

**Determination of fumonisins in maize by High Performance Liquid
Chromatography with fluorescence and ultraviolet detection of *o*-
phthaldialdehyde, naphthalene-2,3-dicarboxaldehyde and dansyl chloride
derivatives**

by

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**A thesis submitted in partial fulfilment of the requirements for the degree
Magister Scientiae in the Department of Chemistry, University of the**

Western Cape

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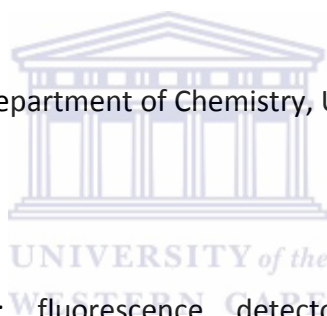
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Abstract

Determination of fumonisins in maize by High Performance Liquid Chromatography with fluorescence and ultraviolet detection of *o*-phthaldialdehyde, naphthalene-2,3-dicarboxaldehyde and dansyl chloride derivatives

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Keywords: Fumonisins; fluorescence detector; ultraviolet detector; *o*-phthaldialdehyde; naphthalene-2,3-dicarboxaldehyde; dansyl chloride; high-performance liquid chromatography; strong anion extraction; immunoaffinity; diode array detector.

Fumonisins, carcinogenic mycotoxins produced by various *Fusarium* species, occur naturally in maize and maize-based food products. They are hazards for animal and human health as they cause cancer in rodents and have been associated with oesophageal cancer and neural tube defects in humans. The most abundant naturally occurring fumonisins analogues in maize are

fumonisin B₁, B₂ and B₃ (FB₁, FB₂ and FB₃). For analytical determination, they mostly require suitable extraction, clean-up and pre or post-column derivatization together with reversed-phase HPLC separation. *o*-Phthaldialdehyde (OPA) had been adopted as the most widely used derivatization reagent for fumonisins as they lack useful chromophores or fluorophores. Alternative derivatization reagents, naphthalene-2,3-dicarboxaldehyde (NDA) and dansyl chloride (DnS-Cl), were investigated in this study. The HPLC system used was equipped with diode array (DAD) and fluorescence detectors to determine UV detection as an alternative following derivatization with OPA, NDA and DnS-Cl. Optimization of the NDA derivatives with working standards resulted in limits of detection (LOD) of FB₁, FB₂ and FB₃ with FLD of 0.11 ng, 0.50 ng and 0.27 ng, respectively, and with DAD 13.8 ng, 12.5 ng and 6.6 ng, respectively. Subsequently naturally contaminated maize samples, collected from subsistence farmers in the Eastern Cape, were cleaned-up with strong anion exchange (SAX) solid phase extraction (SPE) cartridges. The coefficient of variation (CV) for FB₁, FB₂ and FB₃ in maize samples (n=6) were 2.6%, 1.8% and 5.3%, respectively, with FLD compared to 6.0%, 3.4% and 9.5%, respectively, with DAD. Subsequently the NDA derivatization was compared to the OPA derivatization, as well as an alternative sample clean-up with immunoaffinity column (IAC) by analyzing naturally contaminated maize samples (n = 15) ranging in total fumonisin (TFB = FB₁ + FB₂ + FB₃) levels from 106 - 6000 µg/kg. After IAC clean-up of extracted samples, the recoveries for NDA-FLD of FB₁, FB₂ and FB₃ were 62%, 94% and 64%, respectively. NDA proved to be an

effective derivatization reagent for fumonisin in naturally contaminated maize samples following IAC clean-up, except for DAD at TFB levels below 1000 µg/kg. In contrast NDA derivatization following SAX clean-up produced results comparable to OPA only for levels below 1000 µg/kg. FLD and DAD produced comparable results irrespective of the clean-up method or the derivatization agent. The investigation of DnS-Cl as a derivatization reagent resulted in LOD for FB₁, FB₂ and FB₃ of 4.3 ng, 3.9 ng and 2.1 ng, respectively, with FLD and 17.2 ng, 15.6 ng, 15.6 ng, respectively, with DAD. Although sensitive and reproducible derivatives were formed with fumonisin working standards, matrix interferences from maize samples with DnS-Cl rendered this derivatization reagent unsuitable for fumonisin analysis in naturally contaminated maize. In conclusion this study has shown that UV detection can be utilized as an alternative to FLD for fumonisin analysis in naturally contaminated maize irrespective of the clean-up method or the derivatization agent.

13 May 2011

DECLARATION

I declare that '*Determination of fumonisins in maize by High Performance Liquid Chromatography with fluorescence and ultraviolet detection of o-phthaldialdehyde, naphthalene-2,3-dicarboxaldehyde and dansyl chloride derivatives*' is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Full name..... Date.....



Signed.....

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Lastly, I want to thank God for all the blessings he has bestowed on me. I am who I am because I know the Lord *“Know the Lord, He is God, it is He who has made us, and not we ourselves; we are His people and the sheep of His pasture- Psalm 100: 3”*.

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ABBREVIATIONS

FB	Fumonisin
FB ₁	Fumonisin B ₁
FB ₂	Fumonisin B ₂
FB ₃	Fumonisin B ₃
OPA	o-phthaldialdehyde
NDA	Naphthalene-2,3-dicarboxaldehyde
DnS-Cl	Dansyl Chloride
SAX	Strong anion exchange
IAC	Immunoaffinity column
FBTest	FumoniTest
HPLC	High - performance liquid chromatography
FLD	Fluorescence detector
DAD	Diode array detector
UV	Ultraviolet detection
RSD	Relative standard deviation
CV	Coefficient of variation
LOD	Limit of detection
LOQ	Limit of quantification
Std	Standard

The language and style used in the thesis are in accordance with the requirements of *Mycotoxin Research*, *Journal of Chromatography B* and the *PROMEC Unit, MRC*. Each chapter is an individual entity and some repetition between chapters has, therefore, been unavoidable.

CONTRIBUTIONS OF THESIS

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determination of fumonisin mycotoxins in maize: A comparative study of naphthalene-2,3-dicarboxaldehyde and *o*-phthaldialdehyde derivatization reagents for fluorescence and diode array detection. (Journal of chromatography B)

Conference contributions from thesis:

1. Analitika Conference 2010 (Stellenbosch University, South Africa)

Poster: N. Ndube, L. van der Westhuizen, Green IR and G.S. Shephard. UV detection as an alternative to fluorescence detection for HPLC determination of fumonisins in maize.

2. Mycored International Conference 2011

(CTICC, Cape Town, South Africa)

Poster: N. Ndube, L. van der Westhuizen, Green IR and G.S. Shephard. HPLC analysis of fumonisins in maize using ultraviolet detection as an alternative to fluorescence detection.

CHAPTER 1



1.1 Introduction

Toxins are poisonous substances that are produced by living cells or organisms (Medical Dictionary). They are synthesised by plant species, animal or micro-organisms and are generally harmful to a different organism. Mycotoxins are toxic secondary metabolites produced by fungi growing on a range of cereal and food matrixes and many are produced by plant pathogens (Turner et al., 2009). Since the discovery of aflatoxins in 1960, mycotoxins have been found to be responsible for a variety of human and animal diseases (Shephard, 2008). Fumonisin are carcinogenic mycotoxins produced by fungi of the *Fusarium* species, primarily by *F. verticillioides* and *F. proliferatum* and were first isolated by Gelderblom et al., 1988. They are an economically important group of mycotoxins that occur primarily in maize and maize-based products (Shephard et al., 1996).

There are at least 28 chemical analogues of fumonisins, but the most important are the fumonisin B's (FBs) which occur naturally in contaminated maize. The most important FBs are fumonisin B₁ (FB₁), fumonisin B₂ (FB₂) and fumonisin B₃ (FB₃), with FB₁ being the most prevalent (Rheeder et al., 2002). Fumonisin have been reported to show cancer promoting properties in rats (Gelderblom et al., 1996). Fumonisin are not mutagenic (Gelderblom et al., 1991; Knasmüller et al., 1997) nor genotoxic in primary rat hepatocytes (Norred et al., 1992) but FB₁ is hepatocarcinogenic in male BD IX rats (Gelderblom et al., 2001) and B6C3F₁ female mice and nephrocarcinogenic in male Fischer 344 rats (Howard et al.,

2001). Fumonisin is known to cause leukoencephalomalacia in horses and pulmonary edema syndrome in pigs (Howard et al., 2001; Marasas, 2001).

Fumonisin has been associated with the high prevalence of human oesophageal cancer in areas like the former Transkei region in South Africa (Rheeder et al., 1992) and Santa Catarina State, Brazil (Van der Westhuizen et al., 2003), where high levels of fumonisin contaminated foods are part of the normal diet (Rheeder et al., 1992; Sun et al., 2007; Wang et al., 2008). It has been suggested that fumonisins are a risk for inducing liver cancer in humans (Ueno et al., 1997). There has also been a proposed link between fumonisin exposure and neural tube defects in humans (Missmer et al., 2006; Hendricks, 1999; Marasas et al., 2004).



Based on information available at that time, the International Agency for Research on Cancer classified FB₁ as a possible human carcinogen (group 2B) (IARC, 2002). The US Food and Drug Administration (FDA) have recommended that cautionary levels be put in place to reduce human exposure to fumonisins intended for human and animal consumption (FDA, 2001). In 2007, The European Commission (EC) regulated fumonisin exposure at various levels from 4000 µg fumonisins/kg for unprocessed maize, 1000 µg/kg for maize intended for direct human consumption and 200 µg fumonisins/kg for processed maize-based foods and baby foods (EC, 2007).

Due to the problems and risks associated with fumonisin contamination of animal feed and human food, there is a growing need to develop reliable and

sensitive methods for the determination of fumonisins in maize and maize-based foods (Shephard, 1998). Various different methods that have been reported for the analysis of fumonisins include: thin-layer chromatography (TLC) (Gelderblom et al., 1988; Shephard and Sewram 2004), liquid chromatography-mass spectrometry (LC-MS) (Zollner et al., 2006), gas chromatography-mass spectrometry (GC-MS) (Plattner et al., 1990) and liquid chromatography with reversed-phase high performance liquid chromatography (RP-HPLC) being the most widely used current method for fumonisin determination (Shephard et al., 1996). Samples are extracted either with aqueous methanol or acetonitrile and mostly cleaned-up on strong anion exchange (SAX) solid phase extraction (SPE) cartridges or immunoaffinity columns (Sydenham et al., 1996). Fumonisins lack a suitable chromophore that enables them to be detected by UV and consequently need to be derivatized prior to HPLC separation (Shephard, 1998).

Different derivatization reagents have been reported for fumonisin analysis such as maleyl (Sydenham et al., 1990), fluorescamine (Ross et al., 1991), 4-fluoro-7-nitrobenzofurazan (Scott and Lawrence 1992), but *o*-phthaldialdehyde (OPA) still remains the derivatization reagent of choice for most laboratories as it produces highly fluorescent derivatives readily separated by HPLC (Shephard, 1998). Detection of OPA fumonisin derivatives is mainly fluorescence-based at excitation wavelength of 335 nm and emission wavelength of 420 nm (Shephard 1990). However, many laboratories requiring infrequent fumonisin analysis are equipped only with HPLC with an ultraviolet detector (UV) (Ndube et al., 2009).

1.2 Aims and objectives

Fumonisin analysis has been based almost exclusively on fluorescence detection (FLD) and liquid chromatography mass spectrometry (LC-MS). However, there has been a growing need to develop an analytical method for fumonisin analysis by laboratories requiring occasional fumonisin analysis and equipped only with a UV detector. Limited work has been done on the analysis of fumonisins by HPLC-UV detection. Maleyl derivatives analysed by HPLC-UV gave detection limits of 10 µg/g which is inappropriate for naturally contaminated maize samples (Sydenham et al., 1990) and fluorescent derivatives of 4-fluoro-7-nitrobenzofurazan (NBDF) gave higher detection limits of 100 µg/g and also showed limited stability (Scott and Lawrence 1994). OPA, naphthalene-2,3-dicarboxaldehyde (NDA) and dansyl chloride (DnS-Cl), the reagents studied here, have previously only been used as fluorogenic reagents.

The aim of this work was to investigate the degree to which fumonisins present in maize can be determined by high-performance liquid chromatography (HPLC) with both FLD and UV detection of their OPA, NDA and DnS-Cl derivatives. The objective of the study was to determine the extent to which UV may be used as an alternative to FLD as well as the extent to which NDA or DnS-Cl may be employed as alternatives to the widely used OPA derivatization reagent. The specific objectives of this study were:

1. To investigate the scope to which fumonisins in maize could be determined by high-performance liquid chromatography (HPLC) with ultraviolet (UV) and fluorescence (FLD) detection.
2. To optimize the derivatization of fumonisins with NDA and DnS-Cl.
3. To determine and compare differences between FLD and DAD responses of the different derivatization reagents.
4. To evaluate the applicability of the derivatization reagents for the analysis of naturally contaminated maize samples using different clean-up methods.

1.3 Research approach

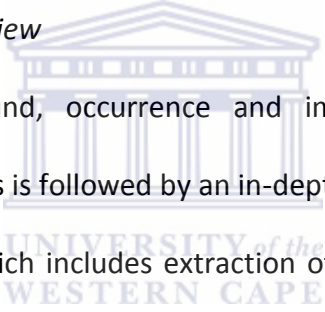
Derivatization of fumonisins is necessary as they lack a suitable chromophore or fluorophore for ultraviolet and/or fluorescence detection. This is best achieved by pre-column rather than a post-column on-line approach, as pre-column derivatization requires less equipment and is much easier to handle. Consequently the initial step of the study was to identify suitable derivatization reagents which might offer a suitable system for UV detection. The well characterized method using OPA was investigated first, where after the reagents NDA and DnS-Cl were evaluated on a comparative basis. As these latter two reagents have found limited use in fumonisin analysis, the derivatization protocol and chromatographic separation were first optimized. RP-HPLC determination of fumonisins mostly follows SAX or IAC clean-up of a suitable extract. Both clean-up methods were used in these studies to test which clean-up method works

best for each derivatization reagent. The HPLC system was equipped with both UV and fluorescence detectors connected in series, to determine the extent to which UV offers an alternative to fluorescence detection of fumonisins and to allow for direct comparison between the two detectors.

1.4 Research structure

The current chapter includes the introductory overview on the research theme, the aims and objectives, research approach and structure of the study. The succeeding chapters will be as follows:

Chapter 2: Literature Review



The background, occurrence and impact of fumonisins will be presented. This is followed by an in-depth description of the analysis of fumonisins which includes extraction of maize samples, clean-up and derivatization. All the derivatization reagents relevant to the study are reviewed in detail including their mechanism or reactions to form derivatives. The review concludes with a literature survey of HPLC detection methods for fumonisins which are used in the study as well as a discussion of other detection methods.

Chapter 3: Ultraviolet detection (UV) of fumonisin B analogues as o-phthaldialdehyde derivatives

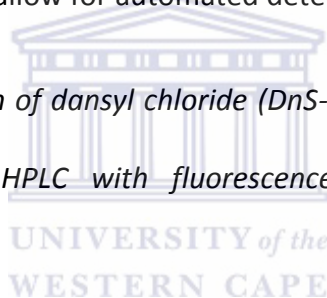
Chapter 3 investigates the degree to which the fumonisin method of Sydenham et al., 1996 could be combined with UV detection of the FB-OPA derivatives. The FLD and developed DAD method is applied to

naturally contaminated maize samples. Comparison of SAX with IAC and FLD and DAD concludes the chapter.

Chapter 4: Optimization of naphthalene-2,3-dicarboxaldehyde (NDA) derivatives for fumonisin derivatization and its applicability to fluorescence (FLD) and ultraviolet (UV) detection

This chapter discusses the extent to which the optimized NDA derivatization method could be used for analysis of South African subsistence maize samples. It aims to provide stable fumonisin derivatives to allow for automated detection of fumonisins with HPLC.

Chapter 5: An evaluation of dansyl chloride (DnS-Cl) for fumonisin derivatization analysed by HPLC with fluorescence (FLD) and ultraviolet (UV) detection



Chapter 5 investigates the extent to which DnS-Cl can be used for fumonisin derivatization in maize samples. The method is optimized to obtain best conditions for pre-column derivatization and HPLC analysis of the derivatives.

Chapter 6: Comparisons of methods, General Discussion, Recommendations and Conclusion

The study is concluded with a concise discussion of the most significant points from all the preceding chapters. A comparison of the derivatization reagents are discussed and as well as how each method

has either advantages or not over the current. Finally, directions for future work are suggested.



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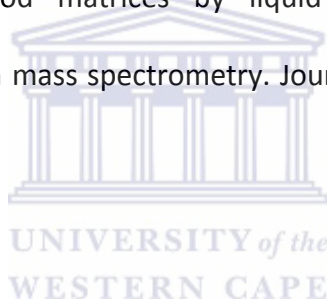
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CHAPTER 2



Literature review

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2.1 Introduction

2.1.1 Background to fumonisins

Fumonisins are mycotoxins that are produced by several species of *Fusarium* mainly *F. verticillioides* and *F. proliferatum* (Marasas, 2001). Fumonisins are known to be an epidemiologically and economically important group of mycotoxins (Shephard, 2000). They were first isolated in 1988 from *F. verticillioides* strain MRC 826 at the Programme of Mycotoxin and Experimental Carcinogenesis (PROMEC) of the Medical Research Council for South Africa (MRC) by Gelderblom et al., 1988. The structure of fumonisins was determined in 1988 in a collaborative study between the PROMEC Unit of the MRC and the Council for Scientific and Industrial Research (CSIR) in Pretoria (Marasas, 2001) and is based on a long eicosane hydrocarbon chain substituted with methyl, hydroxyl and amino groups. Fumonisins are diesters of propane-1,2,3-tricarboxylic acid and 2-amino-12,16-dimethylpolyhydroxyeicosanes in which the C₁₄ and C₁₅ hydroxyl groups are esterified with the terminal carboxyl group of tricarballic acid (Bezuidenhout et al., 1988; Figure 2.1). There are at least four closely related series of fumonisins viz., A, B, C and P that have been isolated (Rheeder et al., 2002). The fumonisin B has three analogues that occur most abundantly in naturally contaminated maize are termed FB₁, FB₂ and FB₃ (Shephard et al., 1996). FB₁ is the most predominant of the fumonisins in the range of 70-80 %, followed by FB₂ and FB₃ which occur at between 15-25 % and 3-8 % respectively (Rheeder et al., 2002).

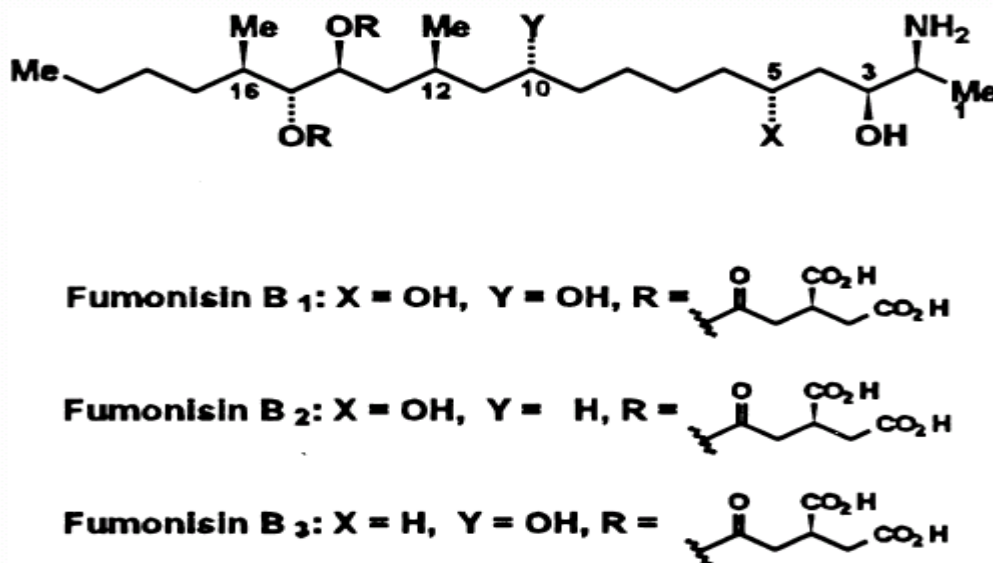


Figure 2.1 Schematic diagram of the structures of Fumonisin B₁ (FB₁), Fumonisin B₂ (FB₂) and Fumonisin B₃ (FB₃)

[Published in: *Shephard, 1998 J. Chromatogr. A 815: 31-39*]

2.1.2 Occurrence of fumonisins

Mycotoxins are produced by one or more specific fungal species, with some species forming more than one mycotoxin (EMAN). FB₁ is a secondary metabolite and therefore its occurrence is caused entirely by the existence of fungal contamination (Miller, 2001). Fumonisins are natural contaminants of cereal grains worldwide (Weidenborner, 2001) but have been found to occur predominately in maize and maize-based products (Shephard et al., 1996; Figure 2.2). Fumonisin contamination has been reported in a variety of food commodities, which include sweet maize at low concentrations of 4-82 µg/kg (Trucksess et al., 1995) and maize beer samples at concentrations of 43-1329

$\mu\text{g/kg}$ for total fumonisins (Shephard et al., 2005). Other commodities where evidence of fumonisin contamination occurs include wheat, rice and cereal-based food (Shephard et al., 1996). FB_2 has recently been reported to be present in red wine (Logrieco et al., 2010), black tea and medicinal plants (Martins et al., 2001). One of highest fumonisin levels in maize intended for human consumption was reported by Rheeder et al., 1992 in the Transkei region, South Africa with a FB_1 level of 117.5 mg/kg. Other areas of high contamination include Santa Catarina State, Brazil and Huainan and Fusui, China (Van der Westhuizen et al., 2003; Sun et al., 2007). The highest levels ever reported for animal feed (330 mg/kg) were found in the US maize screening (Ross et al., 1991).



Figure 2.2 Maize infected with fumonisin producing fungi, *F. Verticillioides*.

[Picture obtained from WFO Marasas, PROMEC Unit]

Exposure assessment studies have been performed in the rural former Transkei region of South Africa. Human exposure in the region of Bizana, an area of relatively low oesophageal cancer, was found to be $3.43 \pm 0.15 \mu\text{g/kg body weight day}^{-1}$ which was lower than in Centane, an area with high oesophageal cancer, which was reported to have a mean exposure of $8.67 \pm 0.18 \mu\text{g/kg body weight day}^{-1}$. Both areas reported results higher than $2 \mu\text{g/kg body weight day}^{-1}$ which is the provisional maximum tolerable daily intake set by the Joint FAO / WHO Expert Committee on Food Additives (Shephard et al., 2007).

2.1.3 Impact of fumonisins

Fusarium verticillioides MRC 826 culture material is highly hepatotoxic and cardiotoxic in rats (Kriek et al., 1981) and was later found to be hepatocarcinogenic in rats and to cause primary hepatocellular carcinoma and cholangiocarcinoma in rats (Marasas 1984). Fumonisin have been reported to cause equine leukoencephalomalacia (ELEM) in horses orally dosed with fumonisin B₁ (Kellerman et al., 1990), and to cause porcine pulmonary edema (PPE) in pigs (Harrison et al., 1990). Laboratory studies have shown FB₁ to be hepatocarcinogenic and nephrocarcinogenic in male rats and hepatocarcinogenic in female mice (Gelderblom et al., 1991; Howard et al., 2001).

The high rates of human esophageal cancer have been associated with high intake of maize contaminated with fumonisins (Rheeder et al. 1992). Due to increased rates of neural tube defects (NTD) in populations along the Texas-Mexico border (Hendricks et al., 1999; Marasas et al., 2004), and typical of areas

where maize forms part of the diet, an investigation was conducted to correlate NTD with the consumption of fumonisin contaminated maize by the mothers. The findings suggested that “fumonisin exposure increases NTD risk, proportionate to dose and up to the threshold level at which death may be more likely to occur” (Missmer et al., 2006). It has also been suggested that fumonisins are a risk factor for liver cancer in humans (Ueno et al., 1997; Marasas et al., 2004). Further elaboration of the human health effects of fumonisins requires the availability of a suitable biomarker of exposure. The biochemical mechanism of action of fumonisins is disruption of *de novo* sphingolipid biosynthesis leading to an accumulation of sphinganine and an increase in the sphinganine : sphingosine ratio in cells and physiological fluids (Van der Westhuizen et al., 2008; Silva et al., 2009 b). Attempts to use this as a biomarker in humans have been unsuccessful but recently a urinary FB₁ biomarker has been validated in the population of the former Transkei (Van der Westhuizen et al., 2011).

Economically, fumonisins impact directly on animal loss, health and veterinary care costs as well as regulatory and research costs which focus in order to determine the impact and severity of mycotoxin problems (Hussein et al., 2001; Wu, 2004; Shephard, 2000). A few countries worldwide have legislated maximum tolerated levels, including Bulgaria, Cuba, France, Iran and Switzerland (FAO, 2003). The Joint FAO/WHO Expert Committee on Food Additives (JECFA) has recommended a provisional maximum tolerable daily intake (PMTDI) of 2 µg/kg body weight for FB₁, FB₂ and FB₃ alone or in combination (Bolger et al., 2001). The US Food and Drug Administration (FDA) has guidance levels for fumonisins in

human food and animal feed and has recommended that levels be put in place to reduce the exposure of fumonisins in maize products intended for human and animal consumption (FDA, 2001a). The European Commission (EC) has regulated fumonisins at various levels from 4000 µg/kg for unprocessed maize to 200 µg/kg for baby foods (EC, 2007).

Analytical methods for all fumonisin analogues in maize and maize-based foods generally rely on reversed-phase high-performance liquid chromatography (RP-HPLC) separation after suitable extraction and clean-up. As fumonisins lack a useful chromophore or fluorophore for HPLC, detection is facilitated by suitable derivatization followed by sensitive fluorescence detection (Shephard et al., 1996). There are currently several methods used to measure the concentration of fumonisins in various matrixes (Shephard, 2008). One of the most commonly used methods for quantitative analysis in maize involves solid-phase extraction (SPE) of solvent extracts, followed by strong anion exchange (SAX) SPE and *o*-phthalaldehyde (OPA) derivatization prior to HPLC separation and quantification of fluorescent OPA-FB₁, FB₂ and FB₃ derivatives (Shephard, 1998).

2.2 Analysis of fumonisins

2.2.1 Introduction

The majority of analytical methods employed in the analysis of fumonisins include sampling and sub-sampling, appropriate extraction, clean-up and concentration and then derivatization prior to instrumental analysis (Shephard et al., 2011). Fumonisin analysis by HPLC requires sample clean-up in order to

remove matrix impurities and to then concentrate the fumonisin (Shephard, 1998). There are different clean-up methods that could be used; these include SAX, immunoaffinity columns (IAC), Q_UEChERS (Quick, Easy, Cheap, Rugged and Safe) and C18 columns, and each clean-up has its own advantages (Bennett et al., 1994; Visconti et al., 1996; Stockenström et al., 1994; Shephard et al., 2011; Zachariasova et al., 2010).

2.2.2 Extraction of samples

The fumonisin structure is quite polar due to the four carboxylic acid groups and an amine group, which makes them readily soluble in polar solvents (Wilkes et al., 1998) and hence amenable to extraction using polar solvents such as methanol : water, acetonitrile : water, methanol : acetonitrile : water and acetonitrile : sodium dihydrogen phosphate and by using different combinations and proportions followed by a clean-up step using SPE on a reversed phase C18 column, SAX or IAC (Cortez-Rocha et al., 2003; Shephard, 2000) it is reasonable to anticipate a good clean product will result.

Food matrixes are generally extracted either by acetonitrile : water (1:1, v/v) (Rice et al., 1995; Bennett et al., 1994) or methanol : water at 70-80 % methanol with optimum results being obtained when using methanol : water (3:1) (Shephard, 1998). Different extraction efficiencies have been reported when using either blending or homogenization. Increased efficiencies were reported by Sydenham et al., 1992 by using methanol : water (3:1) combined with homogenization between 1-5 minutes. In contrast, Bennett et al., 1994 later

obtained better extraction efficiencies with acetonitrile : water compared to methanol : water when shaking was employed for 30-60 minutes.

The European Intercomparison Study showed that the use of higher solvent/sample ratios improved the recoveries of fumonisins (Visconti et al., 1996). The extraction of highly contaminated samples is difficult compared to the extraction of spiked maize which can be attributed to matrix components in the sample (Bennet et al., 1994). The extraction of processed baby foods has been found to be particularly difficult. This could be caused by different factors such as matrix interference. Optimum results for extraction of cornmeal-based infant foods were achieved with 70 % methanol at pH 4 (Sewram et al., 2003).

Comparison of different extraction procedures showed that acetonitrile : water (1:1) gives higher recoveries than methanol : water (3+1, v/v) for all infant formulae even though phase separation during the extraction step with acetonitrile : water showed it to be an inappropriate mixture (De Girolamo et al., 2001). Other approaches that have been reported for the improvement of extraction of fumonisins in maize include the use of extraction solvent mixtures at alkaline pH (Scott and Lawrence 1994), the use of EDTA as extraction solvent (Kim et al., 2002) and increasing the temperature of the solvent used (Lawrence et al., 2000).

2.2.3 Clean-up

Fumonisin sample extracts are normally purified in order to remove unwanted and interfering matrix impurities and to facilitate the concentrate of the fumonisins (Shephard, 1998). The most widely applied purification methods (SAX and IAC column cartridges) will be investigated.

2.2.3.1 Strong anion exchange clean-up (SAX)

The anionic nature of fumonisins is the reason why SAX is the most widely used method for clean-up of maize samples (Maragos et al., 1996). Purification of maize samples with SAX is dependent on the pH or ionic strength of the sample (Shephard, 1998). For optimum recovery results, the pH of the sample extracts must be monitored at 5.8-6.2 with elution flow rate < 2.0 mL/min (Sydenham et al., 1992). SAX cartridges have been reported to provide superior purification over C18 clean-up (Visconti et al., 1996).

2.2.3.2 Immunoaffinity column clean-up (IAC)

IACs are composed of mycotoxin-specific antibodies bound to the sol-gel material and packed in small cartridges. The specificity of the antibody ensures a relatively clean final product (Kruger et al., 1999). Once bound to the antibody, the mycotoxin is eluted by denaturing the antibody using an organic solvent (Trebstein et al., 2008), usually methanol. For this reason, it is not generally recommended that IACs be used more than once. IAC provides much cleaner analyte eluates when compared to SPE clean-up although not completely

selective to the analyte (Kruger et al., 1999). These have been reported to be more robust for fumonisin analysis and are less likely to present matrix interferences compared to other SPE methods (De Girolamo et al., 2001). IAC clean-up is used mainly for mycotoxins from varying diverse matrices (Krska et al., 1998) including fumonisins in highly contaminated maize samples (Kim et al., 2004).

After extraction and clean-up, samples are often analyzed using different chromatographic methods varying from thin layer chromatography (TLC) to high-performance liquid chromatography, which is the most widely used analytical method for the detection of fumonisins.



2.2.4 Derivatization

For sensitive detection of fumonisins by spectrometric methods, derivatization is required to form suitable derivatives that can be easily isolated, separated and detected (Shephard 1998). Since they lack a suitable chromophore or fluorophore they are derivatized prior to HPLC injection using fluorogenic reagents like o-phthaldialdehyde (OPA), naphthalene-2,3-dicarboxaldehyde (NDA) or dansyl chloride (DnS-Cl), with OPA the most commonly used derivatization reagent as it yields highly fluorescent compounds that can be easily separated by HPLC (Shephard, 2000; Bennett and Richard 1994).

The analytical result of fumonisin analysis is greatly affected by the derivatization reagent used viz., fluorescamine formed two reaction products with fumonisins (Sydenham et al., 1990) and maleyl derivatives produced

detection limits of 10 µg/g with HPLC-UV which is unsuitable for naturally contaminated maize (Scott and Lawrence 1994). Other derivatization reagents that have been investigated for fumonisin analyses include 4-fluoro-7-nitrobenzofuran (NBDF) (Scott and Lawrence 1992), 9-fluorenylmethylchloroformate (FMOC) (Holcomb et al., 1995) and 6-aminoquinolyl-N-hydroxysuccinimidylcarbamate (AccQ.Fluor) (Velazquez et al., 2000). The derivatization reagents that will be investigated in this study are OPA, NDA and DnS-Cl.

2.2.4.1 *O*-phthaldialdehyde (OPA) derivatization reagent



Figure 2.3 Structure of *o*-phthaldialdehyde (OPA)

OPA is the most widely used derivatization reagent for pre-column derivatization of fumonisins in most laboratories (Shephard et al., 1996). Fumonisin reacts with OPA in the presence of a sulphur containing nucleophile, most usually 2-mercaptoethanol (ME) to form a highly fluorescent isoindole, the reaction occurring at the free amine moiety as illustrated by figure 2.4.

The combination of OPA with other nucleophiles like OPA/*N*-acetyl-L-cysteine, OPA/ethanethiol and OPA/3-mercaptopropionic acid have been reported, but

were found to form either two reaction products or side reactions with aliphatic amines (Hanczko et al., 2004). Stroka et al., 2002 tested the percentage decay for 2-mercaptoethanol (ME) compared with other nucleophiles and found that 94 % of ME decayed within 2 hours but nucleophiles like 2-thioglycerol showed no decay within the tested time. The disadvantage of using OPA is its instability at room temperature (Williams et al., 2004), even though the use of ME improves the stability of the derivative and thereby its fluorescence peak area and peak height (Stroka et al., 2002). However, OPA/ME is not stable enough as a derivative for overnight or auto-injection analysis (Williams et al., 2004). OPA derivatives have been reported to be stable for up to 4 minutes at room temperature after which they decrease by 5 % after 8 minutes and 52 % after 64 minutes in their specific fluorescence response of the FB₁ derivative. The time-degradation of fumonisin derivatives can be overcome by standardizing the time (< 4 minutes) between the reagent addition and the HPLC injection (Shephard, 1998). Despite its instability problems, the AOAC (Association of Official Analytical Chemists International) approved the OPA derivatization of fumonisins as an official method (Sydenham et al., 1996).

Blank maize samples spiked with FB₁, FB₂ and FB₃ standards at concentrations of 100 to 8000 ng/g produce mean recoveries of between 75% and 85% for individual toxins and detection limits of 50 ng/g have been reported for OPA analysis (Sydenham et al., 1996).

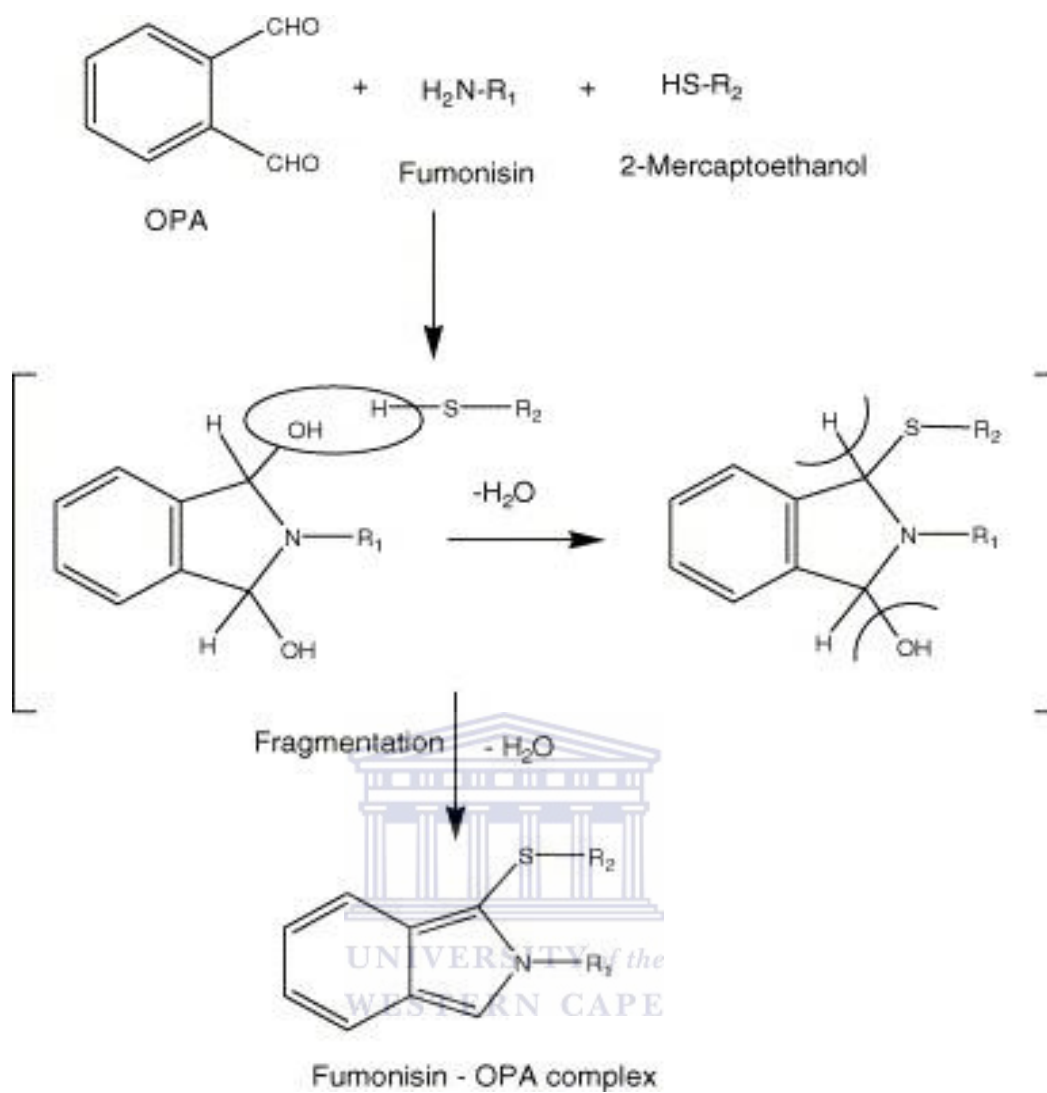


Figure 2.4 Reaction mechanism for the formation of FB-OPA complex

[Published in: Samapundo et al., 2006. *J. Chromatog. B* 1109: 312-316]

2.2.4.2 Naphthalene-2,3-dicarboxaldehyde (NDA) derivatization reagent

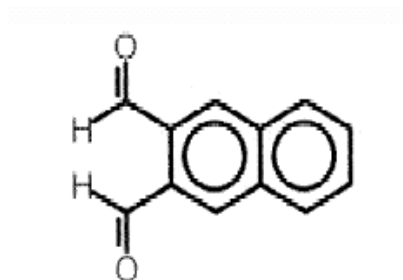


Figure 2.5 Structure of Naphthalene-2,3-dicarboxaldehyde (NDA)

NDA is known to be a useful derivatization reagent for primary amines, amino acids and small peptides and is the second most widely used fluorogenic reagent for the detection of amines (Carlson et al., 1986). It was screened by De Montigny et al., (1987) using several nucleophiles like ME, HSO_3^- and CN^- with alanine as a primary amine. Under fluorescence spectroscopy, CN^- was the most suitable reagent to form derivatives with both high fluorescence intensity and good chemical stability (De Montigny et al., 1987). The NDA/ CN^- reaction was then further tested with other primary amines, amino acids, peptides and proteins at room temperature to produce highly fluorescent and stable 2-substituted 1-cyanobenz [f] isoindole derivatives, which are often used to measure trace levels of biogenic amines in biological matrixes (Bennet et al., 1994; Figure 2.6).

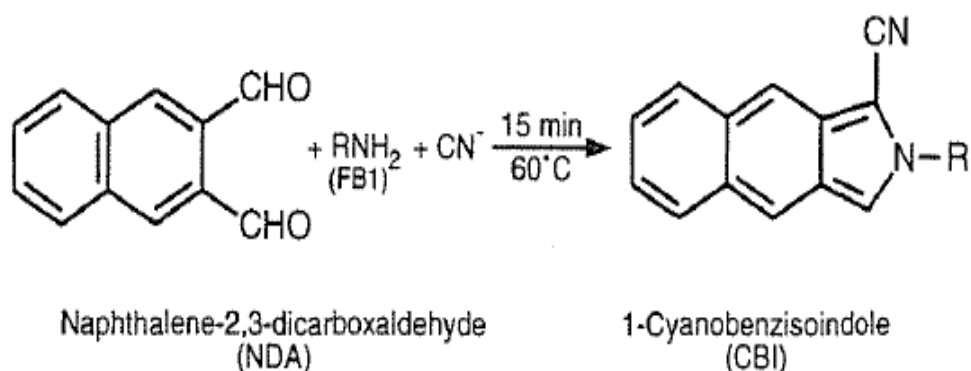


Figure 2.6 Reaction of NDA with FB₁ to form 1-Cyanobenzisindole (CBI) stable derivative

[Published in: *Bennett and Richard 1994 J. AOAC Int. 77: 501-506*]



The above reaction is for FB₁; other fumonisins B analogues follow the same reaction sequence.

Most analytical methods derivatizing fumonisins with NDA and other amines are based on the method by Ware et al., 1993 with minor modifications. Extraction methods used vary as per laboratory, sample type and instrument of analysis. For chromatographic separation, researchers have used similar conditions, viz., sodium borate pH 9, NDA dissolved in acetonitrile or methanol and the addition of cyanide to drive the reaction (Carlson et al., 1986; De Montigny et al., 1987). Chromatographic separation of NDA derivatives is generally achieved with acetonitrile : water : acetic acid (~ 66 : 38 : 1) mobile phase at 1 mL/min using isocratic elution (Bennett et al., 1994; Lino et al., 2007) and fluorescence

detection at an excitation wavelength of 420 nm and emission wavelength of 520 nm (Bennett et al., 1994; De Montigny et al., 1987). This thesis describes for the first time a comparison of NDA with other derivatization reagents using both diode array detection and fluorescence detection on South African maize samples for the most naturally abundant fumonisin B analogues (FB₁, FB₂ and FB₃).

When compared with other derivatization reagents, NDA gives comparable results and can be used as an alternative to OPA as it produces more stable and highly fluorescent derivatives of fumonisins (Ware et al., 1993). NDA is a stable derivatization reagent and it has been reported that after 24 hours the NDA fluorescent signal decreases to 86.4±5.9 % as compared to 63.2±4.8 % of OPA and to 82.8±7.1 % after 48 hours for NDA compared to 57.2±8.3 % for OPA (Cho et al., 2002).

Detection limits of 20 µg/kg for FB₁ and 15 µg/kg for FB₂ were reported by Silva et al., (2009 a) and 23.3 nmol/L and 34.4 nmol/L for amines (Lamba et al., 2008). Bennett et al., 2004 reported recoveries of 92-95 % for FB₁ and FB₂, respectively, at levels of 10 µg using SAX columns and 83-88 % for FB₁ and FB₂ at 10 µg levels using RP C18 columns, whereas Silva et al., (2009 a) reported recoveries of 79 % to 102 % for FB₁ and FB₂, respectively, at spiking levels of 150 µg/kg and 250 µg/kg using LC -MS.

2.2.4.3 Dansyl Chloride (DnS-Cl) derivatization reagent

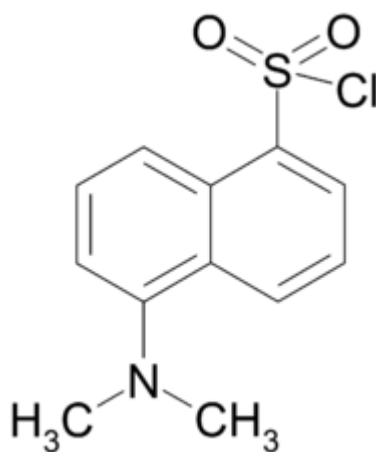


Figure 2.7 Structure of Dansyl Chloride (DnS-Cl)

Dansyl chloride (DnS-Cl) (5-dimethylaminonaphthalene-1-sulfonyl chloride) is a fluorescent labelling reagent for primary, secondary and tertiary amines (Bartzatt, 2001). It was introduced in 1952 by G. Weber to prepare conjugated proteins (Blau et al., 1978). It is often used for the quantitation of polyamines in biological samples (Khuhawar et al., 2001) and in the presence of amino acids, dansyl chloride forms stable fluorescent sulfonamide adducts (Walker et al., 1994) and in the presence of sodium carbonate it allows for the detection of 1 µg amounts of analyte (Bartzatt, 2001).

Sodium carbonate (1 M) has sufficient ionic strength and high pH to enable the binding of DnS to tertiary amines where elimination of either alkyl or aryl substituents is possible (Bartzatt 2001). DnS-Cl derivatives are more stable than OPA and more suitable for detection at pico molar ranges (Minocha et al., 2004)

and are stable for over two hours (Dasko et al., 2006). It has been reported to be a good derivatization reagent for fumonisin analysis, but with the disadvantage of forming analytical interferences with maize samples (Scott et al., 1992; Arranz et al., 2004). The derivatization of amine components with DnS-Cl can produce intense overlapping HPLC peaks due the reaction of DnS-Cl with water to produce hydrolysis products (Kang et al., 2006). Sample clean-up with IAC has been used to eliminate interferences for determination of FB₁ in beer samples (Dasko et al., 2006). Cleaner chromatograms are also obtained by reacting minimum stoichiometric amounts of DnS-Cl or adding triethylamine and tetrabutylammonium hydroxide to separate the hydrolysis peaks from the analyte peak (Kang et al., 2006). Environmental conditions like temperature (Dasko et al., 2006), reaction time, pH and concentration of DnS-Cl can affect the reaction yield (Kang et al., 2006). Hence DnS-Cl methods should be optimized as per laboratory and sample matrix.

Dansyl chloride derivatives can be measured using both ultraviolet and fluorescence detection with UV detection at 286 nm being the most sensitive (Minocha et al., 2004). Recoveries between 89.47-97 % have been reported for the separation of di- and polyamines as their dansyl derivatives using RP-HPLC and methanol : water or acetonitrile : water mobile phase (Marce et al., 1995). Recoveries of amines of 73.8 to 114 % in wine samples with detection limits of 0.08 ng and quantization limit of 0.16 ng where reported by Loukou et al., 2003.

2.3 Detection of fumonisins

2.3.1 Introduction

There are various separation techniques used for fumonisin analysis which vary from chromatography, enzyme-linked immunoassay (ELISA) to electrophoresis methods. Alternative methods for the detection of fumonisin which are often used for confirmation of the presence of fumonisins include gas chromatography (GC), HPLC, liquid chromatography- mass spectrometry (LC-MS), and thin-layer chromatography (TLC) which provides faster analysis time for rapid screening of samples (Shephard 1998) with the ELISA and TLC often being relegated to screening methods. Fumonisin is a polar molecule that is soluble in water and polar solvents are thus suited for analysis by RP-HPLC (Shephard, 1998). This study uses RP-HPLC for chromatographic separation of the fumonisin B analogues.

2.3.2 Chromatographic methods

2.3.2.1 High-performance liquid chromatographic

A world-wide survey found that 90 % of the laboratories that reported results on fumonisin analysis used pre-column derivatization and quantification by HPLC (Shephard et al., 1996). Since fumonisins do not fluoresce or contain a UV absorbing chromophore, most HPLC methods measure fumonisins after derivatization of their free amino group (Shephard, 2000). Shephard et al., 1990 reported for the first time on the quantitative, sensitive and simultaneous

detection of FB₁ and FB₂ in naturally contaminated maize samples using RP-HPLC. The method was based on MeOH : H₂O extraction, SAX clean-up, fluorescence (FLD) detection of OPA derivatives. Collaborative studies using the method resulted in the AOAC International approving it an official method for fumonisin analysis in maize (Sydenham et al., 1996). HPLC-FLD detection of fumonisins is specific and sensitive with fluorescence detection often achieved at an excitation wavelength of 335 nm and emission wavelength of 440 nm (Shephard et al., 1990).

2.3.2.2 Gas Chromatography

Sydenham et al., 1990 confirmed the presence of fumonisins in maize using GC. The method involved acid hydrolysis of the fumonisins to cleave the ester bond and the tricarballic acid thus formed was confirmed by GC-MS. A direct method for fumonisin analysis involved the production of the fumonisin backbone (aminopolyol) by alkaline hydrolysis which was isolated on XAD-2 resin and then converted to the trimethylsilyl derivative for GC analysis (Plattner, 1990). Plattner et al., 1994 later reported that the accuracy and precision of the GC-MS method can be improved by adding deuterium-labelled FB₁ as an internal standard to the sample extract prior to hydrolysis. Fumonisin analysis using GC requires multiple time consuming sample handling steps such as sample hydrolysis, clean-up and derivatization prior to analysis and so has found little application for fumonisin detection (Shephard, 1998).

2.3.2.3 Thin Layer Chromatography

TLC provides a fast and reliable means of screening contaminated samples. The first method developed for fumonisins involved reversed-phase TLC on C₁₈-modified silica plates developed with methanol : water (3:1, v/v) as a solvent (Cawood et al., 1991). This method was later improved by the use of *p*-anisaldehyde solutions or spraying with ninhydrin to visualize the fumonisins (Shephard, 2000). However, the detection limits of 0.5 mg/g which were obtained were not suitable for naturally contaminated maize (Sydenham et al., 1990). When used with fluorecamine reagent spray under UV light, TLC gave better selectivity and sensitivity for fumonisin analysis in naturally contaminated maize samples (Rottinghaus et al., 1992). Improved detection limits were obtained with the use of SAX clean-up rather than reversed-phase C18 cartridges (Stockenström et al., 1994). TLC detection limits were further enhanced with IAC clean-up and scanning fluorodensitometry to 0.1 mg/kg in maize samples (Preis et al., 2000). A TLC method based on pre-derivatization before TLC separation was reported in a collaborative study for FB₁ analysis in maize and gave recovery results of 74.5 % (Shephard et al., 2004).

2.3.3 Immunological methods

Immunological assays have been used to successfully detect mycotoxins since the late 1970s (Pestka et al., 1995). These methods rely on the immunological principle which is based on the interaction between an antigen (analytes of interest) and an isolated antibody raised against the antigen (Shephard, 2008).

These methods include enzyme-linked immunosorbent assays (ELISA) and immunoaffinity columns (IAC). ELISA has been validated for measuring total FB in maize at levels greater than 0.1 µg/g with acceptable precision (Bird et al., 2002). IAC have been developed with specific antibodies for different mycotoxins with recovery results averaging between 99.7 % for FB₁ at fortification levels of 250 µg/kg and 74.8 % for FB₂ at fortification levels of 200 µg/kg (Lino et al., 2007). Immunoassays are still being used for screening commodities and food for fumonisins with new developments in antibodies and immunoassays reported (Shephard et al., 2011).

2.3.4 Mass Spectrometry

2.3.4.1 *Liquid chromatography-mass spectrometry*

LC-MS is a combination of HPLC with MS and is a powerful technique for identification of fumonisin B analogues (Shephard, 1998). The use of LC-MS has enabled sensitive and specific fumonisin methods to be developed (Silva et al., 2009 a). LC-MS/MS has recently been used for multi-mycotoxin screening of 87 mouldy foods sampled from individual homes. The method involves acetonitrile : water : acetic acid extraction with LC-MS/MS-ESI (electrospray ionization) and HPLC-MS/MS detection (Sulyok et al., 2010).

2.3.5 Recent developments in fumonisin detection

The use of multi-mycotoxin analysis has drawn much attention within the toxicology industry with the use of sophisticated instruments such as UHPLC-

MS/MS and LC-MS/MS-ESI. These have recently involved multi-component methods for the simultaneous detection of mycotoxins and pesticides (Romero-Gonzalez et al., 2011; Sulyok et al., 2010). Ofitserova et al., 2005 provided a screening method for five families of toxins, a method suitable for screening beverages, grains and feeds. It involved chromatographic separation with MYCOTOXTM reversed-phase C18 column and post-column separation instrument, Pinnacle PCX (Pickering laboratories) (Ofitserova et al., 2005). A LC-MS/MS method for multi-mycotoxin has recently been reported by Sulyok et al., 2010. The method involves semi-quantitative screening of 87 mouldy samples. From the analysis results, 49 different fungal metabolites were identified, showing the usefulness of multi-mycotoxin analysis. Other rapid screening methods for mycotoxin analysis include fluorescence polarization immunoassay (FPIA) (Maragos, 2009), lateral flow devices (LFD; dipstick) and biosensors (Maragos and Busman 2010).

2.4 Overview of literature review

The fumonisin B analogues were first isolated in 1988 at the PROMEC Unit of the MRC and occur in maize and maize-based foods. They cause ELEM in horses, porcine pulmonary oedema in pigs, and are hepatotoxic and cardiotoxic in rats. They have been linked to the high incidence of esophageal cancer in the rural Transkei area of South Africa where maize is the staple diet. In 2002, the IARC evaluated the carcinogenic risk of FB₁ to humans and classified it as a Group 2B carcinogen (as a possibly carcinogen to humans). The current status of existing

methods available for fumonisin analysis includes extraction, clean-up, derivatization and chromatographic separation. HPLC with fluorescence, MS or tandem MS are still the most used in laboratory-based methods.

The method developed by Shephard et al., 1990 was approved by the AOAC International as an official method for fumonisin analysis in maize. Derivatization reagents that have been tested for fumonisin analysis include OPA, NDA and DnS-Cl which will be studied in this thesis. HPLC with fluorescence detection is the most widely used detection method. The thesis will investigate the applicability of UV as a possible detection method in HPLC alternative to fluorescence.



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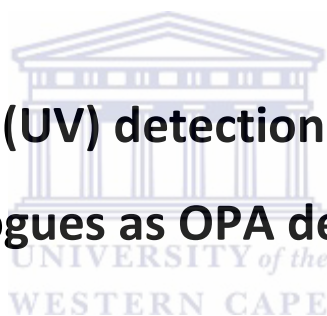
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CHAPTER 3

Ultraviolet (UV) detection of fumonisin B analogues as OPA derivatives



3.1 Introduction

Determination of fumonisins in maize is widely achieved using a validated method involving methanol : water extraction followed by strong anion exchange (SAX) clean-up and derivatization prior to separation by reversed-phase high-performance liquid chromatography (RP-HPLC) with fluorescence detection (Sydenham et al. 1996). As fumonisins lack a useful chromophore or fluorophore, HPLC detection is achieved by suitable derivatization (Shephard et al., 1990). Although a number of fluorogenic derivatizing reagents have been investigated, OPA remains widely used for sensitive and specific analysis of fumonisins as it yields strongly fluorescent derivatives easily separated by HPLC (Shephard, 1998).

On occasion, laboratories equipped with HPLC and ultraviolet (UV) detectors seek to undertake limited fumonisin determinations without the purchase of further instrumentation in the form of a fluorescence detector. The aim of the study reported in this chapter was to investigate the degree to which the fumonisins could be determined using UV detection of the fumonisin-OPA derivatives.

3.2 Materials and Methods

3.2.1 Chemicals

All chemicals used were of analytical grade. Methanol, acetone, sodium hydrogen carbonate (NaHCO_3), acetonitrile, *o*-phosphoric acid (H_3PO_4), potassium dihydrogen phosphate (KH_2PO_4), sodium hydroxide (NaOH), *o*-

phthaldialdehyde (OPA), disodium tetraborate ($\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$), disodium hydrogen phosphate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$), potassium chloride (KCl), sodium dihydrogen phosphate (NaH_2PO_4), hydrochloric acid (HCl), sodium chloride (NaCl), 2-mercaptoethanol (ME) were purchased from Merck. Phosphate buffered saline (PBS) was prepared by dissolving 8.0 g sodium chloride, 1.2 g disodium hydrogen phosphate, 0.2 g potassium dihydrogen phosphate and 0.2 g potassium chloride in a litre distilled water. The pH was adjusted to 7. The OPA derivatization reagent was prepared by dissolving OPA (40 mg) in 1 mL methanol and adding 0.1 M sodium tetraborate (5 mL) solution, 2- mercaptoethanol (50 μL) and vortexing after each solvent addition. The OPA derivatization reagent was used up to 7 days following preparation.

3.2.2 *Fumonisin Standards*

FB_1 , FB_2 and FB_3 standards were isolated at the PROMEC Unit according to the method of Cawood et al. (1991). The FB_3 standard contains approximately 21 - 42% *epi*- FB_3 (Gelderblom et al., 2007). The fumonisin working standards were prepared by diluting a stock with concentration levels of 245 $\mu\text{g}/\text{mL}$, 200 $\mu\text{g}/\text{mL}$, 270 $\mu\text{g}/\text{mL}$ of FB_1 , FB_2 and FB_3 , respectively, with acetonitrile : water (1:1, v/v) to obtain working standard with 55.13 $\mu\text{g}/\text{mL}$, 25.00 $\mu\text{g}/\text{mL}$ and 13.25 $\mu\text{g}/\text{mL}$ for FB_1 , FB_2 and FB_3 , respectively.

3.2.3 *Maize Samples*

Home-grown maize samples intended for human consumption were collected in the rural former Transkei area of the Eastern Cape Province following the 2006 harvest and stored at a temperature (4°C) where fumonisins are stable. The well mixed samples were milled prior to analysis.

3.2.4 *Extraction using strong anion exchange (SAX) clean-up*

Maize samples were extracted using the method of Sydenham et al. (1996) with modifications. A milled maize sample (20 g) was extracted by blending in a homogenizer (Polytron PT 3100, Kinematica, Luzerne, Switzerland) homogenized for 3 min with methanol : water (3:1, v/v; 10 mL). It was then centrifuged at 4°C for 10 min at 500 x g. The supernatant was filtered using a MN 617 (185 mm) filter paper and the pH adjusted to 5.80-6.25 with 1 M NaOH or 1 M HCl. After centrifugation, an aliquot (10 mL) of the supernatant was cleaned-up using SAX cartridges (10 mL, 500 mg packing Bond-Elut, Varian, Harbor City, CA, USA), which were preconditioned with 5 mL methanol followed by another 5 mL methanol : water (3:1) (flow rate \leq 2 mL / min, no air was forced through the column; the column was not allowed to dry through-out the entire clean-up process). The extracted sample (10 mL) was loaded on the SAX and washed with 5 mL methanol : water (3:1) and 3 mL methanol. After washing with methanol, the fumonisins were eluted with acetic acid : methanol (1:99, v/v 10 mL) under gravity. The eluate was evaporated to dryness under nitrogen at $< 60^{\circ}\text{C}$ and stored at 4°C prior to analysis.

3.2.5 Extraction using immunoaffinity columns (IAC) clean-up

Homogeneously mixed samples (20 g) were extracted with 50 mL of the extraction solvent (acetonitrile + methanol + distilled water, 25 + 25 + 50, v + v + v) by shaking on an orbital shaker for 20 min. The extraction solution was centrifuged for 10 min at 500 x g and the supernatant was filtered as described above to avoid the transfer of any solid material. The remaining solid material was re-extracted with an additional 50 mL of solvent in the same manner as described above. The filtered supernatants were combined and a 10 mL aliquot was diluted with 40 mL Phosphate buffered saline (PBS) and the solution mixed well.

3.2.6 IAC cleanup

The PBS diluted extract (10 mL) was passed through the FumoniTest column (Watertown, MA, USA) at a flow rate of 1 to 2 drops per second and the eluate discarded. The column was washed with 10 mL PBS until air came through the column and the eluate discarded. The fumonisins were eluted with 2.5 mL of HPLC grade methanol, at a rate of 1 drop per second. The eluate was evaporated at 60°C using nitrogen gas and stored at 4°C prior to analysis.

3.2.7 Derivatization

Standards (25 µL) with concentrations of 55.25 µg/mL for FB₁, 25.00 µg/mL and 13.25 µg/mL for FB₂ and FB₃, respectively, were derivatized with 225 µL OPA reagent and 10 µL injected. The nitrogen dried samples were re-dissolved in 200

μL methanol and 25 μL was derivatized with OPA (75 μL), vortexed and 20 μL injected onto the HPLC exactly 2 min after mixing.

3.3 Chromatography

RP-HPLC was performed on an Agilent Technologies (Waldbronn, Germany) 1260 Infinity pump, Rheodyne 7725i injector and a Phenomenex (Torrance, CA, USA) Luna C18 5 μm column (150 mm x 4.60 mm). The HPLC instrument was configured with an Agilent 470 (Waldbronn, Germany) 1100 series diode array detector (DAD) and an Agilent 1100 series fluorescence detector (FLD) connected in series. The sequence of the detectors was the DAD first followed by the FLD (to prevent overpressure, the fluorescence detector should always be the last module in the flow system). The mobile phase of methanol : 0.1 M sodium phosphate (77:23, adjusted to pH 3.35 with *o*-phosphoric acid) was pumped at an isocratic flow rate of 1 mL/min. Data was collected and analyzed by Agilent ChemStation software and quantification achieved by comparison of peak areas with those of authentic fumonisin standards.

3.4 Results and Discussion

3.4.1 Peak resolution

As reliability of data derived from a chromatographic analysis depends on the effective separation of the analytes of interest from one another and from additional matrix components, peak resolution was analysed. Complete resolution of the OPA derivatives was achieved with RP-HPLC C18 column and an

isocratic mobile phase of methanol : 0.1 M NaH₂PO₄ (77:23) in less than 20 minutes. FB-OPA derivatives are generally monitored at excitation wavelengths 335 nm and emission wavelengths 440 nm for fluorescence detection (Shephard, 1998) which are wavelengths used in the study for FLD detection.

These wavelengths were tested using fumonisin working standards employing the chromatographic conditions above. Based on the high sensitivity of the DAD at 335 nm it was selected as wavelength for DAD detection. An iso-absorbance plot (software programme which displays chromatographic details in 3D including retention time versus wavelength, from which optimum wavelength can be selected) was then used to confirm the wavelength selection for DAD.

Injection of fumonisin working standards with concentrations of 55.25 µg/mL for FB₁, 25.00 µg/mL and 13.25 µg/mL for FB₂ and FB₃ respectively resulted in an elution order of FB₁, FB₃ and then FB₂. Retention times for FB₁, FB₂ and FB₃ were 4.6, 12.2 and 10.8 min (± 5 %), respectively, for both FLD and DAD (Figures 3.1 and 3.2). Another fumonisin elutes just before FB₃ and is only partially separated from it. This compound has been identified as an epimer of FB₃ (*epi*-FB₃) and is quantified as part of FB₃ (Gelderblom et al., 2007). The two isomers have similar chemical properties and exhibit similar retention times when analysed on a reversed-phase HPLC column. To assume the two isomers have the same chromatographic response factors appears to be reasonable and allows accurate analysis to be performed.

To test for matrix interferences, reagent blanks were analysed and the resultant chromatogram overlaid with that of fumonisin standards. No interferences were observed and the background noise of both the blanks and standards were found to be insignificant for both detectors; therefore no form of baseline correction was necessary. Satisfactory resolution of the fumonisin analogues was achieved with analytes identified using their retention times. Chromatographic resolution of the peaks in the study compared well with previous studies (Shephard et al., 1990).



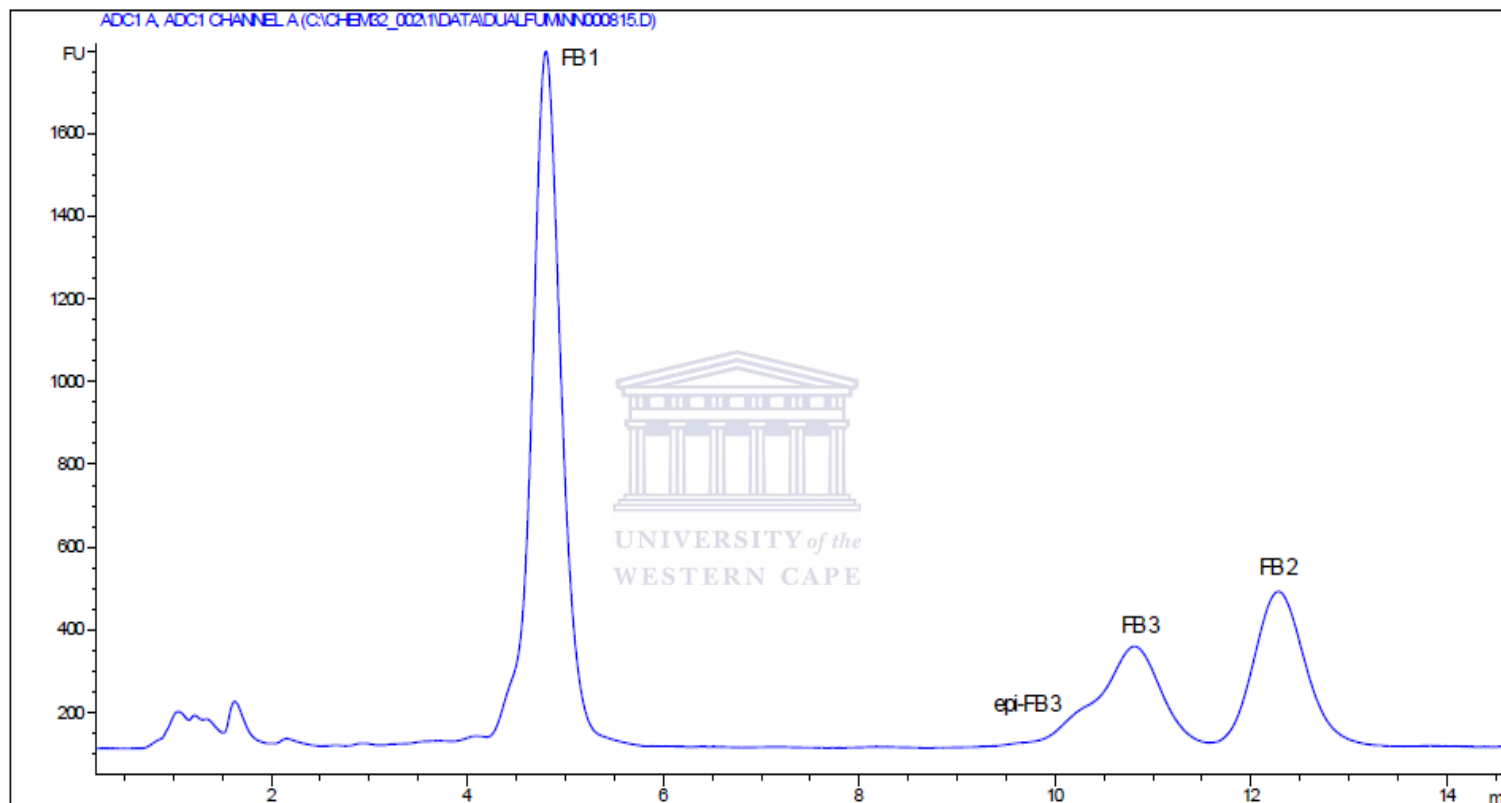


Figure 3.1 Chromatogram of fumonisin working standard detected by FLD

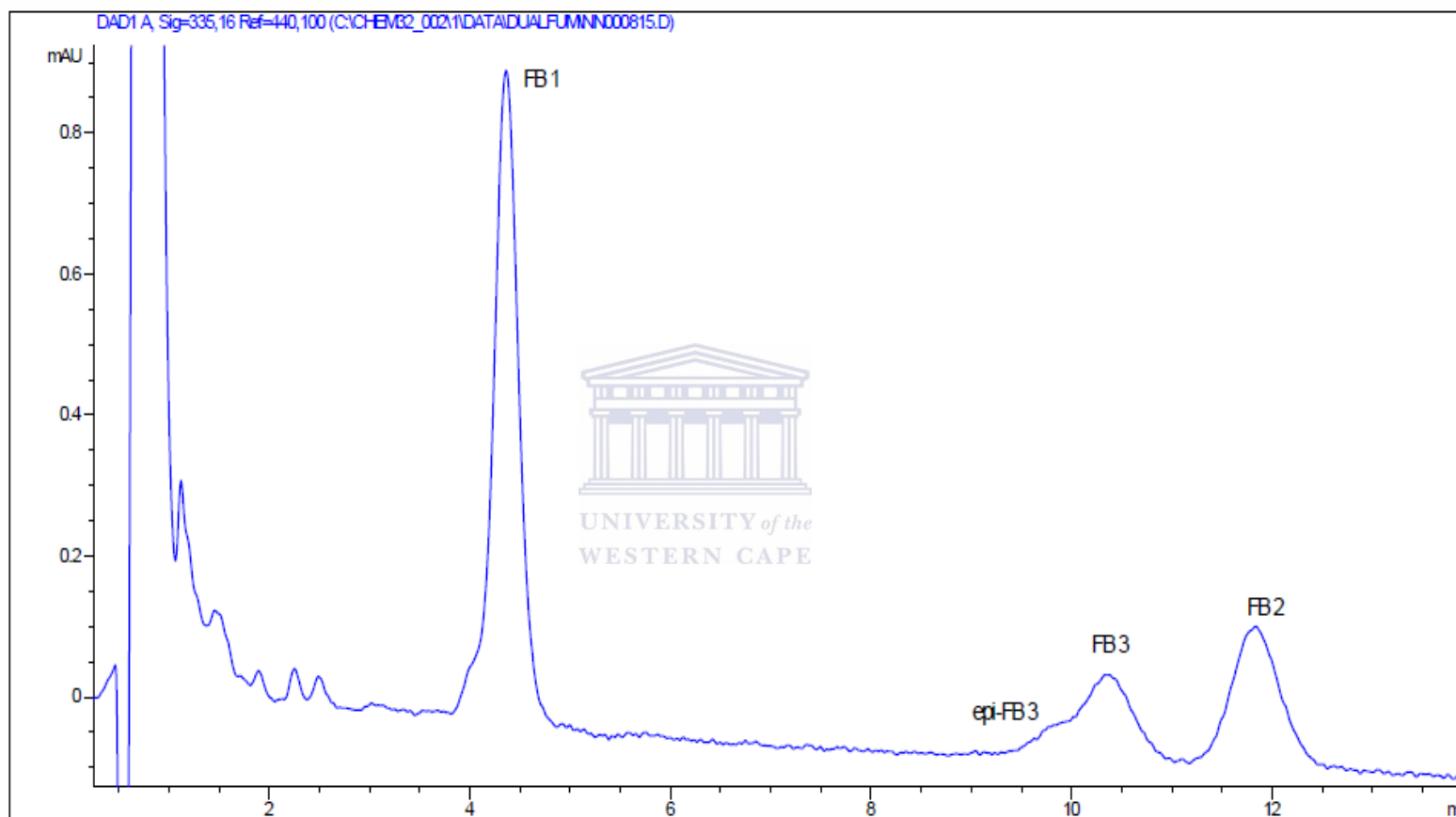


Figure 3.2 Chromatogram of fumonisin working standard detected by DAD

3.4.2 Method precision

Precision of the FB-OPA method was determined in terms of intra- and inter-day analysis. The intra-day precision (daily) was obtained by injecting three consecutive working standards and the inter-day precision (day-to-day) was measured over a period of five days. These parameters were determined to ensure both the repeatability and reproducibility of the standard preparation and derivatization is within acceptable variances (i.e. RSD values for intra-day ≤ 5 %; inter-day ≤ 20 %). Based on the results of the intra-day analysis (Table 3.1), the method's repeatability was good with RSD for the FLD ≤ 1 % and ≤ 3 % for DAD, indicating good precision for the OPA method. The method precision gives an estimation of the variability that can be expected when performing fumonisin analysis using OPA.

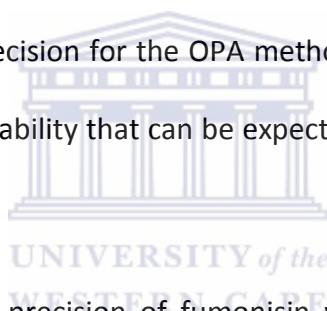


Table 3.1 Intra-day precision of fumonisin working standards (n=3) using standard peak areas

	FLD			DAD		
	FB ₁	FB ₂	FB ₃	FB ₁	FB ₂	FB ₃
Standard 1	35153	13924	11715	16.5	6.7	5.2
Standard 2	35047	13909	11688	16.6	7.0	5.1
Standard 3	35635	14073	11905	17.0	6.6	5.3
Mean	35279	13968	11769	16.7	6.8	5.2
Stdev	313	90	119	0.3	0.2	0.1
RSD (%)	0.9	0.7	1.0	1.6	2.9	1.6

The intermediate precision of the method is reflected in the inter-day results (Table 3.2); the method shows acceptable precision with RSD values $\leq 13\%$ for both FLD and DAD which is suitable because inter-day RSD values should be $\leq 20\%$. The method is generally reproducible in terms of standard preparation, derivatization and injection. These results indicate the OPA method to be reproducible, precise and repeatable. Based on the intra- and inter-day precision results, OPA provides adequate precision for fumonisin analysis.

Table 3.2 Inter-day precision of fumonisin working standards using standard peak areas, (n=18, 3 x std injected/day)

	FLD			DAD		
	FB ₁	FB ₂	FB ₃	FB ₁	FB ₂	FB ₃
Day 1	30349	13563	10343	20.0	9.1	7.0
	30433	13443	10229	20.2	8.2	5.6
	31772	13855	10332	21.4	9.4	7.2
Day 2	32164	13155	10854	20.7	8.8	6.2
	32828	13507	10971	19.5	8.5	6.5
	31717	13067	10771	19.9	8.3	5.9
Day 3	24749	10134	8248	15.4	7.1	5.4
	23923	9811	8044	15.7	8.0	5.5
	23915	9814	8590	15.3	6.9	5.2
Day 4	31630	14336	10964	16.5	6.7	5.2
	32099	14028	10924	16.6	7.0	5.1
	31745	14452	11175	17.0	6.6	5.3
Day 5	35153	13924	11715	16.2	7.1	6.1
	35047	13909	11688	16.5	6.9	5.6
	35635	14073	11905	16.3	6.4	5.3
Mean	30877	13005	10450	17.8	7.7	5.8
Stdev	3808	1644	1224	2.1	1.0	0.7
RSD (%)	12	13	12	12	13	11

3.4.3 Detection limits

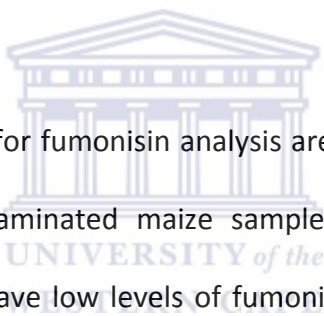
The limit of detection (LOD) and quantification (LOQ) were estimated from the signal-to-noise ratio. The LOQ was obtained at 10:1 signal-to-noise ratio and 3:1 signal-to-noise ratio was used for the LOD. The FB₁ analogue was more reliably detected with lower detection limits (Table 3.3) compared to FB₂ and FB₃. FLD is more sensitive than DAD, this is evident in the reduced detector noise (Figures 3.1 and 3.2) and enhanced analyte signal of the FLD (1700 FU) compared to the DAD (0.8 mAU). The detection limits of the FLD are estimated to be approximately 20-times more sensitive than DAD when one considers the amount injected into the column. It is thus reasonable that based on the detection limits obtained; the method will be able to detect fumonisins at the concentration levels routinely encountered in contaminated maize with adequate accuracy and sensitivity.

Table 3.3 Determination of the detection limits using the amount (ng) injected into the column

		Amount (ng) injected into the HPLC column		
		FB ₁	FB ₂	FB ₃
LOD (s:n=3)	FLD	1.0	1.2	1.2
	DAD	22	20	20
LOQ (s:n=10)	FLD	2.3	2.7	2.7
	DAD	37	48	56

3.4.4 Recoveries

Recoveries were determined by spiking maize samples with 400 µL fumonisin working standards containing 1103, 500 and 270 µg/kg FB₁, FB₂ and FB₃, respectively. The analysis was repeated six times for each concentration level and the recoveries determined using IAC clean-up. Since the OPA method has been validated both in-house (Shephard et al., 1990) and internationally (Sydenham et al., 1996), SAX recoveries were confirmed on only two samples and gave recoveries of up to 75 % as previously obtained by Sydenham et al., 1996. Consequently, further testing of SAX clean-up recoveries was found to be unnecessary.



“True analytical blanks” for fumonisin analysis are difficult to find as fumonisins occur naturally in contaminated maize samples. In this regard, even good commercial maize can have low levels of fumonisins. Therefore, a sample with very low fumonisin levels was used as a blank and the fumonisin levels detected were accounted for in the calculation of the recoveries.

The recoveries measured were lower than expected (Table 3.4) since IAC has been reported to produce results of between 70 – 95 % as previously reported by Visconti et al., 2001. Comparison of the FLD and DAD was acceptable for FB₁; however for FB₂ the recoveries were lower than expected for DAD. The recoveries obtained indicate that the method is acceptable for fumonisin analysis in naturally contaminated maize samples.

Table 3.4 Recoveries with IAC clean-up

	FLD			DAD		
	FB ₁ (µg/kg)	FB ₂ (µg/kg)	FB ₃ (µg/kg)	FB ₁ (µg/kg)	FB ₂ (µg/kg)	FB ₃ (µg/kg)
Blank 1	105	20	12	0	0	0
Blank 2	73	18	8	0	0	0
Mean	89	19	10	0	0	0
Sample 1	963	242	154	727	233	104
Sample 2	935	326	212	982	420	272
Sample 3	783	277	180	796	294	173
Sample 4	770	301	229	784	320	190
Sample 5	807	305	287	919	362	252
Sample 6	683	398	218	730	224	97
Mean	824	308	213	823	309	181
Stdev	106	52	46	105	75	73
RSD (%)	13	17	21	13	24	40
Spiked	1103	500	270	1103	500	270
Recovery (%)	67	58	75	67	46	62

3.5 Method application

The derivatization method was successfully applied to 15 maize samples using SAX and IAC clean-up. Tables 3.5 and 3.6 provide a comprehensive view of the total fumonisin (µg/kg) levels in the maize samples analyzed. In the study all the samples analyzed were contaminated with FB₁, FB₂ and FB₃. IAC clean-up results were not comparable at levels below 500 µg/kg between the two detectors with DAD detecting only at levels above 140 µg/kg for total fumonisins. SAX clean-up provided comparable results at all levels analysed. The results obtained were consistent whether determined by SAX or IAC with both clean-up methods in

terms of comparison of the detectors (Figures 3.3 and 4.4) signifying that OPA is a robust derivatization reagent for fumonisin analysis in terms of clean-up.

Table 3.5 Fumonisin levels ($\mu\text{g/kg}$) in naturally contaminated maize samples cleaned-up with SAX

Sample	FLD				DAD			
	FB ₁	FB ₂	FB ₃	Total	FB ₁	FB ₂	FB ₃	Total
1	70	21	16	106	102	*nd	nd	102
2	162	60	12	234	253	57	nd	310
3	195	74	18	288	176	81	nd	257
4	229	55	13	296	320	48	nd	368
5	153	56	11	220	239	61	nd	300
6	724	320	87	1132	948	331	nd	1279
7	886	382	107	1375	1025	399	46	1470
8	1829	1076	239	3144	2018	1195	21	3234
9	1431	566	166	2163	1708	517	64	2289
10	1717	614	206	2537	1924	669	212	2805
11	1336	432	131	1900	1362	452	133	1948
12	229	55	13	3120	2053	865	246	3164
13	1083	377	118	1577	1316	410	86	1812
14	3189	2086	812	6088	3417	2144	553	6114
15	2368	1034	337	3740	2551	1280	612	4442

*nd- Not detectable

Table 3.6 Fumonisin levels ($\mu\text{g/kg}$) in naturally contaminated maize samples cleaned-up with IAC

Sample	FLD				DAD			
	FB ₁	FB ₂	FB ₃	Total	FB ₁	FB ₂	FB ₃	Total
1	74	22	7	103	*nd	nd	nd	nd
2	134	56	48	238	142	nd	nd	142
3	181	59	20	260	143	nd	nd	143
4	120	29	8	157	nd	nd	nd	nd
5	191	57	16	263	161	nd	nd	161
6	496	185	20	701	491	218	nd	709
7	1132	405	86	1623	1199	391	nd	1590
8	2424	1112	193	3730	2524	1174	106	3804
9	1186	390	134	1711	1223	362	nd	1585
10	1593	436	166	2196	1653	403	175	2231
11	1294	306	111	1711	1377	311	nd	1688
12	3313	1087	289	4689	3371	1154	190	4716
13	1124	260	101	1485	1157	289	nd	1445
14	3919	1032	376	5327	4003	951	291	5246
15	2617	904	373	3894	2753	826	176	3756

* nd- Not detectable

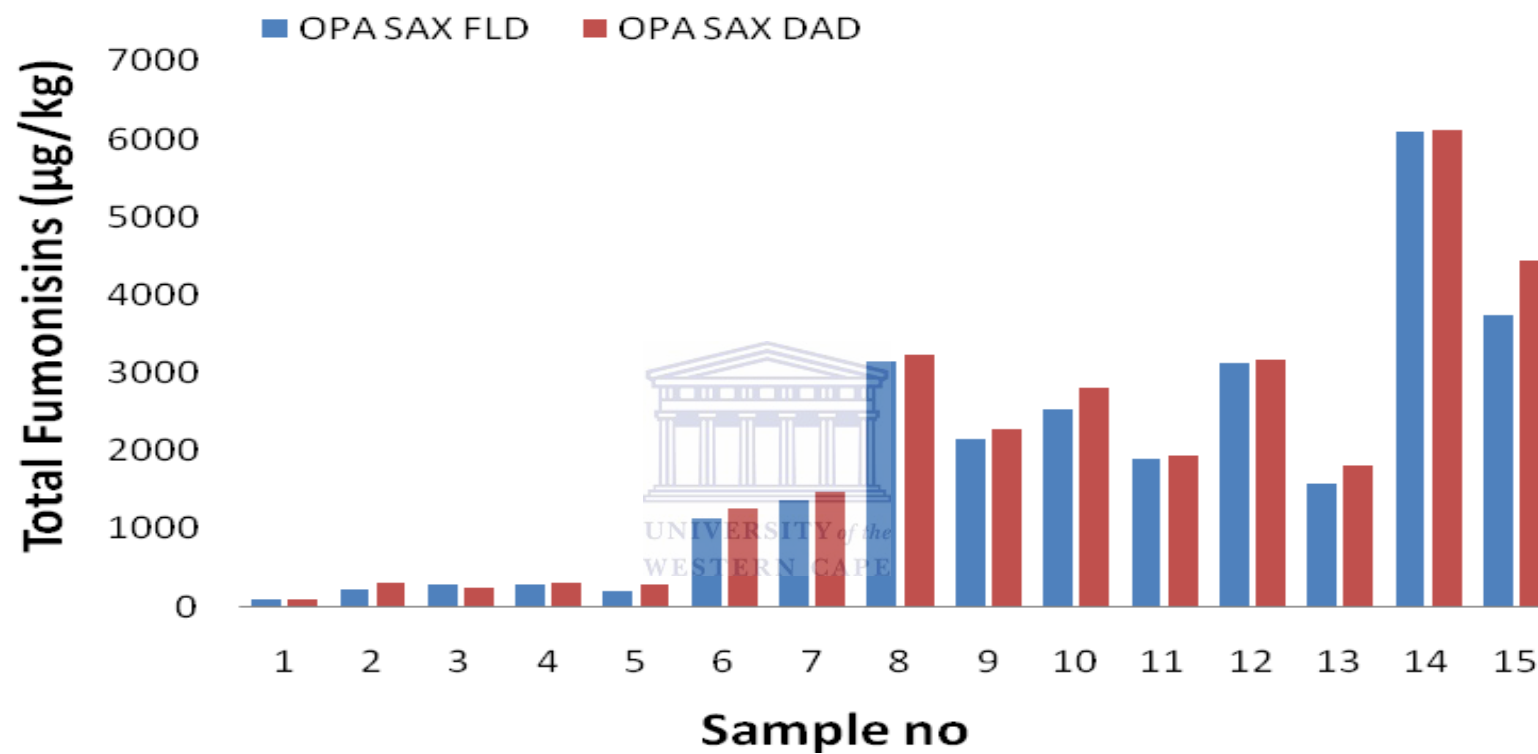


Figure 3.3 Comparison of FLD and DAD using SAX celan-up, Total Fumonsins = $FB_1 + FB_2 + FB_3$

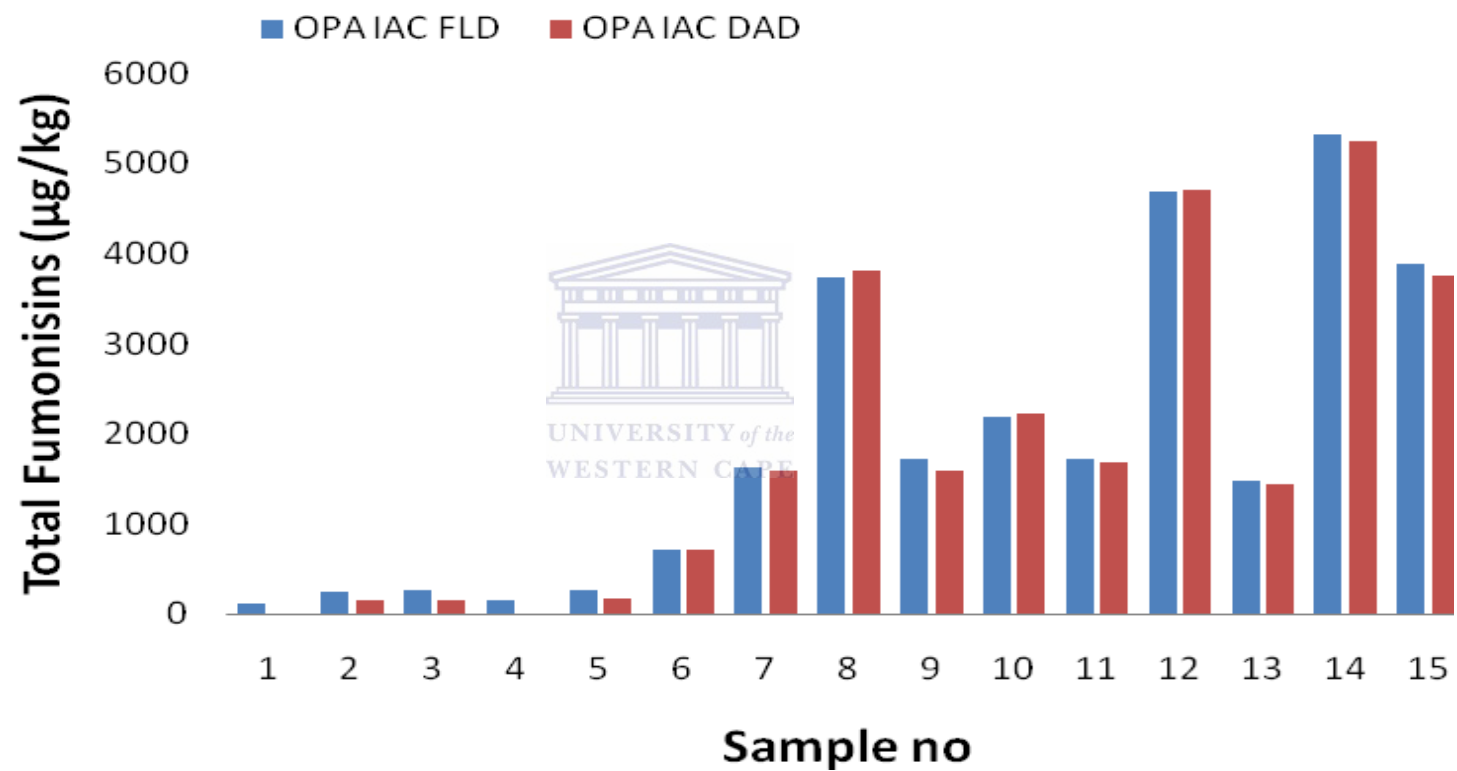


Figure 3.4 Comparison of FLD and DAD using IAC celan-up, Total Fumonsins = $FB_1 + FB_2 + FB_3$

3.6 Conclusion

For chromatographic separation of the fumonisin analogues, OPA was found to be a very effective derivatization reagent providing comparable results for both FLD and DAD. During the study, the detectors were connected in series in order to allow for comparison of the detectors without any variations produced by repeat derivatization and injection. Although most sensitive fumonisin analysis has been done with the use of fluorescence detection, this work indicates that fumonisins in maize can be comparably determined by UV detection.

Simultaneous detection with FLD and DAD shows the FLD to be more sensitive than DAD. However, the two detectors can be used as alternatives to each other for maize samples above 500 µg/kg following IAC clean-up compared to SAX clean-up which provided comparable results at all levels with both detectors. Based on the results obtained and the Official Analytical Chemists International (AOAC) adopting the OPA derivatization of fumonisins as the standard HPLC technique (Wilkes et al., 1998; Sydenham et al., 1996); the method will be used as the reference method in the other studies in the thesis.

References

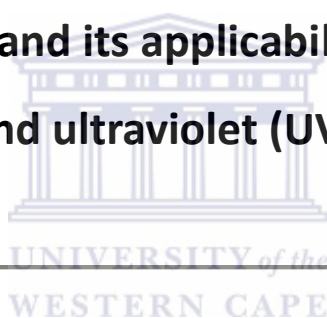
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CHAPTER 4

Optimization of naphthalene-2,3-dicarboxaldehyde (NDA) derivatization reagent for fumonisin derivatization and its applicability to fluorescence (FLD) and ultraviolet (UV) detection



4.1 Introduction

In chapter 2, reviews of the current methods used for naphthalene-2,3-dicarboxaldehyde (NDA) derivatization of fumonisins were described. Most researchers report using similar reaction conditions viz., sodium borate buffer pH 9, NDA dissolved in acetonitrile or methanol and chromatographic conditions, with HPLC and LC-MS being the most commonly used techniques (Ware et al., 1993, Bennett et al., 1994, Silva et al., 2009). The reaction of NDA with the nucleophilic cyanide anion forms stable and highly fluorescent derivatives (Cho et al., 2002) and reacts with the primary amine moiety of fumonisin B₁ to form an N-substituted 1-cyano-2-benz[*a*]isoindole derivative (Bennett and Richard, 1994).

Studies reported in this chapter are aimed at methodology development to optimize both NDA derivatization reaction and instrumentation conditions for determination of FB₁, FB₂ and FB₃ in maize. Initial conditions used were based on the method by Scott and Lawrence (1992) with some modifications. Thus the essential aims were to optimize NDA derivatization for fumonisin determination in naturally contaminated maize following strong anion exchange (SAX) or immunoaffinity column (IAC) clean-up, utilizing diode array detection (DAD) as a practical alternative to fluorescence detection (FLD).

4.2 Materials and Methods

4.2.1 Chemicals

All chemicals used were of analytical grade. Methanol, acetone, sodium hydrogen carbonate (NaHCO_3), acetonitrile, *o*-phosphoric acid (H_3PO_4), potassium dihydrogen phosphate (KH_2PO_4), sodium hydroxide (NaOH), disodium tetraborate ($\text{Na}_2\text{B}_4\text{O}_7 \cdot 10 \text{ H}_2\text{O}$), disodium hydrogen phosphate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$), potassium chloride (KCl), sodium dihydrogen phosphate (NaH_2PO_4), hydrochloric acid (HCl), sodium chloride (NaCl) were purchased from Merck. Napthalene-2,3-dicarboxaldehyde (NDA) was purchased from Invitrogen (Molecular Probes). NDA was prepared by dissolving 4 mg NDA in 8 mL methanol. Phosphate buffered saline (PBS) was prepared by dissolving 8.0 g sodium chloride, 1.2 g disodium hydrogen phosphate, 0.2 g potassium dihydrogen phosphate and 0.2 g potassium chloride in a litre distilled water. The pH was adjusted to 7 with *o*-phosphoric acid.

4.2.2 Fumonisin Standards

The standards were obtained and prepared as described in Section 3.2.2.

4.2.3 Maize Samples

Home-grown maize samples intended for human consumption were collected in the rural former Transkei area of the Eastern Cape Province following the 2006 harvest and stored at a temperature (4°C) where fumonisins are stable. The well mixed samples were milled prior to analysis.

4.2.4 Extraction using strong anion exchange (SAX) clean-up

Maize samples were extracted using the method of Sydenham et al. (1996) with modifications as described in Section 3.2.4.

4.2.5 Extraction using immunoaffinity columns (IAC) clean-up

Extraction of maize samples for IAC clean-up was performed as per manufacturer's instructions with minor modifications as described in Section 3.2.5.

4.2.6 IAC cleanup

IAC clean-up was performed as per manufacturer's instructions with minor modifications as described in Section 3.2.6.

4.2.7 Derivatization



Standards: Working standard (20 μ L) was used, 20 μ L of borate buffer (0.1 M) was added followed by 20 μ L potassium cyanide (65.13 mg/100 mL distilled water) and 40 μ L NDA (4 mg/8 mL methanol). The solution was vortexed and heated at 60°C for 15 minutes, then cooled to 24°C under running tap water. Mobile phase (100 μ L) was then used to dilute the solution and 20 μ L injected into the HPLC system.


Samples: The samples were reconstituted in 200 μ L methanol and 100 μ L of borate buffer (0.1 M) was added followed by 100 μ L potassium cyanide (65.13 g/100 mL distilled water) and 200 μ L NDA (4 mg/8 mL methanol). The solution

was vortexed, heated at 60°C for 15 minutes and cooled to 24°C under running tap water. 500 µL mobile phase was then used to dilute the solution and 20 µL injected into the HPLC system.

4.2.8 Recoveries

The maize samples were spiked with fumonisin working standards (40 µL) directly onto the dry milled maize samples. Since maize without fumonisin was not available, the unspiked maize samples were analyzed for fumonisins and these unspiked levels were taken into account for the calculation of the recoveries.

4.3 Chromatography



RP-HPLC was performed on an Agilent Technologies (Wildbronn, Germany) 1260 Infinity pump, Rheodyne 7725i injector and a Phenomenex (Torrance, CA, USA) Luna C18 5 µm column (150 mm x 4.60 mm) which was configured as described in Section 3.3. The mobile phase was prepared by combining methanol (780 mL) : 0.1 M sodium phosphate (NaH_2PO_4) (220 mL), and the pH of the mixture was adjusted to pH 3.35 with *o*-phosphoric acid. The mobile phase was filtered using 0.45 µm X 47mm filter paper with vacuum and pumped at 1 mL/min flow rate. Data was collected and analyzed by Agilent ChemStation software and quantification was achieved by comparison of peak areas with those of authentic fumonisin standards.

4.4 Results and Discussion

4.4.1 Peak resolution

Figures 4.1 and 4.2 are chromatograms obtained from a 10 μL injection of a fumonisin working standard derivatized with NDA at levels of 55.13 $\mu\text{g/mL}$, 25.00 $\mu\text{g/mL}$ and 13.25 $\mu\text{g/mL}$ for FB_1 , FB_2 and FB_3 respectively using FLD and DAD detection. Analytical separation of the FB-NDA derivatives was performed using isocratic elution with methanol : NaH_2PO_4 (78:22) mobile phase at 1 mL/min flow rate. The NDA derivatives were separated with retention times for FB_1 , FB_2 and FB_3 at 6.31, 14.87 and 11.87 ($\pm 5\%$) min respectively. An examination of the FLD and DAD chromatograms revealed that a number of other peaks (labelled A and B) in addition to the analyte peaks were present.

A reagent blank was prepared to test the interference of these peaks; the resultant chromatogram obtained from the reagent blank was overlaid with that of the standard chromatogram. All the peaks except for analyte peaks were present in the reagent blank chromatogram. This led to the conclusion that the peaks are from the reagents and do not interfere with the quantification or resolution of the analytes. Furthermore, Lillard et al., 1998 also observed peak A as a reagent peak during separation of amine NDA derivatives and suggested that it was formed as a by product of the benzoin condensation (Roach et al., 1987) or other side products (Kwakman et al., 1990) of NDA upon exposure to aqueous buffer conditions. Since the peak is present in completely independent experiments it was ruled out as a laboratory contaminant.

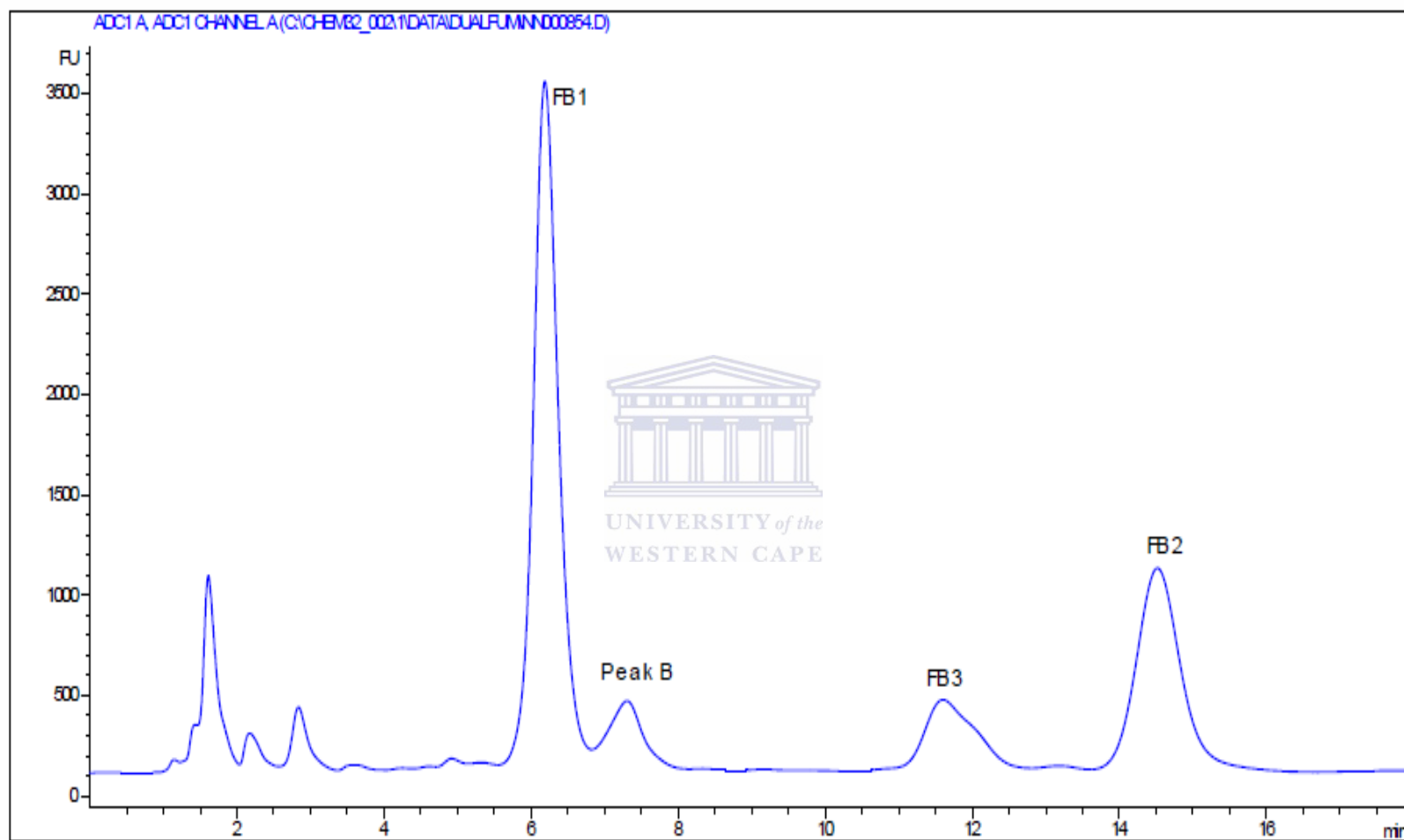


Figure 4.1 Chromatogram of fumonisins working standard detected by FLD

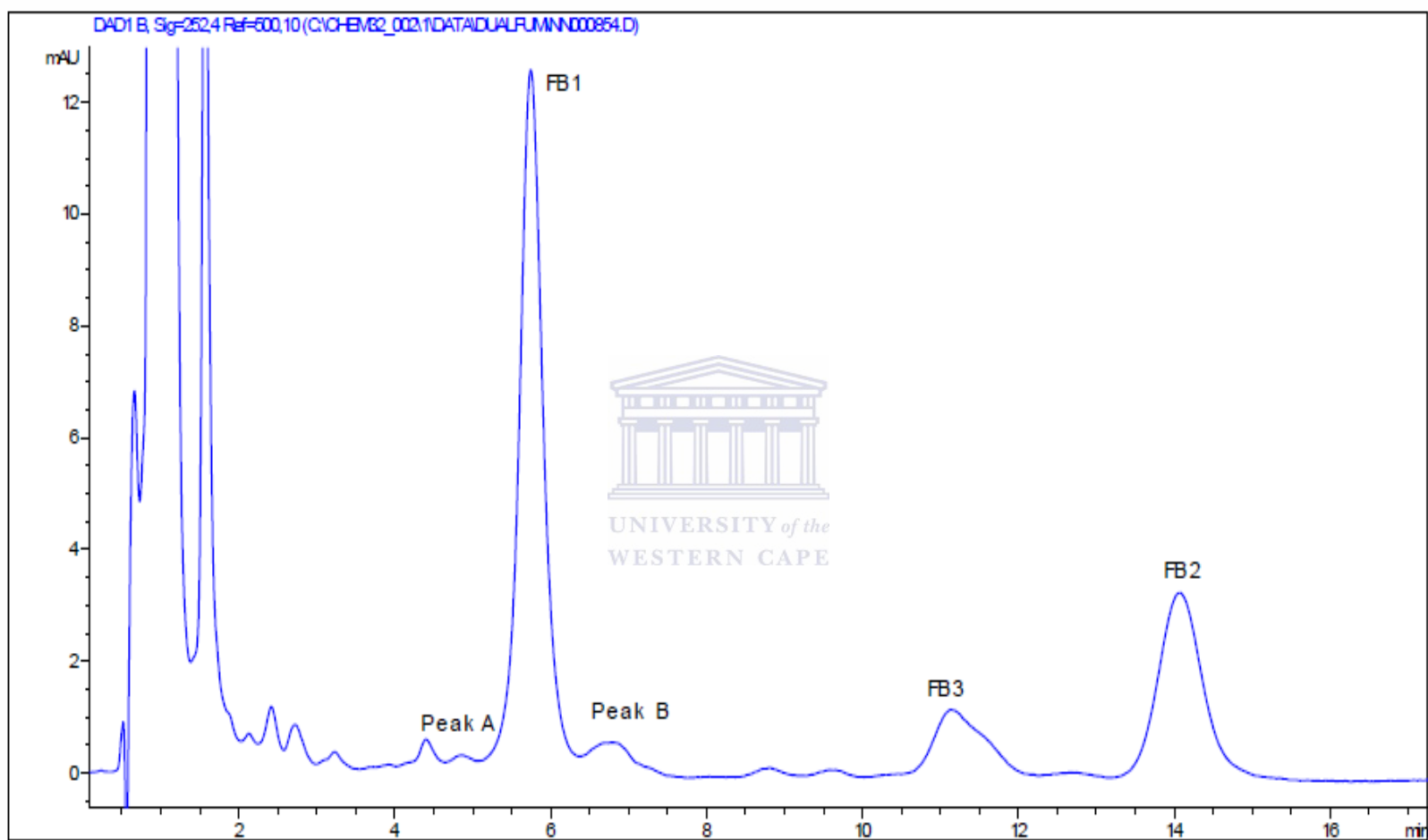


Figure 4.2 Chromatogram of fumonisin working standard detected by DAD

4.4.2 Method precision

Reproducibility was determined by measuring the intra- and inter-day repeatability of the working standards. The intra-day was measured by injecting three working standards in one day and the inter-day was measured over a period of five days. The excellent intra-day (Table 4.1) results obtained for FLD and DAD demonstrates the precision of the derivatization method. Comparison of the results across the two detectors suggests that the FLD has better repeatabilities with RSD values ≤ 2 %. Inter-day (Table 4.2) results of both detectors are higher than their corresponding intra-day results. However the inter-day results are still acceptable with both detectors reporting RSD values ≤ 8 % at $n = 15$ which is below the maximum standard value of 20 %.

Table 4.1 Intra-day precision of fumonisin working standards ($n=3$) using reported as peak areas

	FLD			DAD		
	FB ₁	FB ₂	FB ₃	FB ₁	FB ₂	FB ₃
Standard 1	99819	55625	34884	456	247	151
Standard 2	99512	55075	35001	453	237	154
Standard 3	101148	57079	35005	441	241	159
Mean	100160	55926	34964	450	242	154
Stdev	870	1035	69	8	5	4
RSD (%)	0.9	1.9	0.2	1.8	2.1	2.7

The precision of the method is acceptable and demonstrates both the reproducibility and repeatability of the derivatization method to be satisfactory. Furthermore, it allows for the use of standard peak areas for quantitation during method optimization. The precision results are in accordance with the performance characteristics of FB₁ and FB₂ as regulated by the Commission Directive of the European Commission (EC, 2005).

Table 4.2 Inter-day precision of fumonisin working standards (n=15) reported as peak areas

	FB ₁	FLD		FB ₁	DAD	
		FB ₂	FB ₃		FB ₂	FB ₃
Day 1	114205	53905	32857	482	253	133
	110123	52786	33105	486	253	140
	108985	52055	32996	510	263	140
Day 2	129982	61197	34623	495	239	140
	126829	60671	34852	481	231	140
	128419	60910	35430	479	228	138
Day 3	127202	61118	31727	545	281	160
	122601	58140	29928	547	285	160
	132047	63281	35671	555	278	165
Day 4	124543	63407	30718	505	278	136
	130530	66972	31294	528	287	142
	126289	64486	32361	550	267	155
Day 5	109950	61791	33012	524	273	145
	107691	59853	32758	545	297	140
	110155	61122	31310	523	277	139
Mean	120637	60113	32843	517	266	145
Stdev	9211	4269	1717	28	21	10
RSD (%)	8	7	5	5	8	8

4.4.3 Detection Limits

The limit of detection (LOD) was calculated as the amount of analyte injected resulting in peak heights of three times the maximum noise height whereas the limit of quantification (LOQ) was calculated as the amount of analyte injected giving a peak height ten times the maximum noise peak height. The NDA detection limits indicate that the FLD is 10-times more sensitive than that of the DAD with fumonisin standards (Table 4.3) and 100-times more sensitive with naturally contaminated samples (Table 4.4). Although FB₁ has the lowest LOD, it may be stated that the LODs of the FB₂ and FB₃ analogues are also satisfactory.

Table 4.3 Amount (ng) injected into HPLC Column for standards

		FB ₁	FB ₂	FB ₃
LOD (s:n=3)	FLD	0.11	0.50	0.27
	DAD	13.78	12.5	6.63
LOQ (s:n=10)	FLD	11.03	25.00	13.25
	DAD	55.13	25.00	27.00

Table 4.4 Limits of detection (LOD) and quantification (LOQ) expressed as levels in sample (µg/kg) following IAC clean-up

		FB ₁	FB ₂	FB ₃
LOD (s:n=3)	FLD	0.004	0.03	0.08
	DAD	0.3	170	180
LOQ (s:n=10)	FLD	0.03	0.1	0.4
	DAD	3	300	350

4.4.4 Stability of NDA derivatives

Stability tests were performed by treating both standards and samples to the same environmental conditions over a period of three days. Initial experiments showed a monotonic decrease in response (approximately 10 %) over an 8 hour period when derivatized samples were left at room temperature. This was overcome by storing both standards and samples at - 22°C after derivatization. Improvement in both the repeatability and precision of the method (Table 4.4) was observed as the derivatives under these conditions remained stable after six consecutive injections (~ 120 min) of the same standard.

NDA stability was further evaluated by storing derivatized standards and derivatized maize extracts at -22°C over three consecutive days. The FB-NDA responses (Figure 4.3 A) were stable for 24 hours; after which a decrease in detection (approximately 10 %) was observed in the FLD response. In contrast, the DAD response (Figure 4.3 B) on day 2 and 3 apparently increased 10 % over day 1. Previous reports described increases in FB-NDA response after 24 hours (Bennett et al., 1994, Lino et al., 2006). These results suggest that NDA derivatives are suitable for auto-injection or over-night analysis with working standards being injected between samples to allow for better quantification and to accommodate any derivative instability.

Table 4.5 Stability of FB-NDA standard after six consecutive injections (~ 120 min)

	FLD			DAD		
	FB ₁	FB ₂	FB ₃	FB ₁	FB ₂	FB ₃
Injection 1	63445	37292	9452	256	145	36
Injection 2	70098	41288	10680	289	171	45
Injection 3	67623	39935	10385	276	157	50
Injection 4	66881	39354	10274	293	160	44
Injection 5	63322	36994	9738	255	149	37
Injection 6	64251	37651	9952	262	161	41
Mean	65937	38752	10080	272	157	42
Stdev	2718	1710	451	17	9	5
RSD (%)	4	4	4	6	6	12

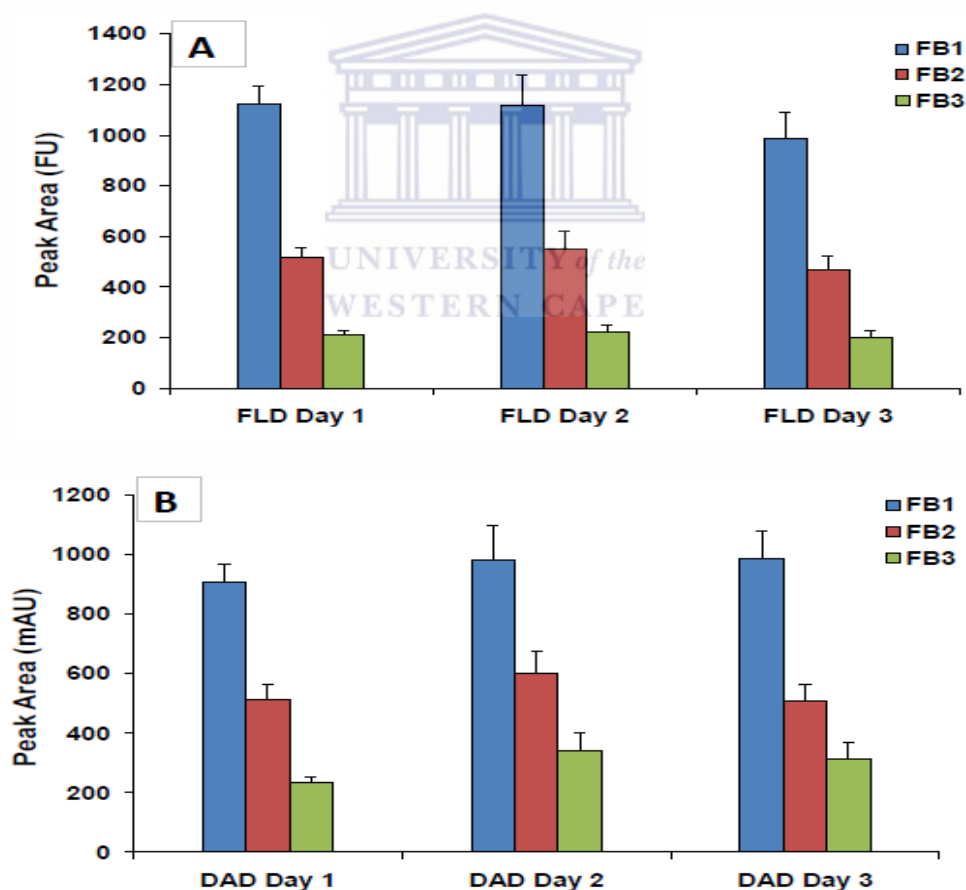


Figure 4.3 Stability of FB-NDA derivatives ($\mu\text{g/kg}$) over period of three days (72 hours). Results calculated from mean ($n=6$) \pm standard deviation. (A) Stability of FLD, (B) stability of DAD

4.5 Robustness

4.5.1 Optimization of mobile phase

Different organic solvents used as HPLC mobile phase components were examined for their suitability to provide the shortest run time without compromising on the resolution of the closely eluting peaks (FB₂ and FB₃). Figure 4.4 shows the effect change in mobile phase composition has on the retention times of the analytes. From the graphic representation, 77 % and 80 % methanol composition causes interferences between FB₁ with peaks A and B, and FB₃ with peak B. Selection of mobile phase was consequently influenced by the separation of peaks A and B from analyte peaks.

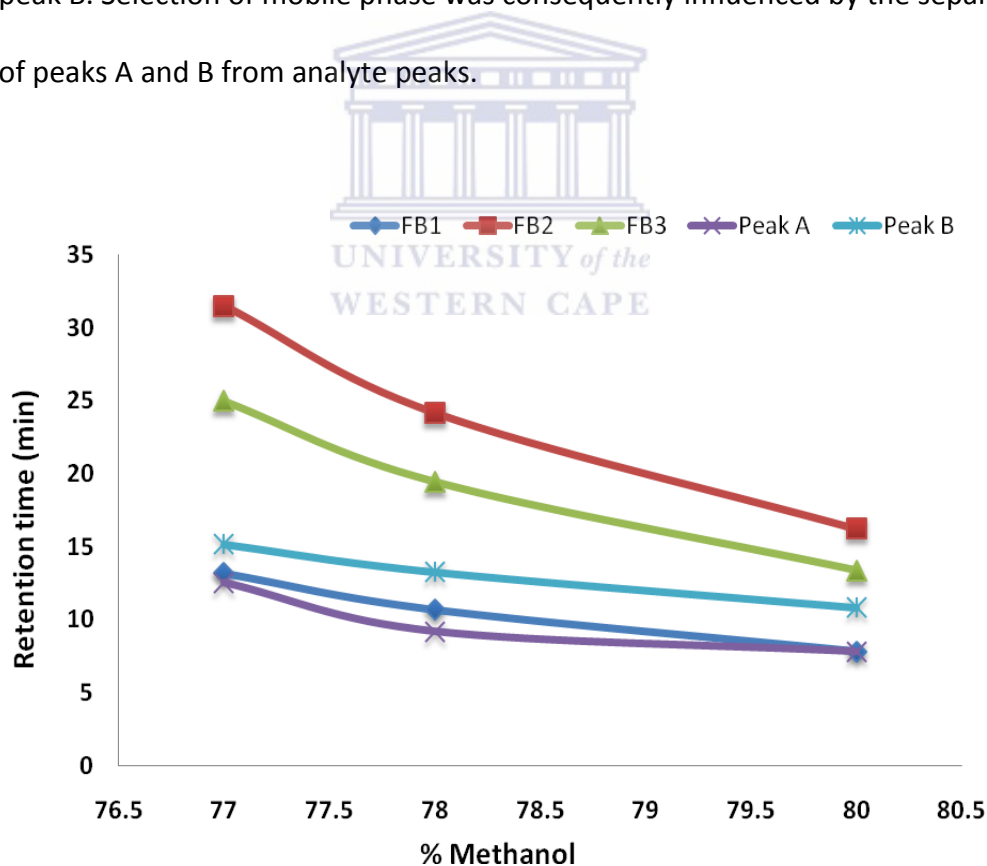


Figure 4.4 Effect of methanol concentration on retention time and interference with reagent peaks

Most studies on NDA use acetonitrile as an organic solvent in their mobile phase because it provides significantly lower retention times and more sensitive results (Lino et al., 2007). Acetonitrile was found to be the best solvent since it provided an excellent baseline resolution coupled with short analysis time.

The results suggested that the elution order of the FB-NDA derivatives is dependent on the type of mobile phase used as the relative retention of the analytes were altered with the use of acetonitrile. Methanol : 0.1 M NaH₂PO₄ (78:22) mobile phase provides an elution order of FB₁, FB₃ and FB₂. However in acetonitrile : water : acetic acid (65:35:1) as eluent, the elution order of FB₂ with FB₃ and FB₃ with its isomer epi-FB₃ was interchanged, a phenomenon which has not previously been reported. Finally, further method optimization allowed for the development of a methanol mixture as the HPLC mobile phase component of choice since it not only yielded comparable results to acetonitrile but is cheaper than acetonitrile which was unavailable during the period of the study.

From the different proportions of methanol and 0.1 M NaH₂PO₄, the best separation of all the fumonisin B analogues within the shortest analysis time was obtained with mobile phase methanol : 0.1 M NaH₂PO₄ (78:22, pH 3.35). This was consequently selected as the mobile phase of choice for the chromatographic separation of NDA derivatives.

4.5.2 Optimization of wavelength

FB-NDA derivatives are generally monitored at excitation wavelengths of 420 and 246 nm and emission wavelengths of 500 and 418 nm (Bennett et al., 1994; De Montigny et al., 1987). These wavelengths were tested using fumonisin working standards. Based on the high sensitivity of the FLD at excitation 420 nm and emission 500 nm; these were selected as optimum wavelengths. The DAD absorption wavelengths (248 nm, 250 nm, 252 nm, 256 nm,) were examined and 252 nm in our hands provided the best sensitivity (Figure 4.5). An iso-absorbance plot (software programme which displays chromatographic details in 3D including retention time versus wavelength, from which optimum wavelength can be selected) was then used to confirm the wavelength selection for DAD.

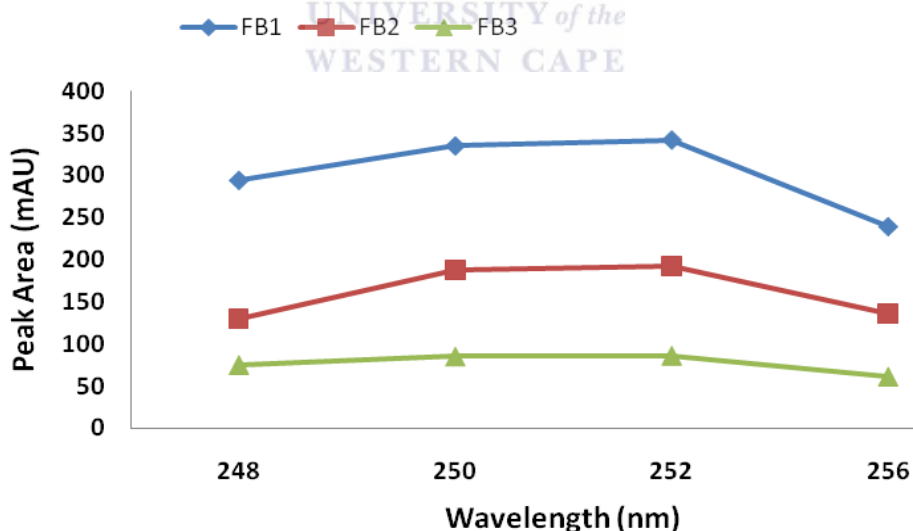


Figure 4.5 Optimum wavelength selections for UV absorbance relative to peak area, results calculated from mean (n=3)

4.5.3 Reaction time

Figures 4.6 A and B demonstrate the effect heating time has on standard peak areas. The results are based on five standards heated for 15 and 30 minutes at 60°C. Both the FLD and DAD showed a decrease in standard area for FB₁ and FB₂ when the reaction time was increased from 15 minutes to 30 minutes. FB₃ was not affected by change in the reaction time as it remained constant during heating. Since FB₁ and FB₂ produced higher standard area when heated for 15 minutes and the fumonisins B analogues are analysed simultaneously, the reaction time for the derivatization of the fumonisins with NDA was selected to be 15 minutes.

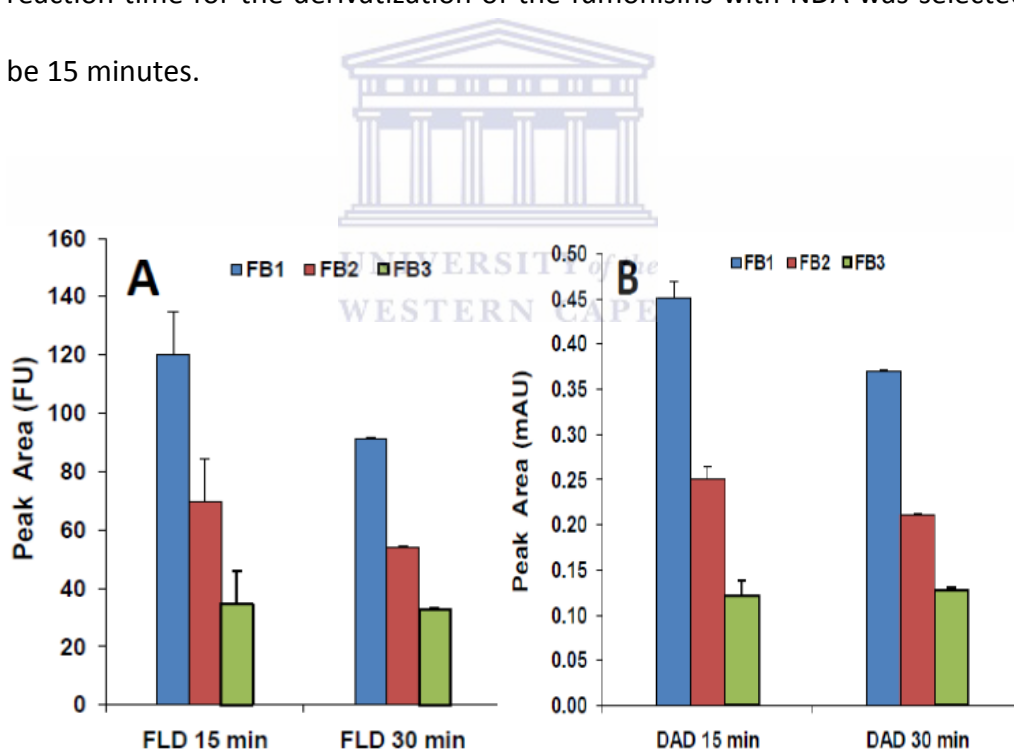


Figure 4.6 Effect of reaction time on peak areas. Results reported as mean (n=5) \pm standard deviation. (A) Comparison of FLD, (B) Comparison of DAD

4.5.4 Effect of reaction temperature

To determine the effect of reaction temperature, experiments were performed at two different temperatures (24°C and 60°C) using fumonisin working standards. Temperature estimations were selected from literature (Lamba et al., 2008; Scott and Lawrence 1992) with all experiments performed for 15 minutes. The FB-NDA derivatization reaction was found to be temperature dependent (Figures 4.7 and 4.8) as the responses increased with increase in temperature. Performing the experiments at room temperature (24°C) resulted in half the reaction efficiency compared to 60°C for both the FLD and DAD. Due to increase in standard peak area when the reaction was heated for 60°C and the improved RSD values, 60°C was selected as reaction temperature for NDA derivatization.

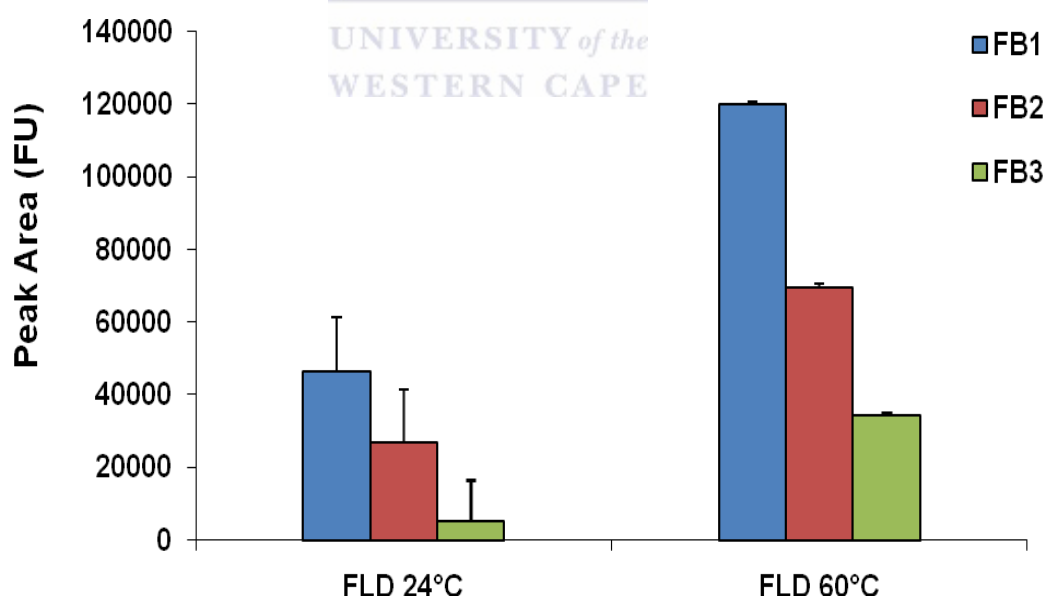


Figure 4.7 Effect of reaction temperature on peak areas. Results reported as mean (n=5) \pm standard deviation for FLD

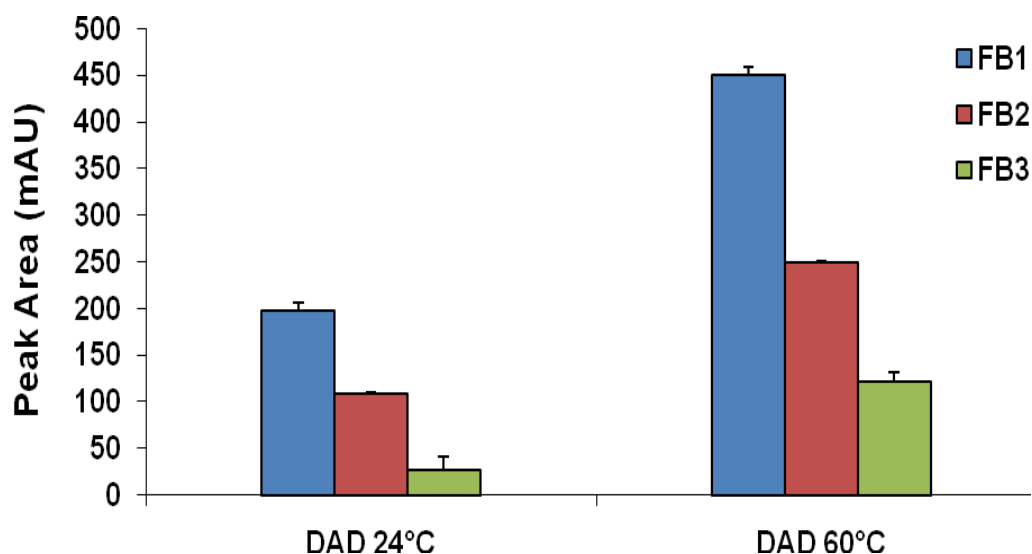


Figure 4.8 Effect of reaction temperature on peak areas. Results reported as mean ($n=5$) \pm standard deviation for DAD

4.5.5 Optimization of buffer concentration

Optimization of buffer concentration was done by performing experiments at three different buffer concentrations (0.05 M, 0.08 M and 0.1 M, all adjusted to pH 9.5). Based on the results of five standard injections at each concentration level, the use of different buffer concentrations affected the fluorescence response of the FB-NDA derivatives. Although no major difference in HPLC responses were observed between 0.05 M and 0.08 M buffers, a steep increase (approximately 10 % from the others) in standard area was observed with 0.1 M (Figure 4.9) with RSD values ≤ 3 % for all fumonisin analogues. The DAD response was not affected by changes in buffer concentration (Figure 4.10). Since the FLD and DAD are run simultaneously, it is convenient to use the same buffer for all their preparations. Given that 0.1 M provided optimum results for FLD and buffer

concentration change not influencing DAD response, 0.1 M was selected as the buffer concentration for fumonisin derivatization.

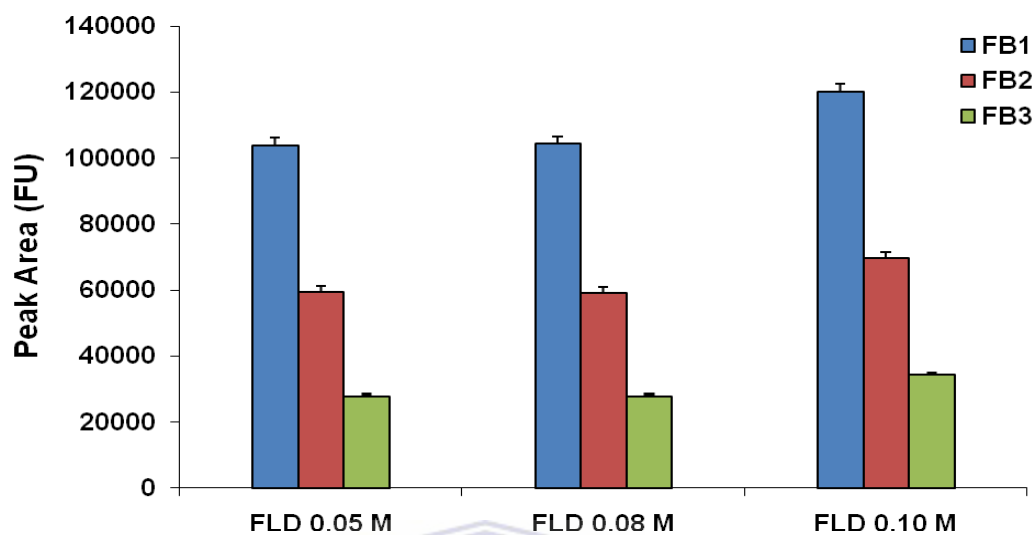


Figure 4.9 Effect of buffer concentration on peak areas for mean (n=5)
± standard deviation for FLD

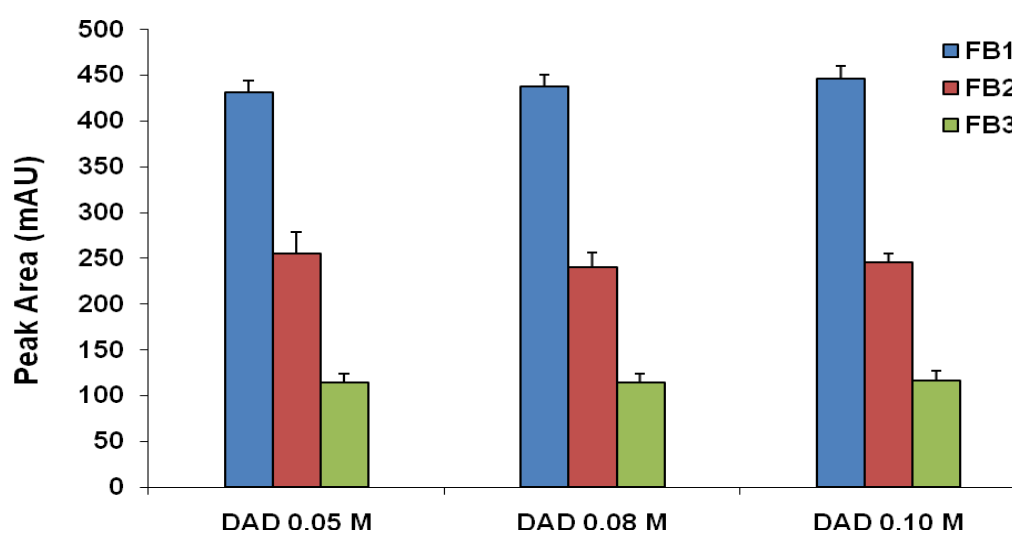


Figure 4.10 Effect of buffer concentration on peak areas for mean (n=5)
± standard deviation for DAD

4.5.6 *Effect of extractants*

To improve the extraction efficiencies, two different extraction solvents were tested for their efficiency to extract fumonisins from maize. Approaches in literature for improving the extraction of fumonisins from maize include the use of EDTA as an extraction solvent (Sydenham et al., 1995, Dombrink-Kurtman et al., 1999, Scott et al., 1996) whereas methanol : water (3:1) has been reported to provide increased extraction efficiencies compared to other solvent mixtures (Shephard, 1998).

Methanol : 0.1 M EDTA (3:1, v/v) was tested as an extraction solvent for its efficiency for fumonisin extraction compared to the widely used methanol : water (3:1, v/v). Table 4.5 provides detailed results on the extraction experiments using both solvents. Results of the extraction with methanol : 0.1 M EDTA (3:1, v/v) as a solvent provided similar results to the methanol : water (3:1, v/v) extraction solvent. Agreement between extraction solvents was excellent with the highest variation noted with methanol : water (3:1, v/v) as it produced RSD values varying from 7 – 10 % compared to the methanol : 0.1 M EDTA (3:1, v/v) with RSD values between 2 – 10 %. Use of methanol : 0.1 M EDTA (3:1, v/v) caused some difficulties as the 0.1 M EDTA precipitated out of solution when it was added to the methanol.

Due to the precipitation of EDTA and it not providing any improvement in results when compared to methanol : water (3:1, v/v), the latter was chosen as the

extraction solvent for the study as used previously for fumonisin extraction (Shephard et al., 1990).



Table 4.6 Solvent extraction efficiency for NDA derivatization of maize samples (20 g / 100 mL) following SAX clean-up

Solvent	Extraction	FLD			DAD		
		FB ₁ (µg/g)	FB ₂ (µg/g)	FB ₃ (µg/g)	FB ₁ (µg/g)	FB ₂ (µg/g)	FB ₃ (µg/g)
MeOH : H ₂ O (3:1, v/v)							
	1	1197	547	191	974	534	197
	2	1150	530	234	924	516	242
	3	1120	526	206	886	534	237
	4	1149	549	208	974	563	233
	5	1144	512	224	842	511	236
	6	980	437	210	841	421	252
	Mean	1123	517	212	907	513	233
	Stdev	74	41	15	61	49	19
	RSD (%)	7	8	7	7	10	8
MeOH : 0.1 M EDTA (3:1, v/v)							
	1	1240	551	188	1216	564	211
	2	1321	571	201	1288	610	247
	3	1261	544	201	1245	582	218
	4	1314	560	213	1289	608	249
	5	1315	557	216	1171	612	267
	6	1285	567	215	1102	610	263
	Mean	1290	558	205	1218	598	242
	Stdev	33	10	11	73	20	23
	RSD (%)	3	2	5	6	3	10

4.6 Recoveries

The accuracy of the optimized method was determined by measuring the percent recoveries of the method. This was achieved by spiking maize samples with 1103 µg/kg, 500 µg/kg and 270 µg/kg of FB₁, FB₂ and FB₃ respectively. In order to optimize recoveries two different extraction solvents (methanol : water 3:1 and methanol : 0.1M EDTA 3:1) were investigated, but little difference was seen between the two and hence methanol : water (3:1) as previously used for maize samples analysed by SAX clean-up was selected (Sydenham et al., 1996). The accuracy and repeatability of the method is generally within acceptable limits for both FLD and DAD following SAX clean-up (Table 4.6). In addition to SAX clean-up, a similar recovery experiment was performed using IAC clean-up. A good basis of comparison between the FLD and DAD methods was thus achieved, even though a decrease in NDA-FB₁ recoveries was observed with DAD following IAC clean-up (Table 4.7).

Initial recovery experiments were done by spiking directly on the eluate of the SAX cartridge to test the accuracy of the clean-up and derivatization method. The results obtained from those experiments were comparable to those of spiking directly on the maize sample. This signifies that little fumonisin is lost in the extraction process, clean-up stage and derivatization; indicating the accuracy and effectiveness of both sample preparation and derivatization for fumonisin analyses when using NDA derivatization. Recovery results of both SAX and IAC clean-up are acceptable according to the values established by the European

Commission which recommends recoveries of between 60 – 120 % for individual FB methods (EC, 2005).

Table 4.7 Fumonisin recoveries (%) from maize samples cleaned-up with SAX

	FLD			DAD		
	FB ₁ (ng/g)	FB ₂ (ng/g)	FB ₃ (ng/g)	FB ₁ (ng/g)	FB ₂ (ng/g)	FB ₃ (ng/g)
Blank 1	295	110	21	175	126	62
Blank 2	341	126	36	203	149	91
Mean	318	118	28	189	137	76
Sample 1	1197	547	191	974	534	197
Sample 2	1150	530	234	924	516	242
Sample 3	1120	526	206	886	534	237
Sample 4	1149	549	208	974	563	233
Sample 5	1144	512	224	842	511	236
Sample 6	980	437	210	841	421	252
Mean	1123	517	212	907	513	233
Stdev	74	41	15	61	49	19
RSD (%)	7	8	7	7	10	8
Spike	1103	500	270	1103	500	270
Recoveries (%)	73	80	68	65	75	58

Table 4.8 Fumonisin recoveries (%) from maize samples cleaned-up with IAC

	FLD			DAD		
	FB ₁ (ng/g)	FB ₂ (ng/g)	FB ₃ (ng/g)	FB ₁ (ng/g)	FB ₂ (ng/g)	FB ₃ (ng/g)
Blank 1	113	67	12	406	703	nd
Blank 2	156	91	18	143	657	173
Mean	135	79	15	275	680	87
Sample 1	835	415	177	834	912	214
Sample 2	843	403	266	812	910	252
Sample 3	803	942	144	716	1227	311
Sample 4	793	749	161	646	1173	242
Sample 5	789	402	135	684	881	258
Sample 6	823	395	244	1100	844	245
Mean	814	551	188	799	991	254
Stdev	23	236	54	165	164	32
RSD (%)	3	43	29	21	17	13
spiked	1103	500	270	1103	500	270
Recovery (%)	62	94	64	48	62	62

4.7 Method application

To demonstrate the applicability of the optimized method, analysis was applied to 15 maize samples collected from the Eastern Cape Province former Transkei area, South Africa. The analysis of the samples was routinely carried out using SAX and IAC clean-up with simultaneous detection with HPLC-FLD and DAD. The same samples were used to allow for comparison between the two clean-up methods. Fumonisin was detected in all the samples with contamination levels varying from 93 µg/kg to 4120 µg/kg for total fumonisins.

Comparison of FLD and DAD utilizing SAX clean-up was good with all samples comparable at all levels (Table 4.8; Figure 4.12). The IAC clean-up however showed poor comparison between the detectors as the results produced low levels at high fumonisin contamination and high levels at low fumonisin contamination with FLD, a trend that was reported by Chu et al., 1994 (Table 4.9, Figure 4.13). Nonetheless, above 1800 µg/kg, the FLD and DAD could compare after IAC clean-up.

Table 4.9 Comparison of HPLC-FLD and DAD following SAX clean-up ($\mu\text{g/kg}$)

Sample	FLD				DAD			
	FB ₁	FB ₂	FB ₃	Total	FB ₁	FB ₂	FB ₃	Total
1	90	17	1	108	120	29	*nd	149
2	166	55	10	231	184	64	25	273
3	229	88	18	336	242	99	38	380
4	271	76	20	367	165	116	47	328
5	172	54	15	240	179	84	25	288
6	733	250	48	1030	663	245	35	943
7	442	176	20	638	370	184	nd	554
8	1712	832	227	2771	1522	809	197	2528
9	507	182	20	709	480	229	nd	709
10	713	248	23	983	663	265	41	969
11	1171	345	58	1575	997	326	129	1452
12	2065	747	201	3014	1875	688	241	2804
13	523	150	14	686	498	161	41	699
14	1754	453	284	2492	1598	465	338	2402
15	318	108	136	562	301	115	111	528

*nd- Not detectable

Table 4.10 Comparison of HPLCFLD and DAD following IAC clean-up (µg/kg)

Sample	FLD				DAD			
	FB ₁	FB ₂	FB ₃	Total	FB ₁	FB ₂	FB ₃	Total
1	59	22	12	93	325	442	*nd	766
2	145	58	16	219	211	347	137	695
3	156	59	10	225	343	387	nd	730
4	148	43	11	202	194	310	nd	504
5	173	53	21	248	403	383	nd	786
6	503	257	25	785	627	802	nd	1429
7	942	327	47	1316	1043	778	151	1972
8	1961	928	127	3016	1944	1277	140	3361
9	1370	370	80	1820	1467	737	243	2447
10	1518	428	168	2115	1631	804	144	2579
11	1133	297	59	1490	1204	690	183	2077
12	2410	825	108	3343	2506	1171	153	3830
13	1015	251	66	1332	1073	627	nd	1700
14	2713	673	278	3664	2797	1073	249	4120
15	1971	676	227	2874	2043	968	205	3215

*nd- Not detectable

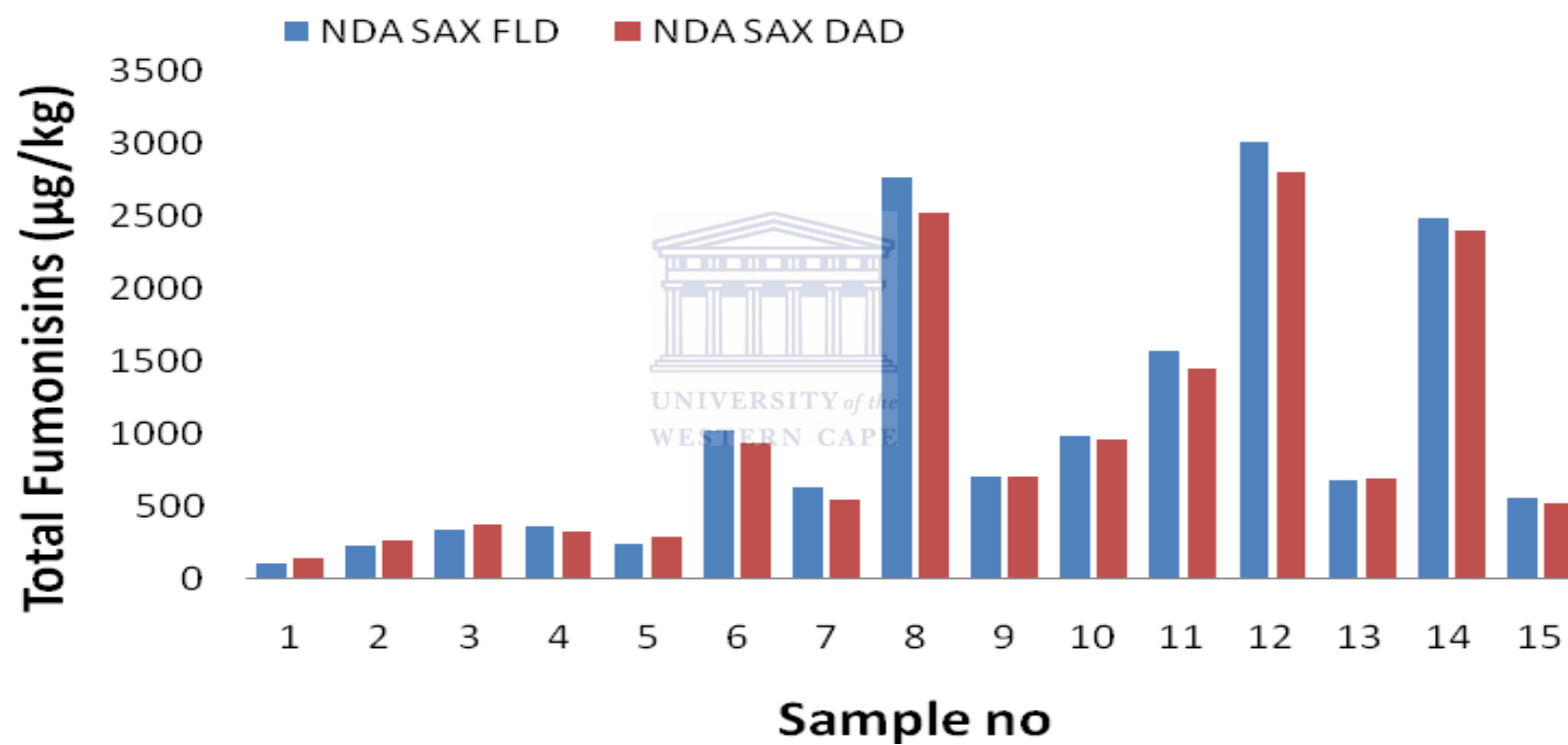


Figure 4.11

Comparison of FLD and DAD following SAX clean-up, Total Fumonisin = $FB_1 + FB_2 + FB_3$

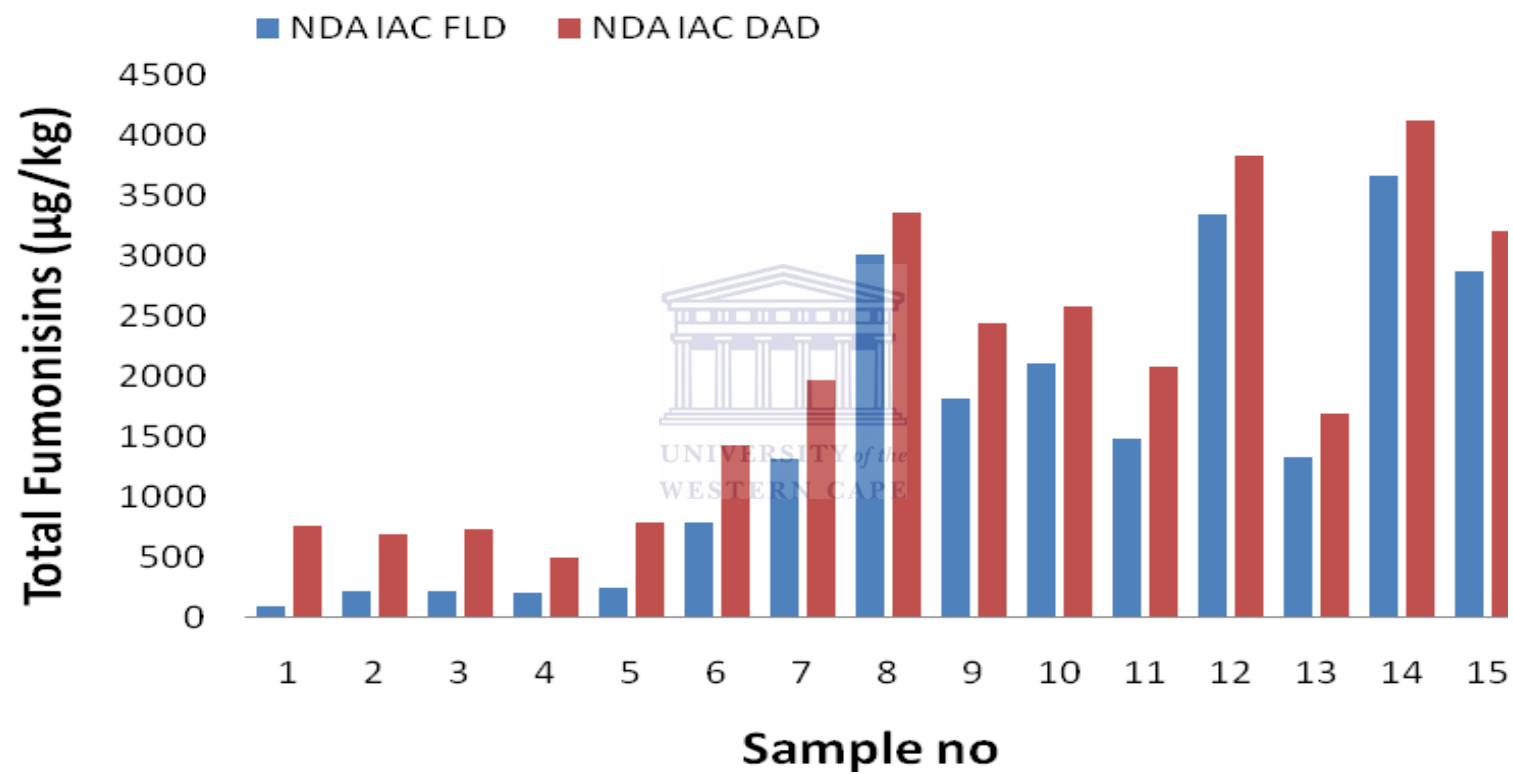


Figure 4.12 Comparison of FLD and DAD following IAC clean-up, Total Fumonisin = $FB_1 + FB_2 + FB_3$

4.8 Conclusion

Based on the chromatographic resolution of the fumonisin analogues in the naturally contaminated samples, the selected optimized conditions are suitable for NDA derivatization and the detection of fumonisins in maize. The reaction of fumonisins with NDA yielded FB-NDA derivatives which were found to be stable, sensitive and selective for both FLD and DAD methodologies.

The derivatization using NDA is fast with total retention time less than 15 minutes. The analytical procedure supports using methanol : water (3:1) as extraction medium and purification using either SAX or IAC allows for simultaneous detection and quantification of FB₁, FB₂ and FB₃. Aside from the difference in limits of detection, a comparative study of FLD and DAD for the analysis of fumonisins in maize demonstrated that the response achieved by both detectors is sensitive enough for the analysis of fumonisin in naturally contaminated samples. Both detectors would be appropriate for quantification purposes with the highest sensitivity achieved by FLD.

Safety

The cyanide anion is a highly lethal and toxic reagent and consequently it is necessary to observe and apply very strict and appropriate treatment conditions for the safe disposal of all waste materials containing the cyanide anion.

References

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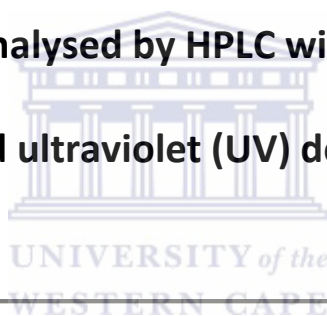
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CHAPTER 5

**An evaluation of dansyl chloride (DnS-Cl) for fumonisin
derivatization analysed by HPLC with fluorescence (FLD)
and ultraviolet (UV) detection**



5.1 Introduction

Dansyl chloride (DnS-Cl) reacts with both primary and secondary amino groups to provide stable derivatives. DnS-Cl derivatives are known to combine the unique feature of being both fluorescent and detectable in the UV region (Loukou et al., 2003). The only HPLC determination of fumonisin (Dasko et al., 2006) utilizing DnS-Cl was FB₁ in beer. In this chapter the extent to which DnS-Cl can be used for the derivatization of fumonisins in naturally contaminated maize samples was investigated.

Initial conditions used were based on the method by Dasko et al., (2006) with modifications. The method was optimized to obtain optimum conditions following strong anion exchange (SAX) and immunoaffinity (IAC) clean-up, pre-column derivatization and HPLC detection with diode array (DAD) and fluorescence detection (FLD). Dansyl chloride reacts with amines by nucleophilic substitution and forms fluorescent dansyl derivatives (Legua et al., 1999) and produced both sensitive and reproducible results with the analysis of biogenic amines (Mo Dugo et al., 2006).

5.2 Materials and Methods

5.2.1 Chemicals

All chemicals used were of analytical grade. Sodium carbonate (Na₂CO₃), sodium chloride (NaCl), *o*-phosphoric acid (H₃PO₄) and dansyl chloride (DnS-Cl) were purchased from Merck. DnS-Cl derivatizing solution was prepared by dissolving

100 mg DnS-Cl in 10 mL acetone. Phosphate buffered saline (PBS) was prepared as described in Section 3.2.1.

5.2.2 Fumonisin Standards

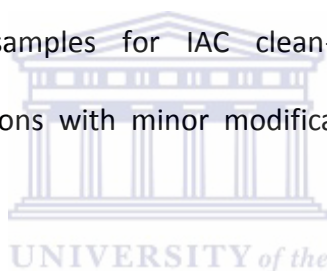
The standards were obtained and prepared as described in Section 3.2.2.

5.2.3 Maize Samples

Home-grown maize samples were collected as described in Section 3.2.3.

5.2.4 Extraction using strong anion exchange (SAX) clean-up

Extraction of maize samples for IAC clean-up was performed as per manufacturer's instructions with minor modifications as described in Section 3.2.4.



5.2.5 Extraction using immunoaffinity columns (IAC) clean-up

Extraction of maize samples for IAC clean-up was performed as per manufacturer's instructions with minor modifications as described in Section 3.2.5.

5.2.6 Derivatization

Standards: Working standard (20 μ L) was added to DnS-Cl (20 μ L, 100 mg/10 mL) followed by 20 μ L NaHCO_3 (2 M, saturated). The solution was vortexed and heated at 40°C for 10 minutes then cooled to 24°C under running tap water. H_3PO_4 (1 M, 20 μ L) was added and 20 μ L injected into the HPLC system.

Samples: Samples were reconstituted in 100 μL methanol and derivatized by adding 200 μL DnS-Cl (100 mg/10 mL) followed by 200 μL NaHCO_3 (2 M, saturated), heated at 40°C for 10 minutes on a heating mantle, cooled to 24 °C and 200 μL H_3PO_4 (1 M) added and 20 μL injected into the HPLC system.

5.3 Chromatography

Reversed-phase high-performance liquid chromatography (RP-HPLC) was performed on an Agilent Technologies (Wildbronn, Germany) and configured as described in Section 3.3. The mobile phase was prepared by combining methanol (740 mL) : 0.1 M sodium phosphate (NaH_2PO_4) (260 mL), and the pH of the mixture was adjusted to pH 3.35 with *o*-phosphoric acid. The mobile phase was filtered using 0.45 μm X 47mm filter paper with vacuum and pumped at 1 mL/min flow rate. Data were collected and analyzed by Agilent ChemStation software and quantification achieved by comparison of peak areas with those of authentic fumonisin standards.

5.4 Results and Discussion

5.4.1 Peak resolution

A typical fumonisin B chromatogram was obtained when fumonisin working standard (containing 55.13 $\mu\text{g}/\text{kg}$ of FB_1 , 25.00 $\mu\text{g}/\text{kg}$ and 13.25 $\mu\text{g}/\text{kg}$ of FB_2 and FB_3 , respectively) was derivatized with DnS-Cl (Figures 5.1 and 5.2). Chromatographic resolution of the dansyl derivatives was obtained with an isocratic elution using MeOH : 0.1 M NaH_2PO_4 (74:26) mobile phase with well

resolved analyte peaks in less than 15 minutes and an elution order of FB₁, FB₃ and FB₂. Retention times of 4.98, 13.01 and 11.83 min ($\pm 5\%$) for FB₁, FB₂ and FB₃, respectively, for both FLD and DAD were attained.

The chromatogram of fumonisin derivatives showed additional peaks as was also seen for NDA. Interference of the peaks were examined by overlaying the chromatograms of reagent blank with working standards, the peaks (A and B) did not interfere with those of the FB analogues. Removal of the peaks proved difficult, with one of the additional peaks probably dansyl dimethylamine, the most abundant product of dansyl reactions (Seiler et al., 1978). Since they did not interfere with both the resolution and quantification of the analyte peaks, no further attempts were made to remove them and they were classified as reagent peaks.



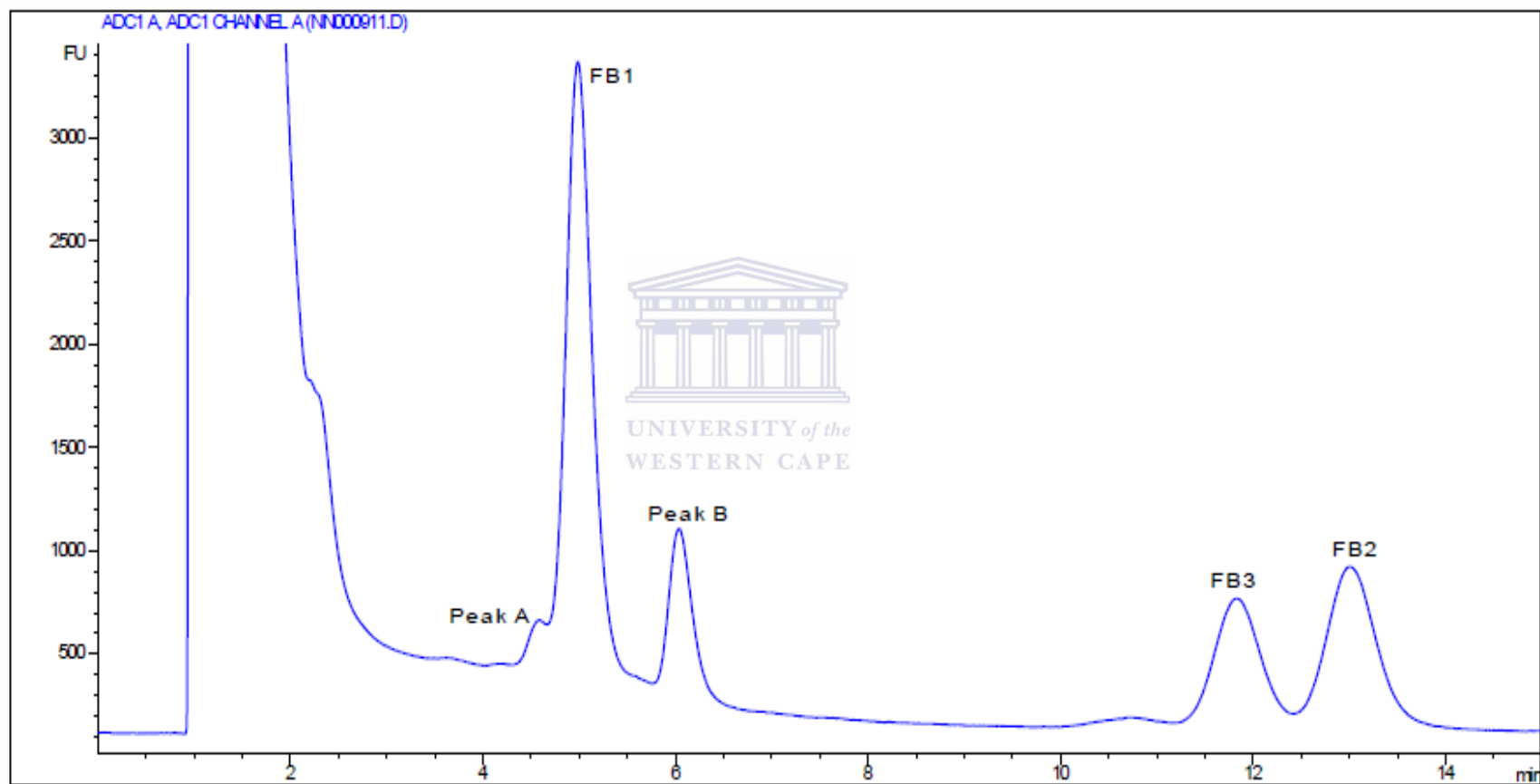


Figure 5.1 Chromatogram of combined fumonisins working standard detected by FLD

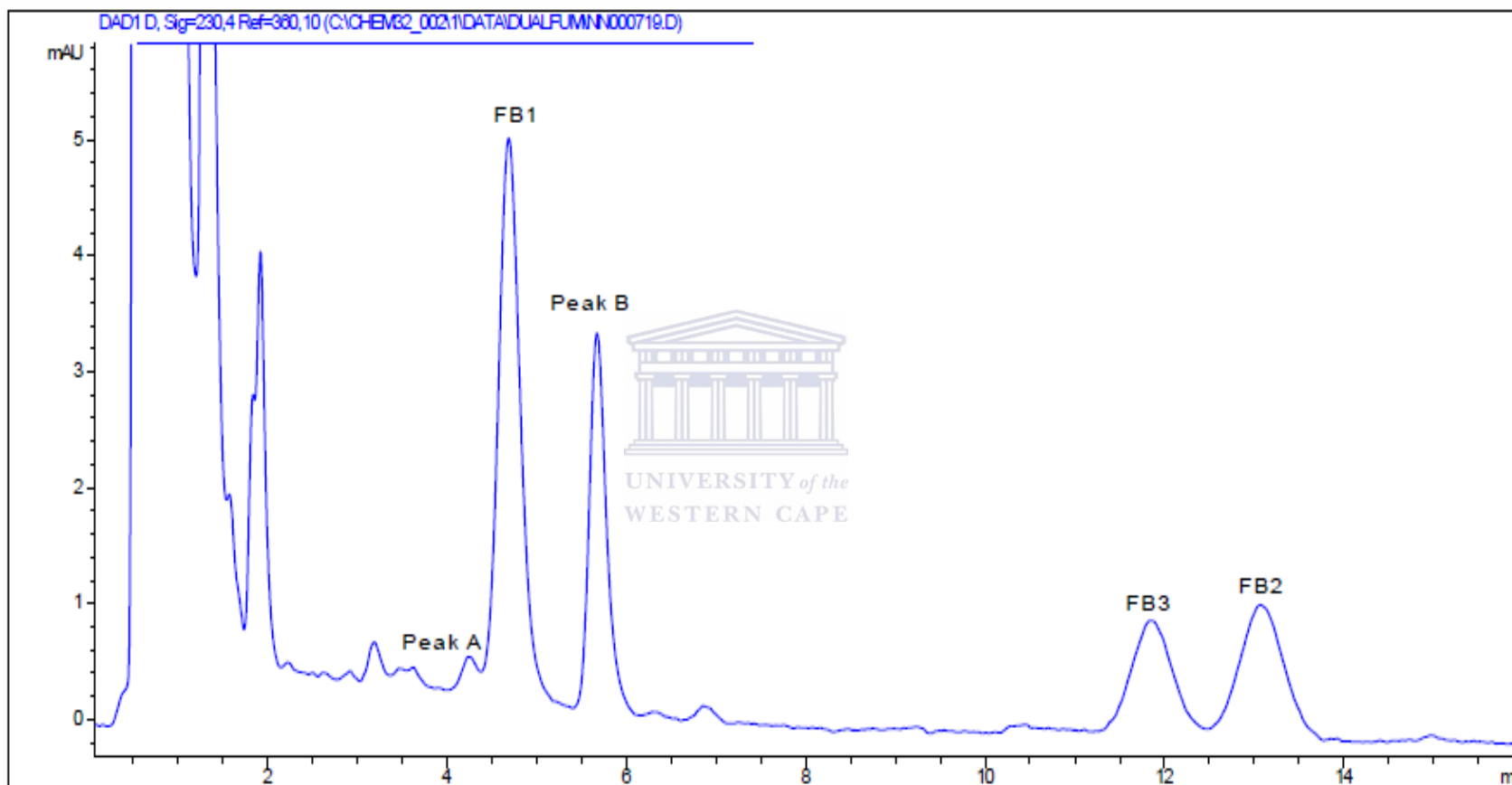


Figure 5.2 Chromatogram of combined fumonisins working standard detected by DAD

5.4.2 Method precision

The repeatability and reproducibility of the method was determined by intra- and inter-day precision and reported as standard peak area and RSD in tables 5.1 and 5.2, respectively. The inter-day precision was obtained by injecting three to four working standards a day. Results obtained by FLD were more reproducible ($\text{RSD} \leq 1\%$) compared to results obtained with DAD ($\text{RSD} \leq 6\%$) (Table 5.1).

Table 5.1 Intra-day precision using peak areas of working standards (n=3)

	FLD			DAD		
	FB ₁	FB ₂	FB ₃	FB ₁	FB ₂	FB ₃
Standard 1	56903	32119	26325	77.3	39.8	31.7
Standard 2	56436	32527	26516	74.2	42.0	31.7
Standard 3	57731	32754	26671	81.3	44.5	35.0
Mean	57023	32467	26504	78	42	33
Stdev	656	322	173	4	2	2
RSD (%)	1.2	0.9	0.7	5	6	6

Inter-day precision was obtained by analyzing three standards per day over a period of four days. Table 5.2 shows inter-day results for FLD and DAD. FLD performed better ($\text{RSD} \leq 12\%$) compared to DAD ($\text{RSD} \leq 16\%$). The results meet

the performance criteria for EU regulatory purposes (EC, 2005), which state that the repeatability RSD must be $\leq 20\%$ for fumonisin levels $\leq 100\text{ }\mu\text{g/kg}$.

Table 5.2 Inter-day precision using peak areas of working standards injected (n=12)

	FLD			DAD		
	FB ₁	FB ₂	FB ₃	FB ₁	FB ₂	FB ₃
Day 1	56903	32119	26325	77.3	39.8	31.7
	56436	32527	26516	74.2	42.0	31.7
	57731	32754	26671	81.3	44.5	35.0
Day 2	60929	28972	20177	104.0	60.3	33.5
	60737	33117	21764	106.7	57.9	36.4
	62663	32104	21315	111.0	56.1	34.5
Day 3	62206	33186	26181	109.5	52.6	39.0
	67248	34381	27639	122.5	51.0	42.6
	63089	35943	28057	115.3	58.6	43.2
Day 4	55809	32432	22626	96.1	59.9	49.1
	53593	31948	21326	89.9	51.9	34.4
	51576	30950	20733	87.3	53.7	34.2
Mean	59077	32536	24111	97.9	52.4	37.1
Stdev	4495	1699	3011	15.9	7.0	5.3
RSD (%)	8	5	12	16	13	14

5.4.3 Detection Limits

The detection limits reached with FLD were generally better than those obtained with DAD (Table 5.3). The LOD values were higher than expected in comparison to other studies (Loukou et al., 2003) with FB₁ and FB₃ determined with comparable sensitivity for both detectors. The values obtained are generally satisfactory for the analysis to be performed using the method. These results indicate that DnS-Cl derivatization reagent provides adequate sensitivity for fumonisin analysis.

Table 5.3 Limits of detection (LOD) and quantitation (LOQ) in terms of amount injected (ng) onto HPLC column

		Amount (ng) injected into HPLC column		
		FB ₁	FB ₂	FB ₃
LOD (s:n=3)	FLD	4.3	3.9	2.1
	DAD	17.2	15.6	16.6
LOQ (s:n=10)	FLD	8.6	6.9	8.3
	DAD	34.5	62.5	33.1

5.5 Robustness

5.5.1 Selection of derivatization solvent

Standard solutions of DnS-Cl are prepared by dissolving the pure compound in either acetone (Dasko et al., 2006) or acetonitrile (Heimbecher et al., 1997). These solvents were tested in this study for their suitability as reaction solvents for the preparation of the dansyl reagent. The reactions of fumonisins with DnS-Cl prepared in acetonitrile were problematic, producing little to no chromatographic peaks suggesting either an incomplete or no reaction between DnS-Cl (prepared in acetonitrile) with the fumonisins. Kang et al., 2006 reported that an increase in DnS-Cl volume in solution can directly increase the intensity of the derivative; therefore the volume of DnS-Cl in solution was increased from 100 μ L to 300 μ L. This improved the reaction, but produced poor chromatographic resolution, reproducibilities and formed unstable derivatives. Analysis of six standards derivatized with DnS-Cl prepared in acetonitrile were scattered with no repeatability. Under the conditions employed, acetonitrile was found to be an inappropriate solvent for DnS-Cl preparation with very low FLD and DAD intensities.

As acetonitrile was shown to be unsuitable, acetone as a reagent solvent was tested. The reaction of fumonisins with DnS-Cl (prepared in acetone) occurred spontaneously with satisfactory chromatographic resolution and reproducible peaks. DnS-Cl prepared in acetone reacted more intensely with the fumonisins

compared to acetonitrile (Figure 5.3). Consequently, DnS-Cl reagent for further use in the study was prepared in acetone.

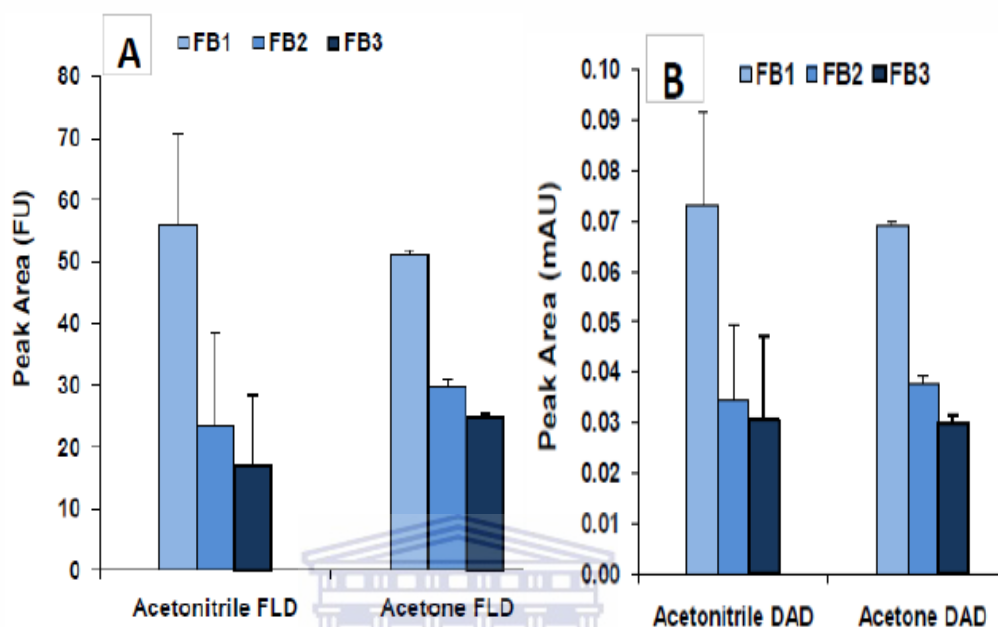


Figure 5.3 Comparison of acetonitrile and acetone as DnS-Cl reagent solvents. Results reported as mean (n=6) \pm standard deviation. (A) Comparison of FLD, (B) Comparison of DAD

5.5.2 Wavelength Selection

Wavelength selection was determined by analyzing working standards at various wavelengths reported in literature (Molins-Legua et al., 1998; Dasko et al., 1996) for their suitability to provide optimum sensitivity and intensity. Optimum results were obtained at excitation wavelength 247 nm and emission wavelength 510 nm for FLD (Table 5.4). For DAD, 230 nm provided highest sensitivity (Table 5.5). Iso-absorbance plots (software programme which displays chromatographic details in 3D including retention time versus wavelength, from which optimum

wavelength can be selected) were used to confirm the optimum wavelength for DAD.

Table 5.4 Effect of wavelength on working standard peak areas for FLD

	Ex 252 Em510	Ex 247 Em 510	Ex 360 Em 510	Ex 365 Em 510
FB₁	38797	50132	6024	47196
FB₂	22190	28519	3627	2737
FB₃	15521	20486	2579	23299

Table 5.5 Effect of wavelength on working standard peak areas for DAD

	230 nm	254 nm	280 nm	335 nm
FB₁	76	67	6	25
FB₂	40	35	ND	14
FB₃	29	24	ND	9

5.5.3 Reaction temperature selection

Temperature experiments were performed at 24°C, 40°C and 60°C for 15 minutes as these are the mostly used temperatures in literature (Smith et al., 1985; Dasko et al., 1996). An increase in standard peak area was observed when the reaction was heated compared to when it was performed at room temperature (24°C). Heating the derivatives improved both the reaction yield and RSD values from 15 % to ≤ 2 % (Figure 5.4). Further increase in temperature to 60°C showed an insignificant increase in response for the FB₂ and FB₃ of the FLD. In contrast, the DAD showed a decrease in response between 40°C to 60°C

except for FB₃; which showed a slight increase in response (Figure 5.5). Since, the FLD results showed increased standard area for FB₁ and DAD showing increased results for FB₁ and FB₂ when experiments were performed at 40°C, this was selected as the optimum reaction temperature.

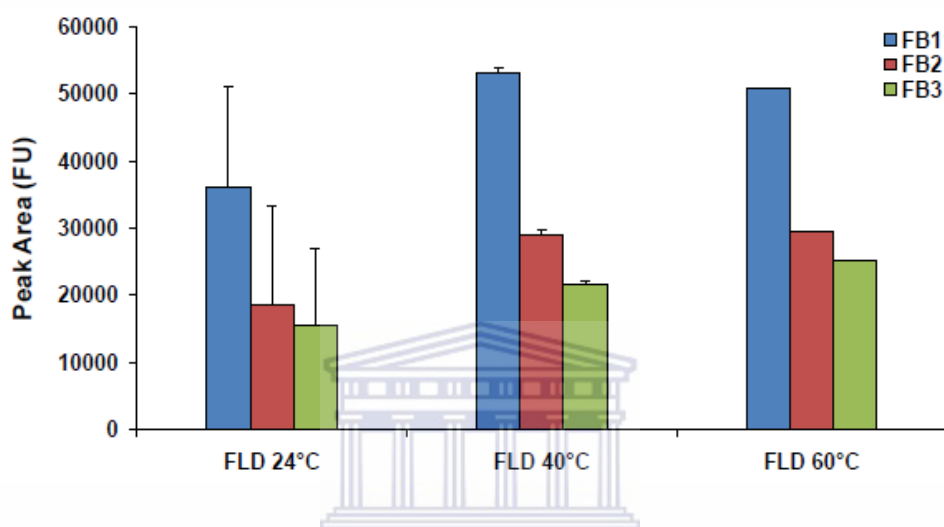


Figure 5.4 Effect of reaction temperature on peak areas of FLD, results calculated on mean ($n=5$) \pm standard deviation

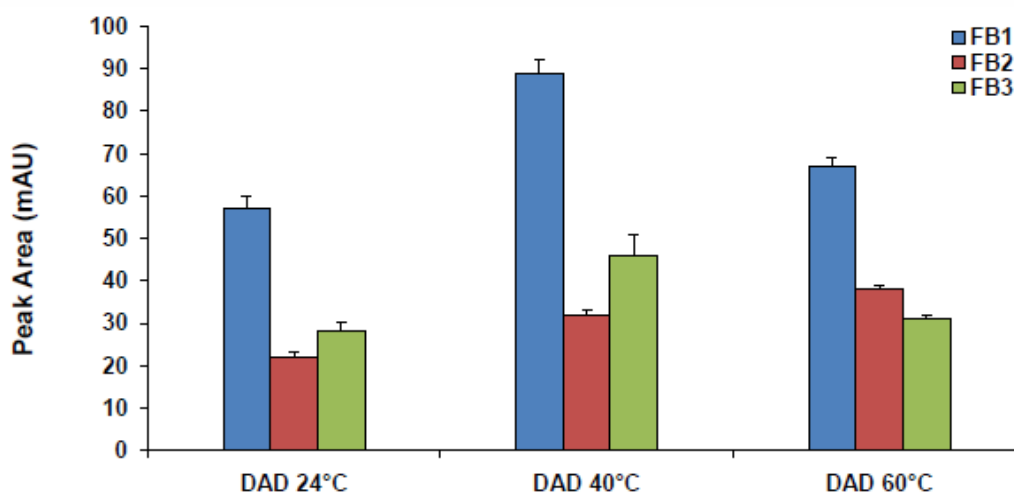


Figure 5.5 Effect of reaction temperature on peak areas of DAD, results calculated on mean ($n=5$) \pm standard deviation

5.5.4 Reaction time selection

Different reaction times (10 min, 15 min and 30 min) were tested, based on the peak areas no major difference in HPLC responses were observed between 15 min and 30 min. Optimum reaction time was obtained at 10 minutes for FLD (Figure 5.6) with a slightly lower response for DAD at 10 min compared to other reaction times (Figure 5.7). To save on analysis time, 10 minutes was selected as the suitable reaction time for DnS-Cl derivatization of the FB analogues.

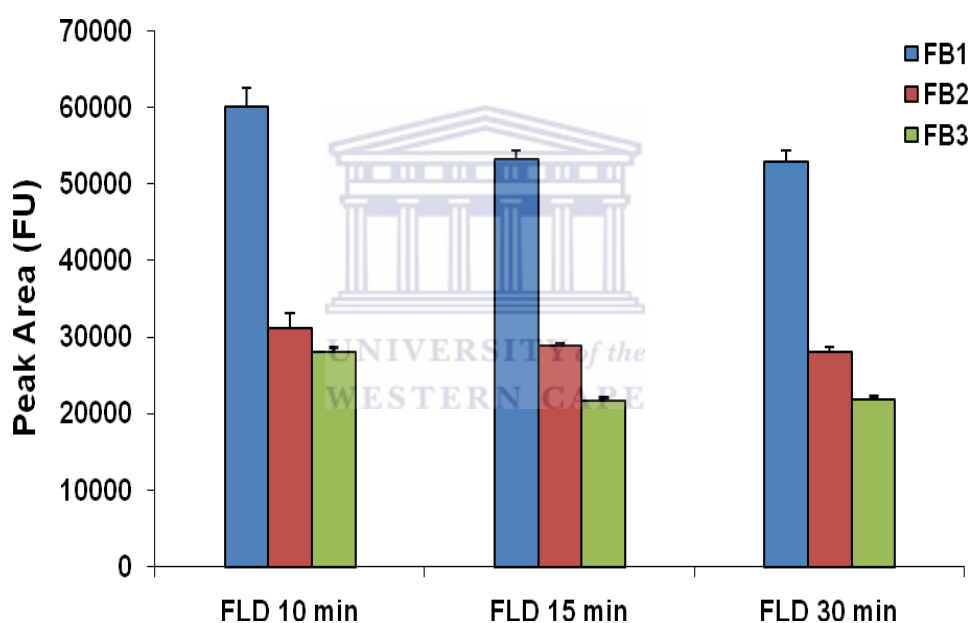


Figure 5.6 Effect of reaction time at 40 °C on peak areas for FLD. Results reported as mean (n=5) \pm standard deviation

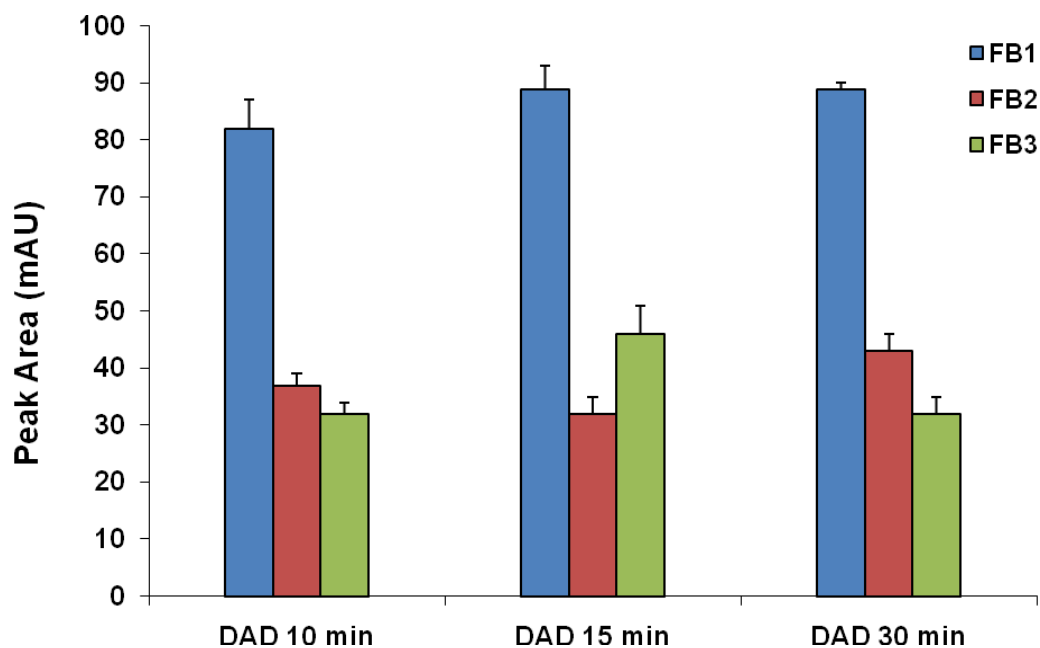


Figure 5.7 Effect of reaction time at 40 °C on peak areas for DAD. Results reported as mean (n=5) \pm standard deviation

5.6 Recoveries

Accuracy of the optimized method was examined by analyzing recoveries of fumonisins. Initial recoveries were determined by spiking directly on the dry milled maize at levels of 1103 $\mu\text{g/kg}$, 500 $\mu\text{g/kg}$ and 270 $\mu\text{g/kg}$ for FB₁, FB₂ and FB₃, respectively, and performing clean-up with IAC. The recoveries obtained with FLD were poor $\leq 30\%$ (Table 5.6). Due to poor chromatography and the presence of larger FB₂ peaks than FB₁, the recoveries for DAD for this experiment could not be calculated. To improve on the results obtained, the derivatization procedure was tested for its ability to derivatize fumonisins in maize samples; this was done by spiking into the 10 mL samples eluted from the SAX prior to the drying step. Recovery results were again poor, with FLD achieving $\pm \leq 40\%$ for the three FB analogues. Apparent recoveries as determined by DAD were

incongruous (10 % for FB₁, 80 % for FB₂ and 77 % for FB₃), as compared to the FLD.

Table 5.6 Determining the recovery of the maize extraction using IAC

	FB ₁ (ng/g)	FLD FB ₂ (ng/g)	FB ₃ (ng/g)
Blank 1	57	0	0
Blank 2	31	18	9
Mean	44	9	4
Sample 1	359	166	83
Sample 2	316	156	84
Sample 3	343	154	83
Sample 4	346	156	78
Sample 5	328	149	72
Sample 6	279	148	69
Mean	328	155	78
Stdev	29	7	7
RSD (%)	9	4	8
Spike	1103	500	270
Recoveries (%)	26	29	27

Table 5.7 Determination of the recovery of the derivatization procedure

	FB ₁ (ng/g)	FLD FB ₂ (ng/g)	FB ₃ (ng/g)	FB ₁ (ng/g)	DAD FB ₂ (ng/g)	FB ₃ (ng/g)
Blank 1	101	31	6	421	742	0
Blank 2	108	36	10	406	831	0
Mean	104	33	8	413	787	0
Sample 1	648	228	123	692	1433	222
Sample 2	628	235	125	415	1146	264
Sample 3	483	176	96	658	1150	181
Sample 4	570	216	107	597	1158	164
Sample 5	563	183	97	507	1040	199
Sample 6	533	176	97	344	1194	214
Mean	571	202	108	535	1187	207
Stdev	61	27	13	138	131	35
RSD (%)	11	13	12	26	11	17
Spike	1103	500	270	1103	500	270
Recoveries (%)	42	34	37	11	80	77

An evaluation of the peak purity for UV absorption was done on FB₁. The results obtained from the peak purity test showed that all calculations were within the calculated threshold (Figure 5.8) and no impurities are present under the analyte peaks. The result thus far suggests that the current derivatization conditions used are not suitable for derivatization of naturally contaminated samples.

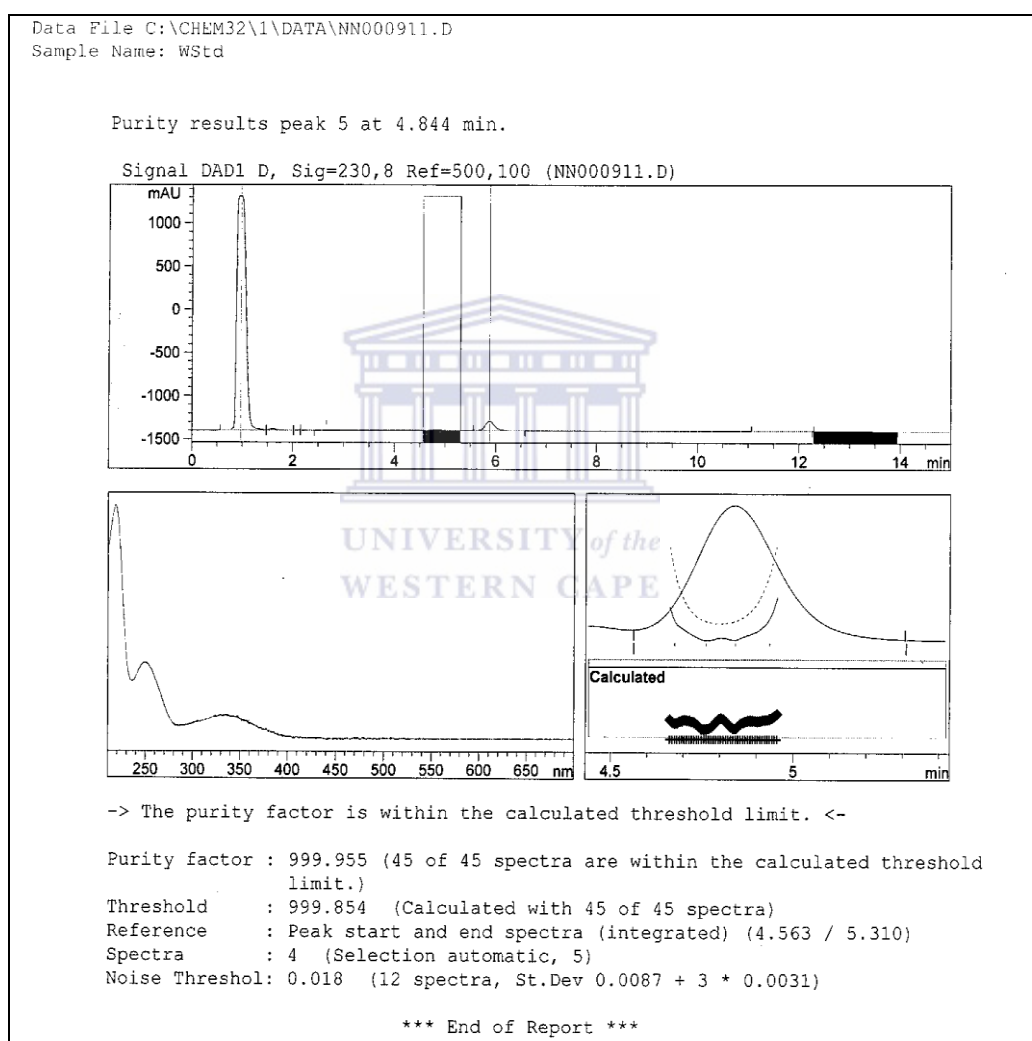


Figure 5.8 Purity peak check results

Figure 5.9 below illustrates the type of chromatogram obtained with DnS-Cl derivatives detected by DAD. Due to poor resolution of FB₁ peak from the

additional peaks in the chromatogram (which could possibly be due to matrix interference) and FB₂ providing higher results compared to FB₁, DAD analysis of DnS-Cl derivatives was abandoned and the study was continued with only FLD.

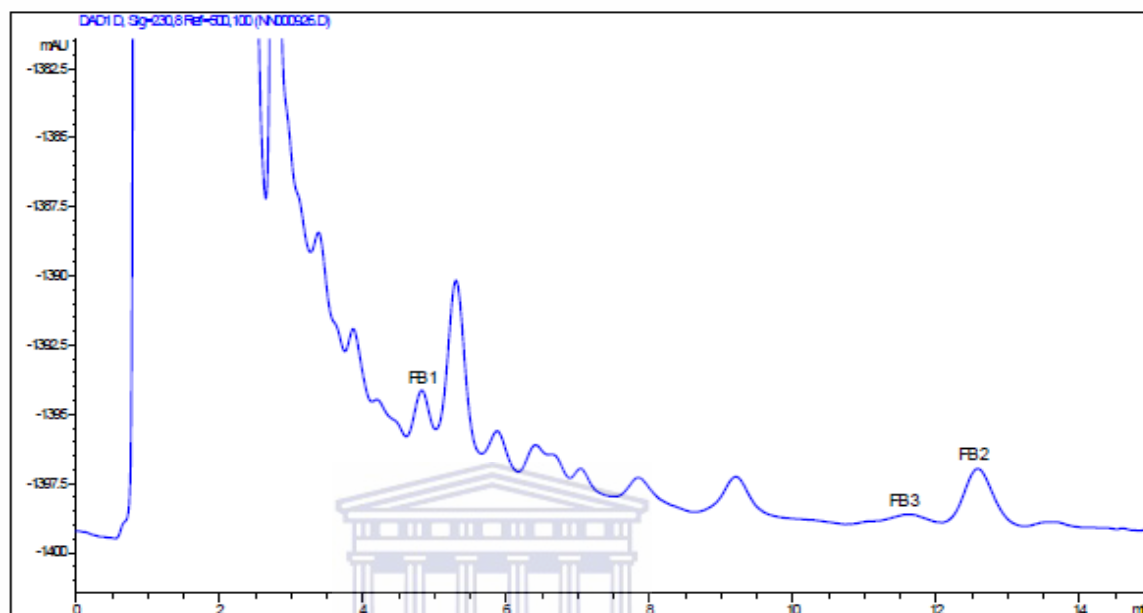


Figure 5.9 Chromatogram of naturally contaminated maize sample with DAD

5.7 Optimization of recoveries using naturally contaminated samples

The fluorescent products obtained by the reaction of fumonisins with DnS-Cl produced low recoveries. To improve on the recoveries obtained, optimization of the reaction was performed by spiking directly on the cleaned-up eluate to allow for both the recoveries and the derivatization process to be examined. The parameters analysed included temperature, time, volume of DnS-Cl and pH as they are known to affect the yield of DnS-Cl derivatives (Kang et al., 2006).

General conditions used:

For optimization of the samples, the following conditions were used unless otherwise stated as per optimization parameter (i.e. during sample optimization, except for the parameter tested, everything was kept constant):

Samples re-dissolved in 100 μL methanol, 200 μL DnS-Cl and 200 μL NaHCO_3 added, heated for 10 minutes at 40°C , cooled to 24°C under tap water and 200 μL H_3PO_4 added and 20 μL injected into HPLC.

5.7.1 Effect of reaction temperature and time

Since both reaction temperature and time can affect the rate of derivative formation and hence HPLC response, optimization of these parameters was undertaken first.

Temperature experiments were performed first; all experiments were performed for 10 minutes at two temperatures (40°C and 60°C). The maximum intensity was obtained at 60°C (Table 5.8). Reaction time experiments were therefore performed at 60°C . Slight differences in HPLC responses were observed between 10 min and 30 min because 10 min provided shorter reaction time (Table 5.9) it was selected as reaction time. Therefore, heating at 60°C for 10 min was selected as optimum temperature and time for sample derivatization. However optimization of the reaction temperature and time did not improve recoveries.

Table 5.8 Effect of reaction temperature on maize derivatized for 15 minutes with DnS-Cl

	40°C			60°C		
	FB ₁ (ng/g)	FB ₂ (ng/g)	FB ₃ (ng/g)	FB ₁ (ng/g)	FB ₂ (ng/g)	FB ₃ (ng/g)
Blank 1	7	0	0	15	7	0
Blank 2	8	0	0	11	4	0
Mean	8	0	0	13	5	0
Sample 1	147	48	24	218	64	41
Sample 2	142	40	22	225	70	40
Sample 3	144	37	20	132	39	22
Sample 4	126	36	20	257	77	43
Sample 5	140	40	21	234	70	40
Sample 6	154	46	24	244	73	45
Mean	142	41	22	218	65	38
Stddev	9	5	2	45	14	8
RSD (%)	6	12	8	20	21	21
Spike	1103	500	270	1103	500	270
Recoveries (%)	12	8	8	19	12	14

Table 5.9 Effect of reaction time on maize derivatized at 60°C with DnS-Cl

	10 min			15 min			30 min		
	FB ₁ (ng/g)	FB ₂ (ng/g)	FB ₃ (ng/g)	FB ₁ (ng/g)	FB ₂ (ng/g)	FB ₃ (ng/g)	FB ₁ (ng/g)	FB ₂ (ng/g)	FB ₃ (ng/g)
Blank 1	7	0	0	15	7	0	12	8	0
Blank 2	13	9	0	11	4	0	15	9	0
Mean	10	5	0	13	5	0	14	8	0
Sample 1	435	189	101	218	64	41	418	153	82
Sample 2	423	198	101	225	70	40	443	182	92
Sample 3	457	192	96	132	39	22	431	176	88
Sample 4	449	187	94	257	77	43	407	158	83
Sample 5	410	170	88	234	70	40	409	173	88
Sample 6	424	191	98	244	73	45	447	180	94
Mean	433	188	96	218	65	38	426	170	88
Stddev	18	10	5	45	14	8	17	12	5
RSD (%)	4	5	5	20	21	21	4	7	5
Spike	1103	500	270	1103	500	270	1103	500	270
Recoveries (%)	38	37	36	19	12	14	37	32	33

5.7.2 Effect of DnS-Cl volume

Given that excess DnS-Cl was already used in the study and that it can either compensate for side reactions (Seiler, 1971; Heimbecher et al., 1997) or increase the response on undesirable reagent compounds (Molions-Legua et al., 1998), the effect of DnS-Cl volume was examined.

DnS-Cl volume in the derivatization reagent was investigated at 100 μ L, 200 μ L and 300 μ L. A sharp increase in response was obtained from 100 μ L to 200 μ L. However, a further increase in volume to 300 μ L resulted in a decrease in response (Figure 5.10). This may be due to the decomposition of the derivatives from the excess DnS-Cl (Tapuhi et al., 1981). Since 200 μ L provided optimum results it was selected for sample derivatization. Calculation of recoveries from the results showed no improvement in recoveries.

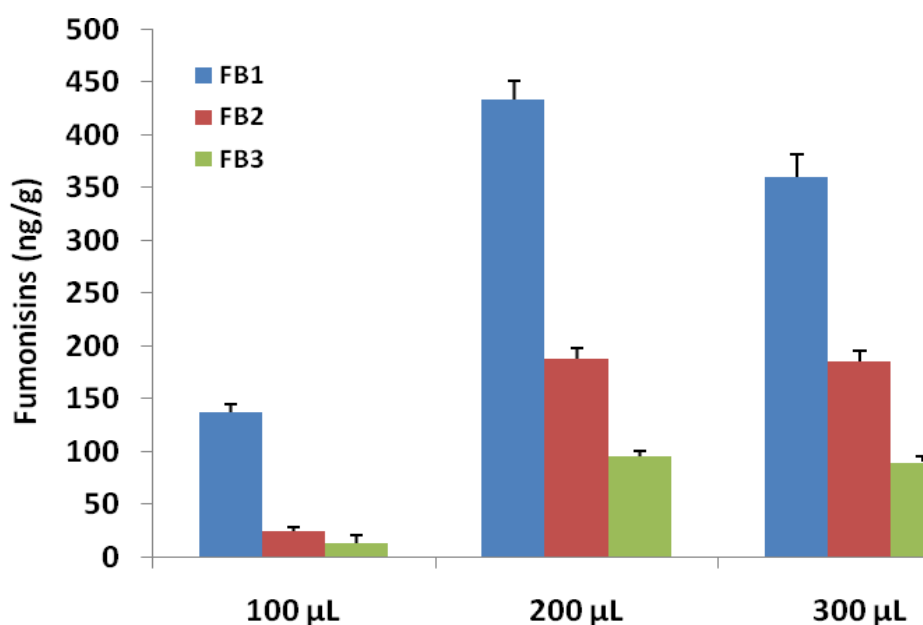


Figure 5.10 Effect of reagent volume (DnS-Cl) on reaction yield, results reported on mean ($n=6$) \pm standard deviation

5.7.3 Effect of pH

Na_2CO_3 was selected as an alternative to NaHCO_3 because it could provide both increased pH, which has been reported to increase the yield of DnS-Cl derivatives (Heimbecher et al., 1997) and provide the basic condition necessary for derivatization. Experiments to test the effect of buffer change and pH were carried out simultaneously (i.e. buffer change from NaHCO_3 to Na_2CO_3 provided a pH change from 8.25 to pH 11.05). The chromatographic resolution of fumonisin working standards with the use of Na_2CO_3 was baseline with increased response for all 3 analogues. However, when the samples were derivatized poor resolution of the peaks was obtained with two chromatographic peaks formed at similar retention times to FB_1 (Figure 5.11). Although identification of the FB_1 peak could be done by the use of retention times, integrating the double peak would be an inaccurate quantification of FB_1 .

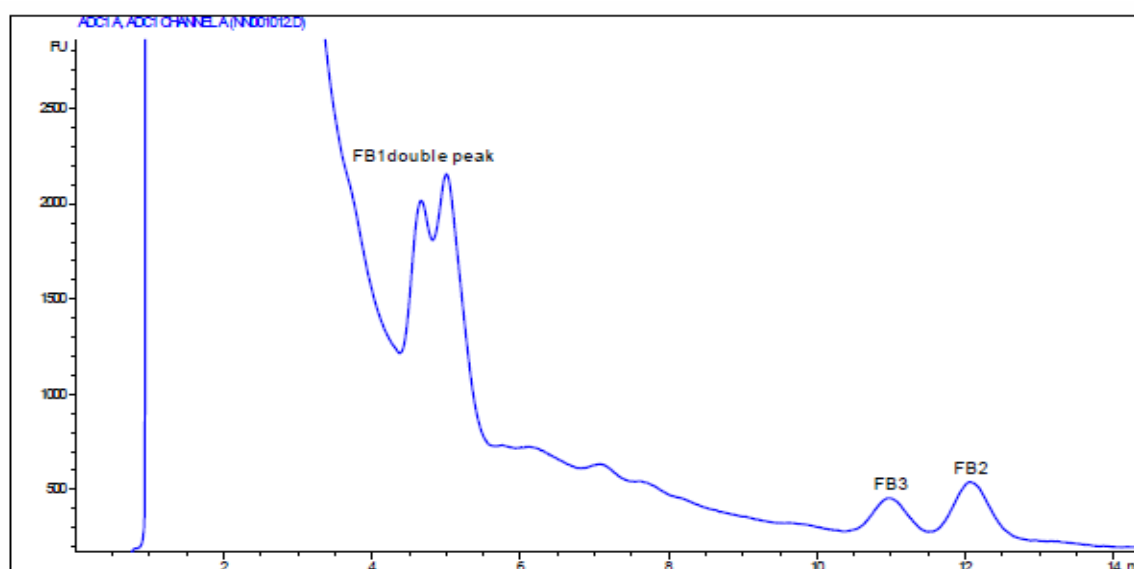


Figure 5.11 Naturally contaminated maize sample with DAD

The source of the double peak was further investigated by preparing working standards using Na_2CO_3 . However, the original FB_1 responses could not be reproduced since its area increased disproportionately to that of FB_2 and FB_3 , suggesting some interference beneath the peak (Figure 5.12) e.g. non-separation from reagent peak A and B (Figures 5.1 and 5.2).

Troubleshooting (i.e. detector settings, cleaning injector port and use of minimal injection volume ($10\ \mu\text{L}$)) of the chromatographic system used indicated that it had no influence on the FB_1 standard area and on the split sample peak. It was thus concluded that DnS-Cl is an inappropriate derivatization reagent for the analysis of fumonisins in maize.

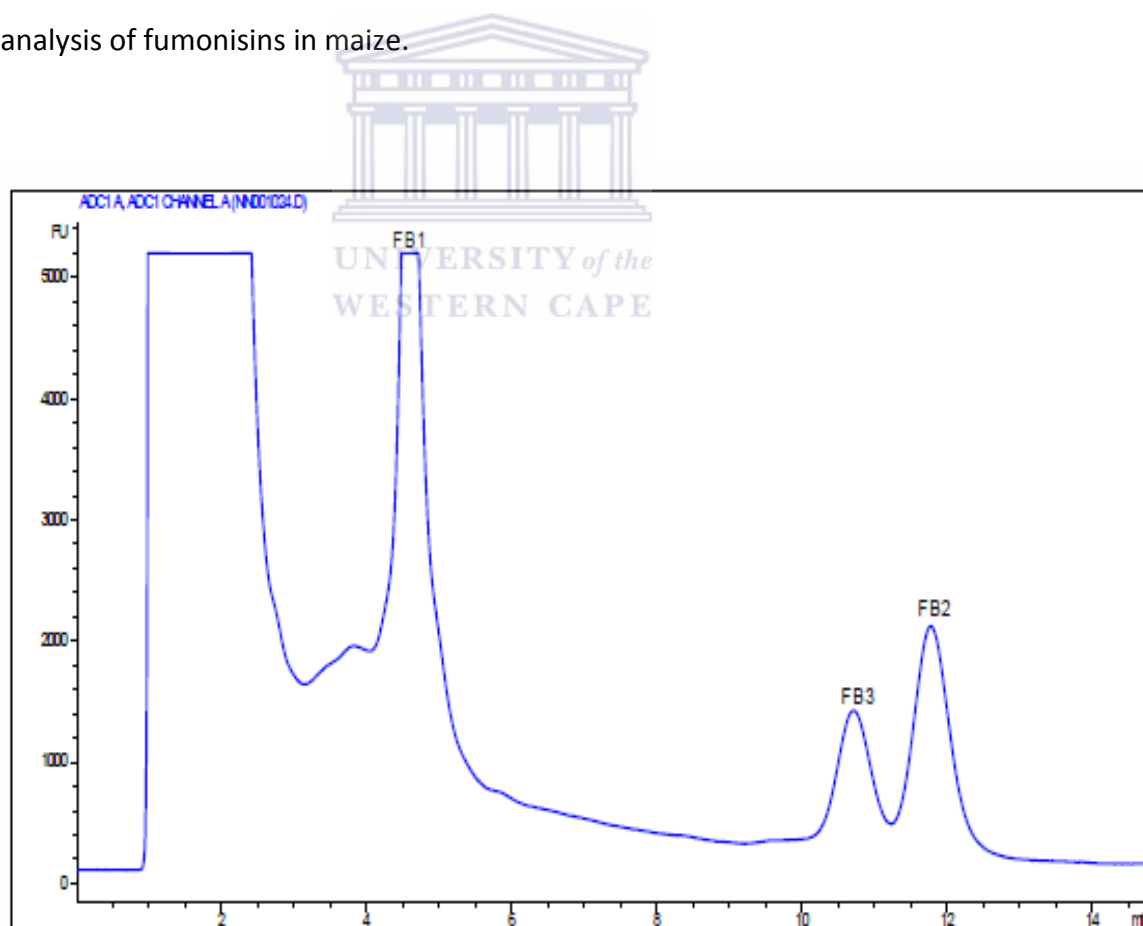


Figure 5.12 Fumonisin working standard derivatized with DnS-Cl and Na_2CO_3 as buffer

5.8 Conclusion

The effectiveness of pre-column derivatization of fumonisins with DnS-Cl, HPLC separation and subsequent application was evaluated. Different experimental conditions in order to improve method performance for fumonisin analysis were used with initial optimization experiments with working standards providing reproducible, repeatable and precise results. Application of the optimized method to naturally contaminated maize samples produced unreliable results for DAD, resulting in the discontinuation of the DAD analysis. Although baseline resolution of the peaks with maize samples was obtained with FLD, the poor recoveries could not be improved even after investigating the influence of DnS-Cl concentration, pH, buffer, derivatization time and temperature.

Generally, it seems that DnS-Cl derivatives are less desirable due to the presence of analytical interferences and suspiciously higher results of FB₂ compared to FB₁. Although all analytes could be identified with FLD with the use of retention times; the recovery results obtained suggests it to be unsuitable for the analysis of naturally contaminated maize samples due to either no reaction or incomplete reaction, and the formation of secondary peaks.

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CHAPTER 6

Comparisons of methods, General Discussion, Recommendations and Conclusion



6.1 Comparison of OPA and NDA

Validation of the methods for recovery and repeatability were applied to 15 maize samples and are reported in Chapters 3 and 4. The same samples were used for both derivatization reagents. From a single extraction, 2 aliquots of the centrifuged extract were independently cleaned-up on SAX, with one eluate being derivatized with OPA and the other with NDA. This allowed accurate comparison between the OPA (FLD and DAD) with NDA (FLD and DAD) by importantly, avoiding variation in the extraction step (Table 6.1).

Although comparison between the detectors was good following SAX clean-up, the results from the two derivatives were comparable only up to 1000 $\mu\text{g}/\text{kg}$. It was found that with concentrations above 1000 $\mu\text{g}/\text{kg}$, the comparison was frequently poor, with NDA being lower than 50% of the OPA (Figure 6.1). This would suggest that certain of the home-grown maize samples studied contained inhibitors to the complete NDA derivatization reaction of their SAX extracts. Due to these poor comparisons, the alternate IAC clean-up method was investigated, since it produces a cleaner extract for derivatization.

In a similar manner to the SAX experiment, single samples were extracted and duplicate clean-ups performed, one for OPA and one for NDA derivatization. For OPA, a good comparison was obtained between FLD and DAD, whereas for NDA, all results were higher with DAD, especially at levels below 1000 $\mu\text{g}/\text{kg}$. The reason for this trend was not apparent (Figure 6.2). Comparison of OPA and NDA derivatives showed much improved results over the previous comparison of the

derivatives after SAX clean-up. In comparing the derivatives using the DAD response, again comparisons above 1000 µg/kg were superior to those below this level.

The OPA results were consistent whether determined either after SAX or IAC clean-up, showing that OPA is a robust derivatization reagent for fumonisin analysis (Figure 6.3). For NDA derivatization (Figure 6.4), IAC clean-up produced much cleaner extracts, which resulted in improved comparison with OPA (Table 6.1). Recent applications of NDA fumonisin analysis with FLD used IAC clean-up (Lino et al., 2006; Lino et al., 2007; Silva et al., 2009). An older method using C18 RP-SPE of fumonisins from mouldy maize reported that compared to OPA, NDA gave higher values at lower contamination levels and lower values at higher contamination levels (Chu et al., 1994).

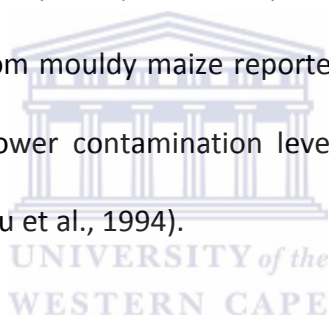


Table 6.1 Total fumonisin levels (FB₁+FB₂+FB₃; µg/kg) in naturally contaminated maize cleaned-up with SAX and IAC, derivatized with OPA or NDA

Sample	FLD				DAD			
	SAX		IAC		SAX		IAC	
	OPA	NDA	OPA	NDA	OPA	NDA	OPA	NDA
1	106	108	103	93	102	149	*ND	766
2	234	231	238	219	310	273	142	695
3	288	336	260	225	257	380	143	730
4	296	367	157	202	320	328	ND	504
5	220	240	263	248	300	288	161	786
6	1132	1030	701	785	1279	943	709	1429
7	1375	638	1623	1316	1470	554	1590	1972
8	3144	2771	3730	3016	3234	2528	3804	3361
9	2163	709	1711	1820	2289	709	1585	2447
10	2537	983	2196	2115	2805	969	2231	2579
11	1900	1575	1711	1490	1948	1452	1688	2077
12	3120	3014	4689	3343	3164	2804	4716	3830
13	1577	686	1485	1332	1812	699	1445	1700
14	6088	2492	5327	3664	6114	2402	5246	4120
15	3740	562	3894	2874	4442	528	3756	3215

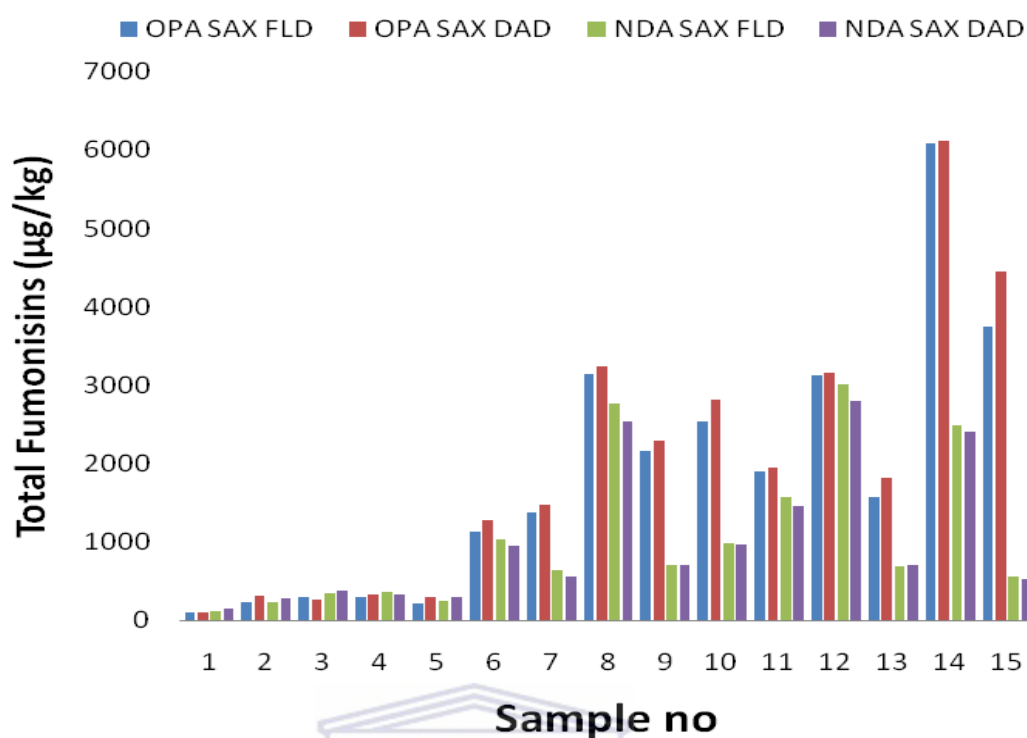


Figure 6.1 Comparison of OPA with NDA following SAX clean-up for FLD and DAD, Total Fumonisin = $FB_1 + FB_2 + FB_3$

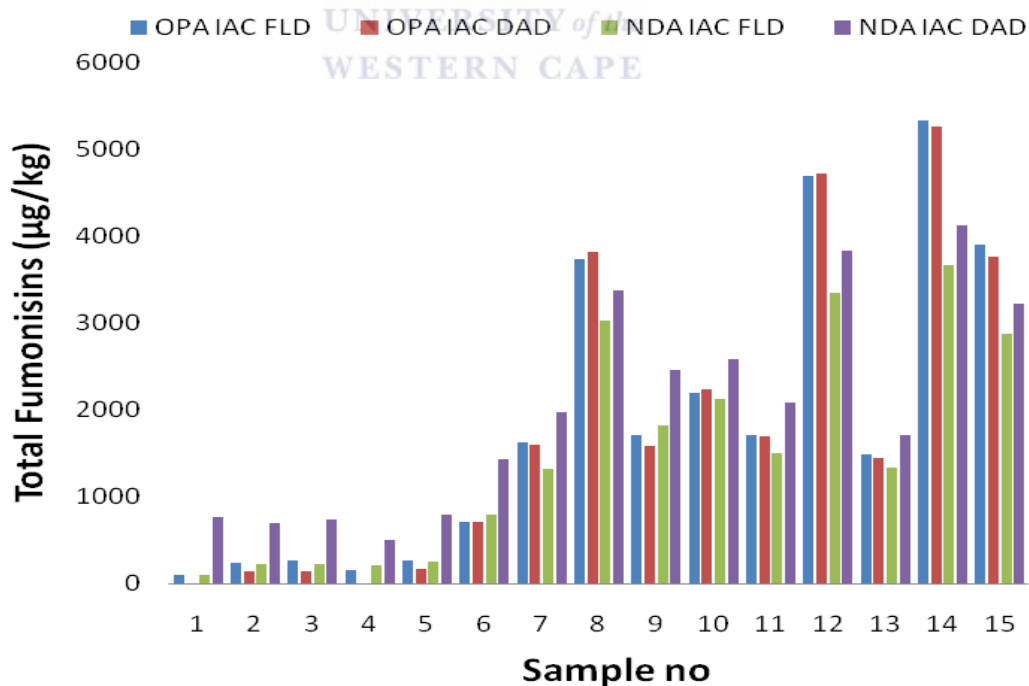


Figure 6.2 Comparison of OPA and NDA following IAC clean-up for FLD and DAD, Total Fumonisin = $FB_1 + FB_2 + FB_3$

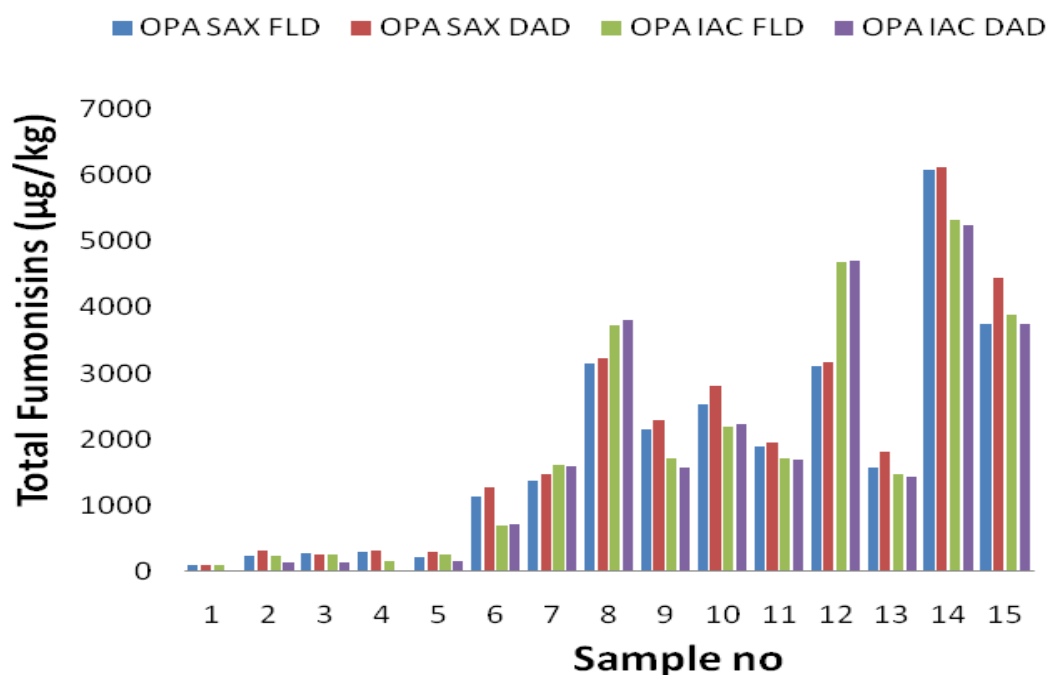


Figure 6.3 Comparison of IAC and SAX with OPA derivatization for FLD and DAD,
Total Fumonisin = $FB_1 + FB_2 + FB_3$

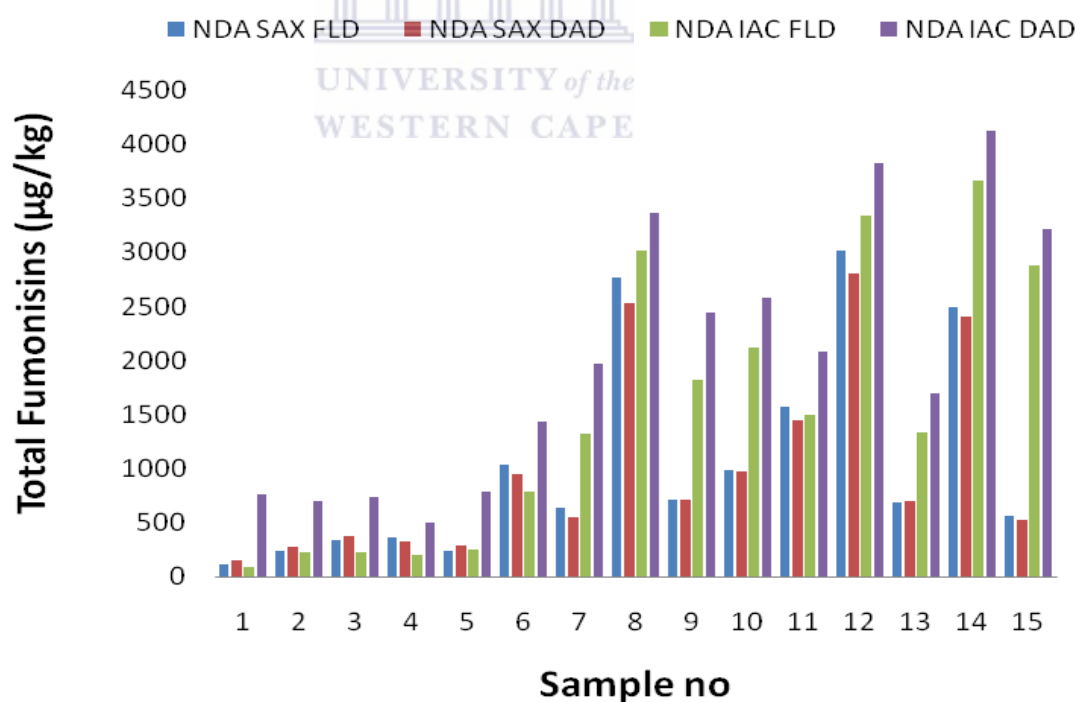


Figure 6.4 Comparison of IAC and SAX with NDA derivatization for FLD and DAD,
Total Fumonisin = $FB_1 + FB_2 + FB_3$

6.2 General Discussion

Chromatographic methods used to analyse fumonisins in maize were investigated with regard to: (1) derivatising reagent (OPA, NDA and DnS-Cl) (2) clean-up method (SAX and IAC) and (3) detection (FLD and DAD). Table 6.2 provides a summary of the optimal conditions used for OPA, NDA and DnS-Cl derivatization of fumonisins in maize.

Table 6.2 Chromatographic parameters for determination of fumonisins as OPA, NDA and DnS-Cl derivatives

	OPA	NDA	DnS-Cl
Column	Luna 5 µm, 75 mm x 4.6 mm	Luna 5 µm, 75 mm x 4.6 mm	Luna 5 µm, 75 mm x 4.6 mm
Mobile phase	MeOH:0.1 NaHPO ₄ (77:23)	MeOH:0.1 NaHPO ₄ (78:22)	MeOH:0.1 NaHPO ₄ (76:24)
Flow rate	1 mL/ min	1 mL/ min	1 mL/ min
Injection volume	Standards – 10 µL Samples – 20 µL	Standards – 10 µL Samples – 20 µL	Standards – 10 µL Samples – 20 µL
Buffer (s)	1M Na ₂ B ₄ O ₇ ·10H ₂ O	1M Na ₂ B ₄ O ₇ ·10H ₂ O	2M NaHCO ₃ 2M Na ₂ CO ₃
Temperature (s)	24 °C	60°C	60°C
Reaction time	2 minutes	15 minutes	10 minutes
DAD wavelength (s)	335 nm	252 nm	230 nm
FLD wavelength (s)	Excitation 335 nm Emission 440 nm	Excitation 420 nm Emission 500 nm	Excitation 247 nm Emission 510 nm

Internal validation of the methods was performed using characteristics such as precision, accuracy, specificity and application to naturally contaminated maize samples. The optimized analytical methods proved to be both selective and sensitive. This is evident in the ability of the methods to produce accurate measures of the analytes in the presence of unknown components and any other products that may be expected to be present in the sample matrix and with comparable fumonisins levels in a wide range of fumonisin contamination. This study emphasized that the use of appropriate fluorometric derivatization procedures is of considerable importance for accurate determination of fumonisins. The robustness of the procedure allowed us to identify some critical steps in the methods for fumonisin analysis in maize. In particular, the extraction of fumonisins from sample matrix, clean-up and derivatization reagent which were all demonstrated to be critical.

The OPA method was found to be sensitive, reliable and reproducible for fumonisin analysis in maize. Although unstable, it has been found to be a suitable derivatization reagent (Shephard et al., 1996). Baseline chromatographic separation was obtained with retention times of the standard peak allowing identification of the analyte peaks. One of the objectives of this study was to determine the extent to which DAD could be used as an alternative to FLD for OPA derivatization. This was achieved by obtaining comparable results between the detectors, although FLD proved to be more sensitive with lower detection limits and significantly higher peak areas. Good comparison between these two detectors was demonstrated with SAX and IAC clean-up.

The NDA method was optimized and validated. NDA provided comparable results to those of OPA and essentially confirming a previous study by Bennett and Richard 1994. It proved to be a suitable alternative for OPA, especially following IAC clean-up, and for laboratories requiring overnight analysis of fumonisins which, due to their high stability, provides an added advantage over OPA. NDA proved to be sensitive as well as sufficiently selective in the application of fumonisin analysis.

Dansyl derivatives are mainly used for amino acid (De Jong et al., 1982; Kang et al., 2006) and biological amine analysis (Loukou et al., 2003; Proestos et al., 2008). Only one study has utilized DnS-Cl for fumonisin derivatization in beer samples and was reported to form stable complexes with satisfactory chromatographic separations (Dasko et al., 2006). Scott and Lawrence, 1992 derivatized fumonisins in maize and found it to form good derivatives. However it also produced analytical interferences with the maize matrix.

As a result of the investigations performed in this study, it was found that DnS-Cl could on the one hand form stable, sensitive and highly fluorescent derivatives with fumonisin standards. However, on the other hand, due to the low recoveries from maize it could not be used for the analysis of naturally contaminated maize samples as previously reported by Scott and Lawrence 1992. This showed that the maize matrix caused analytical interferences with derivatives thereby suppressing the reaction between the fumonisin amino groups with the dansyl chloride reagent. Interferences with the maize were

mainly observed with DAD; therefore the DAD investigations were abandoned. However, DnS-Cl has some potential to be utilized with FLD.

6.3 Recommendations

- Although cyanide has been shown to be a suitable nucleophile for NDA derivatization, it is a highly toxic substance and hence an alternative would be much safer for the environment. Therefore, future research should focus on finding other suitable nucleophiles for NDA derivatization.
- DnS-Cl is known to be a non-specific reagent as it reacts with amino groups of many compounds as well as hydroxyl groups of phenols and some alcohols (Smith et al., 1985). Reaction of fumonisins with DnS-Cl occurs at the more nucleophilic amino functional group, which is where fumonisin derivatization often occurs. Further studies should be conducted to address the issues surrounding the apparent matrix interference of DnS-Cl derivatives on fumonisin and investigate the mechanism between DnS-Cl with fumonisins as they are structurally suited for DnS-Cl reaction.

6.4 Conclusion

This study uniquely investigated three derivatization reagents systematically for fumonisin analysis in South African home-grown maize intended for human consumption with concurrent FLD and DAD detection and with two clean-up methods (SAX and IAC).

This study shows that although FLD is more sensitive, UV detection can be used as a reliable alternative for fumonisin analysis of OPA derivatives. OPA and NDA have proven to be excellent fluorogenic reagents for accurate determination of fumonisins in naturally contaminated maize samples and can be used as alternatives to each other employing both SAX and IAC clean-up with either FLD or DAD. Even though DnS-Cl derivatization could not be applied to maize samples, the study can be the basis for investigating the use of DnS-Cl in other related matrixes. In conclusion this study has shown that UV detection can be utilized as an alternative to FLD for fumonisin analysis in naturally contaminated maize irrespective of the clean-up method or the derivatization agent.



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