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HPLC 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

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ABSTRACT

Fumonisins are mycotoxins produced by various species of *Fusarium* and occur naturally in contaminated maize and maize-based foods. Ingestion of fumonisins has considerable health implications for humans and animals. Since fumonisins lack a useful chromophore or fluorophore, their determination in maize is routinely achieved via HPLC with fluorescence detection (FLD) after precolumn derivatization. This study optimized naphthalene-2,3-dicarboxaldehyde (NDA) derivatization of fumonisins in naturally contaminated maize following strong anion exchange (SAX) solid phase extraction (SPE) clean-up and utilizing diode array detection (DAD) as a practical alternative simultaneously to FLD. The limit of detection (LOD) for fumonisin B_1 (FB_1), fumonisin B_2 (FB_2) and fumonisin B_3 (FB_3) with FLD was 0.11 ng, 0.50 ng and 0.27 ng, respectively, and with DAD it was 13.8 ng, 12.5 ng and 6.6 ng, respectively injected on column. The coefficient of variation (CV, $n = 6$) for FB_1, FB_2 and FB_3 in a naturally contaminated samples obtained with FLD was 2.6%, 1.8% and 5.3%, respectively, compared to 6.0%, 3.4% and 9.5%, respectively, obtained with DAD. Subsequently the optimized NDA derivatization was compared to the widely used *o*-phthaldialdehyde (OPA) derivatization agent as well as alternative sample clean-up with immunoaffinity column (IAC) by analyzing naturally contaminated maize samples ($n = 15$) ranging in total fumonisin ($TFB = FB_1 + FB_2 + FB_3$) levels from 106 to 6000 μg/kg. After immunoaffinity column clean-up of extracted samples, the recoveries of spiked maize samples for NDA-FLD of FB_1, FB_2 and FB_3 were 62%, 94% and 64%, respectively. NDA proved to be an effective derivatization reagent of 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. Aside from the difference in detection limits, FLD and DAD produced comparable results irrespective of the clean-up method or the derivatization agent.

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1. Introduction

Fumonisins are mycotoxins mainly produced by *Fusarium verticillioides* (Sacc.) Nirenberg [1]. Several fumonisin analogues have been reported of which fumonisin B_1 (FB_1) is the most abundant naturally occurring analogue in maize followed by fumonisin B_2 (FB_2) and fumonisin B_3 (FB_3) [2]. Fumonisins have been reported to cause leukoencephalomalacia in horses, pulmonary oedema in pigs and hepatocarcinoma in rats [3,4]. The high incidence of oesophageal cancer in the former Transkei area in South Africa and increased rates of neural tube defects (NTD) in populations along the Texas–Mexico border have been linked to the consumption of high fumonisin contaminated maize [5,6].

Fumonisins have received much attention due to their potential hazards to animal and human health. The International Agency for Research on Cancer declared FB_1 to be a group 2B carcinogen (possibly carcinogenic in humans) [7]. As a result of the latter potential risk, the European Commission placed regulatory limits of 4000 μg fumonisin/kg for unprocessed maize, 1000 μg/kg for maize intended for direct human consumption and 200 μg fumonisin/kg for processed maize-based foods and baby foods for infants and young children [8].

Several methods for the determination of fumonisins in maize utilize reversed-phase high performance liquid chromatography (RP-HPLC) and fluorescence detection (FLD) [9–14]. Direct HPLC analysis of fumonisins is problematic due to their lack of a useful chromophore or fluorophore [9]. For this reason, a number of fluorescent derivatives have been used for the detection of fumonisins, including 9-fluorenylmethylchloroformate (FMOC-CL) [10], 4-flouro-7-nitro-benzofurazan (NBD-F) [11] *o*-phthaldialdehyde

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(OPA) [12], naphthalene-2,3-dicarboxaldehyde (NDA) [13] and dansyl chloride (DnS-Cl) [14].

In a previous study, it was shown that under certain circumstances UV detection may offer an alternative to FLD for OPA derivatives of fumonisins [15]. The present work was undertaken to compare the detection of OPA derivatives of fumonisins with NDA derivatives using diode array detection (DAD) as a practical alternative to the widely used FLD. Reaction of NDA with the nucleophilic cyanide anion forms stable and highly fluorescent derivatives. Due to this stability of NDA derivatives, this has been used as an alternative to the unstable OPA derivatives [16]. The study firstly focused on the optimization of NDA derivatization of fumonisins in naturally contaminated home-grown maize intended for human consumption, followed by clean-up of the maize with strong anion exchange (SAX) solid phase extraction (SPE) utilizing DAD and FLD. The method was then compared to the widely used method of OPA derivatization [17], following both the SAX and the commercial immunoaffinity column (IAC) method [18].

2. Materials and methods

2.1. Chemicals

Fumonisin standards were isolated at the PROMEC Unit according to the method of Cawood et al. [19] and stock solutions were prepared for FB_1, FB_2 and FB_3 at concentrations of 245 μg/mL, 200 μg/mL and 270 μg/mL, respectively, in acetonitrile–water (1:1, v/v). Working standards containing 55.13 μg/mL of FB_1, 25.00 μg/mL of FB_2 and 13.25 μg/mL of FB_3 were prepared. NDA was purchased from Invitrogen, Molecular Probes™. All other reagents were of analytical grade from Merck (Darmstadt, Germany). 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 liter distilled water. The pH was adjusted to 7 with HCl.

2.2. Samples

Samples of home-grown maize were collected from subsistence farmers in the former Transkei region of Eastern Cape Province, South Africa and stored at 4 °C until analysis.

2.3. Sample preparation

2.3.1. SAX extraction

SAX extraction was based on the method by Sydenham et al. [17] with minor modifications. In brief, 20 g of milled maize was extracted by blending in a homogenizer (Polytron PT 3100, Kinematica, Luzerne, Switzerland) for 3 min with methanol:water (3:1; 100 mL) followed by centrifugation (500 × g at 4 °C for 10 min). A 10 mL sample aliquot was cleaned up on a SAX (10 mL, 500 mg packing) SPE cartridge (Bond-Elut, Varian, Harbour City, CA, USA) which had been pre-conditioned with 5 mL methanol and methanol:water (3:1; 5 mL) (flow rate ≤2 mL/min, no air was forced through the column; the column was not allowed to dry through-out the entire clean-up process). Subsequently, the cartridge was washed successively with 5 mL methanol:water (3:1) and 3 mL methanol. The fumonisins were eluted with acetic acid:methanol (1:99; 10 mL) under gravity. The eluate was transferred into 4 mL glass vials and evaporated to dryness under a stream of nitrogen at 60 °C. The dried residues were stored at 4 °C until analyzed.

2.3.2. IAC extraction

The IAC extraction method used was based on the Vicam (Watertown, MA, USA) FumoniTest™ WB HPLC Instruction Manual [18]. In brief, 10 mL extracted sample was diluted with 40 mL phosphate buffer saline (PBS) and mixed well. PBS-diluted extract was passed through the FumoniTest column at a flow rate of 1 or 2 drops per second and the eluate was discarded. The column was washed with 10 mL PBS, until air came through the column and the eluate was discarded. The fumonisins were eluted with 2.5 mL of HPLC grade methanol, at a rate of 1 drop per second. The eluate was dried under nitrogen gas at 60 °C and the dried residues were stored at 4 °C until analyzed.

2.4. Derivatization

2.4.1. Optimized NDA derivatization procedure

The NDA method was based on that of Scott and Lawrence with modification [11]. A 20 μL fumonisin working standard aliquot was derivatized in a screw-cap amber vial by addition 20 μL sodium borate (0.1 M), 20 μL KCN (65 mg/100 mL) and 40 μL NDA (4 mg/8 mL methanol). The vial was capped and the solution was heated for 15 min at 60 °C, thereafter it was cooled to room temperature and stored at −22 °C until analyzed. Mobile phase (100 μL) was added to the solution prior to injection.

2.4.2. OPA derivatization procedure

The OPA derivatization procedure used was based on the Shephard et al. [12] method with minor modifications. In brief, standards (20 μL) were derivatized with 200 μL OPA reagent and 10 μL was injected. The samples were re-dissolved in 200 μL of methanol and an aliquot (50 μL) was derivatized with OPA solution (75 μL) and 20 μL was injected for HPLC analysis exactly 2 min after mixing.

2.5. Chromatography

The RP-HPLC was performed on a system equipped with an Agilent Technologies (Waldbronn, Germany) 1260 Infinity pump, a Rheodyne 7725i injector and a Phenomenex (Torrance, CA, USA) Luna C18 5 μm column (75 mm × 4.60 mm). The column was eluted isocratically at a flow rate of 1 mL/min. Mobile phase used for separation of OPA derivatives was methanol–0.1 M sodium dihydrogen phosphate (77:23; v/v) and for NDA derivatives was methanol–0.1 M sodium dihydrogen phosphate (78:22; v/v). Both mobile phases were adjusted to pH 3.35 with *o*-phosphoric acid. The HPLC system used was assembled with an Agilent 1100 series diode array detector (DAD) and Waters (Milford, MA, USA) 474 fluorescence detector (FLD) connected in series. NDA derivatives were detected at an excitation wavelength 420 nm and emission wavelength 500 nm for FLD, and at 252 nm for the UV detection. For the OPA derivatives, optimal UV detection was obtained at 335 nm, whereas the FLD was set at excitation wavelength 335 nm and emission wavelength 440 nm. Data were captured on Agilent ChemStation software and quantification was calculated by comparing peak areas with those of authentic fumonisin standards.

2.6. Comparison of FLD and DAD, NDA and OPA detection and derivatization of fumonisins in maize samples

Maize samples ($n = 15$) were singly extracted as described in Section 2.3.1 and duplicate clean-ups were performed for NDA and OPA derivatization prior to HPLC analysis, using both FLD and DAD. In a similar manner the same maize samples were singly extracted and duplicate FumoniTest IAC clean-ups were performed for NDA and OPA derivatization.

3. Results and discussion

3.1. Optimizing NDA derivatization

Optimization of the derivatization protocol of fumonisins with NDA was achieved by investigating the sodium borate buffer concentration, reaction time, temperature, detector wavelength and mobile phase solvent. These were investigated in order to establish the optimum parameters for derivatization and HPLC conditions. Different buffer concentrations (0.05 M, 0.08 M and 0.1 M, all adjusted to pH 9.5) were investigated. Although no major difference in HPLC responses was observed between 0.05 M and 0.08 M buffers, the 0.10 M buffer provided optimum response approximately 15% above the others. Since both reaction time and temperature can affect the rate of derivative formation and hence HPLC response, optimization of these parameters was such that they could be studied concurrently (i.e. reaction time (15 min and 30 min) was studied at both temperatures (24 °C and 60 °C)). Optimum response was obtained when the derivatives were heated at 60 °C for 15 min. FB-NDA derivatives are generally monitored at excitation wavelengths 420 nm, 246 nm and emission wavelengths 500 nm, 418 nm [13,16]. These wavelengths were tested using fumonisin working standards, and based on the high sensitivity of FLD at excitation 420 nm and emission 500 nm; these were selected as optimum wavelengths. The DAD absorption wavelengths (248 nm, 252 nm, 256 nm) were examined and 252 nm since in our hands it provided the best sensitivity. An iso-absorbance plot (software programme which displays optimum chromatographic details in 3D) was then used to confirm the wavelength selection for DAD. 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 (FB_2 and FB_3). Acetonitrile provided excellent baseline resolution coupled with short analysis time. Interestingly, this solvent altered the elution order of FB_2 with FB_3 and FB_3 with its isomer epi-FB_3, a phenomenon which has not previously been reported. Since methanol as HPLC mobile phase component yielded comparable results to acetonitrile and is also cheaper, it was selected as the solvent of choice. Method specificity was done by analyzing for interferences and peak purity. A reagent blank was prepared to test the interference of peaks; the resultant chromatogram obtained from the reagent blank was overlaid with that of the standard chromatogram. Peaks in the standard chromatogram other than the analyte peaks, were all accounted for in the reagent blank chromatogram. Therefore the peaks are from the reagents and do not interfere with the quantification or resolution of the analytes. Peak purity tests performed with ChemStation software showed that the peaks were pure and within the calculated threshold.

3.2. Detection limits

The limit of detection (LOD) was calculated as the amount of analyte injected resulting in peak height 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 (Table 1) show that the FLD is more than 100 times sensitive than DAD following IAC. The LOD and LOQ values of the OPA derivatives which were previously reported showed FLDs were approximately 20-times more sensitive than DAD for OPA analysis on fumonisin standards [15].

Table 1
Limits of detection (LOD) and quantification (LOQ) of maize derivatized with naphthalene-2,3-dicarboxaldehyde (NDA) 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

3.3. Reproducibility and 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 a day prior to sample analysis and the inter-day was measured over a period of five consecutive days. Both derivatization reagents achieved excellent repeatabilities (Table 2). CV values calculated from the peak areas indicate the reproducibility and the precision of the method with respect to standards to be good.

The precision of the method relative to maize samples was obtained by extracting the same sample six times. The mean and standard deviation ($n = 6$) of maize derivatized with NDA and detected with FLD for FB_1, FB_2 and FB_3 were 1290 ± 33, 558 ± 10 and 205 ± 11 μg/kg, respectively, and the CVs were 2.6%, 1.8% and 5.3%, respectively. NDA–DAD repeatabilities ($n = 6$) of FB_1, FB_2 and FB_3 were 1218 ± 73, 598 ± 20 and 242 ± 23 μg/kg, respectively, and the CVs were 6.0%, 3.4% and 9.5%, respectively. The accuracy and precision results are in accordance with the performance characteristics of FB_1 and FB_2 as regulated by the Commission Directive of the European Commission [20].

3.4. Stability of NDA

An initial monotonic decrease in response over an 8 h period when derivatized samples were left at room temperature was overcome by storing derivatized samples at −22 °C until injected. Under these conditions the NDA derivatives where shown to be stable after five consecutive injections (∼120 min). NDA stability was further tested by storing derivatized standard and maize samples at −22 °C over three consecutive days (Table 3). The mean ($n = 6$) FB-NDA responses were stable for 24 h; after which a decrease in response (approximately 10%) was observed in the FLD response. In contrast, the DAD response on day 2 and 3 apparently increased 10% over day 1. Previous reports described increases in NDA–FB response after 24 h [13,21]. These results suggest that NDA derivatives are suitable for auto-injection or over-night analysis with working standards injected between samples to allow for better quantification and to accommodate any derivative instability.

Table 2
Coefficient of variation (%) of fumonisin standards derivatized with naphthalene-2,3-dicarboxaldehyde (NDA) and *o*-phthaldialdehyde (OPA).

	Intra-day precision ($n = 3$)			Inter-day precision ($n = 15$)[a]		
	FB_1	FB_2	FB_3	FB_1	FB_2	FB_3
OPA						
FLD	0.89	0.65	1.01	12.33	11.71	12.64
DAD	1.58	2.89	1.64	12.06	11.32	13.01
NDA						
FLD	0.87	1.85	0.20	7.64	7.10	5.23
DAD	1.79	2.09	2.66	5.40	6.93	7.91

[a] 3 injections on 5 consecutive days.

Table 3
Fumonisin levels (μg/kg) of a maize sample derivatized with NDA on day 1 and detected with FLD and DAD on consecutive days. Levels are based on standards similarly treated.

	FLD			DAD		
	FB_1	FB_2	FB_3	FB_1	FB_2	FB_3
Day 1	1123 ± 74	517 ± 41	212 ± 15	907 ± 61	513 ± 49	233 ± 19
Day 2	1118 ± 120	552 ± 70	223 ± 25	982 ± 116	602 ± 72	341 ± 59
Day 3	986 ± 102	468 ± 55	201 ± 24	985 ± 93	508 ± 58	311 ± 59

Values represent mean ± standard deviation of a sample extracted 6 times. For day 2 and day 3 for FB_1 and FB_3 there is no significant difference ($p > 0.05$) and FB_2 marginally significant ($p < 0.05$).

Table 4
Comparative fumonisin recoveries (%) from maize samples cleaned up with SAX, derivatization with NDA and detection with FLD and DAD.

	Spiking level (μg/kg)	FLD	DAD
FB_1	1103	73 ± 9	65 ± 8
FB_2	500	80 ± 10	75 ± 13
FB_3	270	68 ± 8	58 ± 12

Values represent mean ± standard deviation of 6 analyses.

3.5. 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.

The accuracy was obtained by measuring the recoveries for both clean-up methods (SAX and IAC) on maize samples. In order to optimize recoveries two different extraction solvents (methanol:water 3:1 and methanol:0.1 M 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 analyzed by SAX clean-up and OPA derivatization was selected [17]. Recoveries ($n = 6$) for NDA derivatization following SAX clean-up were determined by spiking maize samples at levels of 1103, 500 and 270 μg/kg of FB_1, FB_2 and FB_3, respectively (Table 4). The method accuracy and repeatability are generally within acceptable limits for both FLD and DAD [20]. In addition to SAX clean-up, a similar recovery experiment was performed using IAC clean-up. A good comparison between the FLD and DAD was achieved for both OPA and NDA, even though a decrease in NDA–FB_1 and OPA–FB_2 was observed with DAD when using IAC clean-up method (Table 5).

3.6. Method comparisons

Individual comparison between FLD and DAD produced similar results irrespective of the clean-up method or the derivatization agent utilized in maize samples naturally contaminated with fumonisin ranging from below the LOQ to 6114 μg/kg (Table 6; Fig. 1a and b). This is in contrast to a previous study where the DAD responses were far less sensitive and more varied (CV > 80%) compared to FLD at levels below 1000 μg/kg for maize samples cleaned up with SAX and derivatized with OPA [15].

In the present study, NDA derivatization following SAX clean-up produced comparable results to OPA only up to 1000 μg/kg, whereas at higher levels the comparison was inconsistent as NDA responses frequently resulted in less than 50% of the OPA responses. Hence, despite the optimization and validation of the method described above, it would appear that certain of the home-grown maize samples studied contained inhibitors to the derivatization reaction in their SAX extracts. Therefore, the alternate IAC clean-up method was investigated, since it produces cleaner extracts for derivatization although representing a more expensive alternative. Results obtained with NDA derivatization, following IAC clean-up, resulted in improved comparison with OPA derivatization at all levels analyzed with FLD but not for levels below a 1000 μg/kg with DAD. In the latter case, the fumonisin levels by DAD were elevated above the corresponding OPA levels, possibly due to a co-eluting sample impurity. It is interesting to note that recent applications of NDA to fumonisin analysis by FLD have all used IAC clean-up

Table 5
Comparative fumonisin recoveries (%) from maize samples cleaned up with FumoniTest™ Immunoaffinity columns, derivatization with NDA or OPA and detection with FLD and DAD.

	Spiked (μg/kg)	FLD		DAD	
		OPA	NDA	OPA	NDA
FB_1	1103	67 ± 14	62 ± 3	67 ± 14	48 ± 31
FB_2	500	58 ± 18	94 ± 50	46 ± 33	62 ± 53
FB_3	270	75 ± 22	64 ± 32	62 ± 56	62 ± 19

Values represent mean ± standard deviation of 6 analyses.

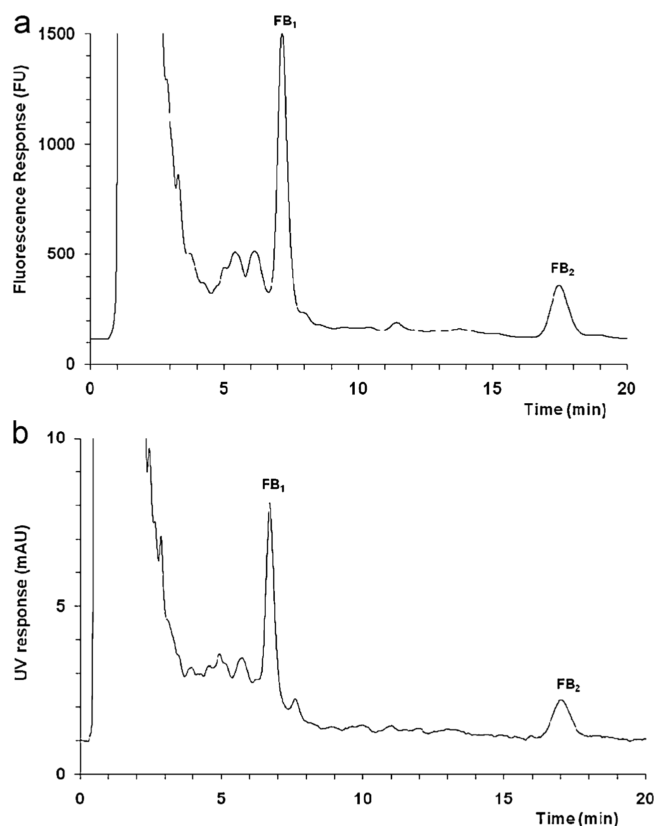

Fig. 1. (a) Naturally contaminated maize sample with 733 μg/kg, 250 μg/kg and 48 μg/kg levels of FB_1, FB_2 and FB_3, respectively. Analysis done by HPLC-FLD after SAX clean-up and NDA derivatization. (b) Naturally contaminated maize sample with 663 μg/kg and 245 μg/kg levels of FB_1 and FB_2, respectively. Analysis done by HPLC-DAD after SAX clean-up and NDA derivatization.

Table 6
Total fumonisin levels ($FB_1 + FB_2 + FB_3$; µg/kg) in naturally contaminated maize cleaned up with SAX or immunoaffinity columns, 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[a]	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

[a] ND = not detected.

[21–23]. An older method using RP (C18) SPE clean-up of mouldy maize reported that NDA gave higher values than OPA at lower contamination levels and lower values at higher contamination levels [24].

4. Conclusion

NDA proved to be an effective derivatization reagent of fumonisin in naturally contaminated maize samples following IAC clean-up, except for DAD at TFB levels below 1000 µg/kg. In contrast NDA derivatization produced comparable results to OPA following SAX clean-up for levels below 1000 µg/kg, except at levels below 1000 µg/kg for IAC. In conclusion, OPA and NDA derivatization of naturally contaminated maize samples subsequent to IAC clean-up compared very well with fluorescence detection. FLD and DAD were comparable irrespective of the clean-up method or derivatization agent.

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