solution prior to being centrifuged. The upper layer was neutralised with 6 N HCl and extracted with dichloromethane and centrifuged.

The lower layer was evaporated to dryness. Cyclopiazonic acid standard used at 105 and 210  $\mu\text{g/kg}$  level to spike peanut samples was recovered at an average rate of 93.3%, with ranges of 89 to 119, and 166 to 221  $\mu\text{g/kg}$  respectively. In a later paper (Lansden, 1984) the same method was used to extract cyclopiazonic acid from peanuts. The recovery of cyclopiazonic acid from peanuts spiked at 68.9, 210 and 955  $\mu\text{g/kg}$  was 85.9% (12.86% relative standard deviation, RSD), 72.9% (6.43% RSD) and 81.4% (0.40% RSD) respectively. The same method was used to extract cyclopiazonic acid from peanuts and corn (Lansden, 1986). The peanut and corn samples were spiked in range 150 to 500 ng/g. The recovery value for peanuts ranged from 89.6 to 94.3% with a relative standard deviation of 9.1 to 30.0%. The recovery value for corn ranged from 71.3 to 99.3% with a relative standard deviation of 6.4 to 30.7%. For both studies, the samples spiked with 250 ng/g cyclopiazonic acid resulted in relative high standard deviation values (30.00% and 30.7% for peanuts and corn, respectively).

Rao and Husain (1985) described an extraction procedure for cyclopiazonic acid from kodo millets that were implicated in 'kodua poisoning' in man. 'Kodua poisoning' is associated with symptoms of depression and complete loss of mobility. The seed sample (500 g) was extracted with chloroform for 48 hours. The solvent was dried over anhydrous sodium sulphate and evaporated to dryness under vacuum. Purification was done by preparative thin layer chromatography with pre-coated silica gel thin layer chromatography plates that were developed with chloroform : methyl ethyl ketone (80:20). Cyclopiazonic acid

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was identified as a violet coloured spot when sprayed with Ehrlich's reagent. The corresponding area on the plate was scraped off, and eluted with chloroform. The chloroform was washed with water to remove the oxalic acid, and evaporated to dryness in a vacuum. Further purification was done on a formamide : oxalic acid impregnated column as described by Holzapfel (1968), and cyclopiazonic acid was crystallised from methanol. In a later paper Rao and Husain, (1987) described the extraction of cyclopiazonic acid from rice, wheat, maize and kodo millets by initially extracting with chloroform, followed by preparative thin layer chromatography as described in Rao and Husain (1985). Recovery experiments were conducted to test the efficiency of the method.

The cyclopiazonic acid extract was analysed by colourimetric analysis. Each substrate was spiked with cyclopiazonic acid in the range 50 µg to 100 µg. The recovery experiment showed 92 to 97% recovery of cyclopiazonic acid.

Goto *et al.* (1987) described the extraction of cyclopiazonic acid from maize, peanut meal and rice, using chloroform and phosphoric acid (100 :1). The extract was thereafter purified on sep-pak silica cartridge column. The recovery of cyclopiazonic acid from maize samples was 82%, at a concentration of level of 1.8 µg/g dry maize.

Chang-yen and Bidasee (1990) described the extraction of cyclopiazonic acid from poultry feed and corn using liquid-liquid extraction. Samples were extracted with chloroform – methanol (85 : 15) solvent, allowed to settle prior to vacuum filtration through Whatman 541 filter paper. The cyclopiazonic acid was back extracted into the aqueous layer by shaking the filtrate with 50 ml of 1 M NaOH. The combined aqueous extract was acidified with 2 M HCl, and then extracted with aliquots of chloroform. The chloroform layer was dried with sodium sulphate, prior to evaporating to dryness on rotary evaporator set at 50 °C. The extract was further purified using a silica gel mini-column, to minimise background absorbance caused by co-extractives from samples. Recoveries from 50 g samples of poultry feed spiked with cyclopiazonic acid ranging from 0.16 to

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1.2 mg/kg averaged 93.8% (n=5) with a reported standard deviation of 3.9 (n=10).

Huang *et al.* (1994) described a rapid method to extract cyclopiazonic acid from isolates of *A. flavus*. The cultures that were grown in vials containing mycological broth, supplemented with sucrose and yeast extract were extracted with chloroform. The mycelial mat was chopped with a spatula in the vial. The vials were then vortexed for 30 seconds and allowed to stand for 5 minutes to allow the chloroform and water layers to separate. The cyclopiazonic acid extract was contained in the chloroform layer. Cyclopiazonic acid was detected in the range 310 to 1 100 µg per vial.

Prasongsidh *et al.* (1997) reported on the incidence of cyclopiazonic acid in milk using liquid-liquid extraction and Sep-pak silica gel cleanup. The milk sample was mixed with sodium bicarbonate-methanol mixture. The milk-methanol mixture was initially defatted with hexane, then acidified with hydrochloric acid to pH 3, prior to extracting with chloroform. The chloroform was passed through a Sep-pak silica gel cartridge. The cyclopiazonic acid was eluted with chloroform-methanol mixture (75:25). The recoveries from milk spiked with 20 ng/ml to 500 ng/ml detectable by capillary electrophoresis were 78 to 81% (% RSD = 2.3 to 6.7%). The recoveries of milk contaminated by 50 ng/ml to 1 µg/ml of cyclopiazonic acid were 70 to 80% (% RSD = 4.1 to 6.8%).

Sobolev *et al.* (1998) used liquid-liquid extraction for the separation of cyclopiazonic acid from fungal cultures. The recovery of cyclopiazonic acid spiked at 10 µg/ml was 98.90%, with a % RSD of 3.27 and when spiked at a level of 100 µg/ml, the recovery was 98.89%, with a % RSD of 3.87 (n=5).

Trucksess (1998) reported that preliminary studies using immunoaffinity columns for the extraction of cyclopiazonic acid from peanuts showed poor recoveries, but good precision.

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The general trend for the extraction of cyclopiazonic acid from maize has been by liquid-liquid extraction or extraction using silica gel cartridges. The recoveries obtained with both techniques compare favourably. There are no reports of the extraction of cyclopiazonic acid from coffee.

- **Moniliformin**

Thiel (1978) described an extraction procedure for moniliformin from fungal cultures grown on sterilised maize kernels. The moulded maize kernels were dried, ground and mixed thoroughly to obtain a homogenous sample. One 40 ml aliquot of distilled water was added to 3 g of dry material in centrifuge tubes. The stoppered tubes were shaken vigorously for one hour on a rotary shaker, and thereafter centrifuged for twenty minutes.

Moniliformin was present in the supernatant solution. The clear supernatant was applied to a Sephadex packed column, which was equilibrated with 0.1 M ammonium acetate solution, to further clean up the extract. Moniliformin was eluted with further addition of 0.1 M ammonium acetate solution.

Moniliformin was present in the eluate between 60 and 120 ml after commencement of sample application. The extract was lyophilised, and the residue retained for further analysis. The recovery of the extraction step was 95% when a spiked sample of ground maize kernels was analysed. Due to background interferences at low concentration of moniliformin, the cleanup procedure was investigated. The lyophilisation step, that simultaneously concentrated the sample and removed the ammonium acetate, was reported to result in a loss of moniliformin. When the cleanup step was included recoveries were of the order of 70%.

Rabie *et al.* (1978) reported on moniliformin as a mycotoxin from *F. fusarioides*. The extraction procedure described involved extracting 50 g of moulded maize sample with chloroform in Soxhlets extractors for 48 hours, followed by extraction with 80% aqueous methanol for a further 48 hours. The methanol extract was



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concentrated and diluted to 100 ml with water, prior to defatting with n-hexane. The methanol extract was then purified using ion-exchange chromatography with the Dowex 1X8 (Cl<sup>-</sup> form) packed columns. The column was eluted with 0.1 M NaCl (760 ml), 0.15 M NaCl (200 ml) and 0.2 M NaCl (800 ml) at a flow rate of 40 ml per hour. The eluate was monitored at 254 nm. The elution curve indicated that moniliformin was eluted in the 0.2 M NaCl fraction. The moniliformin fraction was freeze-dried, and further purified, using charcoal column chromatography. The moniliformin fraction (from the ion exchange column extraction) was dissolved in water, and applied to a column that was packed with decolourising carbon and charcoal previously washed with ethanol, saturated NaCl and water. The moniliformin was eluted with water at a flow rate of 20 ml per hour. An elution curve was developed by monitoring the fraction at 254 nm. The moniliformin fraction was collected and freeze-dried.

Burmeister *et al.* (1979) described a method to extract moniliformin from grits that were inoculated with a suspension of *F. moniliforme*. The fermented grits were extracted twice with methanol, and evaporated to dryness. The residue was dissolved in methanol, and added to four volumes of acetone to remove precipitable impurities. The solution was filtered, and the solvent evaporated.

The residue was re-dissolved in methanol and streaked onto preparative thin layer chromatography plates. The plates were developed in a solvent system containing toluene: acetone and methanol (5:3:2) and examined under ultraviolet-visible light at 254 nm. The zone containing the moniliformin was identified at a  $R_f$  value between 0.25 and 0.30. Silica gel containing the moniliformin, recovered from the plate was eluted with methanol. After charcoal filtration, the solution was reduced to 5 ml, and added slowly to 25 ml of diethyl ether and tiny crystals were observed along an amorphous, brownish precipitate. The crystals were re-chromatographed on thin layer chromatography plates and recrystallised from ether. The reported yield was 480mg of moniliformin from 1 kg of white corn grits.

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Dutton (1981) developed a liquid – liquid multitoxin assay for the separation of acidic, neutral and basic toxins. Initially, the contaminated samples were extracted with a polar solvent and small volumes of water to increase the penetration of the solvent system into the hydrophilic food surface. The slurry was filtered and further extracted for acidic and basic toxins, while the filtrate was extracted for the neutral toxins such as moniliformin.

Shepherd and Gilbert (1986) described an ion pairing extraction technique for the separation of moniliformin from maize samples. The method required extraction of the toxin into aqueous tetrabutylammonium hydroxide (TBAH) and removal of cations from this solution by ion-exchange chromatography. The solution was extracted with dichloromethane, further TBAH was added to the aqueous phase, which was adsorbed onto a hydrophilic matrix, and the tetrabutylammonium moniliformate ion pair extracted into dichloromethane. After evaporation of the organic eluent, the residue was dissolved in aqueous sodium chloride.

Lew *et al.* (1990) described the incidence of moniliformin in European corn. Moniliformin was extracted from the corn samples by extracting 10 g of the ground sample with acetonitrile/water (7:3), followed by filtration. Prior to centrifugation, 50 ml of the filtrate was extracted with chloroform. Thereafter, 10 ml aliquot of the aqueous phase was applied to a Sep-Pak C18 cartridge. Moniliformin passed through the cartridge.

The eluate was evaporated to dryness and re-dissolved in methanol: water (2:1). An acetone precipitation with 50 ml acetone removed interfering acetone-insoluble substances. The filtrate was evaporated and re-dissolved in 500  $\mu$ l methanol for further analysis.

Filek and Lindner (1996) reported the use of strong anion exchange (SAX, 500 mg) SPE columns for the separation of moniliformin from maize and cereals. Initial studies involved the use of mixed bed adsorbents of C18 and SAX material. However, this extraction method was extremely cost intensive. The use of SAX

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columns only was reported to have clean up effects and recoveries, similar to that of using the SAX column only. The method involved extracting the samples with acetonitrile-water (9:1). Ten millilitres of the extract was evaporated to dryness. The residue was dissolved in methanol, degassed by shaking with hexane, and then subjected the solid phase extraction cleanup. The analyte was eluted with 1M hydrochloric acid. The overall recoveries for spiked wheat samples were 70% in the 0.02 to 0.25 mg/kg range.

The general trend for the extraction of moniliformin from food commodities has been by using strong anion exchange (SAX) cartridges. The recoveries compared favourably to the other extraction procedures using Sephadex packed columns.

- **Ochratoxin A**

Zimmerli and Dick (1995) reported the use of immunoaffinity columns for the determination of ochratoxin A in blood, serum, and milk, and selected foodstuff (wine and beer).

Initially silica gel adsorbents were used, however, "serious problems emerged with blank values". The mean recovery of ochratoxin A from serum, blood, milk and selected foodstuff was estimated at 85% with an overall coefficient of variation of 10%.

Mycotoxin test kits have been used for the extraction of specific toxins from contaminated samples. These kits employ one of two methods: thin layer chromatography or immunochemistry. The advantages of the thin layer chromatography method are that several mycotoxins can be detected with a single kit, and immediate visualisation of the toxin, whereas the immunochemistry test kits require further analysis. However, the advantages of the immunochemistry test kits are their ease of use, and the short time required for the extraction, as compared to liquid-liquid extraction techniques (Smith and Henderson, 1991).

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The Vicam column for ochratoxin A analysis uses immuno-affinity chromatography technology. The column contains beads chemically fused to antibodies specific for ochratoxin A. A corn sample was ground and extracted with methanol: water solution. The extract was then run through the column and the ochratoxin A 'sticks' to the column by binding to the antibody beads. The other components in the corn do not interact with the beads and were washed off the column. Ochratoxin A was then removed from the column with methanol, and the extract was ready for thin layer chromatography, high performance liquid chromatography and capillary electrophoresis analysis. Vicam (1996) reported recovery values of 71 to 93% from ochratoxin A spiked coffee samples in the range 0.2 ng/ml to 34.5 ng/ml. The results showed that the percent recovery was higher in the lower concentration range, than in the higher concentration range, with a coefficient of variation of less than 13% throughout the assay range.

Corneli and Maragos (1998) extracted ochratoxin A from spiked coffee samples using solid phase extraction cleanup with a silica column followed by an ochratoxin affinity column. The recovery values for spiked coffee samples in the range 0.2 to 10 ng/ml were 76 to 107%, with a coefficient of variation of 14.1%.

Hurst *et al.* (1998) separated ochratoxin A from artificially contaminated cocoa beans. The sample preparation ranged from extraction of the sample in chloroform followed by a series of liquid-liquid extraction steps, to a direct extraction with a more polar material such as acetonitrile before solid phase extraction with C18 cartridges. Recoveries were conducted at 10, 20, 50 and 100 µg/kg and were determined to range from 87 to 106% from triplicate analysis.

Jorgenson (1998) surveyed pork, coffee, beer and pulses for ochratoxin A. Random samples were purchased from retail stores over the period 1994 to 1995. Beer, roasted coffee and pulses were extracted using immunoaffinity columns. For roasted coffee, the sample was initially extracted with sodium bicarbonate solution and filtered. Twenty millilitres of the extract was diluted with 20 ml of the buffer prior to application on ochratoxin prep columns, purchased from Rhone diagnostics. The recovery of ochratoxin A from spiking experiments were

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60 to 121% at the level 0.5 to 4  $\mu\text{g/kg}$  ( $n=48$ ) for pork samples, 66 to 113% at the level 0.3  $\mu\text{g/kg}$  ( $n=12$ ) for beer, 59 to 83% at the level 5  $\mu\text{g/kg}$  ( $n=6$ ) for roasted coffee, and 78 to 103% at the level 5  $\mu\text{g/kg}$  ( $n=8$ ) for pulses. The results for the natural contamination of ochratoxin A were not corrected for recoveries.

Scott *et al.* (1998) reported surveyed Canadian human blood plasma for the presence of ochratoxin A. The method of extraction involved sample cleanup by C18 solid phase extraction column followed by immunoaffinity columns (Vicam ochratest column). The percent recovery was 85% (standard deviation = 9.7%) at the ochratoxin A level of 2 ng/ml ( $n=31$ ).

Skaug *et al.* (1998) described the extraction of naturally occurring ochratoxin A from human milk samples in Norway. The milk samples were extracted with methanol, chloroform and dilute HCl (1:1:0.1), and the chloroform extract passed through a commercial (Bond elute, Varian) 500 mg silica column. The silica gel was washed with 5 ml chloroform, prior to the application of the chloroform extract. The solvent was evaporated, and the residue was reconstituted in the high performance liquid chromatography mobile phase (10 mM TBAB in a methanol - potassium phosphate buffer, pH 7.5 mixture - (51: 49). The milk was naturally contaminated with ochratoxin A in the concentration range from 10 to 130 ng/ml. The mean recovery, calculated from spiked milk samples in the concentration range 10 to 500 ng/ml, was 75% (64 to 84% range,  $n = 22$ ). The paper reports that 2 to 26% of the samples contained more than 40 ng/ml ochratoxin A, which would cause a daily intake of ochratoxin A from human milk to exceed the suggested tolerance level in Norway (5 ng/kg body weight).

Trucksess (1998) compared the recovery values of ochratoxin A from the extraction of spiked maize samples by liquid-liquid extraction and immunoaffinity columns. Recoveries of the liquid-liquid extraction ranged from 58 to 114%, and recoveries for the immunoaffinity column from two sources, ranged from 58 to 114% for one column, and from 4 to 86% for the other. The between laboratory relative standard deviation was 34% for the liquid-liquid extraction, and 34 to 42% for the two immunoaffinity columns.

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The general trend for the extraction of ochratoxin A from maize has been by liquid-liquid extraction or sep-pak cartridges. The recoveries of the liquid-liquid extraction compare favourably to the sep-pak cartridge methods. The extraction of ochratoxin A from coffee has been by the immunoaffinity column.

### 1.4 Scope of the study

Currently, there is no methodology available for the simultaneous analysis of cyclopiazonic acid, ochratoxin A and moniliformin. Since the mycotoxin - producing fungi are major constituents of the mycoflora of corn, peanuts, cotton, seeds and other agricultural goods in South Africa; cyclopiazonic acid, moniliformin and ochratoxin A represent potential mycotoxin problems in these commodities, that needs to be thoroughly investigated. This study attempts to address this need, by evaluating the potential of several high performance liquid chromatography techniques and capillary electrophoresis for a multitoxin analysis.

Since the discovery of aflatoxins in 1961, normal phase thin layer chromatography, with silica gel plates has been the procedure of choice for mycotoxin analysis. Although the number of publications on new thin layer chromatography methods have declined in recent years, refinement of the technique continues to be published (Scott, 1993). Recent developments include bi-directional thin layer chromatography and 2-dimensional thin layer chromatography. These techniques, however, were not investigated as the techniques are not practical for screening of large number of samples (Lansden, 1986). Matsudo and Sasaki (1995) described oxalic acid treated silica gel plates to be the best for cyclopiazonic acid detection by thin layer chromatography analysis. However, Lansden (1984) indicated that the thin layer chromatography method may suffer from variation in plate-to-plate reproducibility and from inaccuracy due to inconsistent colour development. Therefore repeatability and reproducibility studies were performed for the toxin. Cyclopiazonic acid has been quantified by visual comparison with standard samples (Gorst-Allman and Steyn, 1979) or reflection densitometry (Lansden, 1986). The current study was

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performed using visual comparison only, as the equipment required for reflection densitometry was unavailable.

Various chromatographic modes, for example, reversed-phase, normal phase and ion exchange chromatography have been employed in mycotoxin analysis.

Of these techniques, reversed phase systems are the most commonly used technique for most analysis. Cyclopiazonic acid has been separated by reversed phase high performance liquid chromatography, using zinc sulphate in the mobile phase (Lansden, 1984). However this method required long column equilibrium time (four to six hours), and was thus not investigated. Normal phase conditions for the separation of cyclopiazonic acid by high performance liquid chromatography as described by Goto *et al.* (1987) was used instead. Moniliformin is a highly water soluble and polar compound that may be separated by ion exchange chromatography, reversed phase ion suppression or reversed phase paired ion chromatography. There are numerous problems associated with ion exchange chromatography, such as difficulties with repeatability of compound retention times and long analysis time (Poole and Poole, 1991). Therefore ion exchange chromatography was not used. The pKa of moniliformin is low (pKa = 1.70, Steyn *et al.*, 1978) and hence retention on a reversed phase column would be weak (Smith and Henderson, 1991). Thus analysis of moniliformin by high performance liquid chromatography was not amenable to ion suppression techniques. Therefore paired ion chromatography was the best choice for high performance liquid chromatography analysis of moniliformin (Thiel, 1978). Abarca *et al.* (1994) observed severe peak tailing and column binding for the analysis of ochratoxin A neutral eluents and a C-18 column. Hence, acid was added to the mobile phase to improve the analysis (Abarca *et al.*, 1994).

Capillary electrophoresis is a relatively new technique, with very limited publications in the field of mycotoxicology. Holland and Sepaniak (1993) described a successful multitoxin separation for several mycotoxins. The article focused on trends in the retention of these toxins, resulting from change in buffer composition and pH. Most of the data was generated on high concentration of

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mycotoxin standards ( $1 \times 10^{-4}$  M). In addition, the qualitative aspects of the analysis were evaluated on randomly generated mycotoxin-interferent mixtures, which were not justified on food and feed samples. The conditions described in this paper were optimised for the separation of cyclopiazonic acid, moniliformin and ochratoxin A.

A recent study on capillary zone electrophoresis described by Corneli and Maragos (1998) used laser induced fluorescent detector, with modifications to the excitation and emission wavelengths. This was not investigated, as the capillary electrophoresis instrument was equipped with a fixed excitation wavelength of 488 nm.

The acidic mycotoxins (cyclopiazonic acid and ochratoxin A) were extracted with the routinely used liquid-liquid extraction techniques described by Dutton *et al.* (1981). The use of extraction cartridges and mycotoxin test kits were also investigated, as good recoveries were reported by Vicam (1996).

From the literature review, there are no methods available for the simultaneous detection of cyclopiazonic acid, ochratoxin A and moniliformin. However, there are multitoxin high performance liquid chromatography and capillary electrophoresis methods available for other toxins, e.g., ochratoxin A, zearelenone and citrinin. Therefore, high performance liquid chromatography and capillary electrophoresis were the techniques targeted for a multitoxin assay. The aim of the study was to evaluate the potential of high performance liquid chromatography and capillary electrophoresis for a multitoxin assay, and to optimise the technique that appeared superior.

The project was divided into three phases:

- Current methods for the analysis of cyclopiazonic acid, ochratoxin A and moniliformin were set up, and the repeatability, reproducibility, linearity range and detection limit for the techniques established (**Chapter 2**).



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- High performance liquid chromatography and capillary electrophoresis multitoxin assays were developed, and the technique that appeared superior was optimised. The detection limits, linearity range, repeatability and reproducibility of the multitoxin method to the individual methods were compared (**Chapter 3**).
- The applicability of the developed method using established extraction methods on spiked samples and naturally contaminated samples were established (**Chapter 4**).

In conclusion the experimental work presented and recommendations for future research are presented in **Chapter 5**.

## VALIDATION OF INDIVIDUAL ASSAYS FOR THE ANALYTICAL MEASUREMENT OF CYCLOPIAZONIC ACID, MONILIFORMIN AND OCHRATOXIN A

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### 2.1 Introduction

The work reported in this section involved the setting up of thin layer chromatography and high performance liquid chromatography systems from literature as described by Abarca *et al.* (1994), Faber (1988) and Goto *et al.* (1987), for the three toxins, and to establish the repeatability, reproducibility, linearity and detection limits of the existing methods. The sensitivity obtained for each toxin using the thin layer chromatography method was compared to that obtained using the high performance liquid chromatography system for the corresponding toxin. Ultraviolet-visible spectrophotometric analysis was performed to confirm the  $\lambda_{\max}$  for the toxins.

### 2.2 Materials and methods

#### 2.2.1 Ultraviolet-visible spectrophotometric analysis of cyclopiazonic acid, moniliformin and ochratoxin A standards

The optimum wavelength for each of the three toxins was established using the Varian DMS 100 double beam ultraviolet-visible spectrophotometer. The cyclopiazonic acid, moniliformin and ochratoxin A standards (99.99% purity) were purchased from Sigma (St. Louis, United States of America). The details of the ultraviolet – visible spectrophotometer are described in Appendix 1.

- Mycotoxin standards: All stock solutions were stored at  $-20^{\circ}\text{C}$ , until required. The standards (1 mg or 5 mg) were diluted, in the vials with methanol to obtain a concentration of 1000  $\mu\text{g/ml}$ . These solutions were initially diluted 10-fold to prepare 100  $\mu\text{g/ml}$  of cyclopiazonic acid standard solution. The 100  $\mu\text{g/ml}$  standard solution was subsequently diluted 2-fold to prepare 50  $\mu\text{g/ml}$  cyclopiazonic acid standard.
- The absorbance spectrum for each toxin was obtained using the Varian DMS 100 double beam ultraviolet-visible spectrophotometry, with methanol as a reference solvent from 190 to 350 nm. The experimental  $\lambda_{\max}$  for each toxin

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was compared to that described by Pohland *et al.* (1982) and Rabie *et al.* (1978).

- The corresponding absorptivity constant (*a*) was calculated using the Beer's law equation  $A = abc$ , where the 'a', 'b', and 'c' terms are the absorbance values, path length (1cm) and the concentration of the toxin ( $\mu\text{mol l}^{-1}$ ) respectively.

### 2.2.2 Thin layer chromatography and high performance liquid chromatography analysis of cyclopiazonic acid, moniliformin and ochratoxin A

The details of the high performance liquid chromatograph and the reagent preparation for thin layer chromatography and high performance liquid chromatography analysis of cyclopiazonic acid, moniliformin and ochratoxin A are described in Appendix 1.

- **Thin layer chromatography analysis was performed using the following conditions:**
- cyclopiazonic acid was analysed using the methods described by Goto *et al.* (1987), with toluene:ethyl acetate: formic acid (5:4:1) as the developing solvent, pre-coated silica gel plates (Merck, South Africa), previously sprayed with 2% oxalic acid as the stationary phase, and Ehrlich's reagent as the visualising reagent. The plates were air-dried and viewed under ultraviolet light (254 nm) as a purple spot.
- moniliformin was analysed using the method described by Faber (1988), with methanol: chloroform (2:3) as the mobile phase and pre-coated silica gel plates (Merck, South Africa) as the stationary phase, with 2,4 dinitrophenylhydrazine as the visualising agent. The plates were air-dried and viewed under ultraviolet light (254 nm) as a yellow spot.
- ochratoxin A was analysed using the method described by Abarca *et al.* (1994), with toluene:ethyl acetate: formic acid (6:3:1) as the mobile phase, and pre-coated silica gel plates (Merck, South Africa) as the

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stationary phase. The plates were air-dried and viewed as a fluorescent green spot at 366 nm.

The repeatability, reproducibility and detection limit studies for each of the toxins were performed with respect to the  $R_f$  values and thin layer chromatography spot intensity as follows:

- The repeatability studies were validated by performing twenty consecutive analyses with 5  $\mu$ l of 50  $\mu$ g/ml of commercial standard.
  - The reproducibility data for the toxins were collected over a period of ten days, using 5  $\mu$ l of fresh 50  $\mu$ g/ml standard solutions.
  - The average and the percent relative standard deviation (% RSD) for the repeatability and reproducibility data was calculated using the Microsoft Excel program.
  - The detection limit was visually determined by spotting 5  $\mu$ l of each of the 100, 75, 50, 25, 20, 15, 10, 5, 4, 3, 2, 1, and 0.1  $\mu$ g/ml standard on a thin layer chromatography plate. The intensity of the thin layer chromatography spot was graded, and the lowest standard concentration that produced a spot was the detection limit for the technique.
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- **High performance liquid chromatography**

High performance liquid chromatography analysis was performed using the Waters 600E system controller with a Gilson 401C autosampler and a Waters photodiode array detector connected to a computer with the Waters 991 computer package. The sample volume was 20  $\mu$ l. The mobile phase flow rate was 1 ml per minute.

Cyclopiazonic acid, moniliformin and ochratoxin A standards were diluted as described for the thin layer chromatography analysis. In addition, the following standards were prepared in methanol:

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cyclopiazonic acid : 1, 0.8, 0.7, 0.6, 0.5, 0.25, 0.1 and 0.05 µg/ml

ochratoxin A : 1.0, 0.5, 0.25, 0.13, 0.02, 0.01, 0.005 and 0.001 µg/ml

moniliformin : 5, 2.5, 1.25, 0.63, 0.31, 0.15, 0.10 and 0.05 µg/ml .

Cyclopiazonic acid was analysed using the method described by Abarca *et al.* (1994), using normal phase conditions with ethyl acetate: isopropyl alcohol: (25%) ammonia, (55: 20: 5) as the mobile phase, with the Lichrosorb 10 Si (25 cm x 4.6 mm) column and ultraviolet-visible detection at 284 nm, as no peaks were observed with the photodiode array scan.

Ochratoxin A and moniliformin were analysed using reversed phase conditions with a methanol: isopropyl alcohol: H<sub>2</sub>O (pH 2), (59: 7: 35) mobile phase for ochratoxin A and 10 mM phosphate buffer with 5 mM tetrabutylammonium bromide, pH 7.0 : methanol, (92: 8) as the eluent for moniliformin. The C18: Phenomenex Prodigy 5 ODS-2 (150 mm x 4.6 mm, 5 µm) column was used for ochratoxin A and moniliformin analysis, and detection occurred at 229 nm and 215 nm, respectively.

The repeatability, reproducibility, linearity and detection limit studies for each of the toxins were performed as follows:

- Repeatability studies were conducted to determine the consistency of the retention time and peak area for the cyclopiazonic acid standard by performing twenty consecutive injections of twenty microlitres of 1 µg/ml cyclopiazonic acid and monitoring the retention time and the area count for toxin peak. Reproducibility studies were investigated by injecting fresh samples of twenty microlitres of standard solutions in ten separate analyses over a ten-day period, under the same conditions. The percent RSD values for the retention time and area of the toxin peak were calculated using the Microsoft Excel program.
- Linearity was determined by calculating the R-squared values, using the Microsoft Excel program, for the graph of peak area versus concentration for the toxins. The lowest concentration that produced a peak area that was

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three times the signal of the noise level was the detection limit for the toxin (Ravindranath, 1989).

## 2.3 Results and discussion

### 2.3.1 Analysis of ultraviolet-visible absorbance data

The ultraviolet-visible absorbance spectra of cyclopiazonic acid, moniliformin and ochratoxin A (Figure 2.1) showed distinct  $\lambda_{\max}$  values for each of the three toxins. The experimental  $\lambda_{\max}$  and corresponding absorptivity constant ( $a$ ) compared favourably to the reported values for the toxins (Table 2.1); hence the toxins were of very high purity. All subsequent ultraviolet-visible detection of cyclopiazonic acid, moniliformin and ochratoxin A was monitored at 280 nm, 229 nm and 215 nm respectively.

**Table 2.1: Wavelength with corresponding absorptivity constant ( $a$ ) for 50  $\mu\text{g/ml}$  solutions of cyclopiazonic acid, moniliformin and ochratoxin A in methanol**

Toxins *	Wavelength ( $\lambda_{\max}$ ) (nm)		Absorptivity constant ( $a$ ) ( $\mu\text{mol l}^{-1}\text{cm}^{-1}$ )		References
	Calculated	Reported	Calculated	Reported	
CPA	222	224	1970	1972	Pohland <i>et al.</i> (1982)
	280	275			
	(shoulder)	(shoulder)			
MON	229	229	17919	18 000	Rabie <i>et al.</i> (1978)
	260	260			
OA	215	214	3702	3720	Pohland <i>et al.</i> (1982)
	332	333			

\* CPA = cyclopiazonic acid, MON = moniliformin, OA = ochratoxin A

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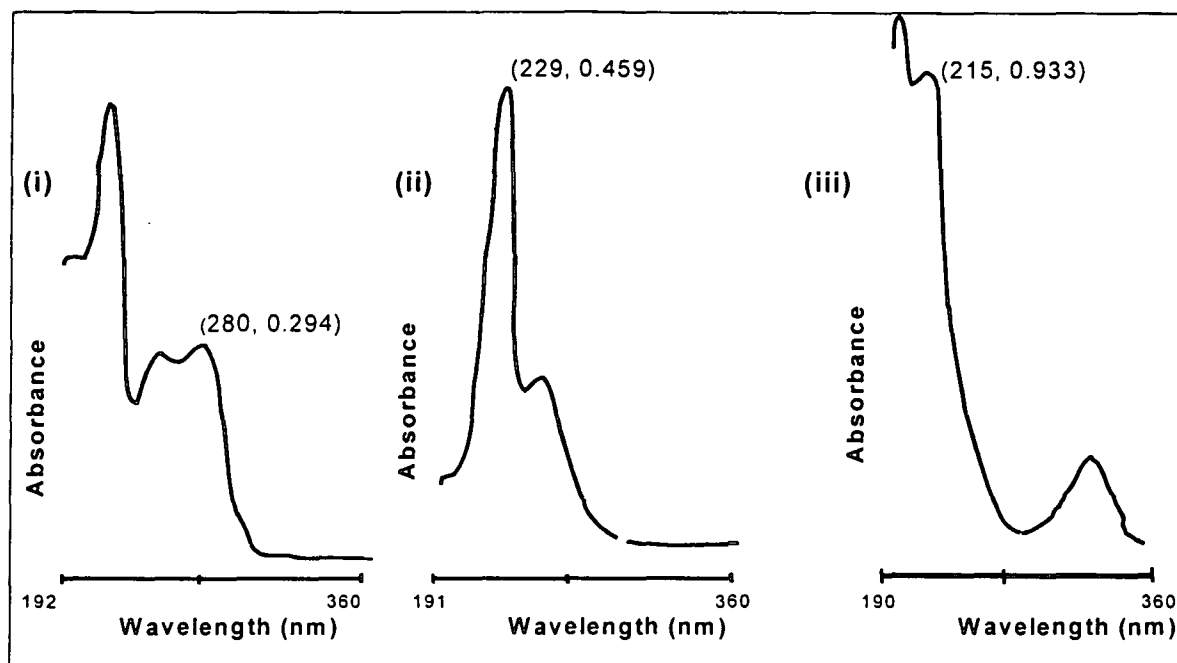


Figure 2.1: Ultraviolet spectra of (i) 50  $\mu\text{g/ml}$  solution of cyclopiazonic acid, (ii) 6.25  $\mu\text{g/ml}$  solution of moniliformin and (iii) 50  $\mu\text{g/ml}$  solution of ochratoxin A in methanol. Analysis conditions as described in Appendix 1.

### 2.3.2 Analysis of thin layer chromatography and high performance liquid chromatography data

- Thin layer chromatography of cyclopiazonic acid

Cyclopiazonic acid was viewed as a purple spot under ultraviolet light (254 nm), after spraying with Ehrlich reagent. The experimental  $R_f$  values for cyclopiazonic acid was 0.621, which showed close correlation with the reported value of Dorner *et al.*, (1983) and Goto *et al.*, (1987), which were 0.68 and 0.63, respectively. In addition, plates that were not treated with oxalic acid, gave the same colour reaction, but extensive tailing was observed, as also observed by Dorner *et al.* (1983).

The repeatability and reproducibility data showed excellent consistency, with an average  $R_f$  values of 0.621 for repeatability and 0.624 for reproducibility. The

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percent relative standard deviation was 0.69 % and 1.60 % for repeatability and reproducibility, respectively.

The intensity of the thin layer chromatography spot decreased proportionally with decreasing standard concentration from 100 µg/ml to 5 µg/ml. The lowest concentration detected using thin layer chromatography was 5 µg/ml for cyclopiazonic acid, which corresponds to 25 ng. This result compared favourably with the detection limit of 26 ng and 25 ng that was reported by Lansden and Davidson (1983), and Lansden (1986), respectively.

- **Thin layer chromatography of moniliformin**

Moniliformin was visualised as a yellow spot when viewed under ultraviolet light, after spraying with 2,4 dinitrophenol solution. The  $R_f$  value was 0.546, which was similar to the reported value of 0.53 (Rabie *et al.*, 1978).

The repeatability (average  $R_f$  = 0.546 with 1.30 % RSD) and day-to-day reproducibility (0.542 with 1.59 % RSD) data showed excellent consistency.

The linearity of the method was examined from 5 µg/ml to 100 µg/ml. The lowest concentration detected visually by thin layer chromatography was 25 µg/ml for moniliformin, which corresponds to 125 ng. This result compared favourably with the detection limit of 100 ng reported by Burmeister *et al.* (1979) and Chelkowski *et al.* (1990) using densitometers. The slight deviation in results could be due to visual detection of the toxins, which is prone to errors (Smith and Henderson, 1991).

- **Thin layer chromatography of ochratoxin A**

On the thin layer chromatography plates that were air-dried, ochratoxin A was viewed as a green spot at 366 nm, with a  $R_f$  value of 0.452. This is in keeping with the  $R_f$  value of 0.40 reported by Howell and Taylor (1981).



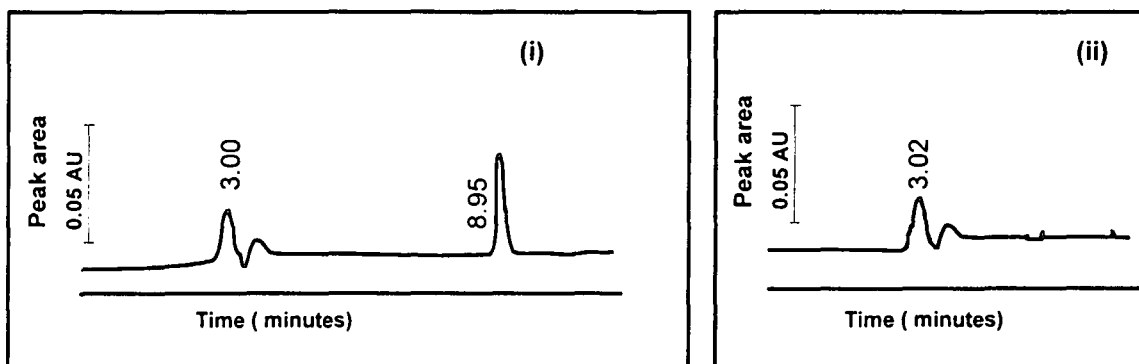
## VALIDATION OF INDIVIDUAL ASSAYS

Repeatability (average 0.453 with % RSD = 1.59) and reproducibility (average 0.452 with % RSD = 1.32) data showed excellent stability.

The method was linear in the range 0.40  $\mu\text{g/ml}$  to 100  $\mu\text{g/ml}$ , with the minimum detectable amount of analyte being 0.40  $\mu\text{g/ml}$ , or 2 ng. This result is in keeping with that described by Kumagai and Arbaca (1994) – 2.5 ng.

- **High performance liquid chromatography of cyclopiazonic acid**

The high performance liquid chromatography chromatogram of cyclopiazonic acid showed peaks at retention time of 3.00 minutes and 8.95 minutes (Figure 2.2a). Injections of a range of cyclopiazonic acid standards indicated that the peak at 8.95 minutes was concentration dependent. In addition, injection of the solvent (methanol) produced a peak at 3.00 minutes (Figure 2.2b). Hence, cyclopiazonic acid was monitored at a retention time of 8.95 minutes.



**Figure 2.2: (i) Chromatogram obtained for the analysis of 5  $\mu\text{g/ml}$  cyclopiazonic acid in methanol, and (ii) blank (methanol).**

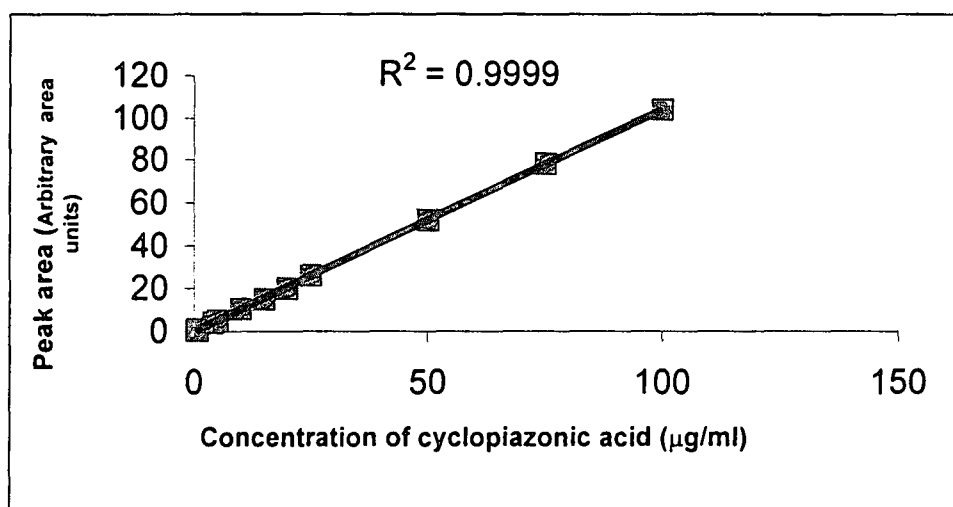
[Analysis conditions : Ethyl acetate, isopropyl alcohol, ammonia (25 %), Lichrosorb 10 Si (25 cm x 4.6 mm) column, 20  $\mu\text{l}$  sample injection, UV-280 nm detection. Flow rate = 1 ml per minute.]

The percentage relative standard deviation for the twenty replicate injections indicated that the method was consistent (1.76 % and 0.82 % for the peak area and retention time, respectively). The reproducibility data also showed excellent

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stability and consistency (1.81 % and 0.84 % for the peak area and retention time, respectively).

The retention times of the standard solutions were consistent and the area for each standard concentration increased proportionately for the concentration range 0.25 µg/ml to 50 µg/ml, as shown by the linear graph (Figure 2.3), with the R-squared value equal to 0.9999. These results are in keeping with the linear response described by Prasonsindh *et al.* (1997). The lowest detectable concentration of cyclopiazonic acid was found to be 250 ng/ml or 5 ng on column, at 280 nm. This result compared favourably with the detection limit of 4 ng reported by Lansden (1984). In addition, Sobolev *et al.* (1998) reported a detection limit of 250 ng/ml or 5 ng for cyclopiazonic acid at 280 nm.

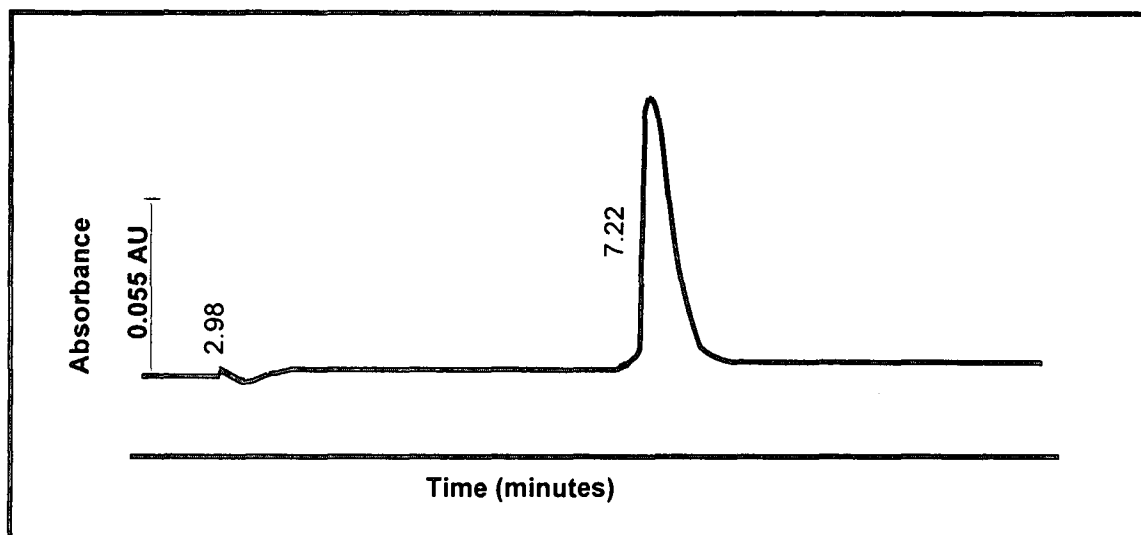


**Figure 2.3: Calibration curve for cyclopiazonic acid standard solutions at 280 nm.**

- **High performance liquid chromatography for moniliformin**

There was minimum absorbance of the solvent (methanol) for moniliformin, as was evident from the very slight deviation from baseline at 2.98 minutes for moniliformin (Figure 2.4). The retention time for the moniliformin peak was 7.22 minutes.

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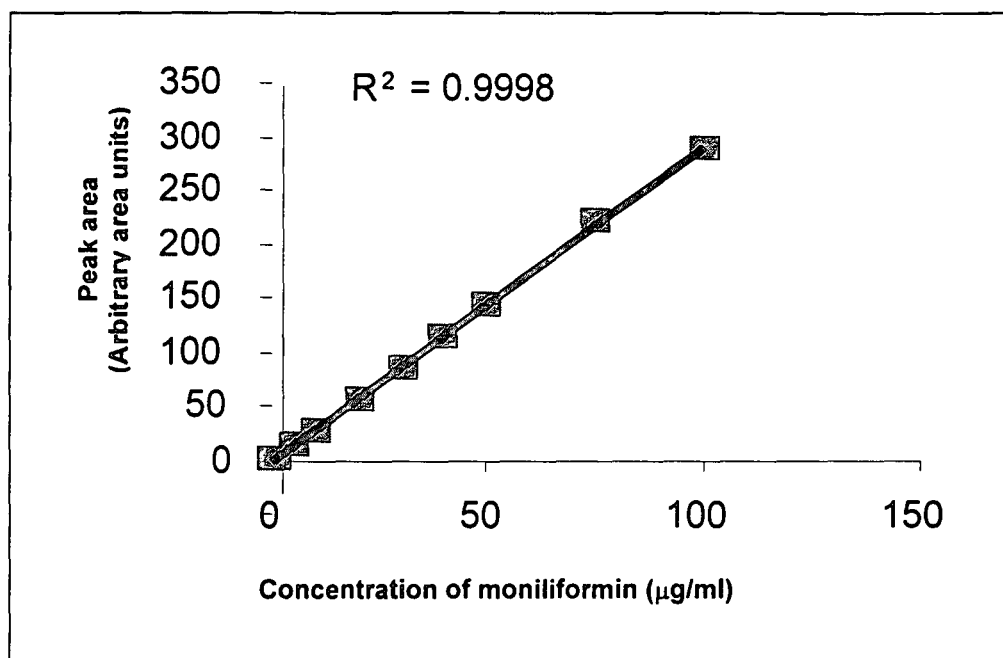
**Figure 2.4: Chromatogram obtained for the analysis of 5  $\mu\text{g/ml}$  moniliformin in methanol.**

[Analysis conditions : Methanol, isopropyl alcohol, water (pH 2), C18: Phenomenex Prodigy 5 ODS-2, (150 mm x 4.6 mm) column, 20  $\mu\text{l}$  sample injection, UV-229 nm detection. Flow rate = 1 ml per minute.]

Repeatability data showed excellent consistency with 1.85 % RSD for the peak area and 0.75 % RSD for the retention time. The day-to-day reproducibility for a ten-day period was 1.90 % RSD for the peak area and 0.71 % for the retention time.

Calibration curve for the range 10 ng/ml to 100  $\mu\text{g/ml}$  was performed, and was linear with a correlation coefficient of 0.9998 (Figure 2.5). The limit of detection (smallest analyte detected at three times the base line noise) was 20 ng/ml at 229 nm, which corresponds to 0.4 ng of moniliformin. Rabie *et al.* (1982) reported a detection limit of 1 ng. The loss of sensitivity may be due to band broadening effects that resulted from the use of a longer column (30 cm), used by Rabie *et al.* (1982), compared to 15 cm used in the present study. In addition, it is highly probable that the difference in sensitivity is a result of improved detector design over the last 20 years or of column packing material.

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**Figure 2.5: Calibration curve for moniliformin standard solutions in methanol at 229 nm.**

- **High performance liquid chromatography for ochratoxin A**

There was minimum absorbance of the solvent (methanol) for ochratoxin A, as was evident from the very slight deviation from baseline at 3.15 minutes for ochratoxin A. The retention time for ochratoxin A was 5.22 minutes (Figure 2.6). The repeatability data showed excellent consistency with 0.91 % RSD for the peak area and 1.04 % RSD for the retention time. The day-to-day reproducibility for a ten-day period was 0.86 % RSD for the peak area and 1.03 % for the retention time.

The method was linear from 0.31 µg/ml to 100 µg/ml, with the correlation coefficient of 0.9998 (Figure 2.7). The detection limit was 0.31 µg/ml or 0.62 ng on column, at 215 nm. This result could not be compared to the literature result, as previous workers do not report the detection limit using ultraviolet-visible detection. Howell and Taylor (1981) reported a detection limit of 0.05 ng with a fluorescence detector at excitement wavelength 333 nm and emission of 470 nm.

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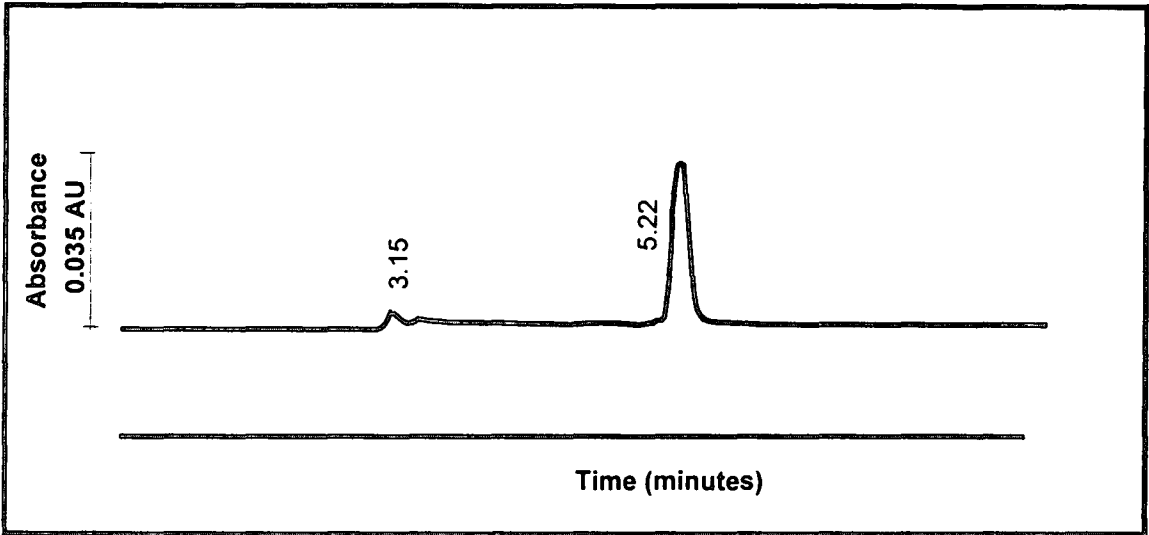


Figure 2.6: Chromatogram obtained for the analysis of 5 µg/ml ochratoxin A in methanol.

[Analysis conditions : 10 mM phosphate buffer with 5 mM tetrabutylammonium bromide (pH 7), methanol (92:8), C18: Phenomenex Prodigy 5 ODS-2, (150 mm x 4.6 mm) column, 20 µl sample injection, UV-215 nm detection. Flow rate = 1 ml per minute.]

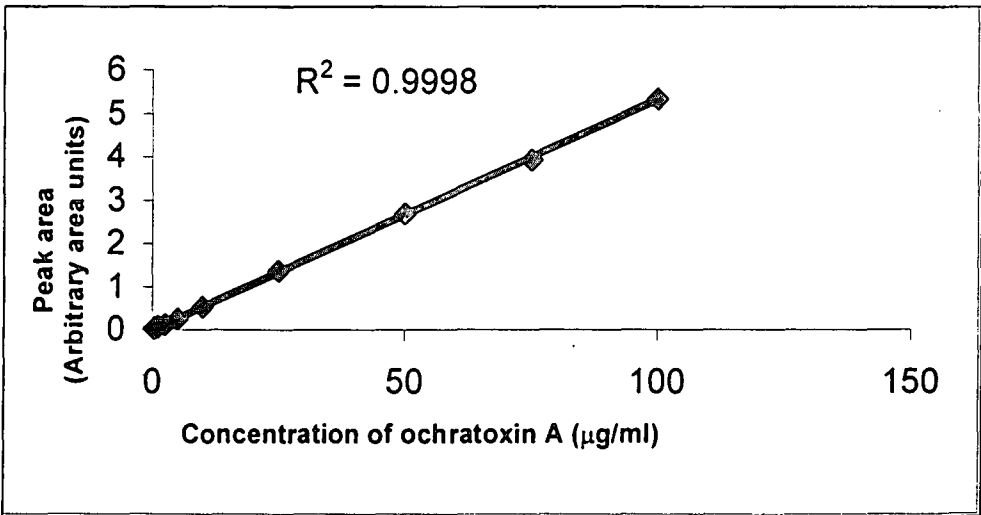


Figure 2.7: Calibration curve for ochratoxin A standard solutions in methanol at 215 nm.

## 2.4 Conclusion

The thin layer chromatography and high performance liquid chromatography methods showed excellent linearity, repeatability and reproducibility for varying concentrations of cyclopiazonic acid, moniliformin and ochratoxin A.

Table 2.2 summarises the detection limits for the three toxins by thin layer chromatography and high performance liquid chromatography methods. The high performance liquid chromatography methods were significantly more sensitive than the thin layer chromatography methods. This result was expected, since the high performance liquid chromatography analysis was performed using high sensitivity detector systems as compared to visual detection by thin layer chromatography. Hence, thin layer chromatography was not investigated any further.

**Table 2.2 : Detection limit for cyclopiazonic acid, moniliformin and ochratoxin A by thin layer chromatography and high performance liquid chromatography**

Mycotoxin	Detection limit	
	Thin layer chromatography	High performance liquid chromatography (on-column)
Cyclopiazonic acid	25 ng	5 ng, at 280 nm
Moniliformin	125 ng	0.4 ng, at 229 nm
Ochratoxin A	2.0 ng	0.62 ng, at 215 nm

The sensitivity of the high performance liquid chromatography methods for the toxins compare favourably to that described in the literature, and was thus adopted for the study, without any further modifications.

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### 3.1 Introduction

The aim of the work in this chapter is to evaluate the potential of high performance liquid chromatography and capillary electrophoresis for the separation of a mixture of cyclopiazonic acid, moniliformin and ochratoxin A.

### 3.2 Multitoxin high performance liquid chromatography

The work reported in this section involved the development of a multitoxin high performance liquid chromatography assay for the separation of cyclopiazonic acid, moniliformin and ochratoxin A by evaluating several systematic method development schemes, based on the various high performance liquid chromatography techniques. The individual high performance liquid chromatography methods were investigated for a multitoxin high performance liquid chromatography assay.

In developing a multitoxin high performance liquid chromatography method, reversed-phase chromatography was initially explored, due to its convenience, wide applicability, and good understanding of operating principles. However, if reversed phase chromatography should fail, due to the diverse nature of the toxins, then alternative strategies such as paired ion chromatography, normal phase and ion chromatography could also be used.

In paired ion chromatography, an organic acid is added to the mobile phase to produce an ion pair complex with the analyte. Two theories are possible, viz., ion pair formation and dynamic ion exchange. In ion-pair formation, the resulting complex behaves as an electrically neutral species, and is separated by standard reversed phase method. In dynamic ion exchange, the ion –pair formation may be described as a process in which the ion pairing reagent is held on the column to create an *in situ* ion exchanger. Thus, the charge on the surface controls the retention and selectivity (Poole and Poole, 1991).

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This charge is increased by any process that increases the column loading. Increasing the concentration of the reagent will shift the equilibrium towards the column, thereby increasing the charge on the column. A long chain ion-pairing reagent will favour partitioning onto the column, thus increasing the charge. Similarly, reducing the mobile phase level of organic solvent will shift the equilibrium towards the column. Therefore, the factors that affect column loading are ion pairing reagent, mobile phase pH, organic solvent concentration in the mobile phase and column temperature. The choice of an ion pairing reagent is not very important, i.e., changing reagent to change selectivity provides little benefit. The concentration of the mobile phase additives are generally 10 to 100 mM. At lower concentrations of ion pairing reagent, (less than 10 mM), small changes in concentration can make relatively large changes in the system performance, but may be too small to be effective. At levels greater than 100 mM, solubility becomes a concern, especially with acetonitrile in the mobile phase. The pH of the mobile phase controls the charge on the sample molecules (Gloor and Johnson, 1977). Generally, it is best to stay at least one pH unit away from the pKa of the compounds to avoid the problems that can occur when a sample molecule is partially ionised. Due to the use of a reversed phase column, mixed retention mechanism occurs (Gloor and Johnson, 1977). Some of the sample molecules will be retained by ion exchange (ion pairing) processes, whereas others will be retained by reversed phase. The result is that the organic-solvent concentration affects the separation in two ways. For non-polar molecules, higher organic solvent levels will reduce retention in the normal reversed-phase pattern. For molecules that are retained by ion exchange, higher organic solvent levels will reduce the concentration of ion pairing reagent on the column, and thus the charge on the column, also resulting in shorter retention times.

The slow equilibrium of the ion pairing reagent on the column means that using gradient elution with ion pairing separations creates an unstable system. Therefore, gradient ion pairing separations are not recommended.



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The choice of organic solvent can affect the selectivity in ion pairing, just as it does in standard reversed phase separations. Acetonitrile is usually the first choice for reversed phase separations, due to its usefulness at low wavelengths and lower viscosity. However, methanol is often the solvent of choice for ion pairing separations because of its superior solubilisation characteristics, compared with acetonitrile. Ion pairing often calls for relatively high concentrations of mobile phase additives, so the risk of precipitation is reduced when using methanol.

The expected change in retention, mobile phase viscosity (which translates to back pressure changes), and selectivity hold for ion pairing just as they do in reversed phase separations. In addition, temperature affects the equilibrium of the ion-pairing reagent between the mobile phase and the column. The temperature can be an important factor in peak shape for ion pairing separations, a phenomenon that does not occur in reversed phase separations. Due to the sensitivity of the system equilibrium to temperature, the column should always be thermostated at approximately 35 – 40 °C.

A technique that has also been explored for the separation of toxins is normal phase chromatography (Goto *et al.*, 1987; Sobolev *et al.*, 1998). A change to normal phase high performance liquid chromatography may be able to separate the toxins, since different retention processes provide different selectivity effects.

In normal phase high performance liquid chromatography, the sample retention is governed by adsorption to the stationary phase. For retention to occur, a sample molecule must displace one or more solvent molecules from the stationary phase. In addition to the displacement effect, polar solvent or sample molecules may exhibit very strong interactions with particular sites on the stationary phase, termed the localisation effect. These two effects, viz., displacement and localisation, are the primary sources of mobile phase selectivity in normal phase high performance liquid chromatography. The primary mobile phase properties which control solvent selectivity have been summarised by Snyder and Kirkland (1988).

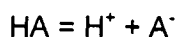
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This classification was based on nonpolar solvent as the diluent (hexane), plus three polar organic solvents non-localising (methylene chloride), basic localising (ethyl ether) and nonbasic localising (ethyl acetate) to vary band spacing for the compounds. Change in column temperature had essentially no effect in normal phase separations (Snyder and Kirkland, 1988).

However, a change from one polar bonded phase to another (e.g., the cyano-group), has a significant effect on band spacing, since interaction between sample molecules and functional groups on the surface of the column packing play a dominant role in determining separation selectivity. Therefore, the two parameters investigated were solvent strength and stationary phase.

Ion exchange chromatography can be used to separate organic acids (HA) that can exist as ions under suitable conditions of pH.



Varying the pH can control the extent of ionisation of acids and bases in an ion exchange chromatography system (Snyder and Kirkland, 1988).

Retention in ion exchange chromatography is usually controlled by varying the concentration of salt or buffer in the mobile phase. Compounds with different pKa values usually can be separated by varying the mobile phase pH to change band spacing. In addition, band spacing can be further altered by varying the temperature, and adding small amounts (5 to 20%) of different organic solvents (Dolan, 1996).

### 3.2.1 Materials and methods

All experiments were conducted with a mixture of 5 µg/ml cyclopiazonic acid, ochratoxin A and moniliformin in either methanol or the mobile phase. The chromatographic system used for all experiments was the Waters 600E system controller with a Gilson 401C autosampler and a Waters photodiode array detector connected to a computer with the Waters 991 computer package. The sample volume was 20 µl. The mobile phase flow rate was 1 ml/minute.

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- **Preliminary investigations**

Multitoxin separation of a mixture of the three toxins was initially investigated using the methods described for the individual high performance liquid chromatography analysis of the three toxins (Chapter 2).

- **Reversed phase chromatography**

The method development strategy described by Kirkland (1996) for the separation of ionisable compounds was adapted for the current study. The strategy involved a series of three phases :

**Phase 1 :**

- The mixture of components was chromatographed using an organic solvent only as a mobile phase and a C18 column.
- The aqueous phase (water) was introduced into the mobile phase at varying concentrations.
- The system was run with a low pH-buffer (10 mM phosphate buffer), at varying organic concentration.

**Phase 2 :**

The column temperature was varied to produce band spacing.

The concentration of the organic solvent was increased to facilitate separation.

An alternate organic solvent was investigated.

**Phase 3 :**

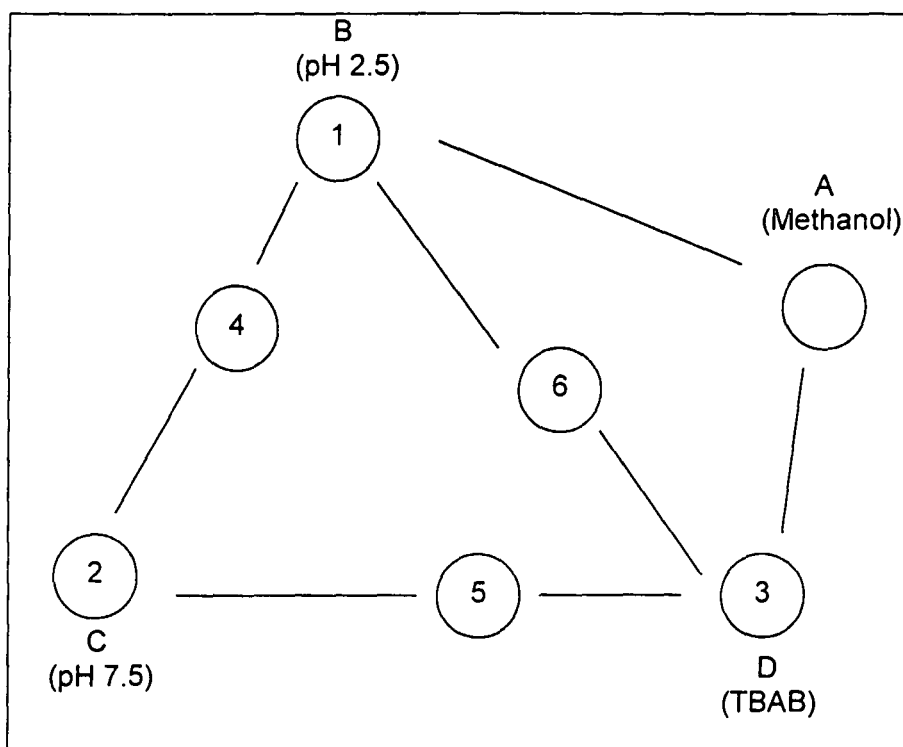
- The Lichrosorb 10 CN (reversed phase), (15 cm x 4.6 mm) column was connected and the separation phases 1 and 2 repeated.

- **Paired ion chromatography**

It was important to implement a systematic strategy for optimising an ion pairing separation, considering the variable. The scheme by Snyder *et al.* (1988) that presents a stepwise approach for optimising a separation was adopted for this study. Figure 3.1 shows the schematic diagram of the optimisation scheme. One organic solvent was used to optimise the separation. The other three compounds of the mobile phase being buffer pH 2.5 (B), pH 7.5 (C), and another buffer of pH

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5.5 containing 100 mM tetrabutylammonium bromide (TBAB), (D). Experiments were performed using different combinations of the buffers B to D, with the amount of methanol (A) varied to obtain the desired separation. The system requires a C18 column, operated at low flow rate (1 ml per minute) to maintain a reasonable backpressure and the column in a column heater. TBAB was used as the ion-pairing reagent.



**Figure 3.1:** Ion pairing method development strategy (Snyder *et al.*, 1988).

According to the strategy, the following phases were implemented in this study:

**Phase 1 :** Various mixtures of low pH buffers and methanol (follow the A-B line in Figure 3.1) was explored.

**Phase 2 :** Switched to the high pH buffer (C)

**Phase 3 :** Intermediate mixtures of mobile phases 1 and 2 to determine the optimum buffer pH (mobile phase 4) were explored.

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**Phase 4 :** Added an ion pairing reagent as a variable to the mobile phase. Explored various blends of the ion pairing reagent (D) with the various buffer systems.

**Phase 5 :** Adjusted column temperature from 25 to 40 °C.

- **Normal phase chromatography**

The selectivity differences available from either reversed-phase or ion-pairing high performance liquid chromatography were insufficient to provide adequate separation of the toxins. Therefore, normal phase chromatography was explored as follows :

**Phase 1:** Varied solvent strength

The mixture was analysed using 100 % hexane only. Various compositions of methylene chloride, ethyl ether and ethyl acetate with hexane was explored for the separation of the three toxins.

**Phase 2 :** Determined the selectivity effects

Performed experiments using different “binary solvent mixtures”. Investigated the effects of flow rate of the mobile phase and column configuration. Investigated the effects of adding additives e.g., TBAB to the mobile phase.

- **Ion exchange chromatography**

There is no recommended set strategy for ion exchange separations. The factors that affect ion exchange chromatography were systematically altered as outlined:

**Phase 1**

Investigated the effect of a change in ionic strength of the mobile phase on sample retention by altering the buffer concentration.

**Phase 2**

Investigated pH effect of the mobile phase.

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### Phase 3

Methanol was added to the mobile phase as the organic solvent.

### Phase 4

Different organic solvents, acetonitrile and ethanol were each added to the mobile phase to change the selectivity of the buffer.

### Phase 5

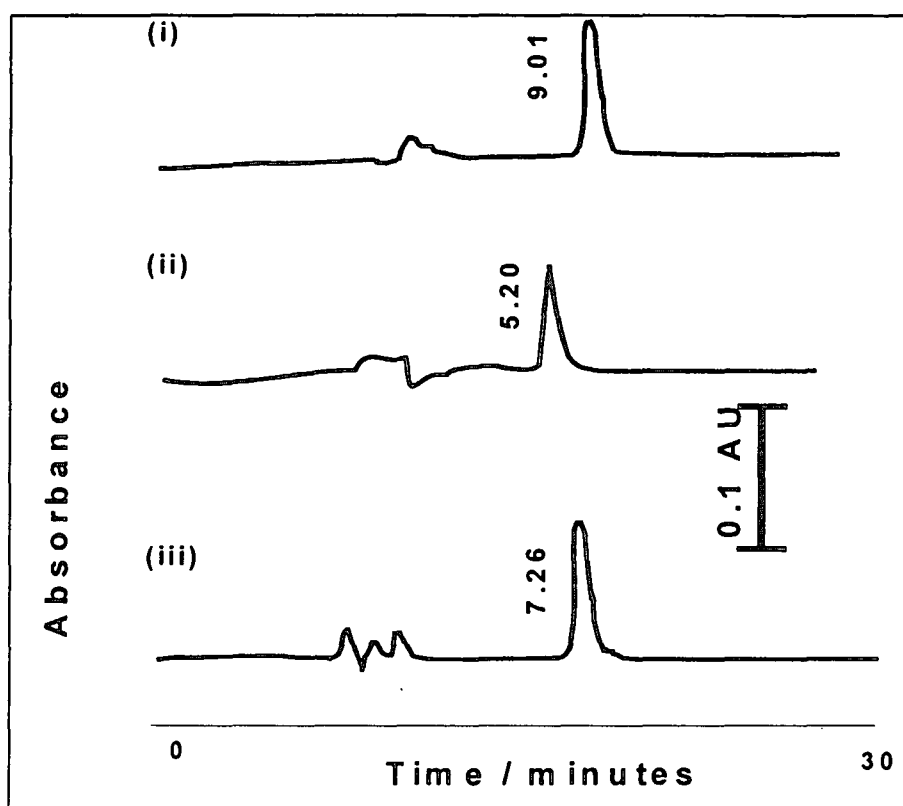
Ion exchange chromatography separations were carried out between ambient temperature and 60 °C.

### 3.2.2 Results and discussion

Preliminary investigations revealed that the individual high performance liquid chromatography methods for cyclopiazonic acid, ochratoxin A and moniliformin were unsuccessful at separating the mixture of toxins. Chromatograms (Figure 3.2) obtained showed poor resolution and distorted peak shapes for all three methods. Spiking experiments indicated that the peaks with retention times 9.01, 5.20 and 7.26 minutes (Figure 3.2) corresponded to cyclopiazonic acid, ochratoxin A and moniliformin, respectively.

Filek and Lindner (1996) reported that due to the polar and ionic nature of the toxins, the selective isolation from different matrix compounds and the chromatographic handling of these analytes presents a major problem. Hence, a method development scheme was investigated.

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**Figure 3.2** Chromatogram obtained for the analysis of a mixture of 5  $\mu\text{g/ml}$  (i) cyclopiazonic acid, (ii) ochratoxin A, and (iii) moniliformin in methanol.

[Analysis conditions : Flow rate = 1 ml per minute.

(i) Ethyl acetate, isopropyl alcohol, ammonia (25 %), Lichrosorb 10 Si (25 cm x 4.6 mm) column, 20  $\mu\text{l}$  sample injection, UV-280 nm detection.

(ii) 10 mM phosphate buffer with 5 mM tetrabutylammonium bromide (pH 7), methanol (92:8), C18: Phenomenex Prodigy 5 ODS-2, (150 mm x 4.6 mm) column, 20  $\mu\text{l}$  sample injection, UV-215 nm detection.

(iii) Methanol, isopropyl alcohol, water (pH 2), C18: Phenomenex Prodigy 5 ODS-2, (150 mm x 4.6 mm) column, 20  $\mu\text{l}$  sample injection, UV-229 nm detection.]

- **Reversed phase chromatography**

A C18 column was used to investigate the mobile phase conditions necessary for the separation of the three toxins. A C18 column was reported to provide the best peak shape, long term reproducibility, and repeatability between column batches [Kirkland *et al.*, 1993, and Kohler and Kirkland, 1987]. Acetonitrile was used as the initial organic solvent, because of its low viscosity, strong elution power and excellent low wavelength ultraviolet transmittance (Kohler and Kirkland, 1987).

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The chromatograms for the mixture showed poor retention, with peaks that eluted at the void. This implied that there was poor interaction between the stationary phase and the sample. Hence, the polarity of the mobile phase was increased (by incorporating water) to facilitate separation. However, there was no improvement in the retention of the compounds. Low pH buffers (10 mM phosphate buffer, at pH 2.5 to 4.5) were investigated first since better reversed phase separations are expected, because the ionisation of column silanols and many acids is suppressed. Further, operating at low pH should produce well shaped, narrow peaks for most acidic compounds (Snyder *et al*, 1988). Thereafter, buffers with higher pH were evaluated (10 mM phosphate buffers, pH 6.5 to 11). The addition of phosphate buffer to the mobile phase was not successful in improving resolution or peak shape. In addition, introducing organic solvent to the buffer system, by the use of gradient elution showed no improvement to the chromatography. Lansden (1984) reported similar problems (poor resolution and severe peak tailing), using gradient elution for the analysis of cyclopiazonic acid in peanuts by reversed phase chromatography. The variation in organic-aqueous mobile phase ratio was unsuccessful at separating the three toxins.

For the investigation of column temperature and an alternate organic solvent (Phase 2), the column temperature was varied from 25 to 45 °C. Altering the column temperature was unsuccessful at separating the mixture of toxins. Increasing the concentration of the acetonitrile from 10 to 90 percent, at different column temperatures showed no improvement in the separation. Methanol was used as an alternate organic solvent. Changing to methanol as the organic solvent did not show any change in the selectivity.

The change in temperature, organic solvent composition and methanol as an alternate solvent was unsuccessful at separating the mixture of toxins. A cyanopropyl column (Phase 3) was tested as an alternate column to the C18 column. The increase in polarity of the stationary phase implies that there would be stronger interaction between the polar toxins and the stationary phase, hence assisting in the resolution of the mixture of toxins. However, this phenomenon



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was not observed. The cyanopropyl column was unsuccessful at separating the mixture of compounds.

In developing a multitoxin assay, by using the strategy described by Kirkland (1996), modifications to organic solvent – buffer concentrations, column temperature (25 to 45 °C), methanol as an alternate organic solvent to acetonitrile, and using reversed phase column with cyano-functionality was unsuccessful in developing a multitoxin high performance liquid chromatography assay system.

Since sufficient retention was still not achieved using reversed phase chromatography, the addition of an appropriate ion-pair reagent, e.g., tetrabutylammonium bromide (TBAB), and changing the chemistry of the stationary phase was investigated. This would increase the retention of acidic compounds such as cyclopiazonic acid and ochratoxin A and hence produce the desired separation (Dolan, 1996).

- **Paired ion chromatography**

The effects of altering the organic solvent concentration and pH and the influence of the ion pairing reagent on the separation of the three toxins on the separation are as follows:

- Effect of organic solvent concentration and pH on the separation of the three toxins

Initial experiments (phases 1 to 3) to determine whether separation could be obtained without an ion pairing reagent was already performed in Section 3.1.2.1 as described by Kirkland (1996) in the optimisation strategy for reversed phase separation. The chromatograms obtained when the organic solvent composition and pH were adjusted showed poorly resolved peaks for all the experiments.

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- Influence of ion pairing reagent on the separation (phase 4)

The ion pairing reagent (tetrabutylammonium bromide) concentration was varied from 10 mM to 100 mM in the low, intermediate and high pH buffer systems. All

experiments produced chromatograms in which the peaks were poorly resolved. However, at buffer pH 7.0 to 7.5, and 20 mM tetrabutylammonium bromide concentration, there was a distinct peak. The toxin mixture was spiked with each of the three toxins, and the peak was identified as moniliformin.

- Effect of altering column temperature (phase 5)

Altering the column temperature from 25 to 45 degrees did not produce the desired separation.

Having followed the strategy for separation of ionisable compound using ion pairing reagent, it was found that by systematically varying the concentration of the ion pairing reagent and column temperature, no effect on the separation of the mycotoxins was produced. Only moniliformin was detected.

- Normal phase chromatography

The phases taken to separate the three toxins by normal phase high performance liquid chromatography were unsuccessful. The chromatograms showed poorly shaped peaks with little or no band spacing. Attempts to change to a cyano-normal phase column showed no change in selectivity.

The addition of TBAB into the mobile phase showed no change in band spacing. Sobolev *et al.* (1998) reported problems with adsorption chromatography for the separation of acidic and neutral metabolites produced by *Aspergillus flavus*. However, when TBAB was added to the mobile phase (heptane: 2-propanol butanol: water: tetrabutylammonium bromide, 2560:900:230:32:8), the chromatographic peaks changed and resolution of all components was obtained. However, in most of the chromatograms, one peak was predominant. When the mixture was spiked, the peak was identified as cyclopiazonic acid.

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All normal phase high performance liquid chromatography experiments were unsuccessful at separating a mixture of the three toxins. Cyclopiazonic acid eluted as a sharp peak, using normal phase chromatography, while ochratoxin A and moniliformin were poorly separated.

- **Ion exchange chromatography**

Ion exchange chromatography is not a very popular technique, largely due to the problems encountered when using these systems. The column variability tends to be more of a problem when using bonded-phase ion-exchange packing, and the columns are less stable than the other chromatographic columns. Ion exchange chromatography is usually favoured for the separation of inorganic ions and large biomolecules (Snyder and Kirkland, 1988).

Therefore, ion exchange chromatography was explored only after reversed phase, paired ion chromatography and normal phase chromatography failed to provide the desired separation.

All experiments resulted in chromatograms that showed poor resolution and poorly shaped peaks. The mobile phase was run at the maximum organic solvent concentration (35%) and highest pH (9.5), in order to improve retention of the toxins. There was no improvement in the separation. However, a peak was obtained using ion exchange chromatography analysis, which was identified to be moniliformin by spiking.

### 3.3 Multitoxin capillary electrophoresis analysis

The work reported in this section evaluated the potential of capillary electrophoresis for the separation and detection of a mixture of cyclopiazonic acid, moniliformin and ochratoxin A. The developed multitoxin assay was optimised for the separation of the three toxins.

Currently, there is no literature available for the multitoxin analysis of cyclopiazonic acid, moniliformin and ochratoxin A by any analytical technique. Capillary electrophoresis offers many advantages over other techniques in terms of high efficiency (plate numbers of up to 200 000) and the facile manner in which retention can be manipulated in pursuit of a desired separation through simple adjustment of buffer composition. Capillary zone electrophoresis (CZE) separates ionic species on the basis of differences in electrophoretic mobility (Heiger, 1992). By incorporating micelles in the capillary system, neutral species can be separated on the basis of their differential distribution between the mobile and micellar phase, and is termed micellar electrokinetic capillary chromatography (MEKC). A survey of the literature indicates that both modes of capillary electrophoresis have been used for the separation of biological samples (Isaaq, 1997; Li, 1993). The use of CZE/MEKC in biological assays can be attributed to the miscibility of ionic and water soluble neutral compounds in these samples.

The buffer systems in biological applications have generally been taken directly from the corresponding traditional gel electrophoretic separations, and evolved empirically as the best for the specific separation. Previous workers (Holland and Sepaniak, 1993, Janini *et al.*, 1996), described the successful use of MEKC for the separation of several toxins. In addition, Holland and Sepaniak (1993) were able to resolve a mixture of ten mycotoxins, including ochratoxin A, using two different micellar buffers. Therefore, initial work in this study was performed using the MEKC mode.

The general trend for the separation of biological compounds by capillary electrophoresis has been by trial and error approaches (Bohs *et al.*, 1995) or univariate separation procedures (Holland and Sepaniak, 1993), in which one parameter is varied at a time, while the other parameters are kept constant. These procedures are often tedious and time consuming. Further, these schemes result in a local optimum rather than a global optimum (Li, 1993). The preferred method would be to use a simultaneous multivariate optimisation approach to obtain the optimum separation. The advantage of the multivariate optimisation is that only a small set of pre-planned experiments needs to be performed. However, multivariate optimisation is often difficult to implement. To solve the problem, a systematic approach was employed in the present study.

There are several multivariate optimisation approaches available for the development of capillary electrophoresis methods. Li (1993) described three overlapping resolution mapping schemes (ORMS) that are dependent on the number of variables to be optimised. The rectangular plot may be used for two variable systems. The scheme is highly flexible, since the criteria for selecting the variables can limit the experimental conditions, to prevent problems of long migration times and high working current. Li (1993) reported the success of the ORMS based on the triangular diagram approach to optimise the  $\alpha$ ,  $\beta$ , and  $\gamma$  cyclodextrins in the buffer systems. The limitation of this scheme is that the migration behaviour of the solutes depends linearly on the three experimental parameters individually, and does not represent non-linear dependence of migration behaviour on the experimental parameters. A third ORMS is based on the cubic diagram approach for the optimisation of three variables, in which the dependence of the individual experimental parameters is represented quadratically, and the interaction terms are represented linearly. This procedure was successfully implemented for the optimisation of pH, sodium dodecyl sulphate (SDS) concentration and TBAB in a buffer system (Li, 1993).

Overlapping resolution mapping scheme seems to be the preferred technique for optimisation, and the choice of ORMS would depend on the number of parameters to be investigated. The first phase in the ORMS was to define the criteria to be used in the selection of optimum separation. Acceptable resolution values of adjacent peaks were set to be between 1.5 and 2.0. Terabe *et al.* (1985) found that the migration range was an important criterion, which determines the final separation of the compounds. An appropriate migration time should be chosen to suit a particular investigation, depending on the type of compounds investigated.

For method development, the applied voltage and injection time were optimised using the optimised buffer system. Column efficiency is a function of applied voltage (Altria, 1998). Therefore it was important to manipulate applied voltage to optimise the efficiency of the system. In capillary electrophoresis only minute volumes of samples are loaded into the capillary in order to maintain high efficiencies. Sample overloading may lead to poor efficiency and is detrimental to resolution (Altria, 1998). For method validation, the stability of the buffer pH, the buffer composition, the nature of the sample and the general instrument conditions required monitoring.

### 3.3.1 Materials and methods

All multitoxin experiments were conducted with a mixture of 5 µg/ml cyclopiazonic acid, ochratoxin A and moniliformin in methanol. The system used was the Beckman P/ACE 5000 capillary electrophoresis system connected to a photodiode array detector. Data was analysed with the Systems Gold software. Separations were performed in a 57 cm (50 cm effective length) x 0.45 µm i.d. fused silica capillary (Beckman Instruments), maintained at 25 °C.

### 3.3.1.1 Micellar electrokinetic chromatography analysis

The concentration of the cyclopiazonic acid, moniliformin and ochratoxin A standard solutions used for the individual analysis were 5 µg/ml. The capillary electrophoresis instrument conditions were:

Applied voltage	:	20 kV, with 65 µA corresponding current
Injection time	:	5 seconds
Detection wavelength	:	254 nm (photodiode array)

### 3.3.1.2 Development of multitoxin capillary electrophoresis method

The experiment was planned by systematically implementing the following phases:

#### Phase 1

- Established the criteria for the separation of the three toxins.
- Performed preliminary experiments to select the experimental range for the variables under investigation.
- Established which ORMS plot to adopt.

#### Phase 2

- The necessary set of experiments for the plot diagram was set up.
- The resolution between each adjacent set of peaks was calculated.

#### Phase 3

- The experimental resolution was fitted into the polynomial equation.
- Designed a multivariate computer program to calculate the coefficients.
- Substituted the coefficients obtained, and calculated the resolution values for the intermediate pH and SDS concentration.

**Phase 4**

- Developed a scheme to transform the data obtained in Phase 3 into resolution plots for each set of adjacent peaks.
- Manipulated the rectangular plots to obtain a final plot of the resolution values for the three toxins.

**Phase 5**

- Located the region of optimum pH and SDS concentration, and establish the optimum conditions for the separation of the three toxins.

In the current study, a maximum time of 30 minutes was set. The concentration of the micelle was adjusted to give a migration time of 30 minutes for the last migrating species. Pre-planned experiments were conducted that covered the widest range for the variables under investigation. The pH ranges investigated for the phosphate buffer was from 6.5 to 7.5. Higher pH buffer systems were investigated using phosphate-borate (1:5) buffer systems, at pH range 8.5 to 12.5. The micelle concentration was varied from 0 to 100 mM in the buffer systems that cover the pH range under investigation. From the preliminary experiments, the resolutions  $R$  between every pair of peaks in the electropherogram obtained by using the nine different buffer systems was calculated using the following equation (Ravindranath, 1989):

$$R = 2 (t_{r2} - t_{r1}) / (w_1 + w_2) \quad \text{Equation 5}$$

Where  $t_{r1}$  and  $t_{r2}$  are the migration times of two adjacent peaks and  $w_1$  and  $w_2$  are the base-widths of the peaks. Migration time and width were recorded in minutes.



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Subsequently, the R-values was fitted into a polynomial:

$$R = a_0 + a_1x_1 + a_2x_2 + a_{12}x_1x_2 + a_{11}x_1^2 + a_{22}x_2^2 + a_{112}x_1^2x_2 + a_{122}x_1x_2^2 + a_{1122}x_1^2x_2^2$$

Equation 6

Where :

$x_1$  corresponds to the pH,

$x_2$  corresponds to the SDS concentration, and

$a_i$ 's are the coefficients, where 'i' is equal to 0, 1, 2, 12, 11, 22, 112, 122, or 1122.

A computer program was used to calculate the coefficient for each R-value obtained using Equation 5. With the known coefficient, the R-value for all the experimental conditions may be calculated. These values are then transformed into a rectangular plot. By overlapping all the rectangular plots and representing each point by the smallest values of resolution among all the individual plots, areas that satisfy the minimum desirable resolution for all the compounds can be established.

For the optimisation of pH and sodium dodecyl sulphate concentration, the range of experimental conditions for this study was : buffer pH in the range 6.5 to 7.5, and SDS concentration in the range 0 to 50 mM. For the optimisation of two parameters, a set of nine experiments was performed at selected points on a rectangular diagram as shown in Figure 3.3 (Li, 1993).

The range of buffers as shown in Figure 3.3 was prepared for evaluation as run buffers. The analytical conditions used were 20 kV applied voltage, 5 seconds injection time and wavelength of 254 nm.

#### 3.3.1.3 Investigation of applied voltage

All experiments were performed using standard instrument conditions, with 10 mM phosphate buffer at pH 7.5 as the running buffer, and a mixture of 50 µg/ml

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cyclopiazonic acid, moniliformin and ochratoxin A standard solution, and the voltage was varied from 15 to 30 kV.

Column efficiency was calculated from the formula described in Equation 7 (Ravindranath, 1989):

$$N = 16 (t_r/w_b)^2$$

Equation 7

Where, N = column efficiency  
 $t_r$  = migration time (minutes)  
 $w_b$  = base width of peak (minutes)

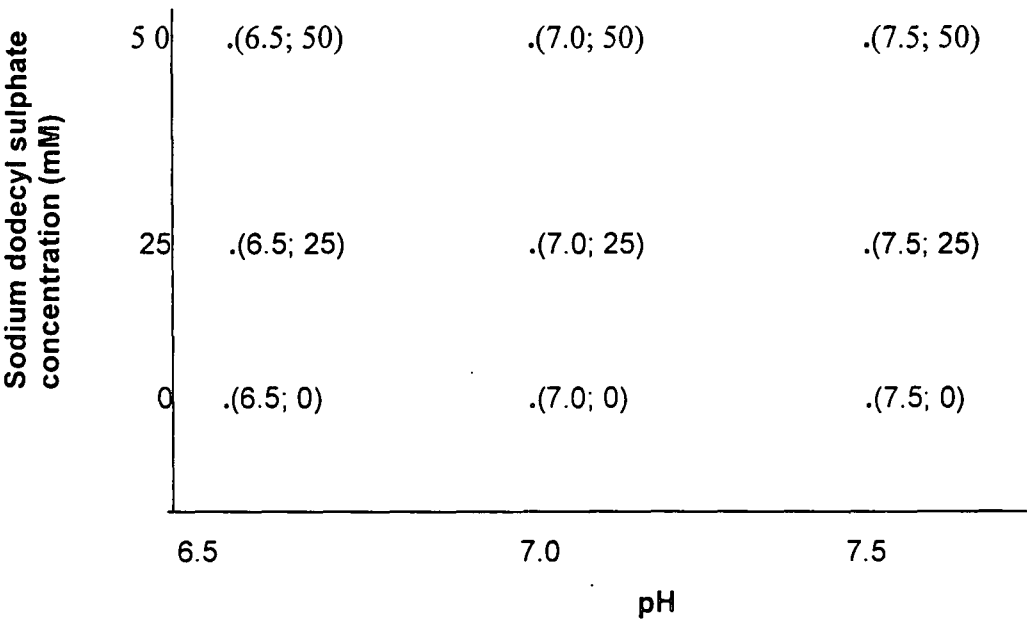


Figure 3.3 : The location of nine experiments chosen from the rectangular plot (Li, 1993). The composition of each point is represented as the pH and sodium dodecyl sulphate concentration for the buffer system.

#### 3.3.1.4 Investigation of injection time

All experiments were performed using 10 mM phosphate buffer at pH 7.5 as the running buffer, with the applied voltage at 15 kV, and the corresponding working current at 40  $\mu$ A, and the injection time was varied from 1 to 10 seconds. Column efficiency was calculated using Equation 7.

#### 3.3.1.5 Method validation

Once the buffer conditions, wavelength, applied voltage and injection time for the multitoxin capillary electrophoresis analysis of the three toxins were optimised, all subsequent analyses were performed using the optimised conditions:

Buffer	:	10 mM Phosphate buffer
Voltage	:	15 kV, with corresponding 40 $\mu$ A current
Injection time	:	3 seconds
Wavelength	:	226 nm

A standard mixture of 1  $\mu$ g/ml cyclopiazonic acid, 5  $\mu$ g/ml ochratoxin A and 5  $\mu$ g/ml moniliformin solution was used for the repeatability and reproducibility studies. The studies were performed as follows:

- Repeatability studies - twenty consecutive injections of the standard mixture were performed, monitoring both the migration time and the area count for the toxin peaks. Relative standard deviation values were calculated for the migration time and peak areas.
- Reproducibility studies - ten injections of the standard mixture over a ten-day period. Both the migration time and peak area of the toxins were monitored.

- The linearity of each toxin was established by initially performing a five-fold dilution of the 50 µg/ml solution to 10 µg/ml, then diluting each toxin as follows:  
cyclopiazonic acid : 3.13, 2.5, 1.25, 1.00, 0.63, 0.5, 0.25, 0.2, and 0.1 µg/ml  
ochratoxin A : 10, 7, 5, 3, 2, 1, 0.5, 0.25, and 0.1 µg/ml  
moniliformin : 10, 8, 5, 4, 3, 2, 1, 0.5, and 0.25 µg/ml.

The R-squared value from the area versus concentration graph for cyclopiazonic acid, moniliformin and ochratoxin A was calculated using the Microsoft Excel program. The detection limit value for cyclopiazonic acid, moniliformin and ochratoxin A corresponded to the peak area that produced a signal that corresponded to three times the signal of the noise level, that is  $S/N = 3$  (Ravindranath, 1989). The absolute amount injected was calculated using the following equation (Li, 1993)

$$\text{Volume} = \frac{\Delta p d^4 \Pi t}{128 \eta L} \quad \text{Equation 8}$$

where : volume = sample of volume injected

- $\Delta p$  = pressure difference across the capillary
- $d$  = inner diameter of capillary
- $t$  = injection time
- $\eta$  = viscosity of running buffer
- $L$  = total length of capillary

### 3.3.2 Results and discussion

#### 3.3.2.1 Micellar electrokinetic chromatography analysis

The MEKC analysis was set up for the three individual toxins as described by Holland and Sepaniak (1993). Electropherograms of cyclopiazonic acid, moniliformin

and ochratoxin A (5 µg/ml of each standard in methanol) are shown in Figures 3.4 (i), (ii) and (iii), respectively.

The migration time for cyclopiazonic acid was 9.426 minutes, 9.570 minutes for ochratoxin A and 14.792 minutes for moniliformin. This order of elution is expected (Heiger, 1992), since moniliformin is a relatively small molecule and is negatively charged, leading to a relatively slower migration towards the cathode. Cyclopiazonic acid and ochratoxin A are relatively larger molecules, with cyclopiazonic acid being the larger of the two compounds. However, the baseline was very noisy. Further, the peak shapes for the toxins were distorted, especially at the peak base. The corresponding current was relatively high (65 µA). In addition, an overlap of the three electropherograms (Figure 3.5) indicated that it was highly probable that co-elution of cyclopiazonic acid and ochratoxin A would occur.

Preliminary experiments confirmed that cyclopiazonic acid and ochratoxin A would co-elute, as the MEKC method failed to produce baseline separation for cyclopiazonic acid and ochratoxin A (Figure 3.6). The cyclopiazonic acid and ochratoxin A peaks were overlapping with a resolution value of only 0.90. The noisy baseline implied that the buffer was interfering with the analytical signal, and resulted in poor sensitivity. Hence, an alternate buffer would have to be investigated.

In summary, the MEKC method was unsuccessful in obtaining baseline resolution. The poor resolution between the cyclopiazonic acid and ochratoxin A peaks, and the noisy baseline suggested that the buffer conditions had to be optimised for the separation of the three toxins.

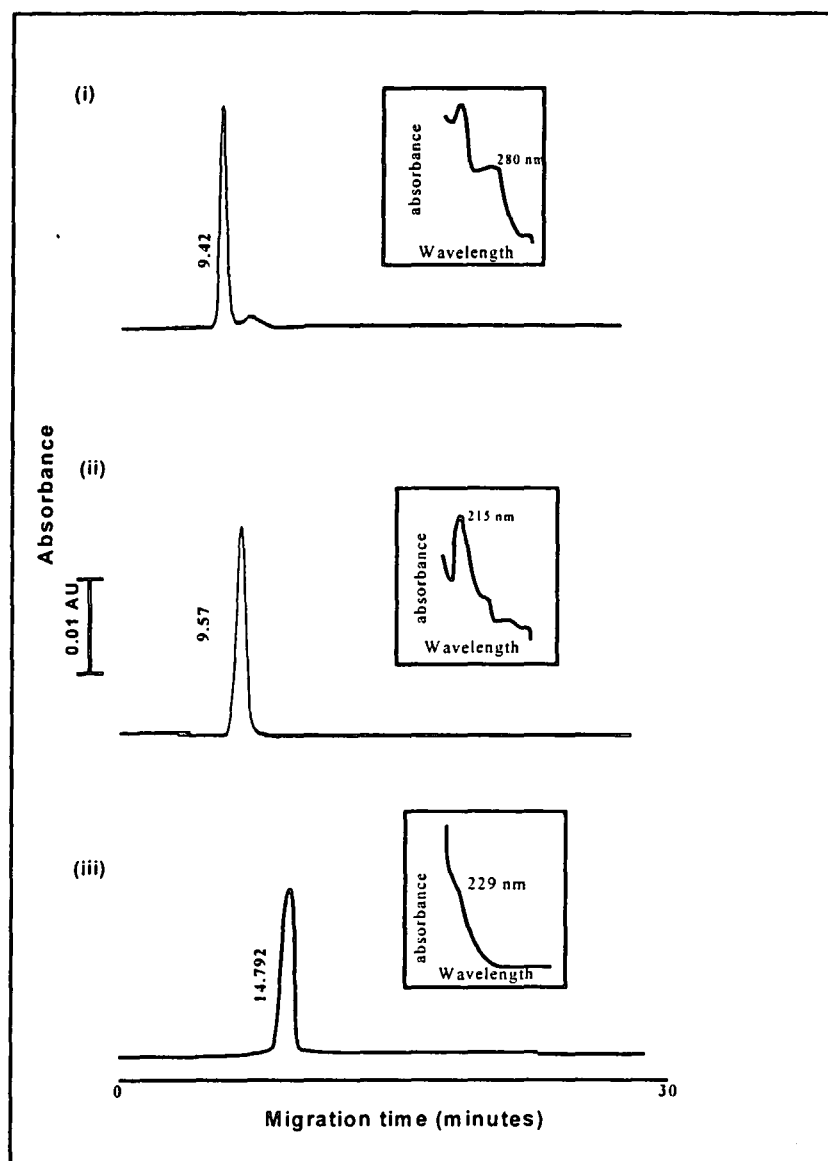
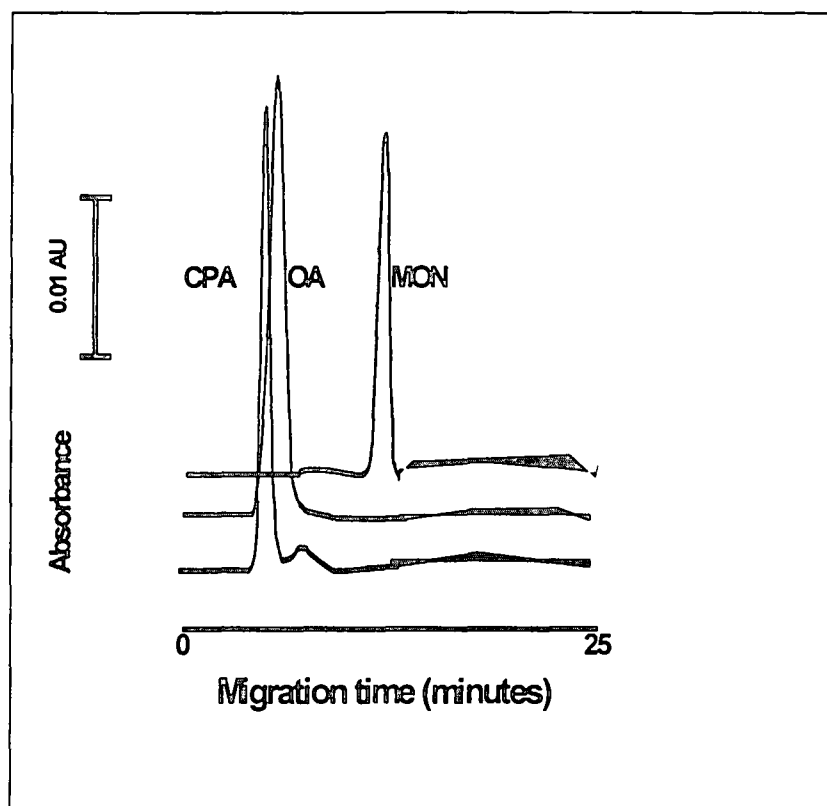


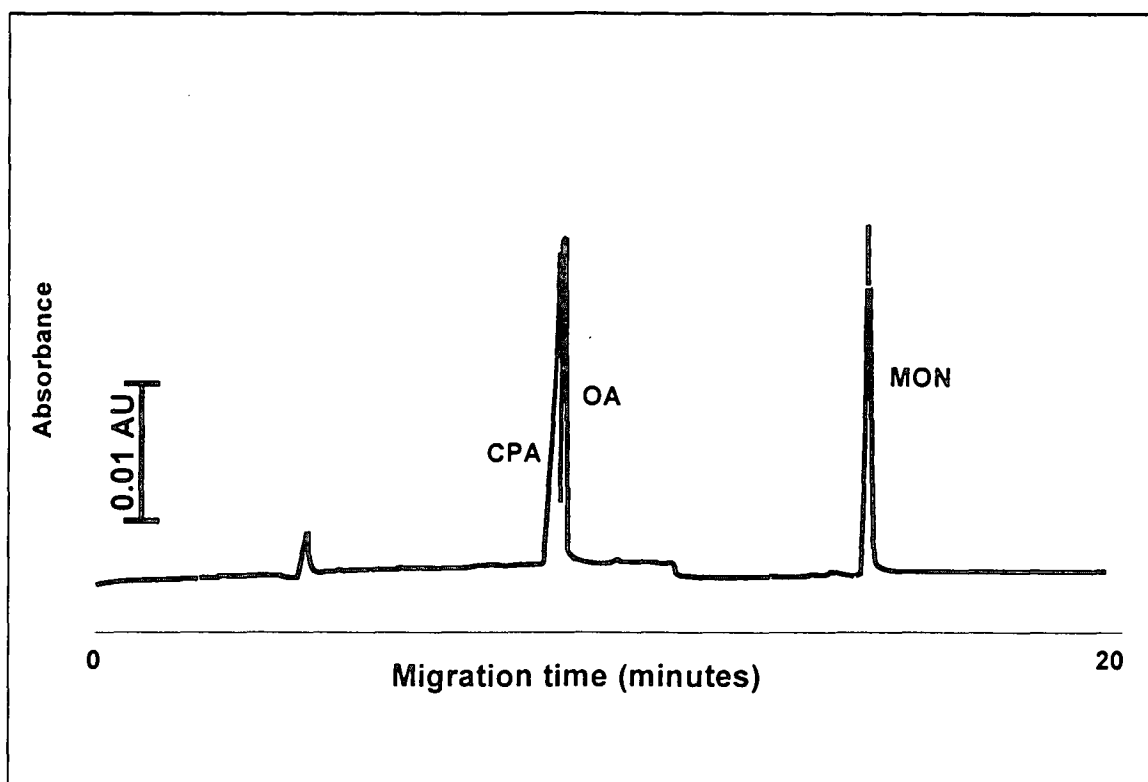
Figure 3.4: Electropherograms of 5  $\mu\text{g/ml}$  solution of (i) cyclopiazonic acid, (ii) ochratoxin A, and (iii) moniliformin by micellar electrokinetic chromatography analysis at 254 nm.

Inset : Photodiode array profile of absorbance versus concentration for the corresponding mycotoxin. [Analysis conditions : 0.05 M sodium dodecyl sulphate in 10 mM phosphate-6 mM borate buffer (pH 9.3), 20 kV applied voltage, 5 seconds injection time.]



**Figure 3.5:** Overlap of three individual electropherograms of 5  $\mu\text{g/ml}$  solution of cyclopiazonic acid, ochratoxin A, and moniliformin by micellar electrokinetic chromatography analysis at 254 nm.

[Analysis conditions : 0.05 M sodium dodecyl sulphate in 10 mM phosphate-6 mM borate buffer (pH 9.3), 20 kV applied voltage, 5 seconds injection time.]



**Figure 3.6:** Electropherogram of a mixture of 5  $\mu\text{g/ml}$  solution of cyclopiazonic acid, ochratoxin A, and moniliformin by micellar electrokinetic chromatography analysis at 254 nm.

[Analysis conditions : 0.05 M sodium dodecyl sulphate in 10 mM phosphate-6 mM borate buffer (pH 9.3), 20 kV applied voltage, 5 seconds injection time.]

### 3.3.2.2 Development of multitoxin capillary electrophoresis method

Initial results indicated that the phosphate-borate systems at high pH resulted in the moniliformin peak eluting very late (longer than the 30 minutes set in the criteria for the separation). In addition, the moniliformin peak was not reproducible in terms of migration time, and had a tendency to 'disappear', i.e., remained undetected even



after a run time of one hour. It could be that moniliformin is very highly ionised, (has a negative charge) hence resulting in extremely long migration time. This unexpected complexation time behaviour was also reported by Bohs *et al.* (1995), where the peak of zearalenone was described to have 'got lost' in the electropherograms using sodium citrate buffers. The chemical background leading to this behaviour was not clear, and was not investigated further. Phosphate buffer systems may be used at the lower pH range (1.3 to 3.1). However, this range was not investigated as the toxins would not be ionised at low pH.

The buffer systems that consisted of relatively high micelle concentration (greater than 50 mM SDS), resulted in a sharp increase in the working current (values greater than 90  $\mu$ A). This often resulted in instrument current errors due to heating effects. The SDS concentration was adjusted to give a working current of less than 60  $\mu$ A, which corresponded to 50 mM SDS concentration.

From the preliminary investigations it was established that the two parameters to be investigated would be pH and SDS. The most appropriate multivariate scheme to adopt for this study was the rectangular plot method (Li, 1993), described in Appendix 2.

For the optimisation of pH and sodium dodecyl sulphate concentration, nine buffer systems were evaluated. The electropherograms for the range of buffers used are shown in Figure 3.7. The resolution of every pair of adjacent peaks in the electropherograms was calculated, (Table 3.1) using the formula indicated in Equation 5.

The next phase was to determine the co-efficients. The computer program that was best suited for the computation of the polynomial equation, after consultation with several statisticians, was determined to be Statsgraphics. A program was designed in which the coefficients ( $a_i$ 's) were calculated (Table 3.2), by fitting the

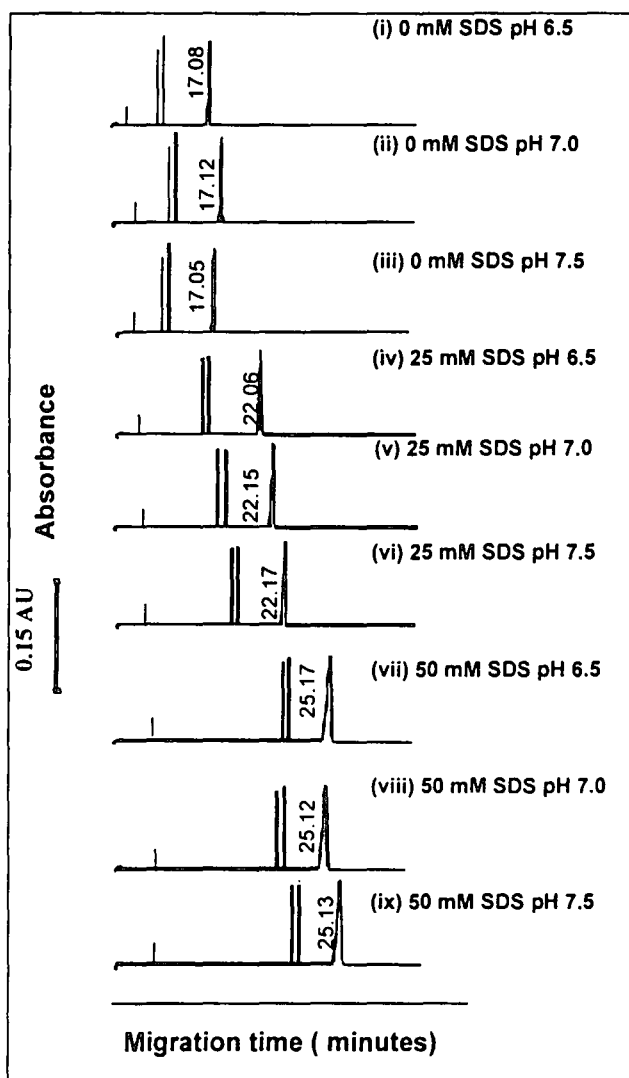
corresponding calculated resolution values of the buffer pH and SDS concentration values to the polynomial equation (Equation 6).

**Table 3.1: Experimental resolution values calculated for cyclopiazonic acid, moniliformin and ochratoxin A**

SDS concentration (mM)	pH	R1*	R2*	R3*
50	6.5	4.04	1.05	2.43
50	7.0	3.56	0.69	2.75
50	7.5	3.37	0.37	3.90
25	6.5	5.38	1.25	4.42
25	7.0	5.15	1.86	2.62
25	7.5	5.08	1.24	3.26
0	6.5	2.02	0.35	5.23
0	7.0	1.87	1.18	4.69
0	7.5	1.77	1.50	3.50

\*R1 values were the resolution between the solvent front (SF) and cyclopiazonic acid, R2 values were the resolution between cyclopiazonic acid and ochratoxin A, and R3 values were the resolution between ochratoxin A and moniliformin.

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**Figure 3.7:** Electropherograms for sodium dodecyl sulphate and pH optimisation studies at 254 nm, with buffer compositions (i) 0mM SDS, pH 6.5, (ii) 0 mM SDS, pH 7.0, (iii) 0 mM SDS, pH 7.5, (iv) 25 mM SDS, pH 6.5, (v) 25 mM SDS, pH 7.0, (vi) 25 mM, pH 7.5, (vii) 50mM SDS, pH 6.5, (viii) 50 mM SDS, pH 7.0 and (ix) 50 mM, pH 7.5.

[Analysis conditions : 20 kV and 5 s injection time. Elution order: solvent front, cyclopiazonic acid, ochratoxin A and moniliformin.]

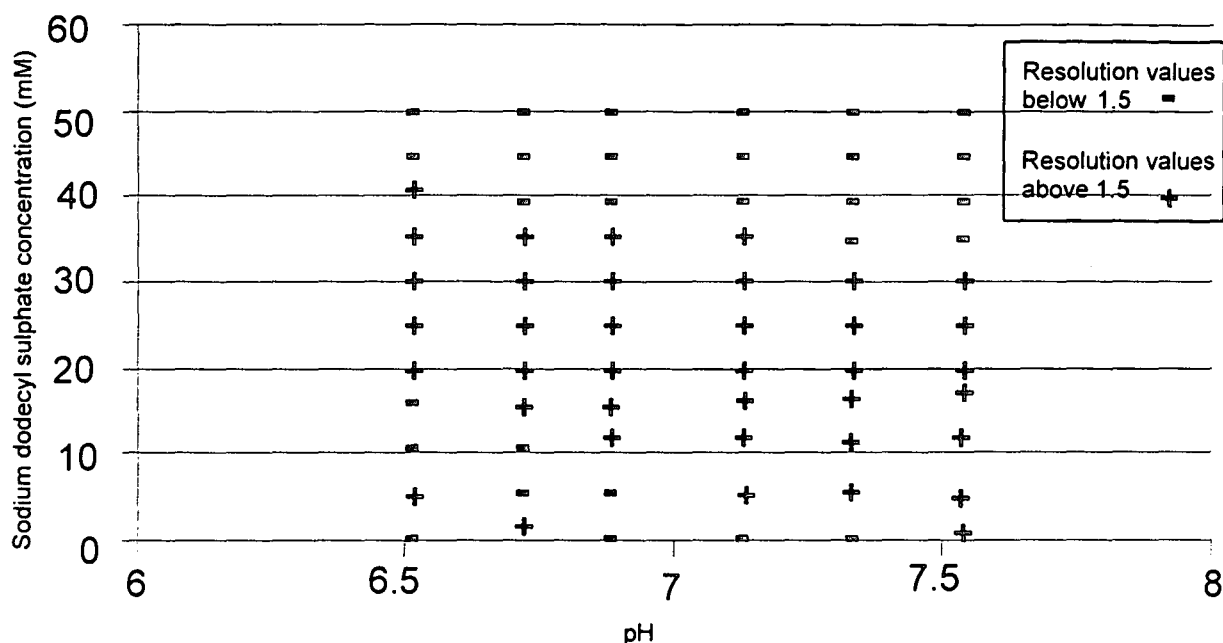
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Table 3.2: Average coefficients (n=3) for the nine buffer systems

SDS concentration (mM)	pH	Coefficients (for cyclopiazonic acid)	Coefficients (for ochratoxin A)	Coefficients (for moniliformin)
50	6.5	7.6200E+00	-6.0420E+01	-6.5840E+01
50	7.0	-1.3800E+00	1.6360E+01	2.2120E+01
50	7.5	1.9908E-01	7.2716E-01	1.5294E+00
25	6.5	4.4497E-03	-9.5625E-02	-2.3726E-01
25	7.0	8.0000E-02	-1.0800E+00	-1.7200E+00
25	7.5	-5.0831E-03	3.5938E-02	4.5106E-01
0	6.5	3.8429E-88	-1.9371E-88	-3.5898E-87
0	7.0	6.2201E-04	-1.2049E-02	-1.3899E-01
0	7.5	-6.4000E-05	9.6000E-04	1.0688E-02

With the known coefficients, the resolution values for all the experimental conditions within the range selected were calculated. The values were manually plotted to transform the data into a rectangular plot. For each of the toxins, a rectangular plot was obtained. Subsequently, by overlapping all the rectangular plots, the areas that satisfy the desired resolution was located.

Figure 3.8 indicates the final overlapped diagram for the three toxins. The diagram indicates that at higher SDS compositions, there is a tendency for co-elution to occur (indicated by -). The optimum range is indicated by the (+) are generally scattered over the SDS concentration below 30 mM, and the entire pH range.



**Figure 3.8 : Final combined overlapped resolution map for cyclopiazonic acid, moniliformin and ochratoxin A, using the calculated resolution values.**

A third parameter was introduced in order to determine the optimum separation conditions, viz., total migration time. Addition of SDS into the buffer system resulted in an increase in migration time. In addition, there was an increase in the working current. The increase in working current may result in heating effects, and poor reproducibility. The migration times of the three toxins were plotted as a function of pH. The plots indicated that the mycotoxins did not exhibit a significant pH effect, as observed by the constant profile of the graphs (Figures 3.9). These results are in keeping with compound of low pKa values.

In addition, the migration time for the last eluting analyte (moniliformin) for the buffer system at 0 mM SDS, at pH 7.5 was 17.05 minutes (Figure 3.7), which was the shortest analysis time, with excellent resolution between the compounds. The

advantage of working with a 10 mM phosphate buffer system only are that the buffer has a low mobility, with large minimally charged ions and good buffering capacity in the working pH range (pH 6.5-7.5), and was hence, chosen to be the optimum buffer for the separation of the three toxins.

- **Evaluation of buffer systems with and without sodium dodecyl sulphate**

The ultraviolet-visible (uv-vis) analysis of the MEKC buffer (0.05 M SDS in 10 mM Phosphate –6 mM Borate buffer, pH 9.3) and the 10 mM phosphate buffer (neat buffer) was performed, in order to establish whether or not there was any interference in the working wavelength region. The uv-vis spectrum of the MEKC buffer [Figure 3.10 (i)], showed absorbance over the wavelength range 190 to 360 nm), whereas the profile of the ultraviolet-visible spectrum for the 10 mM phosphate buffer [Figure 3.10 (ii)] showed negligible absorbance. The absorbance value at 254 nm and 226 nm was 0.0003 absorbance units for the 10 mM phosphate buffer. This was significantly lower to that obtained for the MEKC buffer (0.016 absorbance units). In addition, buffer systems with SDS generated higher working current than the neat buffer systems. This implied that working with a SDS buffer would lead to poor sensitivity for the technique, and increased probability of errors due to heating effects. Therefore, 10 mM phosphate buffer at pH 7.5, with no SDS was found to be the optimum buffer for the separation of the three toxins.

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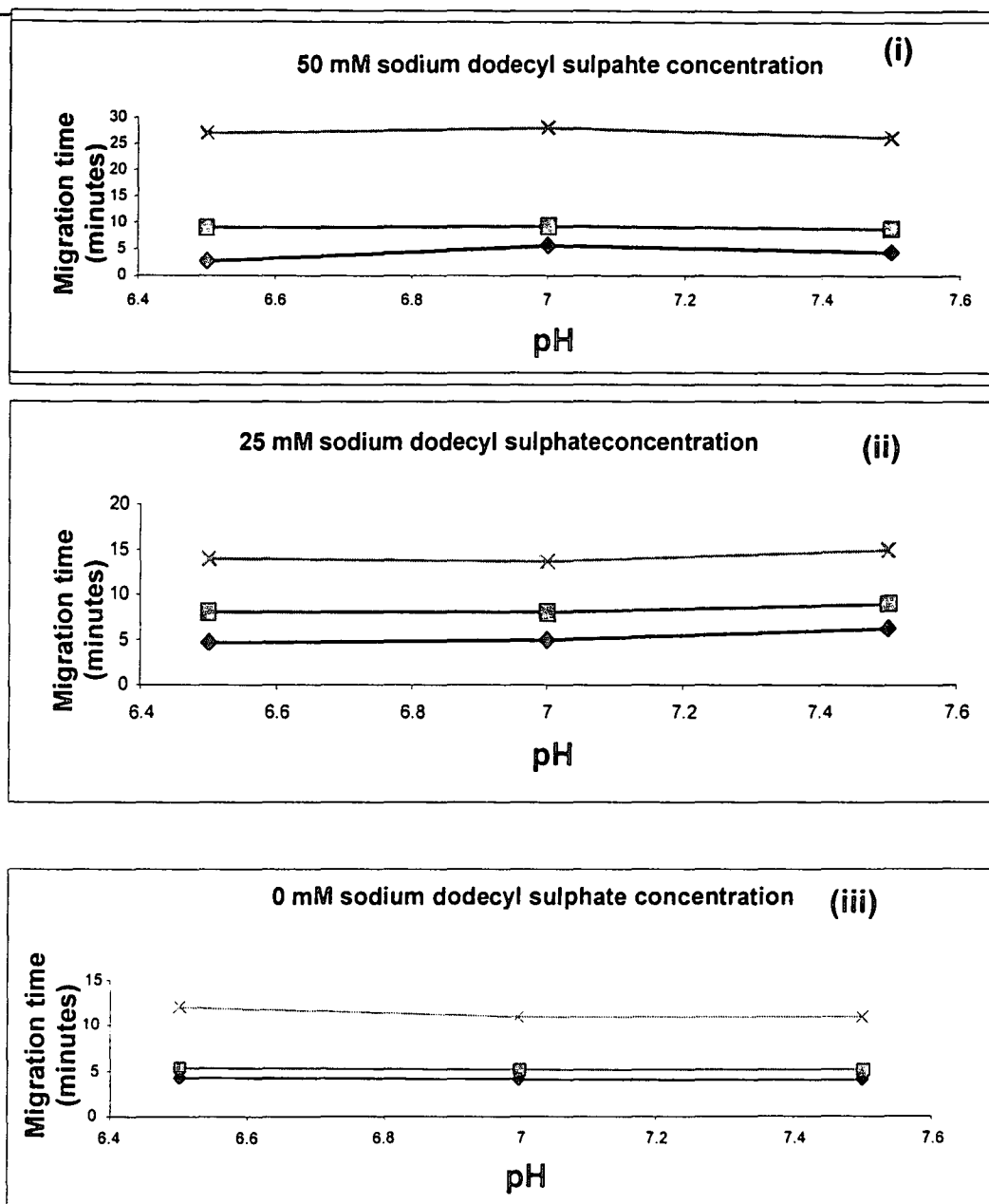


Figure 3.9: Graph of pH versus migration time for (i) 50 mM sodium dodecyl sulphate, (ii) 25 mM sodium dodecyl sulphate and (iii) 0 mM sodium dodecyl sulphate for cyclopiazonic acid, moniliformin and ochratoxin A, showing the pH effect of a compound.

x : Resolution values between solvent front and cyclopiazonic acid, ■ : resolution values between cyclopiazonic acid and ochratoxin A, ♦ : resolution values between ochratoxin A and moniliformin.

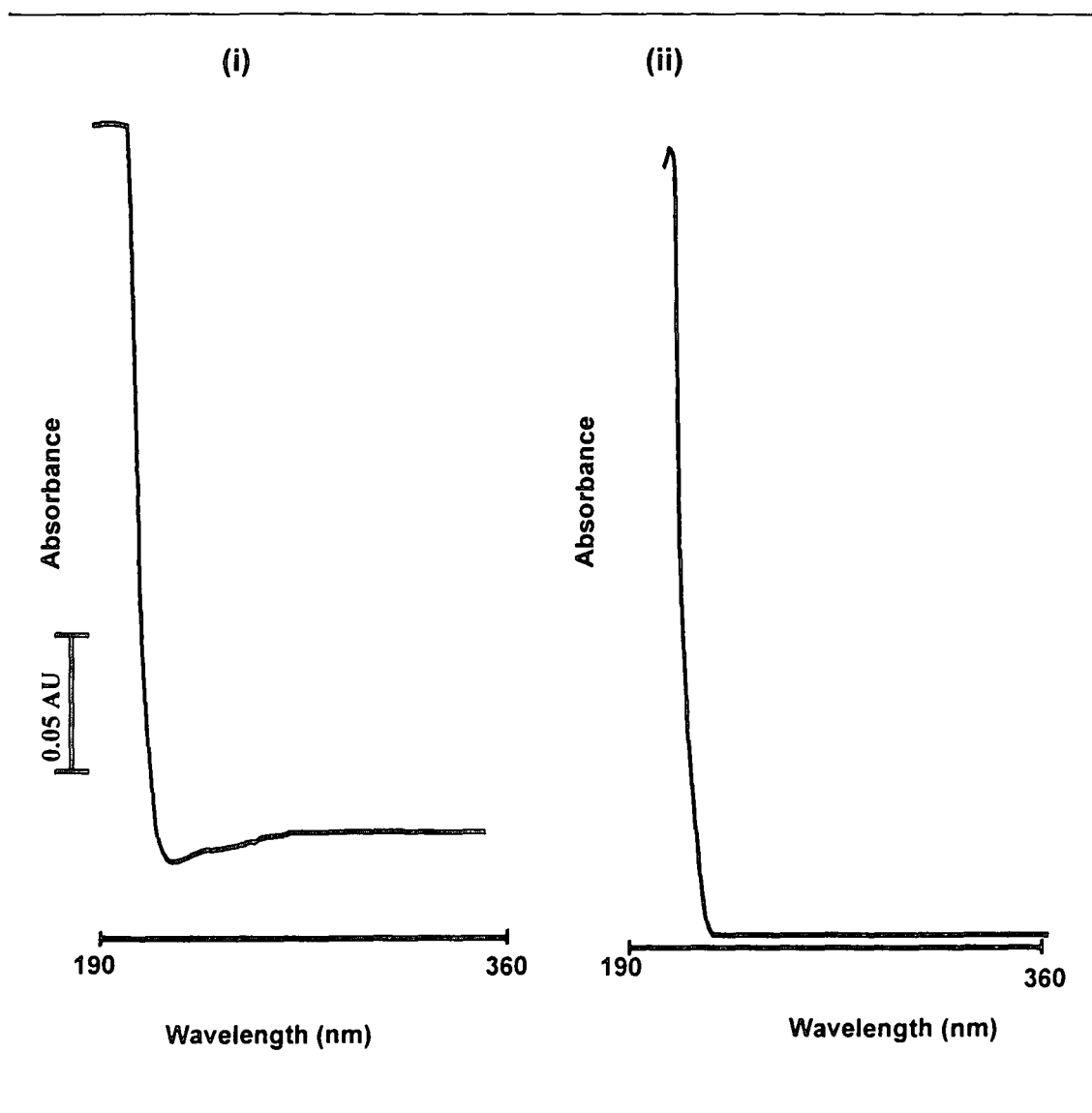


Figure 3.10 : Ultraviolet spectrum of (i) 0.05 M sodium dodecyl sulphate in 10 M Phosphate-6 mM borate buffer, pH 9.3, and (ii) 10 mM phosphate buffer, pH 7.5.



Two articles were published that describe the capillary electrophoresis analysis of mycotoxins by MEKC (Prasongsidh *et al.*, 1998) and CZE (Corneli and Maragos, 1998). The first describes the MEKC analysis of cyclopiazonic acid in milk (Prasongsidh *et al.*, 1998). The optimum buffer conditions were obtained by trial and error and univariate analysis. The buffer system used was 0.05 M sodium deoxychlorate, 0.01 M phosphate and 0.006 M borate at pH 9.3. The variability in capillary electrophoresis in an inter-assay between-day was poor (5.1 %), as predicted for a buffer system with additive. To avoid poor variability, and prevent buffer depletion, buffer replenishment had to be performed after every four injections.

The second paper describes the CZE analysis of ochratoxin A in roasted coffee, corn and sorghum, using a 20 mM phosphate buffer at pH 7.00, with no additives (Corneli and Maragos, 1998). The paper, however does not give any detail of the reasons for the choice of the 20 mM phosphate buffer. The pH 7.00 phosphate buffer solution was reported to have been stable for ten days at room temperature without causing significant fluctuations in migration time. This is in contrast to that reported by Prasongsidh *et al.* (1998) using MEKC analysis. The latter paper highlights the advantage of using a phosphate buffer system, without any additives.

In summary, the overlapping resolution mapping procedure was successfully implemented for the location of the region of optimum separation conditions for the analysis of the three toxins. The optimum buffer system was determined by evaluating the migration time data, pH dependence plots and ultraviolet-visible analysis. The pH and SDS concentration of the buffer corresponding to the optimum resolution between cyclopiazonic acid, ochratoxin A and moniliformin were pH 7.5, and 0 mM SDS with the resolution values of 1.77, 1.53 and 3.31 respectively.

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The ultraviolet-visible spectra obtained for the three toxins (50  $\mu\text{g/ml}$  in the 10 mM phosphate buffer at pH 9.5) were overlaid, to obtain the wavelength region of maximum absorbance (Figure 3.11). The region of maximum absorbance was 210 to 280 nm.

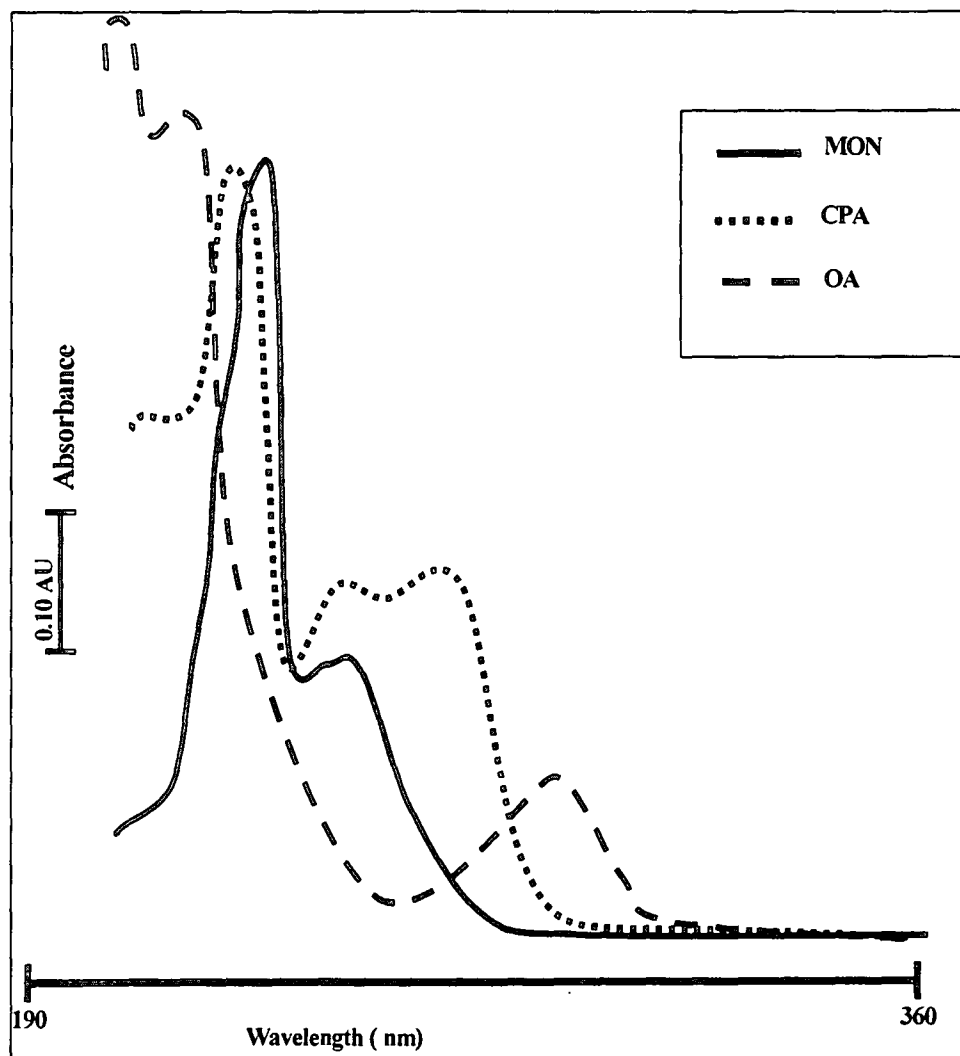


Figure 3.11 : Overlay of the ultraviolet spectra of 50  $\mu\text{g/ml}$  of cyclopiazonic acid, ochratoxin A and moniliformin in 10 mM phosphate buffer at pH 9.5

Thereafter, the areas of the components at the optimum wavelength range were calculated using the Array View (Beckman,1990) program. The wavelength that corresponded to the optimum peak areas for cyclopiazonic acid, moniliformin and ochratoxin A was 226 nm. All subsequent analyses were performed at this wavelength. The optimum wavelength region was 210 to 280 nm. The wavelength that corresponded to optimum peak areas for the three toxins was 226 nm.

#### 3.3.2.3 Investigation of applied voltage

The applied voltage largely affects the speed and quality of a separation. Preliminary investigation of voltage effects on migration time indicated that for the analysis below 15 kV, extremely long migration times (excess of one hour) were obtained.

These results are in keeping with those reported by Altria (1998). For the analysis above 30 kV, high working currents and problems associated with heating effects would have been experienced. However, the instrument was not capable of generating voltages greater than 30 kV. Hence this was not investigated. The voltage range was limited to 15 to 30 kV.

The column efficiency values were consistent, and the % RSD values were below 2%. The graphical presentation of the change in column efficiency with change in applied voltage (Figure 3.12), indicates high column efficiencies. This was expected for capillary electrophoresis analysis since the optical window was directly in the capillary. Hence minimum zone broadening effects due to dead-volume or component mixing (Altria, 1998). High efficiency values in capillary electrophoresis are also due to the flat flow profile obtained (Heiger, 1992). The change in applied voltage from 15 to 30 kV resulted in a general decrease in column efficiency for the three toxins. This is contrary to literature, which indicates that efficiency increases with increasing voltage (Heiger, 1992). The deviation from literature may be explained by the fact that an increase in voltage resulted in an increase in

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temperature (Altria, 1998), hence increasing band broadening effects. This would result in a decrease of column efficiency as observed by Nelson *et al.* (1989). It is also highly probable that higher efficiency would have been obtained at lower applied voltage, but at the expense of analysis time (preliminary studies showed excess of one hour at applied voltage values below 15 kV). The corresponding electropherograms (Figure 3.13), indicate that there were broader peaks at higher voltage, which also indicate the loss of efficiency at higher voltage. Hence, the optimum voltage was taken to be 15 kV.

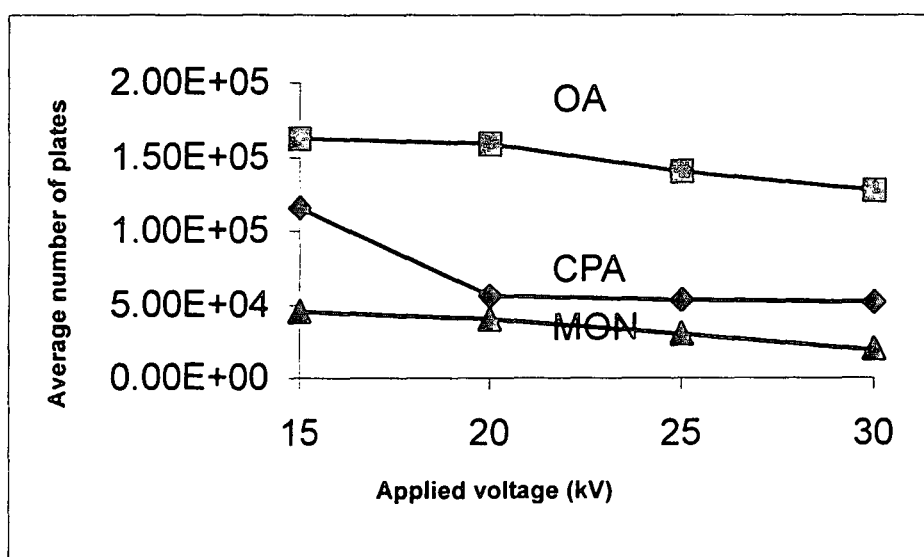
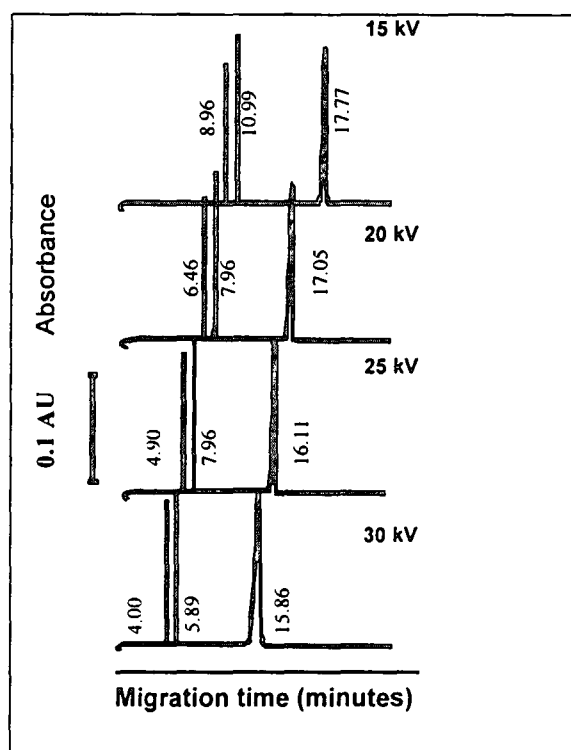


Figure 3.12 : Graph indicating trends for column efficiency at different applied voltages for cyclopiazonic acid, ochratoxin A and moniliformin.



**Figure 3.13 : Electropherograms for cyclopiazonic acid, ochratoxin A and moniliformin for applied voltage studies at 15 kV, 20 kV, 25 kV and 30 kV.**

[Conditions : 10 mM Phosphate Buffer, 5 seconds injection time, UV-226nm. Elution order : cyclopiazonic acid, ochratoxin A and moniliformin.]

The general trend was that as applied voltage was increased, column efficiency decreased. The optimum applied voltage was 15 kV.

#### 3.3.2.4 Investigation of injection time

The injection time employed in capillary electrophoresis analysis determines the volume of sample introduced into the capillary. The increase in peak area is linear with injection time. However the efficiency, which is initially improved, is reduced

with increasing injection time (Altria, 1998). The sample injection time was optimised as a function of column efficiency, by increasing the injection time from 1 to 10 seconds. The graphical presentation of the data (Figure 3.14), indicated that the general trend from 1 to 3 seconds injection time, was a very slight increase in column efficiency. Thereafter, the column efficiency was constant from 3 seconds to 5 seconds, after which there was a drastic decrease in column efficiency for cyclopiazonic acid, and a very gradual decrease in efficiency for moniliformin. These trends may be explained by the fact that injection of large volume of samples onto the column results in sample overload.

Sample overloading has two detrimental effects (Li, 1993), viz., broadening of the peak width, and large sample volumes may intensify field inhomogeneities, and result in distorted peak shape. Both of these would result in a decrease in column efficiency as was seen in Figure 3.15, where the moniliformin peak was sharp for the 1s, 3 s and 5 s injection time. Thereafter the peak became progressively broader with increasing migration time. The small volume requirements of capillary electrophoresis are advantageous since 5 microlitres of sample is sufficient to perform numerous injections, conversely, the small volumes increases sensitivity difficulties for dilute samples.

The optimum injection volume was taken to be 3 seconds. Injection time studies performed by Heiger (1992) produced relative standard deviation values between 1 to 2 %. The relative standard deviation values for this study were between 0.3 to 1.6 %, implying that the precision was in keeping with that of previous workers.

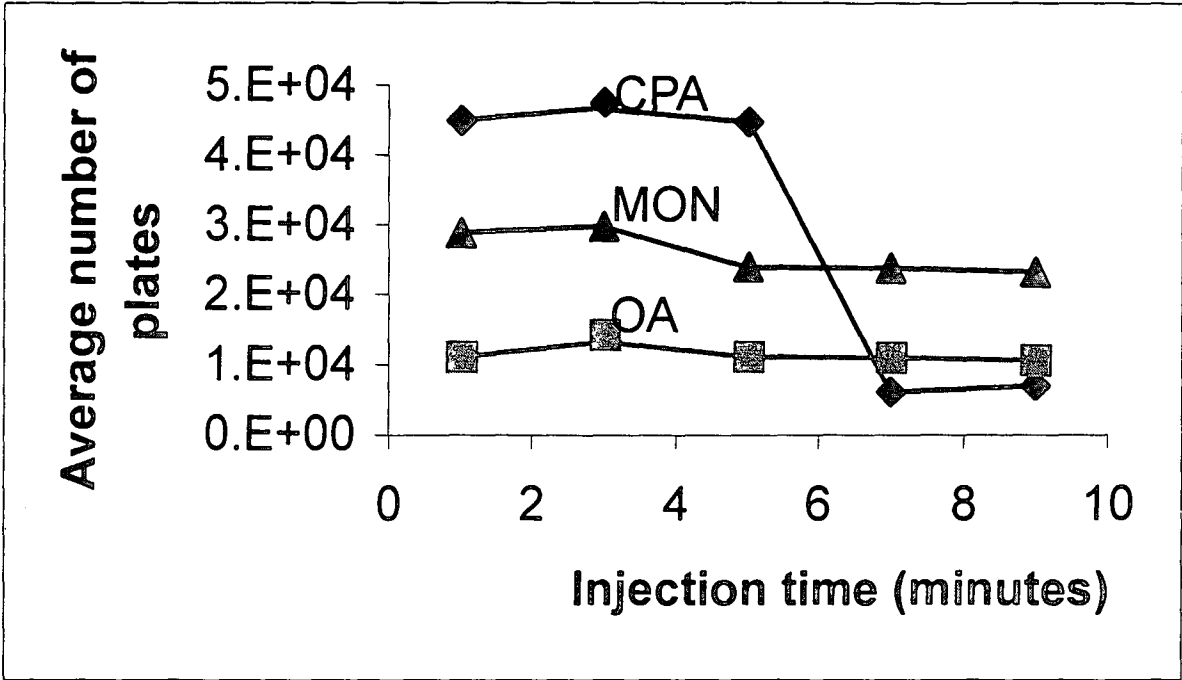
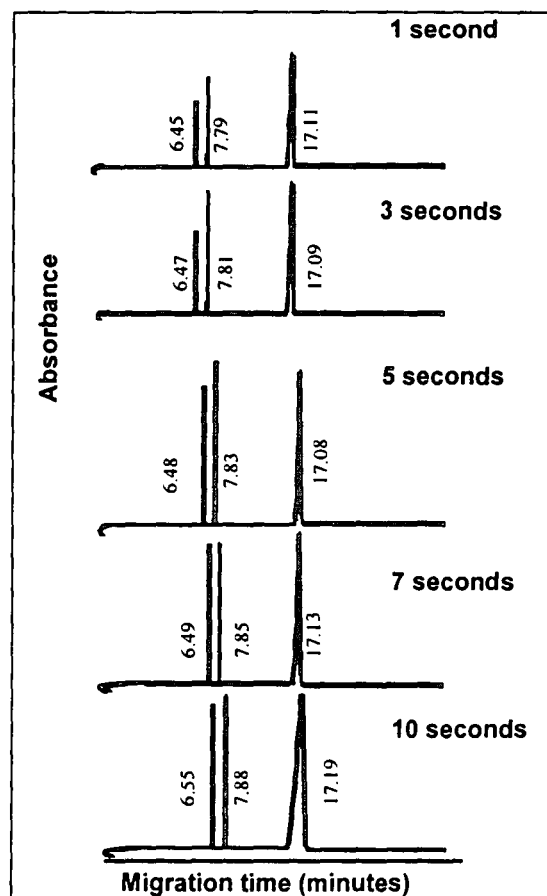


Figure 3.14: Change in column efficiency with varying injection time for cyclopiazonic acid, moniliformin and ochratoxin A.

The general trend was that as injection time increased, column efficiency increased, until column overloading was observed, where the column efficiency decreased with increasing injection time. The optimum injection time was 3 seconds.

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**Figure 3.15: Electropherograms for injection time optimisation studies for cyclopiazonic acid, moniliformin and ochratoxin A at 1 , 3 , 5 , 7, and 10 seconds injection time.**

[Conditions : 10 mM Phosphate buffer, 15 kV applied voltage, and ultraviolet detection- 226 nm. Elution order: solvent front, cyclopiazonic acid, ochratoxin A and moniliformin.]

### 3.3.2.5 Method validation

The stability of the capillary electrophoresis method developed was checked for repeatability and reproducibility by monitoring the migration times and peak areas for cyclopiazonic acid ochratoxin A and moniliformin peaks. Run to run migration time



was in the region of 0.4 to 1.4% RSD for repeatability and 0.18 to 6.16 % for reproducibility. This suggested that the methods were repeatable and reproducible for the toxins.

The high relative standard deviation values for moniliformin (6.16 %) are within the limits set by Shaw and co-workers (1991) for their studies (RSD = 10 %). However, the relative standard deviation value may be improved by the use of an appropriate internal standard, and relative standard deviation calculated for the relative migration time and peak areas of the toxins to the internal standard (Holland and Sepaniak, 1993). One of the most important factors leading to good reproducibility was the conditioning of the column. Base conditioning was performed before and after every run to remove adsorbates and refresh the surface by deprotonation of the silanol groups.

The quantification ranges of cyclopiazonic acid, moniliformin and ochratoxin A were obtained by determining the variation of peak area with the concentration of cyclopiazonic acid, moniliformin and ochratoxin A standard solutions. The peak area versus concentration curves (Figure 3.16) for (i) cyclopiazonic acid (ii), ochratoxin A and (iii) moniliformin were linear over the concentration range used, with R-squared values in the range 0.9968 to 0.9998. These results are in keeping with previous workers (Bohs *et al.*, 1995; Prasongsidh *et al.*, 1998).

The detection limit (at three times the noise level) of cyclopiazonic acid by capillary electrophoresis was 500 ng/ml, 1 µg/ml for ochratoxin A and 2 µg/ml for moniliformin, at 226 nm. Using Equation 8, with a pressure of 50 mbar for 3 seconds,  $0.8904 \times 10^{-2}$  Poise for the buffer viscosity and using a capillary with the following dimensions: 75 µm i.d x 57 cm length (23.0 nl), the absolute amount injected was calculated to be 11.5 pg for cyclopiazonic acid, 23.0 pg for ochratoxin A and 46.0 pg for moniliformin. The detection limit obtained for ochratoxin A is in keeping with that reported by Bohs *et al.* (1995). The reported detection limit (signal to noise level 3:1)

was 1 µg/ml or 15 pg absolute amount injected hydrodynamically. The instrument conditions used were pressure of 50 mbar for 3 seconds, using a 75 µm i.d. x 75 cm total length ( $V = 15.6$  nl) capillary column.

Prasongsidh *et al.* (1997) reported a detection limit of 20 ng/ml or  $0.27 \times 10^{-7}$  g/ml for cyclopiazonic acid using a capillary extended light path (50 µm i.d. x 64.5 cm capillary column), with a pressure difference of 50 mbar, and injection time 7 seconds ( $V = 8.3$  nl). The extended path flow cell was obtained due to a special bubble cell feature, which permitted the accumulation of the sample three times bigger than the one in the inner diameter of the capillary. This feature could not be adapted to the Beckman P/ACE system used in the current study. There are no reported detection limits for moniliformin.

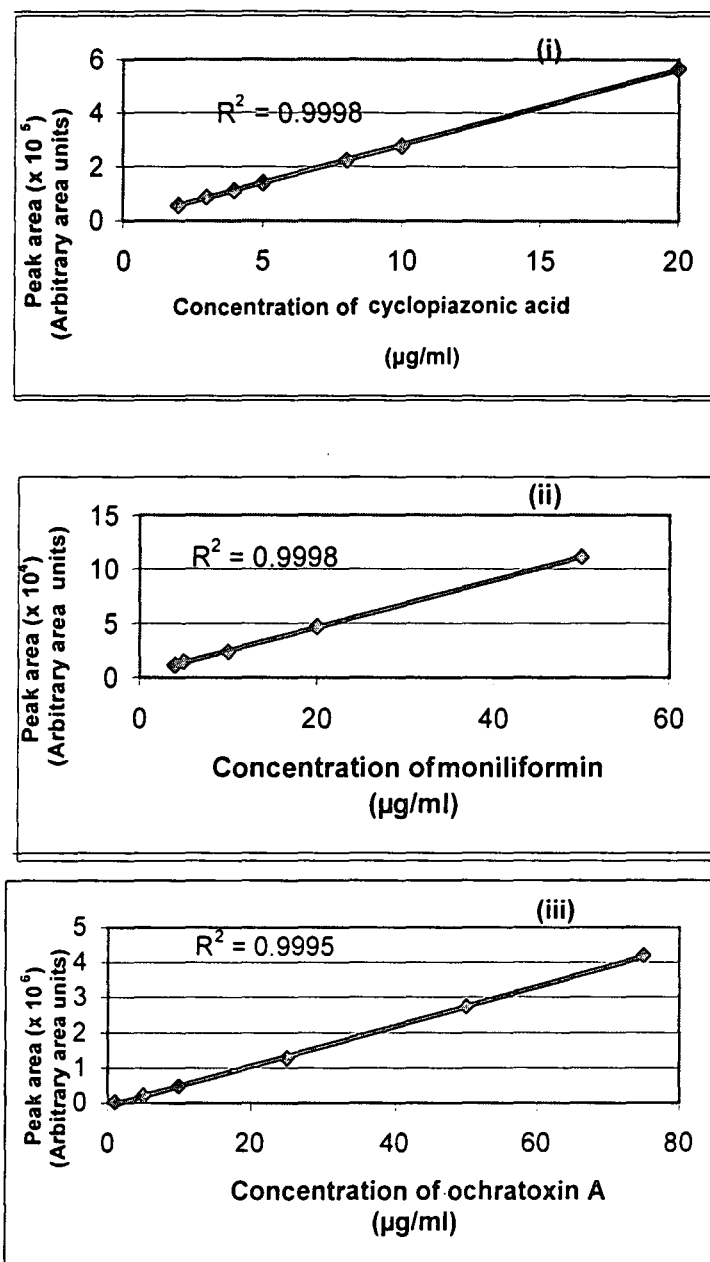


Figure 3. 16 : Calibration curves for (i) cyclopiazonic acid, (ii) moniliformin and (iii) ochratoxin A at 226 nm.

### 3.4 Conclusion

- **Multitoxin high performance liquid chromatography assay**

Reversed phase chromatography, paired ion chromatography, normal phase and ion exchange chromatography were investigated for a multitoxin assay. Cyclopiazonic acid was detected using normal phase chromatography, moniliformin was detected using paired ion chromatography and ion exchange chromatography. However, no technique gave separation for all three toxins, or even two of the compounds. It would seem that because the chemistry of the mycotoxins is very different, a common high performance liquid chromatography method for the detection and separation of these toxins is not viable. Therefore, no further investigation was performed for the separation of these toxins. The study was unsuccessful at developing a multitoxin high performance liquid chromatography method.

- **Multitoxin capillary electrophoresis assay**

The MEKC method described by Holland and Sepaniak (1993) was unsuccessful in obtaining baseline resolution for the mixture of three toxins. Hence, an alternate buffer system was investigated for the separation of cyclopiazonic acid, moniliformin and ochratoxin A. The overlapping resolution mapping procedure was successfully implemented for the location of the region of optimum separation conditions for the analysis of the three toxins. The pH and SDS concentration of the buffer corresponding to the optimum resolution between cyclopiazonic acid, ochratoxin A and moniliformin were pH 7.5, and 0 mM SDS with the resolution values of 1.77, 1.53 and 3.31, respectively. The optimised conditions for the separation of the three toxins were applied voltage of 15 kV, with corresponding 40  $\mu$ A current and injection time of 3 seconds at ultraviolet detection of 226 nm. The developed multitoxin capillary electrophoresis method was repeatable and reproducible. The detection limit for each toxin was 11.5 pg for cyclopiazonic acid (500 ng/ml), 23.0 pg for ochratoxin A (1  $\mu$ g/ml) and 46.0 pg for moniliformin (2  $\mu$ g/ml).

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### 4.1 Introduction

The developed multitoxin capillary electrophoresis method was tested on spiked samples and naturally contaminated samples, and the results were confirmed with the individual high performance liquid chromatography methods.

Several fungi are capable of contaminating a food or feed sample, each fungus capable of producing more than one mycotoxin (Widiastuti *et al.*, 1988). Therefore, it is highly probable that cyclopiazonic acid, moniliformin and ochratoxin A may occur simultaneously in food sample.

From the literature review, there are several methods available for the extraction of these toxins from food samples. Cyclopiazonic acid and ochratoxin A are acidic toxins and are generally co-extracted from maize samples using liquid-liquid extraction (Gorst-Allman and Steyn, 1979 and Dutton, 1983). Currently, reports of clean-up of ochratoxin A extract from coffee samples using the ochratest affinity columns are increasing (Vicam, 1996, Jorgensen, 1998 and Scott *et al.*, 1998).

Due to the very polar character of moniliformin, the selective extraction and separation from polar and non-polar compounds represent the main problem. Solid phase extract using a C18 column, to purify the sample extract from lipophilic components followed by solid phase extraction (SPE) using a strong anion exchange column (SAX) has proved to be a powerful tool. However, the use of C18 and SAX columns becomes extremely costly. Filek and Lindner (1996) described a method to extract moniliformin using SAX columns only, with satisfactory results, thus eliminating the cleanup step with a C18 column.

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**4.2 Materials and method****4.2.1 Extraction of cyclopiazonic acid, moniliformin and ochratoxin A from maize and coffee samples**

**The methods chosen for the extraction of toxins from the maize samples are as follows :**

The extraction of **cyclopiazonic acid and ochratoxin A** (acidic toxins) from maize samples was performed by the liquid-liquid extraction method described in 1981 by Dutton (Figure 4.1). Briefly, the process involved extracting the toxins from 25 g of a maize sample with 50 ml acetonitrile, using a vertical shaker. The salt (KCl) was added to prevent the formation of emulsions. The acetonitrile extract was then defatted, and made basic with the addition of sodium bicarbonate, prior to extraction with chloroform. The chloroform layer was discarded and the remaining aqueous layer was neutralised with sulphuric acid. Cyclopiazonic acid and ochratoxin A were extracted from this aqueous solution with chloroform. The chloroform extract was evaporated to dryness using a rotary evaporator. The toxins were reconstituted in chloroform, evaporated to dryness under high purity nitrogen, and kept in a dark screw cap vial at 0°C until used.

**Moniliformin**, a small polar toxin, was extracted using the method described by Whatman (1991) in which strong anion exchange cartridges (SAX) were utilised. 25 g of the maize sample was extracted with 50 ml of methanol/water (3: 1) for two hours. The slurry was filtered and the pH of the supernatant solution was adjusted to pH 5.8 - 6.5 with 1 M HCl. The SAX cartridges were preconditioned with 5 ml of methanol followed by 5 ml of methanol/ water (3:1). A 10 ml aliquot of the filtered extract was applied to the SAX cartridge at a flow rate of 2 ml per minute.



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The cartridge was washed with methanol/ water (3:1), followed by 3 ml of methanol, before being eluted with 1% HCl in methanol solution. The extract was evaporated under a stream of nitrogen, and kept in a dark screw cap vial at 0°C until used.

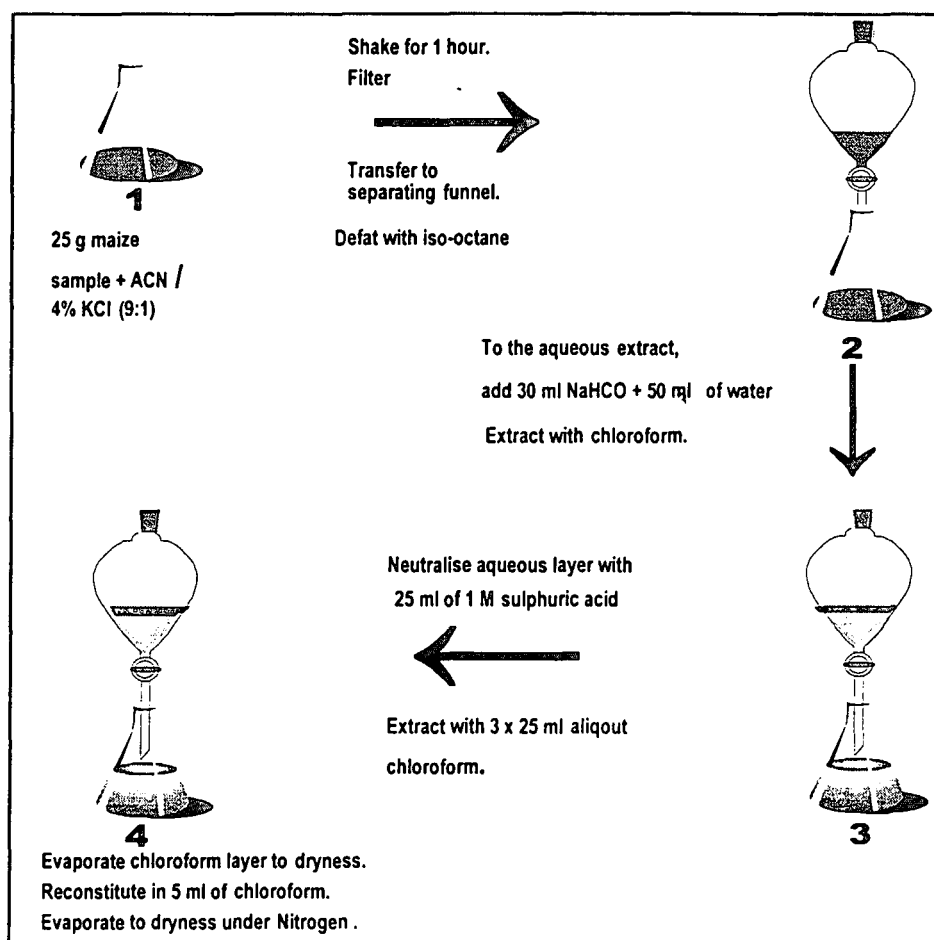


Figure 4.1: Multitoxin liquid-liquid extraction procedure for cyclopiazonic acid and ochratoxin A ( Dutton., 1981).

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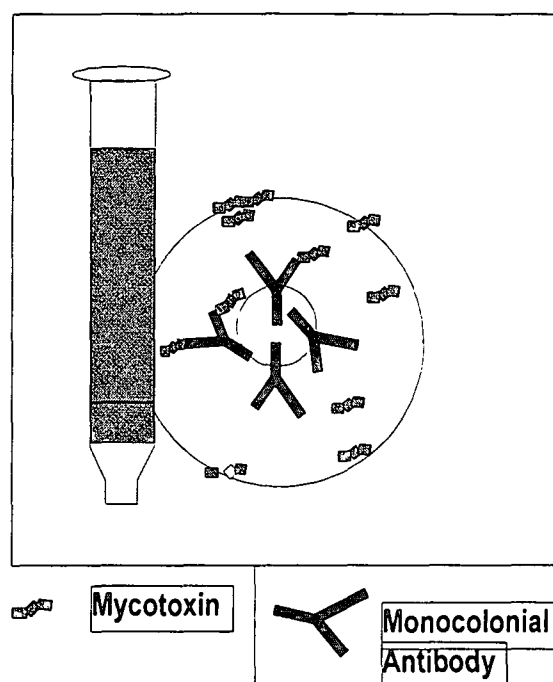
**The methods chosen for the extraction of toxins from the coffee samples are as follows :**

**Ochratoxin A** was extracted from coffee using the Ochratest affinity columns as described by Vicam (1996). This involved blending 25 g of ground coffee prior to mixing with 50 ml of methanol/1% sodium bicarbonate solution. The jar was covered and blended for five minutes. The suspension was filtered using fluted filter paper from Vicam kit. A 10 ml aliquot of the extract was placed in a clean vessel.

Twenty millilitres of 0.01% PBS/Tween 20 solution was added and the mixture homogenised using an ultrasonic bath. The extract was applied to the Ochratest column, which contains antibodies specific for ochratoxins, at a flow rate of 1 – 2 drops per second. The ochratoxin then attached to the antibody, as illustrated in Figure 4.2. After the extract had completely passed through the column, 10 ml of 0.01% PBS/Tween 20, followed by 10 ml Milli-Q water was applied at a flow rate of 1 to 2 drops per second. Ochratoxin A was eluted with 1.5 ml of methanol, at a steady flow rate of 1-2 drops per second. The extract was evaporated, under nitrogen, to dryness.

**Moniliformin and cyclopiazonic acid** were extracted from the coffee samples as described for the maize samples.

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**Figure 4.2 : Mycotoxin- antibody coupling formed in the ochratoxin affinity columns, used for the extraction of ochratoxin A from coffee samples.**

- After the extraction process, the capillary electrophoresis and high performance liquid chromatographic systems were re-calibrated using the external calibration curve technique, and the systems tested for linearity and repeatability, as described in chapters 2 and 3.
- Extraction solvents and glassware were checked to ensure no carry-over of toxins.
- The cyclopiazonic acid, moniliformin and ochratoxin A extracts were analysed using the optimised multitoxin capillary electrophoresis assay. Migration time

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tagging as well as spectral matching calculated by the System Gold program was used for the identification of the toxins.

The correlation coefficients for the spectral match at the corresponding migration times of each toxin were used as an indicator for the presence or absence of the toxins. High correlation coefficients for the spectral match (greater than 0.95) indicated a high probability of toxin contamination, whereas low correlation coefficients for the spectral match (less than 0.8) indicated a low probability or absence of the toxin.

- The capillary electrophoresis results were confirmed using the individual high performance liquid chromatography assays for the three toxins.

#### 4.2.2 Spiked maize and coffee samples, and recovery studies

The developed multitoxin method was tested on spiked maize and coffee samples. Local maize and coffee samples were spiked with 500  $\mu$ l of 50 mg/l, 20 mg/l, 10 mg/l, 5 mg/l and 1 mg/l solutions of a mixture of the three toxins prior to the extraction procedure. The final extracts for cyclopiazonic acid, moniliformin and ochratoxin A were diluted to 500  $\mu$ l with methanol. Hence, the final concentration of the toxins was 25  $\mu$ g, 20  $\mu$ g, 10  $\mu$ g, 5  $\mu$ g and 1  $\mu$ g in 25 g of maize or coffee sample.

The efficiency of the extraction process was investigated by determining the recovery of cyclopiazonic acid, moniliformin and ochratoxin A from the spiked maize, obtained from rural areas. The percent recovery was calculated from the equation as described in Equation 9 (Ravindranath, 1989).

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$$\% \text{ Recovery} = [(C_{\text{calculated}} - C_{\text{untreated}}) / C_{\text{initial spiked}}] \times f \times 100 \quad \text{Equation 9}$$

Where,

$C_{\text{calculated}}$  = Concentration of toxin (mg/l) detected by the experimental technique in the spiked sample.

$C_{\text{untreated}}$  = Concentration of toxin (mg/l) detected by the experimental technique in untreated sample ie natural contamination.

$C_{\text{initial spiked}}$  = Initial concentration of toxin(mg/l) spiked in the sample prior to extraction.

$f$  = dilution factor, where  $f = 1$  for the liquid – liquid extraction, and  $f = 5$  for the ochratest extraction method and the SAX cartridge extraction method.

The extracts of cyclopiazonic acid, moniliformin and ochratoxin A for each matrix was finally pooled together to produce a mixture of the three toxins. The mixtures were diluted to 500  $\mu\text{l}$  with methanol, prior to injecting directly into the high performance liquid chromatography and capillary electrophoresis systems.

#### 4.2.3 Cyclopiazonic acid, moniliformin and ochratoxin A analysis in naturally contaminated maize samples

The toxins were extracted from the maize samples using the chosen extraction methods, in order to test the developed multitoxin capillary electrophoresis assay for the quantification of these toxins. The results were thereafter confirmed by the individual high performance liquid chromatography methods for cyclopiazonic acid, moniliformin and ochratoxin A.

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For this study, twenty maize samples were analysed for natural contamination of cyclopiazonic acid, moniliformin and ochratoxin A. Five maize samples were obtained from the Transkei region in South Africa, which were part of crops that were harvested after the 1997 drought. Fifteen samples were purchased from local suppliers. Twenty coffee samples were analysed for cyclopiazonic acid, moniliformin and ochratoxin A contamination. Five samples were obtained from West Africa, along the Ivory Coast, and fifteen were purchased from local suppliers.

**4.3 Results and discussion****4.3.1 Spiked maize and coffee samples****4.3.1.1 Capillary electrophoresis analysis of spiked maize and coffee samples**

The untreated maize samples did not contain any detectable levels (absence of toxin at characteristic migration time) of cyclopiazonic acid, moniliformin and ochratoxin A. The repeatability and accuracy of the extraction methods were verified by performing recovery experiments using maize and coffee samples artificially contaminated with the three toxins. The results showed that the percent recovery was higher in the higher concentration range than in the lower concentration range with a % RSD of less than 4 % throughout the assay range (Table 4.1). In addition, the correlation coefficients for the spectral match for every toxin was greater than 0.9800.

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Table 4.1: Average recovery (n=3) for cyclopiazonic acid, moniliformin and ochratoxin A from spiked maize samples by capillary electrophoresis

Spiked (µg)	Maize samples						Coffee samples					
	Recovery (%)			RSD (%)			Recovery (%)			RSD (%)		
	CPA*	OA*	MON*	CPA*	OA*	MON*	CPA*	OA*	MON*	CPA*	OA*	MON*
25.00	101.12	90.12	80.15	1.11	1.23	1.55	85.12	80.15	78.56	2.23	2.25	2.36
10.00	100.11	91.12	79.55	1.75	1.56	1.69	85.00	80.13	78.53	2.56	2.26	2.58
5.00	99.75	89.85	79.12	2.05	1.45	1.58	84.23	79.63	78.22	2.55	2.89	2.45
2.50	97.11	83.45	79.03	2.75	2.05	2.06	84.01	79.22	76.23	3.02	3.69	3.06
0.50	97.24	82.15	79.05	3.06	2.25	3.11	80.12	77.23	75.22	3.23	3.75	3.36

\*CPA = cyclopiazonic acid, MON = moniliformin, OA = ochratoxin A

4.3.1.2 Confirmation studies using high performance liquid chromatography

The results obtained by capillary electrophoresis were confirmed using the individual high performance liquid chromatography systems (Chapter 2). The results (Table 4.2) compare favourably with that obtained using the optimised capillary electrophoresis system.

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**Table 4.2: Average recovery (n=3) for cyclopiazonic acid, moniliformin and ochratoxin A from spiked maize samples by high performance liquid chromatography**

Spiked ( $\mu\text{g}$ )	Maize samples						Coffee samples					
	Recovery (%)			RSD (%)			Recovery (%)			RSD (%)		
	CPA*	OA*	MON*	CPA*	OA*	MON*	CPA*	OA*	MON*	CPA*	OA*	MON*
25.00	100.12	89.52	78.85	1.55	1.23	2.22	95.56	85.52	77.23	2.23	2.22	2.565
10.00	100.02	89.10	77.02	1.88	1.56	2.05	95.23	85.56	77.02	2.58	2.25	2.89
5.00	99.55	88.75	76.23	2.23	1.45	2.29	94.23	85.01	76.23	2.01	2.45	2.96
2.50	96.12	82.12	76.11	2.65	2.05	3.25	93.23	84.44	75.23	3.05	2.39	3.01
0.50	95.12	80.15	72.23	3.06	3.05	3.65	93.01	83.23	75.01	3.57	3.54	3.25

\*CPA = cyclopiazonic acid, MON = moniliformin, OA = ochratoxin A

The recovery value obtained for moniliformin from maize samples compare favourably to that reported by Filek and Lindner (1996), (70 % recovery from spiked wheat samples using SPE cartridges in the concentration range 0.02 to 0.25 mg/kg), and Cornelli and Maragos (1998), 79 to 100 % with a % RSD of 10.2% (n=8).

The recovery obtained for the extraction of ochratoxin A from coffee compare favourably to recovery values of 59 to 83 % at levels of 5  $\mu\text{g}/\text{kg}$ , n=6 for roasted coffee reported by Jorgensen (1998). Corneli and Maragos (1998) also reported a mean recovery value of 86 % with a %RSD of 14.1%, n=8, using SPE cartridges, and Vicam (1996) reported a recovery value of 69 to 93% from spiked coffee samples.

In summary, the recovery obtained using the optimised capillary electrophoresis method compares favourably with the individual high performance liquid chromatography methods. Untreated maize and coffee samples did not contain any detectable level of cyclopiazonic acid, moniliformin and ochratoxin A. The percent



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recoveries of the spiked maize and coffee samples were in the range 72 to 101 %, and compare favourably with previous researchers (Cornelli and Maragos, 1998, Filek and Lindner, 1996, and Vicam, 1996).

#### 4.3.2 Cyclopiazonic acid, moniliformin and ochratoxin A in naturally contaminated maize and coffee samples

The potential of the multitoxin capillary electrophoresis assay to separate and detect the mixture of three toxin extracts from a naturally contaminated maize sample was evaluated. Recovery values were not considered for the study of natural contamination of cyclopiazonic acid, moniliformin and ochratoxin A in the maize samples since naturally contaminated commodities are generally more difficult to extract than spiked samples (Bennett and Richard, 1994). Hence, the extraction of the spiked sample cannot be related to the naturally contaminated sample.

##### 4.3.2.1 Capillary electrophoresis analysis of naturally contaminated maize samples

The correlation coefficients for the spectral match at the corresponding migration times of each toxin for the maize samples indicated the absence of ochratoxin A and moniliformin (low correlation values (0.1000 to 0.2000) for all the extracts).

The correlation factors for the spectral match for cyclopiazonic acid in the local samples were in the range (0.1000-0.3500), indicating absence of cyclopiazonic acid. The probability of natural contamination of cyclopiazonic acid in the five rural samples was high, since the correlation coefficients were 0.9990 to 0.9998 at the corresponding migration time for cyclopiazonic acid.

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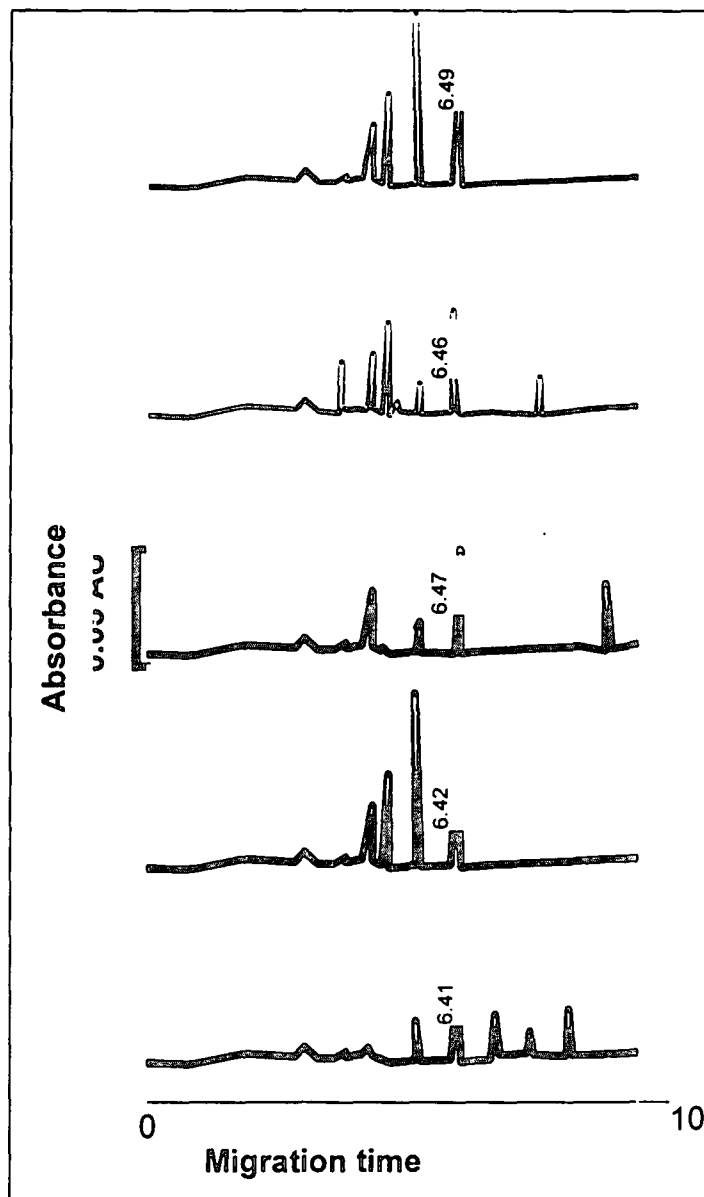
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The level of cyclopiazonic acid contamination in the maize samples was significantly high, with 1181.01 ng/g being the most contaminated sample (Table 4.3). The corresponding electropherograms for the five positive samples obtained from rural area, (Figure 4.3) indicates that cyclopiazonic acid was well resolved and free from the interference of the co-extracted maize matrix.

Table 4.3: Concentration of cyclopiazonic acid (n=3) in rural samples

Sample (Transkei region)	Concentration (µg/ml)	Concentration (ng/g)	RSD (%) Replicate analysis of samples
1	59.1	1181.0	0.12
2	50.0	1000. 0	0.12
3	20.7	415.1	0.37
4	4.6	91.1	0.26
5	3.7	74.1	0.35

## APPLICATION OF THE MULTITOXIN CAPILLARY ELECTROPHORESIS ASSAY



**Figure 4.3: Electropherograms for the cyclopiazonic acid extract from contaminated maize samples.**

[Analysis conditions: 10 mM Phosphate buffer, pH 7.5, 15 kV, 3 s injection time (23.0 nl), UV-226 nm. The migration time for cyclopiazonic acid was 6.41 to 6.49 minutes.]

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The levels of natural contamination of cyclopiazonic acid in maize ranged from 74.1 ng/g to 1181.0 ng/g. Yu and Chu (1998) reported that of the 45 corn samples analysed from West Asia, for the natural contamination of cyclopiazonic acid, 51 % contained cyclopiazonic acid with an average level of 467 ng/g ranging from 25 to 2 777 ng/g.

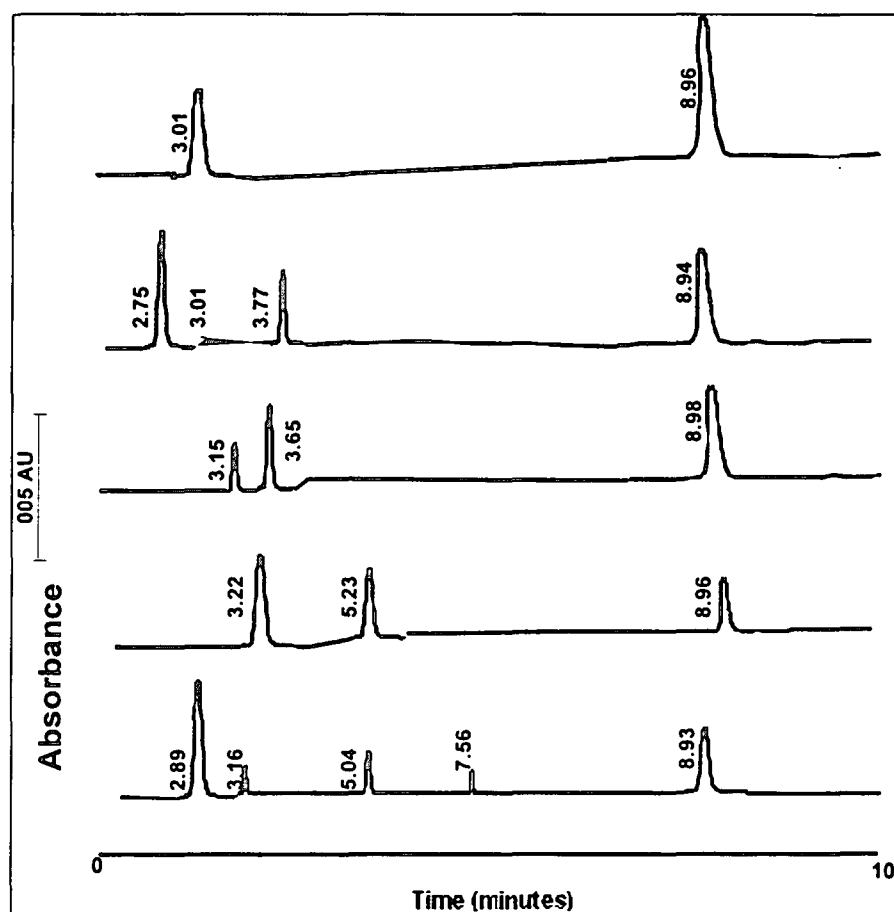
**4.3.2.2 Confirmation of capillary electrophoresis of cyclopiazonic acid, moniliformin and ochratoxin A for the natural contamination of rural maize samples by high performance liquid chromatography**

The individual high performance liquid chromatography parameters for the analysis of cyclopiazonic acid, described in Chapter 2 were used for the study. The extracts showed a significantly high level of cyclopiazonic acid contamination (1177 ng/g being the highest level of contamination) as shown in Table 4.4. The corresponding chromatograms (Figure 4.4) show a well-resolved peak for cyclopiazonic acid.

**Table 4.4: Average cyclopiazonic acid levels (n=3) in maize samples obtained from rural areas**

Samples (Transkei region)	Concentration (µg/ml)	Concentration (ng/g)	RSD (%)
1	58.8	1176.7	0.23
2	50.2	1003.3	0.32
3	20.7	413.3	0.69
4	4.6	92.2	0.89
5	3.67	73.5	0.98

## APPLICATION OF THE MULTITOXIN CAPILLARY ELECTROPHORESIS ASSAY



**Figure 4.4 : Chromatogram obtained for the analysis of cyclopiazonic acid extract from naturally contaminated rural maize samples.**

[Analysis conditions : Ethyl acetate, isopropyl alcohol, ammonia (25 %), Lichrosorb 10 Si (25 cm x 4.6 mm) column, 20  $\mu$ l sample injection, UV-280 nm detection. Flow rate = 1 ml per minute. Retention time of cyclopiazonic acid = 8.93 – 8.98 minutes.]

## APPLICATION OF THE MULTITOXIN CAPILLARY ELECTROPHORESIS ASSAY

### 4.3.2.3 Comparison of capillary electrophoresis and high performance liquid chromatography quantification for cyclopiazonic acid in maize

The concentration of cyclopiazonic acid ( $\mu\text{g/ml}$ ) in the rural maize samples by high performance liquid chromatography and capillary electrophoresis was compared using the F-Test. The F-test provides a simple method for the comparing precision of two sets of measurements. The quantity 'F', which is defined as the ratio of the variances of the two measurements was calculated and compared with the maximum acceptable value of 'F', at a certain probability level. For 5% probability, the critical 'F' value was 6.39. The 'F' value was calculated as the ratio of the variance of the square of the standard deviation of the capillary electrophoresis results and the variance of the square of the standard deviation of the high performance liquid chromatography results. Results for high performance liquid chromatography and capillary electrophoresis quantification compared favourably for the maize samples, (Table 4.5), since the 'F' values were all below the critical value of 6.39.

**Table 4.5 : Comparison of capillary electrophoresis and high performance liquid chromatography (HPLC) results for the maize samples**

Sample (Transkei region)	Capillary electrophoresis ( $\mu\text{g/ml}$ )	RSD (%)	HPLC ( $\mu\text{g/ml}$ )	RSD (%)	F Test
1	59.07	1.11	58.83	1.22	<b>0.83</b>
2	49.98	1.02	50.17	1.20	<b>0.69</b>
3	20.74	1.03	20.67	1.78	<b>0.45</b>
4	5.47	1.67	4.61	1.55	<b>1.16</b>
5	3.69	1.45	3.67	1.67	<b>0.75</b>

APPLICATION OF THE MULTITOXIN CAPILLARY ELECTROPHORESIS ASSAY

4.3.2.4 Capillary electrophoresis analysis of naturally contaminated coffee samples

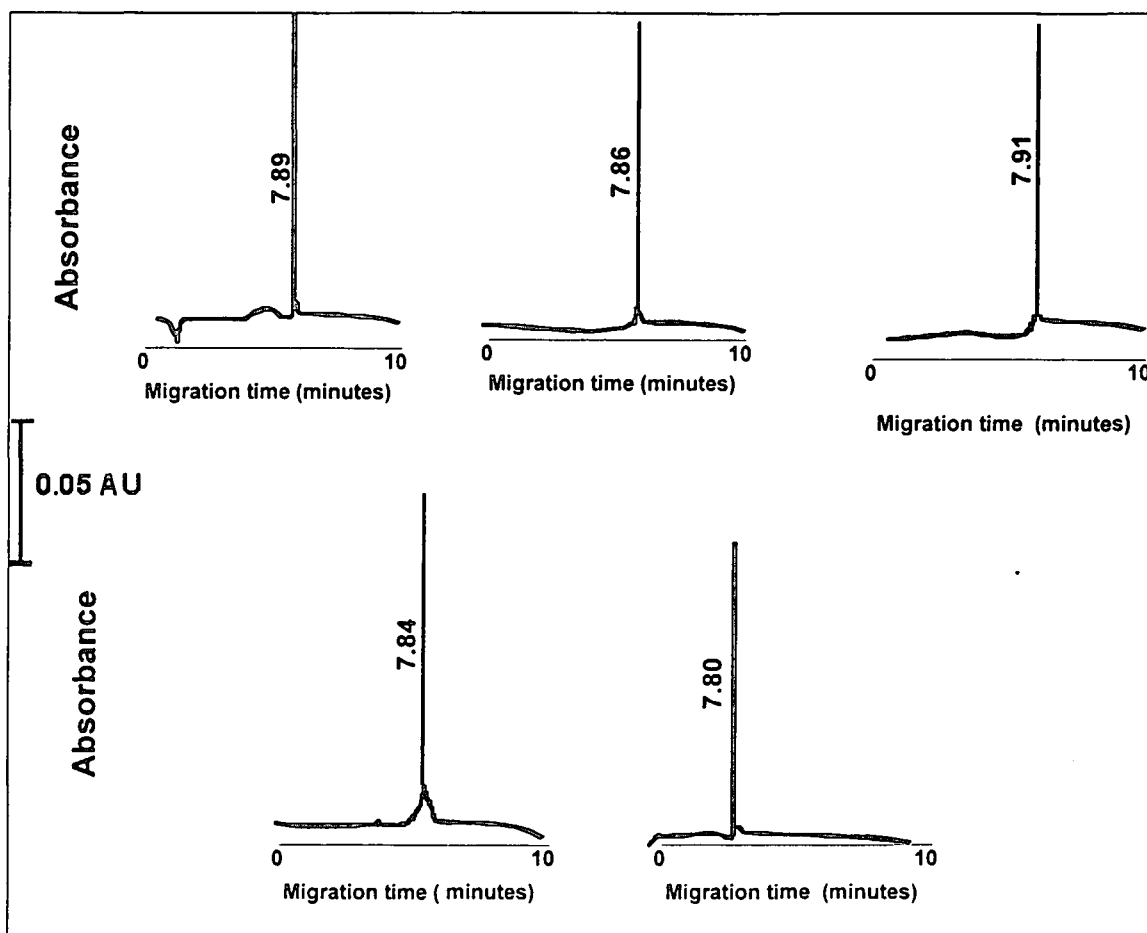
The twenty coffee samples were analysed for the contamination of cyclopiazonic acid, moniliformin and ochratoxin A. Peak identification was confirmed using retention time tagging and spectral matching. For the coffee samples, the cyclopiazonic acid and moniliformin extracts produced low correlation coefficients (0.1011 to 0.2546) at the corresponding migration times. There was no detectable level of ochratoxin A in the fifteen local samples as indicated by the low correlation coefficient (0.1221-0.3425); however, the probability of positive ochratoxin A levels in the five samples from West Africa was high with correlation coefficients in the range 0.9995 to 0.9999.

The natural contamination of ochratoxin A in coffee ranged from 20.2 to 44.0 ng/g (Table 4.6). The representative electropherograms (Figure 4.5) shows a sharp well-resolved peak for ochratoxin A, with negligible co-extracted compounds.

Table 4.6: Average levels of ochratoxin a (n=3) in coffee by analysed by capillary electrophoresis analysis

Sample (West Africa)	Concentration (µg/ml)	Concentration (ng/g)	RSD (%) (Replicate analysis of samples)
1	2.2	44.0	0.12
2	1.9	38.0	0.12
3	1.0	20.2	0.36
4	1.1	22.0	0.25
5	1.5	30.0	0.35

## APPLICATION OF THE MULTITOXIN CAPILLARY ELECTROPHORESIS ASSAY



**Figure 4.5 : Electropherograms for West African coffee samples contaminated with ochratoxin A.**

[Analysis conditions: 10 mM Phosphate buffer, pH 7.5, 15 kV, 3 s injection time (23.0 nl), UV-226 nm. The migration time for ochratoxin A was 7.80 to 7.91 minutes.]

Moss (1998) reported levels of 10 ng/g for instant coffee in European countries, and a survey performed in the United Kingdom involving 100 samples indicated a contamination range of 0.1 to 8 ng/g. Hence, the concentrations observed in this study were very high, and are of concern.



APPLICATION OF THE MULTITOXIN CAPILLARY ELECTROPHORESIS ASSAY

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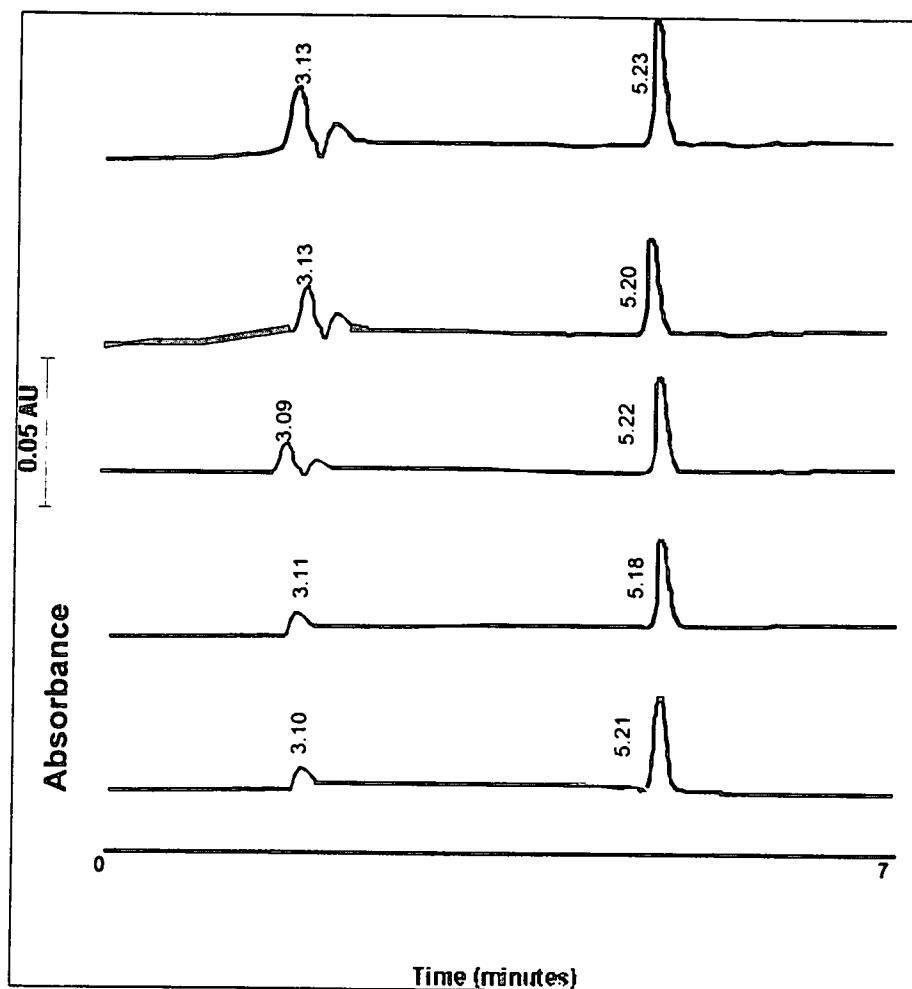
4.3.2.5 Confirmation of capillary electrophoresis results by high performance liquid chromatography

Table 4.7 shows the consistent level of ochratoxin A from triplicate runs for the West African coffee samples, with RSD values below 1%. The corresponding chromatograms (Figure 4.6) show a well-resolved peak for ochratoxin A, with a void peak at 3.00 minutes, and negligible co-extracted compounds.

Table 4.7: Mean level of ochratoxin A (n=3) in coffee analysed by high performance liquid chromatography

Samples (West African)	Concentration (µg/ml)	Concentration (ng/g)	RSD (%) Replicate analysis of samples
1	2.2	44.0	0.95
2	1.9	37.9	0.96
3	1.0	20.1	0.88
4	1.1	21.7	0.95
5	1.5	29.9	0.90

## APPLICATION OF THE MULTITOXIN CAPILLARY ELECTROPHORESIS ASSAY



**Figure 4.6 : Chromatograms obtained for the analysis of ochratoxin A in West African coffee samples.**

[Analysis conditions : 10 mM phosphate buffer with 5 mM tetrabutylammonium bromide (pH 7), methanol (92:8), C18: Phenomenex Prodigy 5 ODS-2, (150 mm x 4.6 mm) column, 20  $\mu$ l sample injection, UV-215 nm detection. Flow rate = 1 ml per minute. Migration time for ochratoxin A = 5.18 to 5.23 minutes.]

## APPLICATION OF THE MULTITOXIN CAPILLARY ELECTROPHORESIS ASSAY

## 4.3.2.6 Comparison of capillary electrophoresis and high performance liquid chromatography quantification for ochratoxin A in coffee

The concentration of ochratoxin A ( $\mu\text{g/ml}$ ) in the maize samples obtained from rural areas using high performance liquid chromatography and capillary electrophoresis were compared using the F-test, calculated by the Microsoft excel program (Table 4.8). The high performance liquid chromatography and capillary electrophoresis quantification results compare favourably for coffee extracts, since the 'F' values were all below the critical value of 6.39.

**Table 4.8: Comparison of capillary electrophoresis and high performance liquid chromatography concentration values for the West African coffee samples**

Sample (West African)	Capillary electrophoresis ( $\mu\text{g/ml}$ )	SD*	High performance liquid chromatography ( $\mu\text{g/ml}$ )	SD*	F-test
1	2.2	1.12	2.2	1.11	1.01
2	1.9	1.33	1.9	1.52	0.76
3	1.0	1.45	1.0	1.67	0.75
4	1.1	1.02	1.1	1.00	1.01
5	1.5	1.03	1.5	1.02	1.01

\*SD = standard deviation

**APPLICATION OF THE MULTITOXIN CAPILLARY ELECTROPHORESIS ASSAY**

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**4.4 Conclusion**

The maize and coffee samples obtained from local vendors showed no natural contamination of cyclopiazonic acid, moniliformin and ochratoxin A. The rural maize samples showed relatively high levels of cyclopiazonic acid contamination (74.1 – 1181.0 ng/g). The five coffee samples that were obtained from a West African source were contaminated with ochratoxin A. The contamination of ochratoxin A in the West African coffee samples were in the range 30.0 – 44.0 ng/g. The amount of variance between the maize and coffee samples could be due to their difference in extrinsic and intrinsic parameters. The high level of cyclopiazonic acid in the rural maize samples, and ochratoxin A in coffee samples, are cause for concern. Hence, these commodities require frequent monitoring.

The capillary electrophoresis results compare favourably with the high performance liquid chromatography results for both the applications. The capillary electrophoresis peaks show higher efficiency as indicated by the sharp peaks for cyclopiazonic acid and ochratoxin A compared to the broad peaks obtained by high performance liquid chromatography. The capillary electrophoresis results for quantification of ochratoxin A in the coffee and cyclopiazonic acid in maize compares favourably with high performance liquid chromatography as shown by the F-test results. This implies that the multitoxin capillary electrophoresis assay was successful in detecting and separating cyclopiazonic acid and ochratoxin A from the complex maize and coffee extract.

## CONCLUSIONS AND RECOMMENDATIONS

This study involved the development of analytical techniques for the simultaneous determination of cyclopiazonic acid, moniliformin and ochratoxin A. Currently, there is no methodology available for the multitoxin analysis of cyclopiazonic acid, moniliformin and ochratoxin A by any analytical technique.

The project was divided into three phases. The first phase of the project involved setting up the current methods for the analysis of the toxins, viz., thin layer chromatography and high performance liquid chromatography, and to establish the repeatability, reproducibility, linearity range and detection limit for the techniques. The results indicated that the existing methods were repeatable and reproducible with a relative standard deviation of less than 2 %. The high performance liquid chromatography methods for the individual toxins were linear with a R-squared value greater than 0.9997. The high performance liquid chromatography methods for the three toxins were significantly more sensitive than the thin layer chromatography methods (Table 5.1). This trend was expected, since high performance liquid chromatography is a more developed technique, using high-resolution instrumentation. The detection limits obtained for the techniques compare favourably to that of previous researchers.

**Table 5.1: Comparison of the sensitivities for cyclopiazonic acid, moniliformin and ochratoxin A by thin layer chromatography and high performance liquid chromatography**

<b>Mycotoxin</b>	<b>Sensitivity of thin layer chromatography</b>	<b>Sensitivity of high performance liquid chromatography</b>
Cyclopiazonic acid	5 µg/ml (25 ng)	250 ng/ml (5 ng on column) at 280 nm
Moniformin	25 µg/ml (125 ng)	20 ng/ml (0.4 ng on column) at 229 nm
Ochratoxin A	0.40 µg/ml (2 ng)	0.31 µg/ml (0.62 ng on column) at 215 nm



## CONCLUSIONS AND RECOMMENDATIONS

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The second phase of the project was to develop a multitoxin method using high performance liquid chromatography and capillary electrophoresis. The first stage evaluated the potential of high performance liquid chromatography for the simultaneous analysis of cyclopiazonic acid, moniliformin and ochratoxin A. Initially, the existing high performance liquid chromatography techniques for each of the three toxins were tested for the separation of a mixture of the three toxins. The chromatograms for the individual high performance liquid chromatography methods for cyclopiazonic acid, ochratoxin A and moniliformin showed poor resolution and distorted peak shapes for all three toxins. Hence, the methods were unsuccessful at separating the mixture of the toxins. Thereafter, various high performance liquid chromatography method development schemes were investigated. The high performance liquid chromatography techniques studied were reversed phase-, paired ion-, normal phase- and ion exchange chromatography. In evaluating each of the different techniques for the simultaneous analysis of cyclopiazonic acid, moniliformin and ochratoxin A, only one toxin was detected per technique. It was highly probable that the other toxins were either eluting at the void, or remaining in the column for an extended period, hence eluting as an extremely broad peak, undetected by the photodiode array detector. Modifications to the techniques, using various method development strategies were unable to resolve the problem. Hence, the various techniques evaluated failed to separate the mixture of the three toxins. This highlights the difficulty of separating a mixture of structurally different compounds by high performance liquid chromatography.

The second stage of this phase was to evaluate capillary electrophoresis for simultaneous analysis of cyclopiazonic acid, moniliformin and ochratoxin A. The capillary electrophoresis techniques used were micellar electrokinetic chromatography and capillary zone electrophoresis. Micellar electrokinetic chromatography was investigated first, as previous researchers reported successful separation of a range of toxins (Holland and Sepaniak, 1993 and Janini *et al.*, 1996). However, baseline separation was not achieved for cyclopiazonic acid and ochratoxin A.

## CONCLUSIONS AND RECOMMENDATIONS

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The implementation of the overlapping resolution mapping scheme for the optimisation of pH and SDS concentration indicated that SDS was not required for the separation of the three toxins. Hence, capillary zone electrophoresis was used for all other work in the study. The optimised capillary zone electrophoresis conditions for the separation of the toxins were 10 mM phosphate buffer, 15 kV applied voltage with corresponding 40  $\mu$ A current, 3 seconds injection time (23 nl injection volume), and UV- detection at 226 nm.

The repeatability, reproducibility and linearity of the developed multitoxin assay by capillary electrophoresis compare favourably with the individual high performance liquid chromatographic systems, with the relative standard deviation below 2 %. The detection limit for capillary electrophoresis (Table 5.2) indicated that this method was less sensitive than the high performance liquid chromatography methods.

**Table 5.2: Detection limits of cyclopiazonic acid, moniliformin and ochratoxin A by capillary electrophoresis at 226 nm**

<b>Mycotoxin</b>	<b>Capillary electrophoresis (on column detection limit)</b>
Cyclopiazonic acid	500 ng/ml or 11.5 pg
Moniliformin	2 $\mu$ g/ml or 46.0 pg
Ochratoxin A	1 $\mu$ g/ml or 23.0 pg

The advantages of capillary electrophoresis, however, are that the method development time for capillary zone electrophoresis was significantly lower than high performance liquid chromatography for the separation of the three toxins. Other advantages observed with this technique, were low sample consumption (23 nl as compared to 20  $\mu$ l in high performance liquid chromatography), low running costs and minimum generation of organic waste.



## CONCLUSIONS AND RECOMMENDATIONS

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Considering the separation efficiencies as currently achieved it is apparent that capillary zone electrophoresis is a promising alternative to high performance liquid chromatography for the determination of toxins in food.

The next phase of the project evaluated the application of the developed multitoxin capillary electrophoresis assay. This was performed using established extraction methods on spiked and naturally contaminated maize and coffee samples. The recovery values obtained using the individual high performance liquid chromatography methods compare favourably with the developed capillary electrophoresis method. The high performance liquid chromatography and capillary electrophoresis quantification results compare favorably for the maize and coffee extracts, since the 'F' values were all below the critical value of 6.39. The rural maize samples showed relatively high levels of cyclopiazonic acid contamination (74.1-1181.0 ng/g). The five coffee samples that were obtained from a West African source were contaminated with ochratoxin A. The extent of contamination of ochratoxin A in the West African coffee samples was in the range 30.0-44.0 ng/g.

The capillary electrophoresis method developed is novel, rapid and compares favourably with other published protocols. However, the sensitivity of the capillary electrophoresis method should be further investigated. Current trends in this regard, involve on-line detection, using the mass spectrometer.

The limitation of the extraction procedures chosen is that there are two separate extraction procedures. To date, there are no reports on the simultaneous extraction of the three toxins. However, cyclopiazonic acid and ochratoxin A have been successfully extracted from the maize sample, with good recoveries in the current study. Future work should involve developing a simultaneous extraction method for the three toxins.



## CONCLUSIONS AND RECOMMENDATIONS

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The high levels of cyclopiazonic acid in the rural maize samples, and ochratoxin A in coffee samples, are cause for concern. Hence, systems should be put in place for the frequent monitoring of these toxins in food commodities.

## Experimental Techniques

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Appendix 1 describes the details of all chemical and instruments used during this investigation. Included in this section are the instrument conditions employed together with the preparation of the mobile phase and buffers required for thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and capillary electrophoresis (CE) techniques.

- **pH measurements**

All pH measurements were made using a Model 691 pH meter (Metrohm), calibrated daily with buffers at pH values of 4.00 and 7.00 (Saarchem).

- **Ultraviolet and visible absorbance measurement**

Absorbance spectra of the dye solutions were measured on a Varian DMS 100 double beam UV-VIS spectrophotometer. Spectra were recorded between 350 and 900 nm using a pair of matched quartz cuvettes with a pathlength of 1 cm. Milli-Q water was used as the reference solution.

- **Thin layer chromatography**

All TLC manipulations were performed under hood where possible, especially since in the dry form mycotoxins tend to be electrostatic by nature, resulting in the tendency to disperse in working area. Accidental spills of toxins were swabbed with 1 % sodium hypochlorite solution.

The **2 % oxalic acid solution** was prepared by diluting 2 g of oxalic acid to 100 ml with water. The silica gel TLC plates soaked with oxalic acid were prepared by soaking the thin layer chromatography plate in 2 % oxalic acid solution, then baking in an oven at 105 °C for one hour. Care was taken when spotting the plates, to ensure that the homogenous layer on the plate was not cracked or damaged.

## Experimental Techniques

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The spray reagent for cyclopaizonic acid visualisation (**Erhlich's reagent**) was prepared by mixing 1 g of 4-dimethyl aminobenzaldehyde, 10 ml water, 20 ml concentrated HCl and diluting to 100 ml with 95 % ethanol. The TLC plate was sprayed with Erhlich's reagent, and air-dried before exposing to concentrated HCl fumes. The spray reagent for moniliformin visualisation - **1 % 2,4 – dinitrophenylhydrazine solution (2,4 DNP)** was prepared by diluting 1 g of 2,4 – dinitrophenylhydrazine and 50 ml concentrated  $\text{H}_2\text{SO}_4$  to 100 ml with water.

- **High performance liquid chromatography**

The chromatographic system used for all experiments was the Waters 600E system controller with a Gilson 401C autosampler and a Waters photodiode array detector connected to a computer with the Waters 991 computer package. The sample volume was 20  $\mu\text{l}$ . The mobile phase flow rate was 1 ml/minute. The HPLC system was allowed to equilibrate with each mobile phase for fifteen minutes at a flow rate of 0.5 ml/minute prior to each experiment. At the end of the day, the system was flushed with acetonitrile : water (50:50) for thirty minutes at 1 ml/minute.

All solvents used were high performance liquid chromatography grade. All buffers and solvents were degassed prior to analysis. Degassing was performed by vacuum filtration, using a 0.45-micron filter paper (Millipore), and on-line sparging with Helium ultra-pure gas from Fedgas (South Africa). All buffers were prepared in Milli-Q water (Millipore Corporation).

The **10 mM Phosphate buffer** was prepared by dissolving 0.55 g di-sodium hydrogen orthophosphate anhydrous ( $\text{Na}_2\text{HPO}_4$ ), and 0.397 g sodium dihydrogen orthophosphate dihydrate ( $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ ) in a beaker containing 200 ml of Milli-Q water. The pH of this solution was approximately 7.50. The pH of the buffer solution was adjusted with 0.1 M HCl to pH 7.0. This solution was quantitatively transferred to a 250 ml volumetric flask and made up to mark. The flask was placed in an ultrasonic bath to ensure complete homogenisation, and then subsequently cooled to room temperature.

## Experimental Techniques

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The 5 mM tetrabutylammonium bromide (TBAB) salt in phosphate buffer was prepared similar to 10 mM Phosphate buffer, except that 1.511 g of TBAB salt was added together with the buffer salts prior to adjusting the pH of the solution.

The buffers required for the multitoxin paired ion chromatography investigation were prepared by dissolving the required quantity of reagents, as shown in Table 1, prior to adjusting the pH values with either sodium hydroxide solution or acetic acid solution.

The solutions required for Table 1 were prepared as follows :

- **50 mM phosphoric acid ( $\text{H}_3\text{PO}_4$ )** : The buffer was prepared by dissolving 2.5551 g di-sodium orthophosphate anhydrous ( $\text{Na}_2\text{HPO}_4$ ), and 1.5397 g sodium dihydrogen orthophosphate dihydrate ( $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ ) in a beaker containing 200 ml of Milli-Q water. The pH of this solution was approximately pH 7.50. The pH of the buffer solution was adjusted with 0.1 M HCl to pH 7.0. This solution was quantitatively transferred to a 250 ml volumetric flask and made up to mark. The flask was placed in an ultrasonic bath to ensure complete homogenisation, and then subsequently cooled to room temperature.
- **5 mM phosphate buffer**: 1:10 dilution of 50 mM phosphate buffer.
- **50 mM acetic acid** : 0.75 ml of acetic acid, 99.95 % (BDH grade) diluted to 250 ml
- **5 mM acetic acid** : 1 : 10 dilution of 50 mM acetic acid.
- **0.2 M tetrabutylammonium bromide (TBAB)**: 16.1200 g tetrabutyl ammonium bromide salt (BDH) diluted to 250 ml with water.
- **0.2M NaOH**: 2.1753 g NaOH pellets (BDH) diluted to 250 ml with water.

## Experimental Techniques

**Table 1 : Buffer composition at various pH for the multitoxin paired ion chromatography high performance liquid chromatography systems.**

Buffer	COMPOSITION
Buffer, pH 2.5	50 ml of 50 mM phosphoric acid and 50 ml of 50 mM acetic acid, adjust to pH 2.5 with 0.2 M sodium hydroxide (~1.5 ml)
Buffer, pH 7.5	50 ml of 50 mM phosphoric acid and 50 ml of 50 mM acetic acid, adjust to pH 7.5 with 0.2 M sodium hydroxide (~5.7 ml)
Ion-pairing agent	0.2 M TBAB in buffer (50 ml), 25 ml of 5 mM phosphoric acid and 25 ml of 5 mM acetic acid, adjust to pH 5.00 with 0.2 M sodium hydroxide (~3.5 ml).

- **Capillary electrophoresis**

The system used was the Beckman P/ACE 5000 capillary electrophoresis system connected to a photodiode array detector. Data was analysed with the Systems Gold software. Separations were performed in a 57 cm (50 cm effective length) x 0.45  $\mu$ m i.d. fused silica capillary (Beckman Instruments). The capillary was equilibrated with 0.1 M NaOH (2 minutes), water (2 minutes), followed by the buffer (2 minutes). The temperature of the capillary was set at 25 °C.

At the end of the day, the capillary was flushed with 0.1 M NaOH ( 3 minutes), 1 M NaOH (3 minutes), 0.1 M NaOH (3 minutes), water (5 minutes) followed by air (5 minutes).

The 50 mM SDS in 10 mM phosphate-6mM Borate buffer, pH 9.3 was prepared by dissolving 2.1234 g di-sodium hydrogen orthophosphate anhydrous ( $\text{Na}_2\text{HPO}_4$ ), 1.5896 g sodium dihydrogen orthophosphate dihydrate ( $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ ), 2.2078 g of sodium

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borate ( $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ ) and 14.4123 g of sodium dodecyl sulphate in a beaker containing 800 ml of Milli-Q water. The pH of the buffer solution was adjusted with 0.1 M NaOH to

pH 9.3. This solution was quantitatively transferred to a 1 l volumetric flask and made up to mark. The flask was placed in an ultrasonic bath to ensure complete homogenization, and then subsequently cooled to room temperature.

The **phosphate – SDS buffers for the overlapping resolution mapping scheme investigation** were prepared by dissolving sodium di-hydrogen orthophosphate anhydrous ( $\text{NaH}_2\text{PO}_4$ ), sodium dihydrogen orthophosphate dihydrate ( $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ ) and SDS in the required ratios as shown in Table 2, in a beaker containing 400ml of Milli-Q water. The pH of the buffer solution was adjusted with either 0.1 M HCl or 0.1 M NaOH to the required pH. This solution was quantitatively transferred to a 500 ml volumetric flask and made up to mark. The flask was placed in an ultrasonic bath to ensure complete homogenization, and then subsequently cooled to room temperature.

**Table 2: Buffer preparation for varying phosphate: SDS ratios**

SDS Conc. (mM)	pH	Mass of Di-sodium hydrogen phosphate (g)	Mass of Sodium di-hydrogen phosphate (g)	Mass of SDS (g)
0	6.5	1.0647	0.7805	0.000
0	7.0	1.0645	0.7804	0.000
0	7.5	1.0645	0.7804	0.000
25	6.5	1.0644	0.7803	3.6007
25	7.0	1.0645	0.7803	3.6003
25	7.5	1.0645	0.7802	3.6004
50	6.5	1.0647	0.7803	7.2004
50	7.0	1.0644	0.7803	7.2001
50	7.5	1.0646	0.7804	7.2004

### Experimental Techniques

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- **Extraction procedure - Liquid-liquid extraction and Ochratest affinity columns (Vicam)**
- 4 % KCl solution - 4 g of KCl (AR grade) diluted to 100 ml with water
- 1 M sulphuric acid (10 ml  $\text{H}_2\text{SO}_4$ , 99.99 % purity diluted to 100 ml with water)
- 1 % methanolic HCl (1 ml of concentrated HCl diluted to 100 ml with methanol)
- 1 % Sodium Bicarbonate solution – 1 g sodium bicarbonate AR grade diluted to 100 ml with water.
- Methanol/ 1% sodium bicarbonate (70 ml of methanol + 30 ml of water)
- Phosphate buffer saline (PBS) – 8.0157 g of sodium chloride + 1.2089 g of  $\text{Na}_2\text{HPO}_4$  + 0.2134 g  $\text{KH}_2\text{PO}_4$  + 0.2341 g + 0.2134 g KCl in milli-Q water, adjusted to pH 7.00 with concentrated HCl.
- PBS / 0.01 % Tween 20 (polyoxyethylene – sorbitan monolaurate) : 0.1 ml of Tween 20 + 1000 ml PBS.

## APPENDIX 2

### Rectangular Plot Method

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This appendix describes the theory used for the rectangular plot optimisation scheme, for the separation of the three toxins.

#### ♦ Theory of the rectangular plot method

For the optimisation of two parameters, a set of nine experiments is performed at selected points on a rectangular diagram (Figure A2-1). Once the experimental range was established from the preliminary experiments, the composition for the other seven sets of experiments would be determined as combinations of the minimum, mid-way and maximum values. For example, the pH and the corresponding composition of the sodium dodecylsulphate buffer for the mid-way experiment would consist of 50 % of the maximum pH and 50 % of the maximum sodium dodecylsulphate concentration.

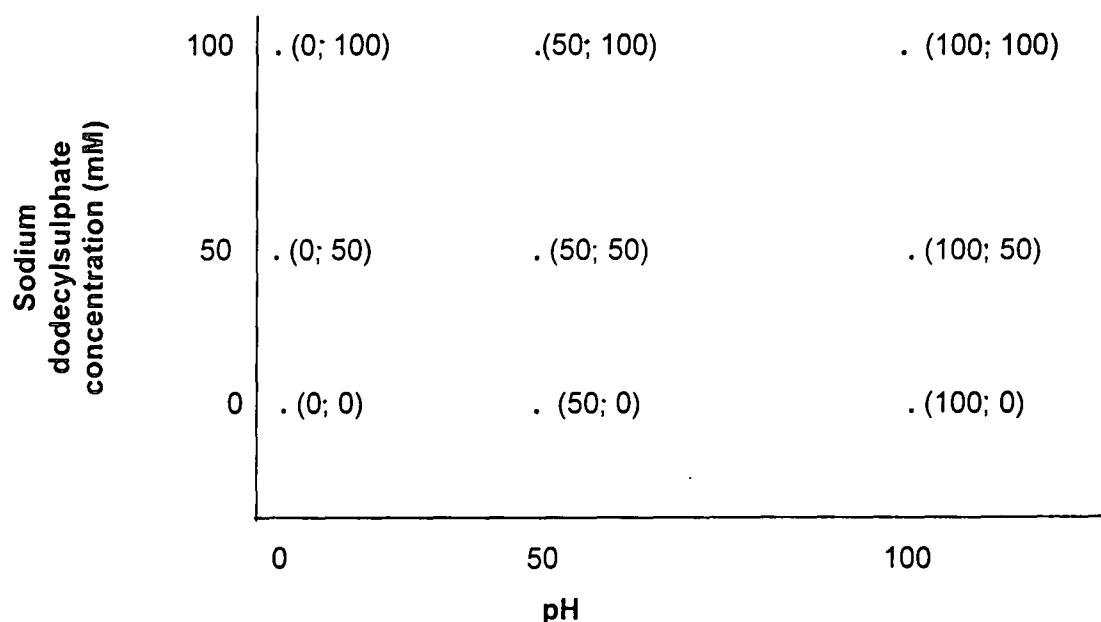


Figure A2-1: The location of nine experiments chosen from the rectangular plot. The composition of each point is represented as the percentage maximum of the pH and sodium dodecyl sulphate concentration for the buffer system.



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