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Review

Chromatographic determination of the fumonisin mycotoxins

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Abstract

The fumonisins are a recently identified group of fungal toxins, occurring worldwide in naturally contaminated maize, which have elicited considerable attention over the past decade due to their association with the animal disease syndromes, equine leukoencephalomalacia and porcine pulmonary oedema, and their reported association with oesophageal cancer in rural areas of Transkei, South Africa and Linxian County, China. This paper reviews the development of sensitive chromatographic analytical methods for the determination of these toxins in a range of mainly maize or maize-based food matrices. Initial attempts at gas chromatographic determination of these toxins were supplanted by the successful development of liquid chromatographic methods based on solid-phase extraction (SPE) of solvent extracts, followed by precolumn derivatisation and HPLC determination using fluorescence detection. The most widely used method involves strong anion-exchange (SAX) SPE and the use of *o*-phthaldialdehyde as derivatising agent. In contrast, the development of thin-layer chromatographic methods enables large numbers of samples to be screened economically. The recent advances in liquid chromatography–mass spectrometry have resulted in the development of suitable methods for fumonisin analysis without the need of derivatisation. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Review; Food analysis; *Fusarium*; Fumonisins; Mycotoxins; Sphingolipids; Toxins

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1. Introduction

The fumonisin mycotoxins, originally isolated in 1988, are produced by several species of the genus *Fusarium*, of which *F. moniliforme* and *F. proliferatum* are the most important as they are worldwide pathogens of maize [1,2]. Although an increasing number of structural analogues have been isolated from fungal cultures [3], the most important analogues found in naturally contaminated maize for which analytical methods have been developed are fumonisin B_1 (FB_1), fumonisin B_2 (FB_2) and fumonisin B_3 (FB_3) [4]. They are diesters of propane-1,2,3-tricarboxylic acid (tricarballylic acid,

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TCA) and various 2-amino-12,16-dimethylpolyhydroxyeicosanes in which the hydroxyl groups on C_{14} and C_{15} are esterified with a terminal carboxyl moiety of the TCA (Fig. 1). In addition, various partially [5] and fully hydrolysed forms are known, the latter being found in maize subjected to alkaline hydrolysis, as in the making of Mexican tortillas [6,7].

Studies on the major naturally occurring analogue, FB_1, have shown it to be the causative agent for the fatal disease syndromes, leukoencephalomalacia in horses [8] and pulmonary oedema and hydrothorax in pigs [9]. FB_1 has also been shown to be nephrotoxic [10], hepatotoxic and hepatocarcinogenic in laboratory rats [11] and to produce toxic effects in broiler chicks [12] and turkey poults [13]. FB_1 is cytotoxic to several mammalian cell lines due to the induction of apoptosis [14,15]. The effect of fumonisins on human health is not certain, but it has been statistically linked to the incidence of oesophageal cancer in the Transkei region of South Africa [16] and in Linxian County, China [17]. On the basis of existing toxicological evidence, the International Agency for Research on Cancer (IARC) has declared that *F. moniliforme* toxins are possibly carcinogenic to humans (class 2B carcinogens) [18].

The problems and risks associated with fumonisin contamination of animal feed and human food have resulted in the development of precise, reliable and sensitive methods for the determination of fumonisins in maize and maize-based food and feeds. In addition, methods have been published aimed at addressing the possibility of fumonisin carry over into other food matrices, such as milk, meat and beer. Although fumonisins have also been determined in physiological samples such as plasma and urine, these determinations have largely been performed in conjunction with experimentation into the toxicokinetics and deposition of these mycotoxins in animals after relatively high doses. Much of this methodological development has involved chromatographic methods and is reviewed here. The development of alternative methods, such as enzyme-linked immunosorbent assay (ELISA), has provided fast analysis times for the rapid screening of samples, but lies outside the scope of this review [19–22]. Although methods have been published for the determination of fumonisins by capillary zone electrophoresis, these have not found widespread application [23–25].

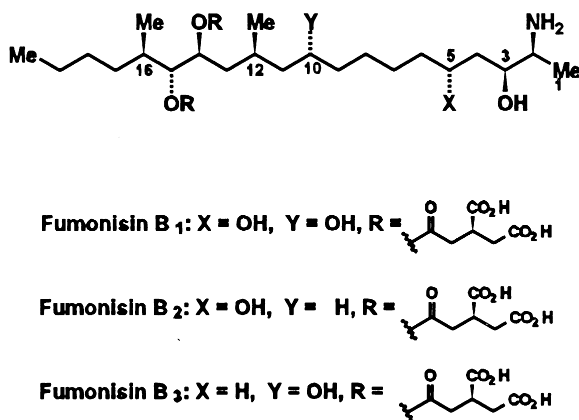

Fig. 1. Chemical structures for the three main fumonisin analogues, FB_1, FB_2 and FB_3, found in naturally contaminated maize.

2. Development of gas chromatographic methods

Although the isolation of the fumonisins had involved extensive column chromatography on normal- and reversed-phase (C_{18}) silica columns, as well as detection of the isolated toxins by thin-layer chromatography (TLC) [1], initial development of an analytical method focused on the application of fused-silica capillary GC techniques. One publication described the confirmation of the natural occurrence of the fumonisins in maize and involved acid hydrolysis of the fumonisins to cleave the ester bonds and the subsequent detection of the resultant TCA following its esterification with isobutanol [26]. Confirmation of the presence of TCA in the hydrolysed samples was obtained by gas chromatography–mass spectrometry (GC–MS). Of more direct use in the determination of individual fumonisins, Plattner et al. [27] reported a method in which the fumonisin backbone (aminopolyol) produced by alkaline hydrolysis was isolated on XAD-2 resin and then converted to the trimethylsilyl derivative for GC analysis. This method, using MS with either chemical or electron ionisation, was applied to the quantitative determination of FB_1 in heavily contaminated

maize samples associated with field cases of equine leukoencephalomalacia. Subsequent capillary GC studies failed to resolve the hydrolysed backbones of FB_2 and FB_3 as their silyl derivatives, but achieved suitable separation of all three aminopolyols as the trifluoroacetyl derivatives [28]. The accuracy and precision of this GC–MS method was improved by the use of deuterium-labelled FB_1 as an internal standard added to the sample extract prior to hydrolysis [29]. The labelled FB_1 was produced in liquid culture using methyl-D_3-labelled methionine as precursor, which results in the labelling of the methyl groups on the fumonisin backbone. Since the fragment ion in the main peak of the electron ionisation spectrum did not contain any of the labelled atoms, electron capture negative chemical ionisation of the trifluoroacetyl derivative was used for quantification as it gave rise to abundant molecular ions. This method is capable of achieving a 10 ng/g detection limit.

Although these GC–MS methods have been used as important confirmatory methods, most of the data on natural occurrence has been generated using the HPLC methods described in Section 3 as they are easier to apply and require less sophisticated equipment. The advances being made in liquid chromatography–mass spectrometry (LC–MS) have also shifted the emphasis away from these GC methods, which require multiple sample handling steps, such as sample hydrolysis, clean-up and derivatisation, prior to analysis.

3. Development of liquid chromatographic methods

The fumonisin mycotoxins are polar molecules which are soluble in water and polar solvents and are thus ideally suited for determination by reversed-phase HPLC. As they lack a significant UV chromophore and are not inherently fluorescent, sensitive detection at the low levels necessary for the analysis of naturally contaminated food samples requires derivatisation of sample extracts. In a recent worldwide survey of fumonisin levels in maize and maize-based products, over 90% of laboratories that reported results used precolumn derivatisation and HPLC for quantitation [4].

Extraction of these polar compounds from food matrices has generally been achieved using either acetonitrile–water (1:1, v/v) [30–32] or methanol–water mixtures containing 70–80% methanol [31,33,34], although a recent report details the use of supercritical fluid extraction from maize using carbon dioxide with 5% aqueous acetic acid as modifier [35]. Aqueous acetonitrile (with 30–60 min shaking) has been reported to give superior extraction efficiency compared to aqueous methanol [31,32], whereas other workers reported slightly better efficiencies for methanol–water (3:1, v/v) using homogenisation for between 1 and 5 min [34]. In a recent European intercomparison study for the determination of fumonisins in maize it was reported that participating laboratories achieved better mean recoveries using shaking (85% and 86% for FB_1 and FB_2, respectively), compared to blending which achieved comparatively low mean recoveries of 70% and 69%, respectively [36]. Different workers have also reported using various solvent–sample ratios from 2:1 to 10:1, without a clear comparison of the recoveries achieved with these different ratios. The European intercomparison study for the determination of fumonisins in maize found better extraction recoveries at higher solvent–sample ratios [36]. It has been noted that fumonisins in naturally contaminated samples are more difficult to extract than fumonisin standard added as a spike to maize, possibly due to an association with other sample matrix components [31]. In this regard, it has been reported that in certain substrates, such as maize bran flour and mixed baby cereal, poor recoveries are obtained using these extraction solvents and that for the bran flour, but not the mixed cereal, the use of methanol–borate buffer, pH 9.2 (3:1, v/v) as extractant gave acceptable recoveries [37].

Prior to HPLC analysis, the crude extracts must be purified to remove matrix impurities and to concentrate the fumonisins. This step has been achieved either by solid-phase extraction (SPE) on reversed-phase (C_{18}) or strong anion-exchange (SAX) cartridges, or by immunoaffinity columns. SAX cartridges achieve superior purification over reversed-phase cartridges [31,36,38], but require monitoring of the pH of the sample extract (above 5.8 for adequate retention on the SAX cartridge), and careful control of elution flow-rates at not more than 1

ml/min for reproducible recoveries [34]. A recently published application of this technique to the extraction of FB_1 from rice found that pH appeared to be critical and that for rice, a pH of 6.2 rather than 5.8 was required [39]. Although they have found wide application in the determination of fumonisins [4], SAX cartridges cannot be used for the determination of the hydrolysed aminopolyol moieties of the fumonisins, which lack the anionic carboxylate groups needed for the SAX clean-up procedure. On the other hand, large variations in recovery from C_{18} cartridges have been noted which were surmised to be due to interaction of the fumonisins with active sites on the sorbent [31]. In addition to reversed-phase and SAX cartridges, SPE sorbents containing both these functionalities have been reported to yield good recoveries of fumonisins over a wide pH range [40], while the sequential use of first a C_{18} and then a SAX cartridge has been applied to the clean-up of rodent feed [41]. Reversed-phase (C_{18}) SPE cartridges have been applied to the purification of the aminopentol produced by the hydrolysis of FB_1 in maize [6,42] and milk [43], while the hydrolysis product in wort samples in beer brewing were also analysed on C_{18} media after a prior clean-up on SAX SPE cartridges [44]. Alkali-processed maize extracts, containing both fumonisins and hydrolysed fumonisins, were initially passed down SAX cartridges to isolate the fumonisins, and then the initial column effluent and its wash were cleaned-up on C_{18} cartridges to separately isolate the hydrolysed moieties [7]. A similar method was used for spiked beef samples, only the hydrolysed moieties were isolated on a XAD-2 resin [45]. As an alternative to SPE, immunoaffinity columns, consisting of antibodies reactive with fumonisins, provide a more selective purification of sample extracts and are commercially available. These columns have been applied to the determination of FB_1 and FB_2 in maize [46], in milk [43], in beer [47] and in sweet corn [48]. Care must be exercised in the use of these columns, as they have a limited capacity which should not be exceeded, otherwise the sample must be diluted and reanalysed.

Purification of physiological samples for fumonisin determination has followed the same principles as for food matrices. Both plasma and urine samples were cleaned-up on SAX SPE cartridges with minor modifications to the above methods [49,50]. The protein in plasma was precipitated with methanol and, after centrifugation, the supernatant was applied to the SAX cartridge, while urine samples were strongly diluted prior to application in order to prevent recovery losses. Faecal extracts were cleaned-up on C_{18} cartridges which allowed simultaneous isolation of the fumonisins and their partially and fully hydrolysed moieties [5].

Sensitive detection of the fumonisins generally requires the formation of a suitable derivative. Various precolumn derivatisation techniques involving reaction of the primary amine group have been reported. The first HPLC method published was developed for fumonisin quantification in culture extracts [51]. It involved the formation of the maleyl derivatives and the separation of these by reversed-phase HPLC with UV detection, which gave a detection limit of 10 μg/g and was hence unsuitable for use in naturally contaminated maize [26]. Fluorescamine derivatisation and fluorescence detection yielded the necessary sensitivity [30,52], but reaction with this reagent resulted in the formation of two reaction products (an equilibrium mixture of a lactone and its hydrolysed analogue) and this was considered undesirable [26]. *o*-Phthaldialdehyde (OPA) has proved a sensitive reagent for the fumonisins using precolumn derivatisation and isocratic reversed-phase HPLC with fluorescence detection [33,34]. A recent survey of fumonisin levels in maize worldwide showed this reagent to be the method of choice in most laboratories reporting results [4]. The derivatisation reaction with OPA and 2-mercaptoethanol is rapid and reproducible at room temperature in a borate buffer (pH 9–10) but the method suffers from the disadvantage of the limited stability of the fluorescent reaction products. These derivatives were found to be stable for a period of 4 min after preparation, whereafter a decrease (5%) in fluorescence response of the FB_1 derivative was noted after 8 min with further decreases thereafter. This problem is readily overcome by standardising the time (about 2 min) between reagent addition and HPLC injection. Detection limits for the method are of the order 50 ng/g or better. Fig. 2 illustrates a typical reversed-phase HPLC separation of the OPA derivatives of the three main fumonisin analogues.

Naphthalene-2,3-dicarboxaldehyde with KCN

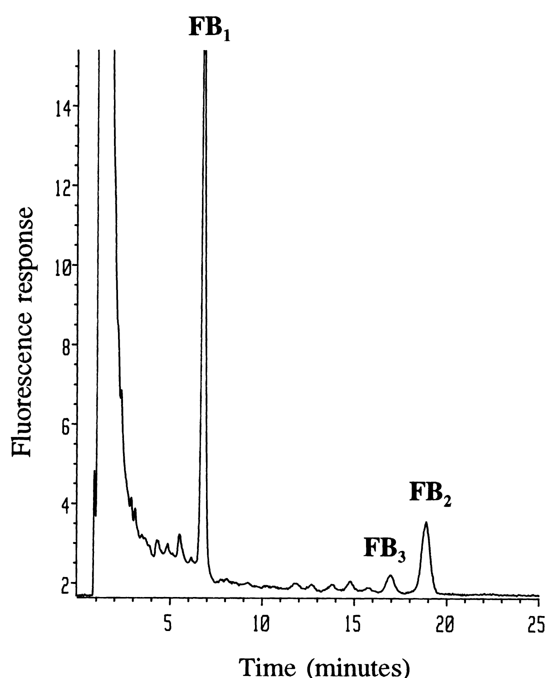

Fig. 2. Typical reversed-phase separation of the OPA derivatives formed by precolumn derivatisation of the main fumonisin analogues extracted from contaminated maize.

formed a highly fluorescent derivative which was relatively stable over 24 h and which allowed detection of 50 pg of FB_1 standard [31,53]. This reagent has been incorporated in a method for the determination of FB_1 and FB_2 in milk which has a sensitivity of 5 ng/ml [54]. Another derivatisation reagent reported to be useful as an alternative to the above is 4-fluoro-7-nitrobenzofurazan (NBDF), which gave a detection limit of 100 ng/g, but also showed limited stability [55]. It was similarly reported that 1-dimethylaminonaphthalene-5-sulphonyl chloride (dansyl chloride) formed a good derivative but was not useful for maize due to analytical interferences [55]. 9-Fluorenylmethyl chloroformate (FMOC) has been used as a sensitive reagent for fumonisin determination in rodent feed, forming derivatives which were stable for at least 72 h and which had a detection limit of 200 ng/g [41]. Another derivatising reagent which has been used and which yields stable derivatives is 6-aminoquinolyl N-hydroxysuccinimidylcarbamate, although the initial report gives an apparent detection limit of 260 ng/g, considerably above that achieved with OPA [56].

As fumonisins are an important contaminant of maize, considerable effort has been spent in the assessment of the performance characteristics of methods for their determination in maize. Soon after the publication of the first analytical techniques for fumonisins in maize, a small collaborative study between four laboratories was conducted to test the fluorescamine procedure. In addition, two laboratories included GC–MS and OPA methods in their results, which showed acceptable agreement in the range 4–1800 μg/g [57]. The OPA method using methanol–water (3:1, v/v) for extraction and SAX cartridges for clean-up was studied collaboratively under the auspices of the Commission of Food Chemistry of IUPAC [58]. Naturally contaminated maize samples were analysed by eleven laboratories from six countries. For FB_1 concentrations ranging from 2000 to 200 ng/g, within-laboratory repeatability varied from 7.7 to 25.5% R.S.D. and between-laboratory reproducibility from 18.0 to 26.7% R.S.D. Corresponding figures for FB_2 were 12.5 to 36.8% R.S.D. for within-laboratory repeatability and 28.0 to 45.6% R.S.D. for between-laboratory reproducibility. A follow-up collaborative study, run as a joint IUPAC and AOAC International project, using 'blank' maize spiked with FB_1, FB_2 and FB_3 standards at concentrations ranging from 100 to 8000 ng/g produced mean recoveries of between 75% and 87% for the individual toxins and resulted in the method being adopted first action by AOAC International [59]. An European intercomparison study for the determination of fumonisins in maize, conducted under the auspices of the European Commission, Measurements and Testing Programme, was carried out by 24 laboratories who analysed a blank and a spiked maize sample, contaminated at the level of 2000 ng/g FB_1 and 1000 ng/g FB_2 [36]. In addition, laboratories conducted a recovery experiment, in which the blank maize was spiked within each laboratory. Although no method was stipulated to participants, most used modifications of the method adopted first action by AOAC International. After correction for individual laboratory recoveries, within-laboratory repeatabilities for FB_1 and FB_2 were 10 and 11%, respectively, while

between-laboratory reproducibilities were 11 and 13%, respectively.

Although the great majority of reports on fumonisin determination by HPLC (apart from LC–MS described in Section 5) have described pre-column derivatisation with fluorescent derivatives for sensitive detection, other systems and detectors have been investigated. A recent publication described the application of postcolumn in-line derivatisation [60]. The fumonisins were separated by ion-pair chromatography and the derivatising mixture of OPA and N-acetyl-L-cysteine in a pH 9 borate buffer was pumped into the column eluate prior to a 5-m reaction coil. Overall detection limit by this method was reported to be 80 ng/g. Another report has shown that amperometric electrochemical detection of the OPA/*tert*.-butyl-thiol derivative is not as sensitive or stable as fluorescence detection of this same derivative [61]. In order to avoid sample derivatisation, evaporative light scattering detection has been employed for the analysis of fumonisin analogues in maize culture samples and in the preparation and isolation of purified fumonisins [62,63].

4. Development of thin-layer chromatographic methods

Although the only officially recognised method of fumonisin determination involves HPLC [59], a need exists for the rapid screening of maize samples for fumonisin contamination. This requirement can be met by the application of immunoaffinity based techniques such as ELISA [19–22]. However, some laboratories prefer to apply TLC methods which can also be partially automated.

The first TLC methods for the determination of fumonisins were developed during the original isolation of fumonisins from maize cultures of *F. moniliforme* MRC826 as a means of monitoring column eluates. The methods involved reversed-phase TLC on C_{18}-modified silica plates developed with methanol–water (3:1, v/v) as solvent and also normal-phase TLC on silica plates using chloroform–methanol–water–acetic acid mixtures [1,64]. After development, fumonisins were visualised by spraying with ninhydrin or *p*-anisaldehyde solutions. Although useful for monitoring column effluents and analysis of fungal cultures, the detection limit of this method (0.5 mg/g) prevented it from being used for the determination of fumonisins in naturally contaminated maize [26]. This detection limit was reduced to 0.1 mg/g by the use of a two-stage sequential development process for the silica TLC plate in which the lower section of the plate was removed between stages, and the fumonisins quantitated by instrumentalised spectrophotodensitometry after spraying with *p*-anisaldehyde solution [65].

The use of fluorescamine as a spray reagent for visualising the fumonisins under UV light improved the sensitivity and selectivity of TLC and enabled it to be used for naturally contaminated maize [66]. The fumonisins were extracted with acetonitrile–water (1:1, v/v) and an aliquot of the extract was diluted with 1% KCl prior to application to a C_{18} SPE cartridge. After washing the cartridge with 1% KCl and acetonitrile–1% KCl (1:9, v/v) to remove some matrix interferences, the fumonisins were eluted with acetonitrile–water (7:3, v/v). The purified extract was chromatographed by reversed-phase TLC using methanol–4% aqueous KCL (4:1, v/v) as solvent. The fumonisins were visualised by successive spraying of 0.1 *M* sodium borate buffer (pH 8–9), a solution of 0.04% fluorescamine in acetonitrile and, after 1 min, a solution of 0.01 *M* boric acid–acetonitrile (4:6, v/v). The plate was read under long wavelength UV light. Although a detection limit of 100 ng/g was reported for this method in maize-based feeds, considerable matrix interferences are frequently present at levels below 1000 ng/g which hamper the visual discernment of the individual chromatographic bands. These interferences can be greatly reduced by sample clean-up using SAX SPE cartridges [38].

Other reported TLC methods have also used SAX SPE cartridge clean-up of sample extracts. The presence of fumonisins in maize and maize-based food products was confirmed by reversed-phase high-performance TLC and subsequent visualisation (detection limit of 250 ng/g) with a spray reagent of 0.5% vanillin in 97% sulphuric acid–ethanol (4:1, v/v) which, after heating at 120°C for 10 min, revealed FB_1 as a blue-purple spot [67]. A high-performance TLC method was developed for determination of FB_1 in rice [39]. As in the case of

maize, C_{18} clean-up was not as efficient as that achieved with SAX SPE cartridges, which, when combined with the novel visualisation technique of dipping the developed plate into a 0.16% acidic solution of *p*-anisaldehyde, yielded a detection limit of 250 ng/g when quantified by scanning fluorodensitometry.

5. Development of liquid chromatographic–mass spectrometric methods

The determination of fumonisins in naturally contaminated maize by HPLC generally requires the use of derivatives to provide the necessary sensitivity of detection. However, the advances currently being made in the interfacing of MS to HPLC and the development of numerous commercial LC–MS systems has resulted in the application of this technique to the analysis of food and feed samples for fumonisins without prior derivatisation. The use of LC–MS also provides strong confirmation of the presence of the fumonisins.

The initial application of MS to the determination of the fumonisins relied on the use of GC separation of the derivatised, hydrolysed forms of the fumonisin analogues as discussed in Section 2. Other reports have dealt with the use of direct sampling methods and fast-atom bombardment (FAB-MS) [27,29,68]. The determination of fumonisins in naturally contaminated maize using an internal standard of deuterium-labelled FB_1 and SAX cartridge clean-up of sample extracts prior to FAB-MS analysis, yielded a detection limit of approximately 100 ng/g, with precision restricted near the detection limit by the fact that FAB-MS by its nature gives a signal at each m/z value [29]. A recent publication describes the use of on-line capillary LC and FAB-MS to detect both partial and fully hydrolysed FB_1 in maize and maize screening samples from the US [69].

Initial comparison of a thermospray (TS) interface using flow injection analysis for sample introduction and an electrospray (ES) interface with infusion of the sample at a flow-rate of 1 μl/min showed that electrospray was a useful technique for the characterisation of nanogram quantities of FB_1 in that it provided a strong signal for the molecular ion and little fragmentation [68]. By contrast, TS–MS was reported to give multiple fragment ions and lacked sensitivity at sub-microgram levels [68]. However, it has more recently been reported that the detection limit of TS-MS can be decreased to 2 ng by using negative-ion mode and an aqueous ammonium acetate and acetonitrile mixture for the mobile phase and as such could be suitable for detection of fumonisins in maize [70]. Other workers have used a particle-beam interface for confirmation of fumonisin production in liquid cultures after separation of their methyl esters by reversed-phase HPLC [71]. The advantage of the particle beam interface is its ability to provide electron and chemical ionisation spectra, which provide greater structural information than atmospheric pressure ionisation techniques such as electrospray.

Electrospray and atmospheric pressure chemical ionisation (APCI) interfaces have recently undergone considerable commercial development and an increasing number of papers deal with their application to fumonisin detection. Initial methods were developed for the analysis of FB_1 standard on a reversed-phase column eluted with water–acetonitrile–acetic acid mixtures [72]. The column eluate was subjected to ES-MS which gave strong signals for the molecular ion and its alkali salts. Sensitivity was in the 100 pg range. A ES-MS method for the determination of fumonisins in commercial maize meal with detection limits of 100, 20 and 50 ng/g for the three main analogues (FB_1, FB_2 and FB_3, respectively) has recently been described [73]. The method used a polymeric hydrophobic column packing, volatile mobile phase of acetonitrile and aqueous ammonium acetate and selected ion monitoring in the positive mode. These same workers further optimised the analysis method by using immunoaffinity columns for a more efficient sample extract clean-up, by using microbore reversed-phase columns for a more efficient chromatographic separation and by using rapid sampling cone-skimmer potential switching to acquire both protonated molecular ions and fragment ions [74]. This method was applied to the determination of fumonisins in laboratory rodent feed with a limit of quantification of approximately 1 ng/g, although endogenous levels in commercial feed always exceeded 10 ng/g in all feeds analysed. Another sensitive method for determination of FB_1 and FB_2 in maize and maize

products utilises ES tandem MS (MS–MS) [75]. Samples containing a deuterated FB_1 internal standard were subjected to SAX cartridge clean-up and MS analysis on a triple quadrupole instrument with either selected reaction monitoring or selected ion monitoring. The method had a quantitation limit of 0.8 ng FB_1/g. Further MS–MS analysis on an ion trap instrument has been used to detect and characterise impurities in a purified sample of FB_1 and to characterise fragmentation pathways available to these toxins [76].

6. Conclusion

The application of the above methods to maize and maize-based foodstuffs has already generated a large quantity of valuable data concerning the fumonisin contamination of the human food supply [4]. These data, when combined with data on relevant food consumption patterns, provides information on human exposure to these carcinogens and lays the basis for risk assessment studies. The availability of accurate, sensitive and reliable methods has made possible the regular, routine determination of fumonisins in maize. If legislative control of fumonisin levels is introduced, these methods will provide the basis for the monitoring of compliance with legislative requirements.

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