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Review

Sample preparation and high-resolution separation of mycotoxins possessing carboxyl groups

Jon G. Wilkes, John B. Sutherland*

National Center for Toxicological Research, Jeffersen, AR 72079, USA

Abstract

The chromatographic analysis of carboxyl-containing mycotoxins, such as fumonisin B_1, ochratoxin A, and citrinin, presents a continual challenge. Toxins must first be extracted from foods or tissues and then cleaned up before chromatographic separation and detection. Liquid–liquid extraction efficiencies for some carboxylic mycotoxins are marginal for spiked samples and uncertain for incurred residues. Immunoaffinity columns may be useful for concentrating mycotoxins from samples before chromatography. In almost every case, more than one analytical method must be used to confirm the identification of the mycotoxin. The fumonisins are especially troublesome to analyze because they are relatively insoluble in organic solvents, they are not separated easily by gas chromatography, and they do not respond to the usual absorbance or fluorescence detectors used in liquid chromatography. Fluorescence derivatization and electrospray liquid chromatography–mass spectrometry have now made it possible to detect trace levels of mycotoxins. The purity of mycotoxin standards for toxicological studies can be determined by liquid chromatography with either an evaporative light scattering detector or electrospray mass spectrometer. New developments in capillary electrophoresis, nonporous microsphere liquid chromatography, and detection methods for low-volatility compounds show promise for improving the analysis of mycotoxins in the future.

Keywords: Reviews; Mycotoxins; Fumonisin B_1; Ochratoxin A; Citrinin

Contents

*Corresponding author

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1. Introduction

In 1987, J.C. Frisvad and U. Thrane [1] of the Technical University of Denmark published an important general method for the chemotaxonomy of fungi based on ‘‘organic solvent extracts of fungal cultures . . . ’’ They were able to detect 182 mycotoxins and other fungal metabolites by using reversed-phase HPLC with UV photodiode array detection. The method was described as suitable for ‘‘ . . . all important groups of mycotoxins and other fungal secondary metabolites . . . ’’ Of the 182 mycotoxins and secondary metabolites they listed, 22 contained carboxylic acid moieties.

The most compelling reason for reviewing the analysis of carboxyl-containing mycotoxins now is the discovery of the fumonisin mycotoxins. Although they are now known to be epidemiologically important and economically significant, the fumonisins [2–4] were not identified until 1988. The molecules of fumonisins contain four carboxylic acid moieties but lack UV chromophores (Fig. 1). Because of the large number of COOH groups, the fumonisins are highly water soluble and are retained in the aqueous phase of a typical liquid–liquid extraction.

For 18 years, scientists of the South African Medical Research Council [5,6] struggled to identify the agent responsible for moldy corn poisoning, a cluster of lethal symptoms observed in horses fed with corn contaminated by *Fusarium moniliforme*. Moldy corn, and beer made from it, were also known to be associated epidemiologically [7,8] with human esophageal cancer. However, until recently, the aqueous phases from liquid-liquid extraction clean-ups of corn cultures were routinely discarded, because nearly all mycotoxins (other than moniliformin and the AAL toxins) known at that time [9] would partition into the organic fraction. Examination of the aqueous phases by HPLC with UV detection [4] would have shown only normal peaks associated with the corn matrix. None of the known *Fusarium* mycotoxins, even including the mutagen fusarin C

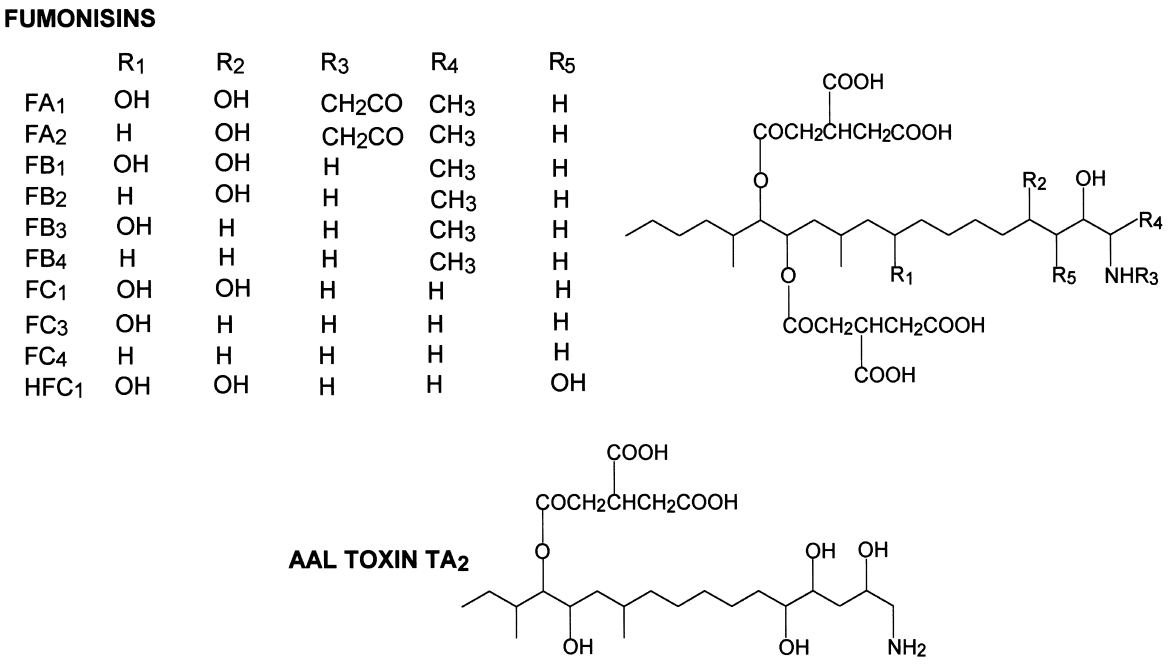

Fig. 1. Molecular structures of the fumonisins and AAL toxin. 'Hydrolyzed' fumonisin C_1 is designated HFC_1. Hydrolyzed indicates the addition to FC_1 of oxygen as an extra hydroxyl group rather than the hydrolytic displacement of a tricarballylic acid side chain.

[2], could account for the carcinogenicity of an *F. moniliforme* strain (MRC 826) that was associated with the toxic effects.

In 1988, Gelderblom et al. [3] successfully isolated the first of the fumonisins and identified the structure (Fig. 1). Again, the fumonisins could not have been detected by the HPLC method of Frisvad and Thrane, although it detects most other mycotoxins. A relatively recent article on the analysis of *Fusarium* mycotoxins by GC, with FTIR spectroscopy and MS [10], discussed the impressive diversity of 13 different mycotoxins produced by *Fusarium* spp. but did not mention the fumonisins. Unless the carboxyl groups have been esterified, the fumonisins have insufficient volatility for GC analysis, and so they were not found in this GC study even though they would have been detected by MS or FTIR.

The AAL toxins, which are produced by *Alternaria alternata* f. sp. *lycopersici* [11], a host-specific pathogen of tomato plants, are structurally analogous to the fumonisins (Fig. 1). A reversed-phase HPLC method described as ''suitable . . . for the separation and detection of major *Alternaria* mycotoxins in foodstuffs'' [12] detected five mycotoxins, but not the AAL toxins. If they had been present, AAL toxins presumably would not have been co-extracted with the other five mycotoxins and would not have registered on the UV detector.

The second section of this review will deal with sample extraction, clean-up, and concentration methods appropriate for most of the carboxyl-containing mycotoxins and those appropriate for the highly water-soluble fumonisins and AAL toxins. The next section will consider representative LC methods for the majority of nonpolar carboxyl-containing mycotoxins. The fourth will cover GC methods for mycotoxins. The fifth will consider electrophoretic and LC separations appropriate for trace levels of the fumonisins and AAL toxins in biological and agricultural matrices. The final major section will discuss LC methods for purity analyses of fumonisin standards; some of the less-sensitive methods may be useful for preparation of pure analytical standards or validation of toxin batch purity for toxicological studies.

This review is intended to illustrate issues related

Table 1
Carboxyl-containing mycotoxins found in the general literature

AAL toxins	Mycophenolic acid
Butenolide (4-acetamido-4-hydroxy-2-butenoic acid γ-lactone)	Naphthalic anhydride
Citrinin	β-Nitropropionic acid
Diplodiatoxin	Ochratoxin A
Fumonisins	Roseotoxin B
Fusaric acid	Sambucinic acid

Table 2
Miscellaneous carboxyl-containing fungal metabolites of uncertain toxicity separated by Frisvad and Thrane

Asterric acid	Gladiolic acid
Carlosic acid	Hadacidin, Na^+
Cyclopaldic acid	Hydroxyisocanadensic acid
Dipicolinic acid	Orsellinic acid
Fulvic acid	Stipitatic acid
Gibberellic acid	

Source, Ref. [1].

to the analysis of carboxylic acid-containing mycotoxins (Tables 1 and 2) and to report recent advances, but not necessarily all variations on previously published methods. The review will not cover mushroom toxins or most microfungal metabolites known to have limited toxicity to most humans. Citrinin, an antibiotic, is highly nephrotoxic in all animal species studied [9] and will be included. Its molecular structure and those of two other organic soluble, carboxylic acid mycotoxins are presented in Fig. 2. Methods for preparative-scale purification of mycotoxin standards will not be covered in this review. Also omitted will be TLC methods and off-line strategies, e.g., fraction collection followed by MS probe analysis, radiometric detection, or NMR analysis. Non-chromatographic immunochemical methods will be mentioned only for comparison

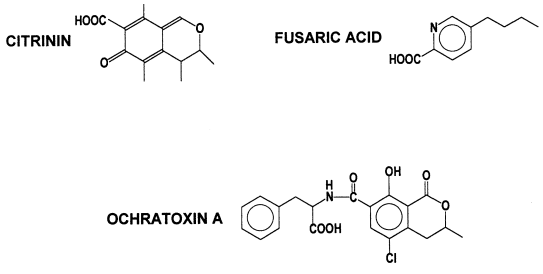

Fig. 2. Molecular structures of major organic-soluble mycotoxins reviewed.

with approaches using high-resolution analytical separations.

2. Extraction and clean-up of samples

2.1. General considerations

The major aspects of mycotoxin analysis, including sampling and subsampling, extraction (spiked vs. incurred residues; matrix effects; supercritical fluid extraction), clean-up/concentration (liquid–liquid or solid-phase extraction; immunoaffinity chromatography), plus others not relevant to this review, such as immunochemical assays for rapid screening in the field, have been summarized well by Scott [13]. Citing Trenholm et al. [14], Scott emphasized that mycotoxins spiked into test samples are more easily recovered for analysis than those incurred as natural residues. Substantial differences in extraction efficiency also depend on the matrix in which the toxins are bound.

Rapid technological advances, combined with the difficulty of selecting a single method suitable for diverse situations, challenge health regulatory authorities. The European Commission has established a consortium involving up to 30 laboratories from different countries. The consortium aims to develop, improve, and validate analytical methods and sampling plans, to harmonize analytical results between European Union member states, and to prepare suitable matrix-matched certified reference materials (CRMs) for studies of mycotoxins in foods and feeds. A generalized plan for producing a mycotoxin/matrix CRM, consisting of a rather complicated flow diagram involving validation steps with both spiked and incurred residues, has been reported by Boenke [15]. The development of a mycotoxin/matrix CRM requires criteria and norms to be met by

any new analytical method proposed for that analyte in that particular matrix. The certification study for an ochratoxin A/wheat flour CRM was the first one among the carboxyl-containing mycotoxins to be completed. A 1995 European Union panel discussion [16] identified future research priorities, including ''sampling protocols . . . for . . . fumonisins in grain, . . . corn grits, flour, polenta, and extruded corn'' and ''set up of special extraction studies in order to judge the accuracy, precision, and traceability of the extraction step in analytical procedures, in particular to study the presence and/or effects of bound residues of mycotoxins.'' Sampling, extraction efficiency, and detection are all major issues for analysis of mycotoxins in biological and biomedical matrices.

2.2. Organic-soluble mycotoxins

2.2.1. Techniques for extraction and clean-up

Little current work is being done to develop new analytical methods for most of the carboxylic acid mycotoxins. The ubiquitous nature of mycotoxigenic fungi, and the multitude of toxins produced by some individual strains, argue the need for a broadly sensitive screening assay. A potential generic method for mycotoxin analysis will be discussed, followed by a few specific methods that illustrate important considerations for sample extraction and clean-up.

The sample preparation method of Frisvad and Thrane [1] involves sequential extractions of culture material with chloroform–methanol and acetone–ethyl acetate mixtures, which are filtered and combined. A final methanol–light petroleum extraction removes most fungal lipids. In the last step, ''The lower secondary metabolite-containing methanol phase'' is analyzed. Any fumonisins or AAL toxin would already have been discarded. Percent recoveries of toxins from fungal extracts were not measured, which was not significant for Frisvad and Thrane at the time because they were using mycotoxin profiles primarily for fungal identification. They expressed the opinion that ''it would be difficult, if not impossible, to propose a general effective clean-up procedure for foods and feedstuffs containing trace amounts of mycotoxins.'' A study of extraction efficiencies and other factors involved in separation and detection will be necessary to adapt their method for general analysis of mycotoxins.

2.2.2. Fusaric acid and citrinin

Samples for fusaric acid analysis can be prepared by liquid–liquid extraction, in which the target analyte alternates between the organic and aqueous layers because of pH adjustment of the aqueous fraction [17]. This commonly used technique succeeds because fusaric acid is a Brønsted–Lowry acid (see Fig. 2). The selectivity of this extraction technique for organic acids also commends it for other carboxyl-containing mycotoxins.

Citrinin, which is produced by several species of *Aspergillus* and *Penicillium*, has a structure shown in Fig. 2. It has been recovered for analysis from biological fluids by Phillips et al. [18]. Starting with a 1 *M* HCl–ethyl acetate extraction, 95+% recovery of citrinin is possible from plasma; the extraction step is not required for bile or urine.

2.2.3. Ochratoxin A

Ochratoxin A (Fig. 2) deserves special attention because of its great toxicity, its importance as a health threat [19], and the peculiar analytical challenges it presents. An 85+% extraction efficiency was achieved by Valenta et al. [20] for ochratoxin A from the urine of swine but only 54–64% from the feces. The method included a liquid–liquid extraction (chloroform–0.1 *M* sodium hydrogen carbonate), in which the ochratoxin A was retained in the high pH aqueous layer, followed by a second liquid–liquid extraction, in which the pH of the aqueous phase was lowered to 2.0 and the ochratoxin A was driven back into chloroform. The chloroform was evaporated to dryness and the analyte was reconstituted in toluene. This was loaded onto a silica gel cartridge, washed with toluene–acetone, and extracted from the solid phase with toluene–acetone–formic acid. Again, the ionic characteristics of ochratoxin A were used repeatedly to achieve the aim of the extraction/clean-up procedure. The paper describes procedural modifications used for extraction of the toxin from feces. It includes a helpful discussion of the irreproducibility problems that occurred when more polar solvents were substituted for the solid-phase washing step in an attempt to eliminate interferences from the feces. Such ana-

lytical problems are multiplied as the matrix becomes more difficult or as the method is adapted for use with a larger variety of target analytes.

De Koe [21] reported the minimum performance characteristics required for ochratoxin A extraction from a generic matrix. For analyte concentrations of less than 1 μg/kg, 50–120% recoveries sufficed; above that level, the acceptable recovery window narrowed to 70–110%.

The results of a 20-laboratory study [22] of methods for determining ochratoxin A show a range in apparent recoveries from 43 to 128% of a freeze-dried pig kidney material containing 10 μg/kg ochratoxin A. The extremes lay well outside the European minimum performance criteria [21] and demonstrated [22] a ''clear need to improve analytical performance, particularly with respect to the extraction efficiency from this type matrix.'' Extraction efficiency may be a problem with ochratoxin A in any solid matrix.

2.3. Water-soluble mycotoxins

In 1995, Rice and co-workers [23], by adding 50 ml of 50% acetonitrile–water to 10 g of ground corn culture material and shaking the flask for 30 min, achieved 89–96% recoveries of incurred residues of fumonisin B_1 (FB_1). Similar results were also obtained for FB_2 and FB_3. Comparative efficiency studies, using either methanol–water or pure water for extraction, proved that the acetonitrile–water solution achieved extraction efficiencies in 30 min that were equalled by methanol–water only after 8 h. Recoveries from acetonitrile–water extractions of samples of corn and poultry feed, spiked at two levels with a *Fusarium*-contaminated corn extract, ranged from 91–94% for FB_1 to 90–100% for FB_2 to 81–93% for FB_3. The coefficients of variation were typically 3–7%. However, when comparing these encouraging recoveries for FB_1 to the disappointing ones discussed earlier for ochratoxin A [22], it is important to realize that the fumonisin levels being analyzed were about three orders of magnitude greater than those of ochratoxin A and reflect the differences in naturally occurring abundances of the two toxins. Ochratoxin A appears to be almost an order of magnitude more toxic than the fumonisins [19,24].

Results of a European Commission multi-laboratory intercomparison study have been reported by Visconti et al. [25,26]. In the second paper, which includes more details, the analytes were FB_1, FB_2, and FB_3. The participants used similar methods for analytical separation and detection but different methods for sample extraction and clean-up. The differences provide a qualitative, and sometimes a statistical, basis for comparison of techniques. All participants used methanol–water (3:1) as an extraction solvent, but four of them repeated the analyses with acetonitrile–water (1:1) (as in the method of Rice et al. [23]). Some participants blended the samples with the extraction solvent at high speed for a few min, whereas others shook the suspension slowly for 30 min. Some of them pooled consecutive extractions from the same material. Some used a higher solvent-to-corn ratio for extraction than that used in the method described by Shephard et al. [27]. After the extraction step, most workers passed a portion of the extract through a strong anion-exchange (SAX) cartridge, but a few substituted a C_{18} cartridge for this clean-up step.

Average recoveries of FB_1 and FB_2 for participants who used blending were 62 ± 6 and 60 ± 6%, respectively. The corresponding recoveries for those who used shaking for 30 min were 85 ± 12 and 86 ± 14%. Shaking was more efficient but less reproducible than blending, other factors being equal. Acetonitrile–water was more efficient but considerably less reproducible (up to ±20% in one case) than methanol–water. The irreproducibility conclusion for acetonitrile–water is uncertain because only four participants used this technique, only one of the four submitted a complete data set for statistical comparison, and the same four used C_{18} rather than SAX clean-up, which may have confused the cause of the measured deviation. (Note that the study of Rice et al. [23] showed mid-single digit coefficients of variation for fumonisins recovered by acetonitrile–water.) Finally, the C_{18} cartridges produced dirtier extracts than the SAX columns, which could explain the irreproducibility of results with acetonitrile–water extraction. Consecutive extractions, longer extraction times, and a higher solvent-to-corn ratio all improved extraction efficiency. Visconti et al. [26] pointed out that the results of their intercomparison study ''could not lead to a certification

exercise . . . [because, under European standards, that] . . . requires average recoveries higher than 70%'' [28,29]. Clearly, extraction recoveries and method reproducibility both must be improved to produce a certifiable method.

Selim et al. [30] recently reported the use of organic-solvent-modified CO_2 for static supercritical fluid extraction (SFE) of FB_1 from grain dust. Under optimized conditions for SFE, they reported that the FB_1 recovery was 40 times [sic] more efficient, faster (20 vs. 150 min), and more reproducible (R.S.D.=3–5% vs. R.S.D.=6.5%) than the solvent extraction method of Gelderblom et al. [3]. It is hard to know how to interpret an apparent 40-fold increase in recovery efficiency compared to a method which, in other investigators' hands, appears to give at least 60% recovery. The fumonisin in the sample they studied [30] was not an incurred residue, but rather a simulated sample artificially contaminated by adding a dried culture extract of *F. moniliforme* to nominally fumonisin-free corn dust.

In contrast, Rice et al. [23] reported their extraction efficiencies for spiked FB_1 relative to the maximum amount recoverable after many hours of liquid–liquid extraction by assuming 100% extraction after that much time. Their plots of recovery vs. extraction time appear to approach a hyperbolic limit. However, it is possible that significant amounts of incurred mycotoxin may be tightly bound in solid matrices.

Since efficiency and reproducibility of recovery are major unresolved issues in the determination of fumonisins, the method developed by Selim et al. [30] should be investigated further to address the issue of incurred fumonisin residues and the ability of any method to extract them from grain or other solid matrices.

2.4. A strategy for the clean-up of samples for multi-target analysis

Immunoaffinity cartridges were used by Scudamore et al. [31] for clean-up and subsequent analysis of three classes of mycotoxins found in pet foods. The authors found that a single extraction technique was suitable for ochratoxin A and four of the aflatoxins. Extraction was followed by a combined clean-up, achieved by connecting in tandem two immunoaffinity columns, one for each of the two mycotoxin classes. A single separation/detection method was also found suitable for the co-analysis of these two rather different mycotoxin classes in real-world samples. Reporting limits (3 times detection limits) for combined analyses, which were not optimized for one or the other class, varied from 0.5 to 1.0 μg/kg. Recoveries from spiked pet food samples averaged 103% each for aflatoxins B_1 and B_2; 84 and 88% for aflatoxins G_1 and G_2, respectively; and 52% for ochratoxin A. The excellent quality of clean-up using immunoaffinity columns is illustrated in Fig. 3, the HPLC trace of a birdseed reference sample containing 7 μg/kg ochratoxin A. (Reprinted with permission, Crown Copyright 1997.)

Scudamore et al. [31] also detected two fumonisins, FB_1 and FB_2, in the pet food. These require procedures, extraction solvents, affinity cartridges, and pre-column fluorescent labeling completely different from those used for ochratoxin A and the aflatoxins. A fumonisin-specific immunoaffinity cartridge was used for the clean-up. The reporting limits for FB_1 and FB_2 were 10 and 25 μg/kg, respectively, which was much better than the minimum sensitivity required. Average recoveries of FB_1 and FB_2 from spiked pet food samples were 94 and 67%, respectively.

Immunoaffinity clean-up also promises to be more selective. Trucksess and Wood [32] and Scott and Trucksess [33] recently summarized immunochemi-

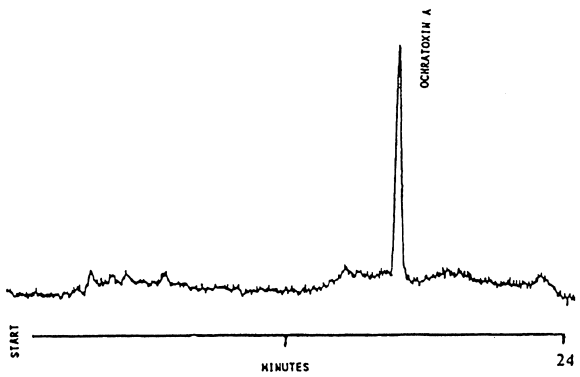

Fig. 3. HPLC separation of a sample of domestic bird food (reference M3736), containing 7 μg/kg ochratoxin A. Extracts were cleaned up using linked immunoaffinity columns; HPLC detection was optimized for ochratoxin A. (Reprinted by permission from Ref. [31], Crown Copyright 1997.)

cal methods adapted for mycotoxin analysis in foods. These concise, informative, and clear papers are particularly recommended for analysts unfamiliar with biochemical methods. The papers include a critique of commercially available immunoaffinity clean-up cartridges for fumonisins and ochratoxin A and discuss matrix interferences. The first paper also mentions a recently developed immunoaffinity cartridge that has multi-toxin selectivity [34]. If the preparation and analysis are also compatible, the co-analysis of diverse mycotoxin classes should be possible, either by using single cartridges with multitoxin selectivity or by using tandem series of cartridges with differing selectivity.

3. Chromatographic analysis of carboxyl-containing mycotoxins

3.1. Single-target chromatographic methods

Several textbook chapters [9,35,36] describe excellent methods for the separation of individual mycotoxins, not including the fumonisins and AAL toxin. These methods should suffice for most narrowly defined analyses of particular toxins. A greater challenge will be to develop practical methods that are not limited to an individual fungal metabolite or a single family of structurally similar compounds.

3.2. Evaluation of a multi-target chromatographic method

A potential advantage of chromatographic methods is their usefulness for simultaneous detection of a wide variety of mycotoxins. The method of Frisvad and Thrane [1] was developed for analysis of 182 structurally diverse targets. Important elements in the separation and detection components of this broad-spectrum method include the use of a rugged HPLC column (Nucleosil, 5 μm, C_{18}); use of an extreme, but shallow, mobile-phase concentration gradient (acetonitrile+0.05% trifluoroacetic acid–water, 10:90 to 90:10 over 40 min in two linear gradients with different slopes); and use of a UV diode-array detector (monitored only at 225 and 254 nm). Minor variations in chromatographic retention with each analysis were compensated by calculating a retention index for each target analyte, defined relative to the retention times of seven alkylphenones included as internal standards.

The quality of separation of this method may be inferred from a table listing the retention index for each mycotoxin. The indices varied from 662 to 1546 near the end of the 40-min separation. Thus, each retention index unit corresponds to about 1.5 s. An examination of the table shows as many as three different mycotoxins having the same retention index. Coelution events occur with greater frequency near the beginning of the separation. As expected, the majority of the carboxylic acid mycotoxins appear in the early, aqueous portion of the separation. Retention indices of carboxyl-containing compounds also considered here are as follows: hadacidin Na^+, 674; butenolide (4-acetamido-4-hydroxy-2-butenoic acid γ-lactone), 677; dipicolinic acid, 677; β-nitropropionic acid, 678; carlosic acid, 692; stipitatic acid, 693; fusaric acid, 715; orsellinic acid, 746; gibberellic acid, 747; hydroxy-isocanadensic acid, 771; gladiolic acid, 779; cyclopaldic acid, 843; citrinin, 919; asterric acid, 984; mycophenolic acid, 984; ochratoxin A, 1086; and naphthalic anhydride, 1440. Since most retention indices had standard deviations between 1 and 3 units, the first four compounds listed and several other pairs further down the list would have been difficult to distinguish on the basis of chromatographic retention alone. Moreover, the multitude of other peaks from non-carboxyl-containing mycotoxins may also interfere.

Differences in UV absorption characteristics at 254 and 225 nm can be used to differentiate some coeluting compounds [1]. To increase specificity for identification of unknowns, Frisvad and Thrane also added retardation factors relative to griseofulvin for two different TLC eluents. Apparently this HPLC–diode array UV method, by itself, lacks sufficient specificity for unequivocal identification of all mycotoxins.

Carboxylic acid analysis with HPLC benefits from the use of base-deactivated columns for reasonable chromatographic peak shape. The single chromatogram shown by Frisvad and Thrane (Fig. 4, reprinted with permission) did not exhibit obvious peak tailing or other asymmetry, but none of the mycotoxin families in this figure contain carboxyl groups. Therefore, the method might require modification for optimal use with carboxyl-containing mycotoxins.

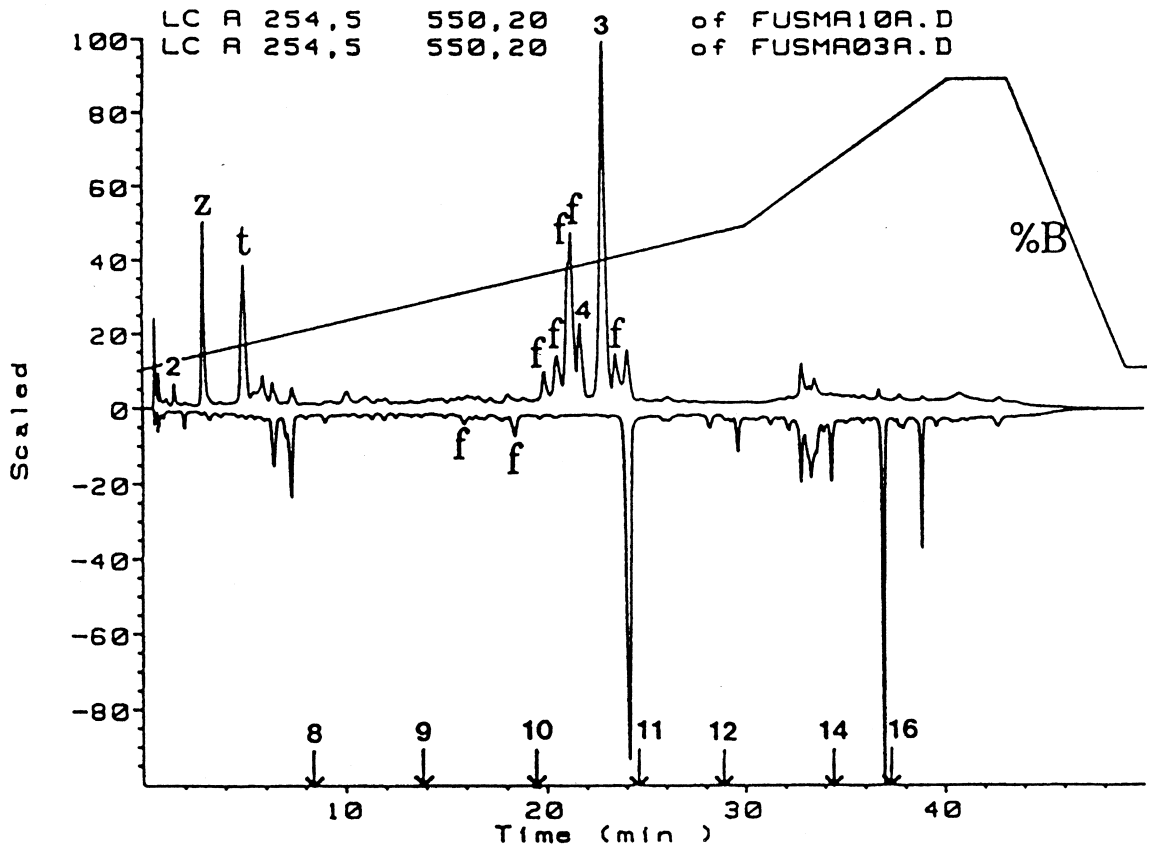

Fig. 4. HPLC traces of mycotoxin extracts from two different *Fusarium* species, the second of which is multiplied by −1. The retention times of the alkylphenone internal standards, analyzed before the two extracts, are marked as arrows on the time axis. The numbers above the arrows are the number of carbon atoms in each alkylphenone. Mycotoxins from biosynthetic families are: f, fusarins (4, fusarin C); z, zearalenones (3, zearalenone); t, trichothecenes (2, deoxynivalenol). Several peaks represent unknown chromophore families. (Reprinted by permission from Ref. [1].)

The authors reported that the only compound of those tested which showed extensive peak broadening was terrestric acid, which is not a carboxylic acid. The trifluoroacetic acid buffer used in the organic component appeared to suppress carboxylic acid ionization adequately.

Possible methodological pitfalls mentioned by Frisvad and Thrane [1] could be irreversible binding of secondary metabolites to the reversed-phase analytical column or reduced sensitivity to type A trichothecenes, which have weak UV chromophores. For the latter reason, the authors did not detect sterols and would not have detected fumonisins or AAL toxins [37]. Frisvad and Thrane did not determine limits of detection or recoveries for any of the target analytes. Nevertheless, they felt that their approach "... should be of great value in developing multi-mycotoxin methods for food and feedstuffs ... [particularly for] groups of mycotoxins, e.g., alkaloids, acidic mycotoxins and terpene mycotoxins."

3.3. Analysis of carboxyl-containing mycotoxins not included in the multi-target method

3.3.1. Diplodiatoxin, roseotoxin B, and sambucinic acid

Diplodiatoxin, $C_{18}H_{28}O_4$ with a molecular weight of 308 [38], contains no strong UV chromophores. It

could be separated from other compounds, but UV monitoring at 225 and 254 nm would not be satisfactory. In addition to the carboxylic acid moiety, the molecule contains one keto carbonyl, one hydroxyl group, and one carbon–carbon double bond. Pre-column fluorescence labeling of the hydroxyls is theoretically possible, by using 7-methoxycoumarin-3-carbonyl azide or 7-methoxycoumarin-4-carbonyl azide in dichloromethane, to form carbamic esters [39]. Alternatively, post-column reaction of the carboxylic acid function of diplodiatoxin with 4-bromomethyl-7-acetoxycoumarin in 0.1 *M* borate buffer (pH 11) in a heated reaction coil (50°C) should lead to a strongly fluorescent derivative [40]. This method is, in principle, suitable for any of the carboxylic acid mycotoxins which require derivatization and are not thermally labile. It is compatible with gradient elution, gives a fluorescence quantum yield independent of mobile phase composition, and has detection limits in the low femtomole range.

Roseotoxin B is a toxic cyclodepsipeptide (*cyclo*-2-hydroxy-4-pentenoyl-*trans*-3-methylprolyl-L-isoleucyl-*N*-methylvalyl-β-alanyl-*N*-methylalanine) [9]. It can be separated by conventional reversed-phase HPLC but has poor sensitivity for UV detection at 254 nm [41]. The mass detection limit for roseotoxin by the method of Engstrom et al. was only 250 ng. Also, the separation did not resolve roseotoxin B from a different mycotoxin, rubratoxin B. According to Edwards and Lillehøj [42], cyclic peptides separated by acetonitrile–water show reasonable sensitivity by diode array UV absorption measured at 212 nm.

Sambucinic acid, a minor metabolite of *Fusarium sambucinum*, was discovered by Rösslein et al. [43], who elucidated its structure as a carboxyl-containing compound otherwise similar to the trichothecenes and sesquiterpenoids. In announcing its discovery and structure elucidation, Rösslein et al. did not report the separation techniques used for isolating sambucinic acid from the large-scale fermentation broth. The molecular structure contains no conjugation, but has one double bond remote from the carboxyl group. One hydroxyl group would be available to form a carbamic ester, using 7-methoxycoumarin-3-carbonyl azide or 7-methoxycoumarin-4-carbonyl azide in dichloromethane, as discussed above for diplodiatoxin [39]. Sambucinic acid would present some of the same analytical challenges as the fumonisins and AAL toxins.

3.3.2. Ochratoxin A and citrinin

An analytical separation for ochratoxin A and its methyl ester, using ion-pair HPLC at pH 7.5 with fluorescence detection, has been developed by Breitholtz et al. [44,45]. The authors reported no problems associated with this type of separation; they continued to use the method in further work [46].

A different method for ochratoxin A, using immunoaffinity column clean-up and an enhanced fluorescence detection technique, was published by Zimmerli and Dick [47]. For the analytical separation, the authors chose a reversed-phase C_{18} column with an acidic buffer. They rejected ion-pair separation because the retention times proved hypersensitive to minor variations in methanol concentration. They also preferred the chemical stability of traditional C_{18} HPLC columns, although these columns did not allow co-analysis of citrinin with the ochratoxin A.

The fate of ochratoxin A during the processing of various meat products has recently been studied [48]. Unfortunately, the interesting and detailed quantitative results in this paper were not accompanied by a description of the methods used to obtain them.

Ion-pair HPLC with fluorescence detection was used by Franco et al. [49] to determine citrinin in fungal cultures and cheese extracts. The authors reported that retention times could be stabilized by using 5.7×10^{-4} *M* tetrabutylammonium hydroxide, adjusted with HCl to pH 5.5, as a mobile phase. The peak for citrinin was symmetrical and appeared at about 11 min. Postcolumn acidification of the mobile phase with 0.2 ml/min of 1 *M* HCl increased citrinin fluorescence by a factor in excess of three orders of magnitude so that the necessary sensitivity was obtained. Franco et al. advanced the combination of ion-pair HPLC and fluorescence detection ''to achieve the best possible selectivity in complex samples.'' They are currently adapting the method [49] for simultaneous determination of citrinin, ochratoxin A, and several non-carboxylic acid mycotoxins produced by *Aspergillus* and *Penicillium* species. The objections to the use of ion-pair chro-

matography for acidic mycotoxins appear to have been overcome.

3.3.3. Fumonisins and AAL toxins

The analysis of AAL toxins and the fumonisins will be considered separately from the organic-soluble mycotoxins in Section 5 and Section 6 below.

3.4. Immunochemical methods (technological competitors to chromatography)

Immunochemical methods are attractive because they are rapid and inexpensive, compared to chromatographic methods, and because they may also be usable in the field. In some cases, immunochemical response may detect metabolites as well as the target compound. Alan Patey of the United Kingdom Food Analysis Performance Assessment Scheme, in a personal communication of August 4, 1997 [50], described discrepancies between sulfamethazine assays using TLC or HPLC and enzyme-linked immunosorbent assays (ELISAs), which were consistently much too high. The cause of the systematic overestimates by ELISA has not been determined. This should serve as a warning of possible problems, especially where analysis is made of tissue extracts or other samples that may contain cross-reacting chemicals, such as metabolites or biosynthetic analogues.

Fusaric acid, a mycotoxin produced by several *Fusarium* species, increases melatonin levels in the weanling rat and in pineal cell cultures; the effect shows a dose-related response [51]. The quantitative data were derived from ELISA- and HPLC-measured levels of melatonin in serum and in pineal gland extracts. The ELISA results were validated by spiking a melatonin standard into the pineal gland extracts. The ELISA data typically showed a standard deviation of more than one-third the magnitude of the measurement: e.g., 35.5 ± 12.8 pg/g. Also, the HPLC results were typically half the magnitude of the corresponding ELISA measurement.

Immunochemical and analytical assays may have different selectivities. If immunochemical methods can be improved (made sufficiently rugged) and designed for specificity related to toxicological significance, they may relegate the more expensive chromatographic approaches to research-related applications involving unknown targets and (when combined with MS) to regulatory enforcement applications requiring legal proof.

4. GC methods for analysis of mycotoxins

4.1. GC analysis of organic-soluble mycotoxins

A few carboxylic acid mycotoxins may be analyzed directly by GC. Naphthalic anhydride has been analyzed in urban aerosols by GC–MS [52], not because it is a fungal metabolite but because it is one of many polycyclic aromatic hydrocarbons associated with industrial pollution. Butenolide, a *Fusarium* toxin, has been determined in wheat, barley, and rice at levels down to 10 ng/g by GC with an electron-capture detector [53]. GC–FTIR spectroscopy was used to analyze 13 different *Fusarium* mycotoxins, without derivatization, on a DB-5 30 m$\times$0.25 mm I.D. column [10]. However, no fumonisins or other carboxyl-containing mycotoxins were included.

Most mycotoxins are not volatile and must be derivatized to make them amenable to GC [13]. This approach is widely used for trichothecenes, nearly all of which lack carboxyl groups. Several derivatization methods for mycotoxin GC analysis have been developed [54].

The trimethylsilyl (TMS) derivatives of mycophenolic acid and its methyl and ethyl esters can be analyzed by GC with a flame ionization detector (FID) [55]. Doerfler et al. [56] published an excellent study of the biosynthesis of mycophenolic acid, addressing issues of its function during sporulation of *Penicillium brevicompactum*. They added [1-^{14}C]acetate to fungal cultures at various intervals. Then they used radiogas chromatography and radiogas chromatography–MS as well as NMR, photomicroscopy, scanning electron microscopy, and wet-chemistry techniques to study the fungal development. Mycophenolic acid was produced at all developmental stages, but its concentration increased when the hyphae began to aggregate together into pellets.

Electron affinity (electron capture) packed-column GC has been used to determine β-nitropropionic acid (as its pentafluorobenzyl derivative) in mold and

moldy cheese [57]. For peaks at least 1 min wide at half height, eluting at a retention time of 15 min, the authors obtained detection limits of 1–3 μg/g. No doubt, a modern capillary GC method for β-nitropropionic acid would be much more sensitive.

4.2. GC analysis of fumonisins

Sydenham et al. [58] first showed that fumonisins could be analyzed by GC and GC–MS. They hydrolyzed the two tricarballylic acid (TCA) side chains of FB_1 and re-esterified them for GC–MS confirmation. Unfortunately, since only the esterified TCA chains could be detected and not the aminopolyol backbone, which contains the molecular variations of the most common fumonisins, this method would not be adaptable for general fumonisin analysis. It was offered as a screening method before more informative techniques had been developed.

TMS-derivatized fumonisin B_1 has been determined by GC-FID and GC–MS, using a 5 m×0.53 mm I.D. DB-5 capillary column [59]. TMS-FB_2 eluted before TMS-FB_1 (retention times=14.5 and 16 min, respectively) and produced the narrow, well-formed peaks customary with capillary GC. The electron ionization spectra of the TMS-fumonisins showed the expected large amounts of fragmentation. The highest mass ion observed for TMS-FB_1 had a mass-to-charge ratio (m/z) of 578, over 200 mass units less than the molecular ion. Since the focus of this paper was evaluation of a submerged culture technique for producing FB_1 in laboratory-scale quantities, the detection limits found for TMS-fumonisins by the GC and GC–MS analyses were omitted.

5. HPLC and CE analysis of trace levels of fumonisins and AAL toxins

5.1. Reasons for analyzing trace levels of fumonisins in foods

Fusarium moniliforme, which is known to produce fumonisins and other mycotoxins, has been shown by Bacon and Hinton [60] to colonize corn endophytically without causing symptoms. This fungus is able to infect the cells of the plant and persist well beyond the seedling stage with no observable disease symptoms. Although Bacon and Hinton suggested that the symptomless state ''could contribute to the total mycotoxin contaminants of maize both before and during kernel development'', they did not assay for mycotoxins.

When the production of fumonisins by 25 strains of *F. moniliforme* was analyzed by HPLC with precolumn fluorescence derivatization [61], the individual strains were found to differ greatly in the amounts synthesized. Although the amount of fumonisin produced by a strain was related to the ergosterol content, there was no correlation with biomass. The fumonisin assay used, however, could not have registered *N*-acylated fumonisin variants (or any other secondary amines).

These two papers [60,61] present significant but ambiguous findings. Trace analysis sensitive to *all* fumonisin mycotoxins is required to resolve the ambiguities, but this requirement is not easily met. The fumonisins and AAL toxins present a cluster of distinct analytical challenges. They are sufficiently toxic to require trace analytical methods that sometimes necessitate concentration steps to obtain sensitivity. They have low volatility. The fumonisins and AAL toxins are not amenable to high-temperature GC analysis without derivatization. Both mycotoxin classes lack a strong UV chromophore and so cannot be directly determined by HPLC-UV. They are formed or sequestered in complex matrices (food, tissue, physiological fluids) so that analytical selectivity is required to differentiate them from environmental co-extractants, yet their molecules do not contain distinctive moieties useful for selective detection. The fumonisins contain four, and AAL toxins two carboxyl moieties, which necessitate use of a strong acid buffer, such as trifluoroacetic acid (TFA), for good reversed-phase HPLC peak shape; but TFA degrades fumonisin whenever a sample is concentrated [62], even if it is buffered and evaporated under vacuum at low temperature. For all these reasons, methods for the analysis for fumonisins are still being developed 10 years after their identification.

5.2. HPLC methods for fumonisins with precolumn fluorescence derivatization

The first derivatization methods for HPLC analysis of fumonisins used maleic anhydride with UV

detection or fluorescamine with fluorescence detection [58]. Maleic anhydride produced a 10-μg/g detection limit, which is not sensitive enough for incurred residues, and also had matrix-related limitations. Fluorescamine produced two separate peaks for FB_1, representing the acid alcohol and lactone derivatives of the fluorescent complex, and in others' opinion [63] was therefore not suitable for quantitation.

Eventually, reaction with *o*-phthaldialdehyde (OPA) was adopted as a more sensitive approach. Although this method has a 50-ng/g detection limit [27], the OPA derivatives of the fumonisins are unstable. To obtain quantitatively valid results, samples must be injected onto the HPLC column, at reproducible intervals, no later than 4 min after the addition of the reagent. Analyses delayed for 1 h result in a 50% loss of signal [64]. Even with the instability problem, OPA derivatization of fumonisins has become a standard HPLC technique [65–71] and has been adopted as an Association of Official Analytical Chemists International (AOAC) approved method [72] for determination of FB_1, FB_2 and FB_3.

Other fluorescence derivatization reagents have also been used, including 4-fluoro-7-nitrobenzofurazan (NBD-F) [69,73] and (9-fluorenylmethyl)chloroformate (FMOC). Using FMOC, Holcomb et al. [74] demonstrated 200-ng/g detection limits for FB_1 in spiked rodent feed, less than 1 min derivatization time at room temperature, over 72 h stability of the FMOC–FB_1 complex, and reasonable chromatographic separation. Any problems with the use of FMOC for derivatization are not obvious. Naphthalene-2,3-dicarboxaldehyde (NDA) has been used for the HPLC determination of FB_1 and FB_2 in milk at levels down to 5 ng/ml because the fumonisin–NDA derivative is relatively stable [75–77]. Derivatization with fluorescein isothiocyanate (FITC) [78] has also been proposed; the major disadvantage of FITC is the 3 h needed for complete reaction.

5.3. Comparison of ELISA techniques with HPLC fluorescence techniques

Maragos and Richard [75] compared the sensitivity of the NDA-fluorescence method and a method adapted from a commercially available ELISA for determining fumonisins in milk. Although the immunoassay was sensitive to 250 ng/g of fumonisin in milk, the HPLC method was sensitive to as little as 5 ng/g of fumonisin in milk. The authors hypothesized that milk fats and proteins might have affected the immunoassay.

Using an ELISA as the only analytical technique [79], the carryover of fumonisin B_1 into the milk of cows has been measured. The concentration detection limit in milk and plasma was reported as 0.5 μg/kg (=0.5 ng/g), which is a six-fold improvement over that found with Scott et al.'s HPLC techniques using OPA and NBD-F [80]. The ELISA method, however, necessitated the use of a mathematical transformation to establish a usable calibration curve. Scott et al. did not use an ELISA but incorporated an immunoaffinity clean-up column for fumonisin analyses and a solid-phase extraction (SPE) cartridge for the aminopentol fumonisin 'backbone' (AP_1) before analysis by HPLC. Even using such a selective clean-up, they observed an artifact peak, which co-eluted with AP_1, in milk samples that had been stored at 4°C for too long.

In their 1994 paper, Maragos and Richard [75] compared their NDA-derivatization method to an ELISA for determination of fumonisins B_1 and B_2 in milk. For the ELISA test, they reported a ''concentration that inhibits color development by 50% (IC_{50})'' of 1200–1600 ng of FB_1/ml. An IC_{50} is not a detection limit, and it would be difficult to infer a detection limit from it. Whatever the number signifies, Maragos and Richard considered the ELISA test insufficiently sensitive for milk sample screening.

In contrast, Sutikno et al. [81] recommended a polyclonal sheep antibody-based ELISA for screening of corn, animal feed, and human food followed by HPLC (without MS) for confirmation. The authors found consistently higher values (1.3–2.9 times) for total fumonisins in all samples with their polyclonal antibody ELISA than they found for FB_1 alone by HPLC. From this, they hypothesized the probable presence of FB_2, FB_3, or structurally similar compounds that might cross-react with the ELISA. The somewhat higher values were a dramatic improvement over those obtained by earlier ELISA methods [82], using monoclonal antibodies, in which fumonisin concentrations were determined over 400-fold greater than corresponding HPLC-

determined values. For the HPLC determinations, Sutikno et al. [81] used a version of the isocratic separations of Sydenham et al. [58] and Shephard et al. [27] with detection of the OPA derivatives. These separation methods should have been able to detect FB_2 and FB_3.

The results of a total fumonisin analysis using polyclonal antibody ELISA have been compared to the results of OPA fluorescence–HPLC analysis for 20 corn samples, 18 of which proved to be naturally contaminated with FB_1, FB_2, and/or FB_3. [83] Most of the ELISA determinations were somewhat greater than the corresponding ones for HPLC, as expected, but two were actually lower. The differences between the two assays were much smaller than those previously reported for other mono- and polyclonal antibody systems. The authors concluded that their polyclonal antibody ELISA would be acceptable for initial screening of fumonisins in corn.

The issues of selectivity and matrix-altered sensitivity remain significant for ELISA methods. Further improvements will be required before more-rapid, less-expensive ELISA techniques can be substituted for chromatographic quantitation of fumonisins. However, if accurate quantitation by ELISA can be combined with appropriate confirmation by either LC–MS or CE–MS, then fluorescence derivatization for routine analysis of fumonisins by HPLC might become unnecessary.

5.4. CE methods for analysis of fumonisins

Methods using CE have the virtue of dramatically reducing the consumption of organic solvents when compared to HPLC methods. CE combined with electrospray MS detection has been used for analysis of FB_1 [84]. Using uncoated columns, the system produced separation efficiencies of 44 000 theoretical plates/m. Quantitation was accomplished relative to tetramethylated FB_1 used as an I.S. The concentration detection limit was 156 ng/g for an estimated injection mass of 1.1 pg and with a signal-to-noise ratio of 10. Quantitative anomalies included a standard curve that reached a plateau with high masses of toxin as well as poor reproducibility (relative standard deviations in spiked corn extracts were 2–30% for the I.S. and 6–35% for the analyte). A subsequent study [85], however, mentioned that the original method would not separate FB_1 from FB_2 and thus is inadequate for general purpose analysis.

Holcomb and Thompson [85] succeeded in separating the FMOC derivatives of FB_1 and FB_2 by CE. The minimum detectable amount (2× background) for FB_1 from feed was 500 ng/g; recoveries of spiked extracts averaged 87% over a range of 2–20 μg/g. Relative standard deviations over the same range varied from 1.4 to 12.6%. This paper shows electropherograms in which the retention time of FB_1–FMOC varied from 15.5 min in a spiked extract to 16.2 min in a standard. Quantitative CE results agreed closely with parallel HPLC results. However, replicate CE analyses showed two to three times greater variability than the corresponding HPLC determinations.

Maragos et al. [76] achieved similar quantitative figures of merit for fumonisins, using CE with pre-column FITC derivatization. Quantitation required a sigmoidal calibration curve, but as little as 50 ng/g FB_1 could be detected. No obvious anomalies in either separation or detector response were observed in the electropherograms shown.

5.5. Mass spectrometric techniques

5.5.1. Electrospray LC–MS of fumonisins

Korfmacher et al. [86] first demonstrated the sensitivity of electrospray MS for ionization of directly infused solutions of fumonisin B_1. Doerge et al. [87] later used electrospray LC–MS in the first reported HPLC separation and analysis of underivatized fumonisins. Using a hydrophobic polymeric column, with a gradient of aqueous ammonium acetate and acetonitrile, they separated FB_1, FB_2, and FB_3 completely in less than 11 min. Curiously, the second eluting of the three isomers, FB_3, showed significantly greater band-broadening than the other two. On-column detection limits for fumonisins spiked into corn meal extracts were between 20 and 100 ng/g and the linearity of response was good. The figures of merit did not reflect sample losses associated with extraction and clean-up, and the experimental methods did not permit acquisition of recovery data. In-source collision-induced dissociation (cone voltage fragmentation) did not produce fragmentation of the protonated molecules $[M+H]^+$ to give additional confirmatory information. The general suitability of electrospray LC–MS for de-

tection and quantitation of underivatized fumonisins, in the presence of potential matrix interferences, was demonstrated.

An electrospray LC–MS method was used by Josephs [88] for detecting and characterizing minor contaminants in a relatively pure (>98%) sample of FB_1. Two different mass spectrometers served as LC detectors: an ion trap, capable of multi-stage tandem (MS^n) experiments, and a triple quadrupole. As before, the electrospray ion source produced only protonated molecular ions, $[M+H]^+$ for the fumonisins. However, 35-eV argon collision-induced dissociation in the triple quadrupole yielded abundant fragmentation of diagnostic value for elucidation and confirmation of structure. Multiple and sequential collisions inside the argon collision cell did not allow distinguishing the particular pathways by which ion fragments were formed. The ion trap, by its MS^n capability, provides ways to investigate fragmentation pathways and to distinguish singly charged from multiply charged species. The information acquired from these fundamental studies was used to develop a precursor-ion mode LC–MS–MS method. This method could detect and characterize, in a single chromatographic run, all compounds with the characteristic fumonisin backbone that generated a product ion at m/z 352. For simultaneous generality and sensitivity of the assay, the acquisition used a data-dependent software algorithm to control the triple quadrupole mass spectrometer. The combination of electrospray ionization with two sophisticated MS systems proved a flexible and elegant, but expensive, solution for fumonisin analysis.

Lucas et al. [89] used electrospray quadrupole tandem MS for qualitative and quantitative analysis of FB_1 and FB_2 in corn and corn products. With deuterated FB_1–D_6 as an I.S., they obtained a routine quantitation limit for incurred residues of 400 pg (0.8 ng/g) for FB_1 with a signal-to-noise ratio of 10:1. Rather than data-dependent MS–MS, they used time-dependent selected-reaction monitoring for qualitative analysis of the two target analytes in the corn extracts. For quantitation, a second chromatographic run was conducted, using selected-ion monitoring of protonated molecules of the two analytes and the I.S., which was incorporated at the approximate levels of fumonisins found during the initial qualitative analysis. All experiments were conducted on incurred residues; extraction efficiencies were not reported. Of six samples of corn grits and two of corn meal, all contained between 1 and 200 ng/g of one or both fumonisins. Fourteen other samples of corn flakes, popcorn, baby food, and sweet corn showed little or no fumonisin contamination.

Electrospray LC–MS methods appear to be excellent for qualitative and quantitative analysis of fumonisin mycotoxins. Their only apparent limitation, for general purpose analysis, is the cost of the instrumentation.

5.5.2. Other MS and LC–MS methods for fumonisins and AAL toxins

Most of the other work using MS and LC–MS methods was done when analysts were struggling to find reasonable and definitive methods for analysis of fumonisins. This work is mainly of historical interest but may be of practical use in some laboratories.

Liquid secondary-ion mass spectrometry (liquid SIMS) was used in early work [2,90] to elucidate the structure of FB_1. Chen et al. [91] used a variety of mass spectrometric techniques plus continuous-flow SIMS, preceded by a C_{18} microcolumn with reversed-phase gradient HPLC at 3 μl/min, for separation, detection, and identification of FB_1 from liquid cultures of *Alternaria alternata* f. sp. *lycopersici*. They also used reversed-phase, isocratic HPLC–ion-spray MS and MS–MS (1 ml/min flow-rates with a 9:1 split), GC of hydrolyzed toxins by the method of Plattner et al. [90], and glycerol FAB-MS for confirmation of identity. All three of these methods appear to be suitable for analysis of both fumonisin and AAL toxin. Analytical figures of merit were not reported, but the methods were applicable for FB_1 concentrations between 5 and 140 μg/g.

The feasibilities of thermospray, fast-atom bombardment (FAB), and electrospray ionization modes for mass spectrometry of FB_1 was examined by Korfmacher et al. [86]. The study did not include on-line HPLC separation, but thermospray and electrospray sample introduction involved liquid flows (1.25 ml/min for thermospray and 1 μl/min for electrospray). With thermospray MS, both the spectral quality and the sensitivity for FB_1 were unacceptably poor. The protonated molecule at m/z 722 was only 10% of the base peak; flow injections of 1 μg each gave weak responses. With electrospray MS, the protonated molecule was the base peak; the

only other significant ions were Na^+ adducts of the molecular ion. A clean electrospray mass spectrum was obtained by infusion of a 5-ng/μl FB_1 solution.

The flow-rate limitations of electrospray MS sources have now been overcome. Best results were obtained by Korfmacher et al. with FAB-MS and FAB-MS–MS. The latter of these techniques has been advocated [63] for non-chromatographic screening of foodstuffs and crops.

After derivatizing fumonisins to their tetramethyl esters, Young and LaFontaine [92] used reversed-phase HPLC with a particle-beam interface to yield chemical ionization and electron ionization mass spectra. They added L-serine and other polyfunctional amino acids as post-column buffers; L-serine produced a 100-fold increase in sensitivity. L-Serine was preferred to L-glutamic acid because of its lower mass, higher solubility, and greater ease of cleaning. More volatile buffers (e.g., ammonium acetate), usually added as carriers in particle beam-MS, did not increase the sensitivity and neither did L-serine methyl ester. Although Young and LaFontaine did not report a limit of detection, calibration curves contained data points for approximately 10 ng of material. The necessity for frequent disassembly and cleaning of instruments, caused by the continuous flow of L-serine solution, probably represents a significant disadvantage.

On-line capillary LC with FAB-MS has been used [93] to distinguish naturally occurring structural isomers of partially hydrolyzed FB_1. Abbas and Riley [94] evaluated the presence and toxicity of fumonisins and AAL toxins in *Alternaria alternata*; analytical confirmations were performed by C.J. Mirocha (who was acknowledged but not listed as a co-author), using continuous-flow FAB-MS. These papers exemplify the utility of LC–MS and LC–MS-MS for examination of mycotoxin contaminants in agricultural matrices.

6. Purity analyses of fumonisins for analytical standards or toxicological studies

6.1. Mycotoxin purity analysis: a distinctive analytical challenge

The purity of a mycotoxin preparation is important for toxicological studies because it is necessary to determine that any effects observed in the study are due to the toxin itself, not to other compounds present. Purity studies of analytical standards or raw ingredient batches added to feed for animal toxicity studies do not require the same high degree of chromatographic detector sensitivity as that needed for trace analysis. The most important qualities for detection in purity analysis are quantitative integrity and universality of response to all potential contaminants.

Marasas, one of the discoverers of the fumonisins, in 1994 [6] described the FB_1 standard used in his laboratory as over 98% pure. At that time, this FB_1 product was referred to colloquially as the South African gold standard. Shortly thereafter, HPLC chromatograms of this fumonisin standard made using an evaporative light-scattering detector (ELSD) [95] showed several impurity peaks, which together amounted to greater than 5% of the total. The South African gold standard was not 98% pure, but a new technology better suited to assess fumonisin standard purity had become available.

6.2. Limitations of fluorescent-labeling techniques for fumonisin purity analysis

Derivatization of FB_1 for purity analyses by HPLC introduces quantitative uncertainty through partial, indiscriminate attachment of the fluorescent or UV-absorbing tag to different components of the mixture. To minimize these effects, the reagent tag must be added to the sample in great excess. The excess reagent typically appears in the chromatogram as a huge, broad peak, which obscures any impurities eluting near it. (See the chromatograms of maleyl- and fluorescamine-derivatized FB_1 shown by Sydenham et al. [58]).

The inappropriateness of relying exclusively on fluorescence or other labeling techniques for analysis of purity can be further illustrated by the recent discovery of *N*-acetylated fumonisin B_1 (FB_1 acetyl amide, or FA_1; see Fig. 1) [96], a relatively minor contaminant in biosynthetic FB_1 batches purified for long-term, low-dose rodent toxicity studies. In FB_1 acetyl amide, the primary-amine target of labeling techniques is already occupied so that purity analyses based on labeling would miss this contaminant. Therefore, several scientists have begun to investigate methods for mycotoxin purity determination that

would be different from the pre- or post-column labeling methods used for ultra-trace analysis of incurred residues in foods or tissues.

6.3. HPLC with evaporative light-scattering detection for purity analysis

The evaporative light-scattering detector (ELSD) for HPLC is relatively new. The analytical signal is obtained from light reflected off the low-volatility residual particles of dried aerosol droplets formed by nebulizing the HPLC eluent. Because structurally dissimilar low-volatility solutes produce similar mass responses, an ELSD placed downstream from a good quality separation can indicate sample purity by the peak areas of separated contaminants and the main constituent.

Wilkes et al. [97] first demonstrated the ability to separate underivatized fumonisins by HPLC and detect them using an ELSD. Although the sensitivity achieved was not sufficient for trace analysis in food (the detection limits were about 3 μg/g), the flat baselines produced during gradient elution chromatography allowed the development of a separation analogous to that of Frisvad and Thrane [1]: i.e. a reversed-phase, large-range solvent composition gradient suitable for separation of both hydrophobic and hydrophilic compounds.

The LC–ELSD system meets some, but not all, of the criteria for optimal purity analysis. One significant characteristic of the ELSD is that the response, although it is reproducible, is not a linear function of particle mass. Therefore, even the peak areas of impurities and the masses they represent cannot be related proportionally to the peak area and mass of the primary constituent. By using a log–log calibration curve and adjusting for differences between the retention time of the main component used to generate the calibration and the retention time of each impurity, it is possible to calculate a compensated relative mass for each chromatographically separated impurity [98]. By adding up the contributions of all of the separated impurities and comparing the total to the total mass injected, an upper bound for sample purity can be calculated. Obviously, contaminants unresolved from the major component cannot be distinguished from it. Also, high volatility contaminants will evaporate with the solvent droplets and not be detected. These two effects combine to underestimate contamination.

This problem of volatile contaminants was observed in a batch of fumonisin B_1 prepared for use in a toxicological study. Evans discovered, by NMR analysis, that 46 mol.% of the 'purified' fumonisin B_1 sample was pyridine, a compound too volatile to be observed by HPLC–ELSD [99]. This analysis led to the rejection of the batch and a change in the clean-up procedure used by the producer. Hansen then developed an HPLC–photodiode array UV method to quantify pyridine; a subsequent batch of fumonisin B_1, containing $0.140 \pm 0.003\%$ w/w of pyridine, also was rejected [100]. FB_1 rodent toxicology studies would be compromised if a compound already known to cause kidney and liver damage and depression of the central nervous system [101,102] were inadvertently added with the fumonisin to rodent feed.

The ELSD is not a universal detector. Nevertheless, the ability of HPLC–ELSD to detect, with similar signal/mass sensitivity, virtually all low-volatility constituents commends the system for purity analysis.

6.4. Use of electrospray LC–MS for fumonisin purity analysis

Musser, of the United States Food and Drug Administration, determined [96] analytical figures of merit for positive- and negative-ion electrospray LC–MS for fumonisin purity analysis. He purified and weighed quantities of FB_1, FB_2, both half and fully hydrolyzed FB_1, and FB_1 acetyl amide (which he had discovered in *Fusarium* cultures while producing gram-quantity FB_1 to be purified for toxicological studies). By positive-ion electrospray MS, the first three compounds showed equimolar responses. Fully hydrolyzed FB_1 produced a molar response approximately twice that of the first three. In the negative-ion electrospray MS mode at pH 4.5, the molar response for FB_1 acetyl amide was about three times that of FB_1. These numbers illustrate the broad, but not universal, nature of the electrospray MS response for fumonisins. The differential sensitivities show that the method is not ideal for generic purity assays, but it would be useful when a comprehensive list of potential impurities with their relative response

factors had already been determined, e.g., for quality assurance of toxin batches prepared for research.

6.5. Comparison of electrospray LC–MS and LC–ELSD purity determinations

Two batches of FB_1, one much less pure than the other, were analyzed by both electrospray LC–MS and LC–ELSD by Wilkes et al. [98]. For the cleaner sample, LC–ELSD- and electrospray LC–MS-based purities were 97.3 ± 0.08 and $94.0 \pm 0.4\%$, respectively. The previously discussed limitations on LC–ELSD purity analysis explain why the 97% value should be considered an upper bound. High solvent backgrounds with electrospray MS necessitate the omission of low-mass ions from acquisitions, which could lead to overlooking volatile small molecule contaminants. Most of the analytical characteristics of electrospray LC–MS are useful for the detection and quantification of contaminants, but the method may even overestimate contamination relative to fumonisin B_1. These characteristics include the abilities to distinguish non-isobaric impurities co-eluting with the sample main component, to ionize both high- and low-volatility species, and to give a response for each analyte that varies linearly with mass. Since the efficiency of electrospray ionization is significantly greater for some common impurities than for FB_1, if the low-mass constituents can be safely ignored, sample purity calculated by electrospray LC–MS may be regarded as a lower bound. For the 'dirty' fumonisin samples, the LC–ELSD-calculated upper bound for purity was 73%, whereas the electrospray LC–MS lower bound was 52% [98].

When samples are of sufficient purity for use in toxicological studies, the LC–ELSD and LC-ES–MS methods converge toward agreement. The ELSD is much less expensive than a mass spectrometer, but the mathematical manipulations required to interpret its non-linear response are time-consuming. The electrospray LC–MS is much more expensive and uses an ionization mode in which structure-dependent discrimination is a well-known phenomenon. Neither instrument produces an unequivocal purity result, but either one is an improvement over the methods previously available.

Truncated chromatograms of the 'dirty' FB_1 sample, acquired using gradient elution reversed-phase LC with an ELSD and positive-ion electrospray MS, are shown in Figs. 5 and 6, respectively. (Reprinted with permission.) The HPLC columns and gradients were not identical, but there is a striking qualitative correspondence between the two traces.

Fig. 5. Truncated Varex LC–ELSD chromatogram from a 4-μl injection of a 'dirty' FB_1 sample dissolved in water at 3.03 μg/μl. (Reprinted by permission from Ref. [98].)

When Plattner et al. [103] used LC–ELSD and electrospray LC–MS for fumonisins in corn samples, they reported usable mass detection limits for the ELSD as 10–50 ng (which is insufficient sensitivity for incurred residues, without sample clean-up and a 10–100-fold concentration); for electrospray LC–MS the mass detection limit was less than 1 ng injected. This excellent article discusses a number of other factors that affect the performance of electrospray MS for fumonisins.

6.6. LC–CRI-MS and LC–PDPID as possible alternative methods

Chemical reaction interface mass spectrometry (CRI-MS) was developed by Markey and Abramson [104] for element- and isotope-selective studies of

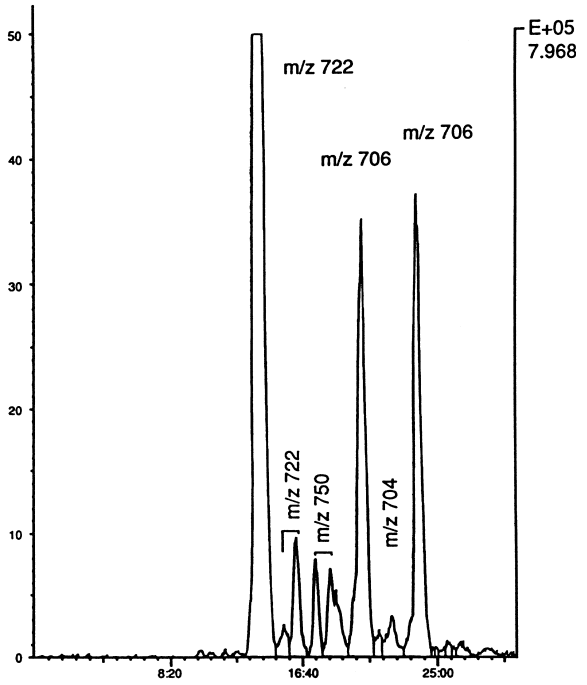

Fig. 6. Truncated electrospray LC–MS reconstructed ion chromatogram, over the range of m/z 563–566 plus 700–760, of the same 'dirty' FB_1 sample as Fig. 5 (Reprinted by permission from Ref. [98].)

drug and toxin metabolism. The CRIMS interface digests chromatographically separated analytes by atomizing them in a microwave plasma. The elements are reacted with a reagent gas (e.g., SO_2) to produce small, stable molecular products (e.g., CO_2 from sample carbon and NO from sample nitrogen), which are leaked into the mass spectrometer to give a response (at m/z 44 for CO_2 and m/z 30 for NO). If interferences from solvents and volatile buffers are removed, CRIMS can be used as a linear, non-discriminating HPLC detector [105] suitable for detection of all low-volatility organic compounds. The HPLC–CRI-MS system uses a solvent-and-volatiles-removal interface to reduce background upstream of the reaction interface and mass spectrometer; the latter becomes a mass detector for low-volatility organic compounds.

The pulsed-discharge photoionization detector (PDPID) (Valco Instruments, Houston, TX, USA) gives universal, linear, and nearly uniform molecular molar response over a dynamic range in excess of four orders of magnitude [106]. It has a mode of operation [107] with greatly reduced or no response for water and acetonitrile but with excellent sensitivity (picogram levels) for most other organic compounds. The concept of the LC–PDPID shows great promise for addressing a variety of analytical issues, including the purity analysis problems discussed here. It might even be usable for screening food and tissue extracts if highly selective immunochemical clean-up procedures are included in the method.

7. Perspectives

Mycotoxins containing carboxylic acid moieties present a number of analytical challenges related to extraction and clean-up from food and other biological matrices, chromatographic separation, and detection. The problems are exacerbated for the fumonisins and AAL toxins, which are not directly amenable to GC and cannot be directly measured by HPLC UV or fluorescence detectors.

Liquid–liquid extraction efficiencies for some carboxylic mycotoxins are marginal for spiked samples and uncertain for incurred residues. One study using supercritical fluid extraction for fumonisin in corn reported 40-fold (sic) efficiency increases relative to a standard liquid–liquid technique.

Immunoaffinity clean-up techniques with high resolution chromatography show particular promise. Recent advances using tandem or mixed selectivity immunoaffinity cartridges demonstrate the feasibility of multi-target mycotoxin assays. Immunochemical (ELISA) techniques not tied to chromatographic separations represent a technological competitor that promises rapid, cheap, sample screening for mycotoxins in food. However, significant questions of matrix interference and selectivity affect the quantitative integrity of ELISA techniques.

The best solutions to separation and detection challenges include precolumn fluorescence derivatization with HPLC for trace level detection and electrospray LC–MS for trace level detection and confirmation. Electrospray LC–MS and HPLC with evaporative light scattering detection are commercially available systems suited to assess the purity of mycotoxin standards. Because no system is perfect for general purity analysis, several systems with

complementary characteristics should be used in critical applications, such as the certification of materials destined for toxicology studies.

The need remains for improved separation and detection of carboxylic mycotoxins. Few workers have adapted more rapid or efficient separations (e.g., nonporous microsphere HPLC or electrophoresis) for mycotoxin analysis. Experimental HPLC detection systems are being developed which have a linear, uniform or near-uniform molar response for low-volatility, organic compounds. These should be particularly useful for purity analyses of mycotoxin standards.

8. Disclaimer

The opinions expressed in this review are those of the authors and do not reflect official positions of the United States Food and Drug Administration.

9. List of abbreviations

AAL	class of mycotoxins produced by *Alternaria alternata* f. sp. *lycopersici*
AAL	mycotoxin produced by *Alternaria alternata* f.
AOAC	Association of Official Analytical Chemists International
AP_1	aminopentol 'backbone' of a fumonisin type B mycotoxin
CRIMS	chemical reaction interface mass spectrometry
CRM	certified reference material
ELISA	enzyme-linked immunosorbent assay
ELSD	evaporative light-scattering detector
FB_1, FB_2, or FB_3	fumonisin mycotoxin B type 1, 2, or 3, respectively
FID	flame ionization detector
FITC	fluorescein isothiocyanate
FMOC	(9-fluorenylmethyl) chloroformate
IC_{50}	analyte concentration that inhibits its color development of an ELISA by 50%
I.S.	internal standard
MS^n	multi-stage tandem mass spectrometry
m/z	mass-to-charge ratio
NBD-F	4-fluoro-7-nitrobenzofurazan
NDA	naphthalene-2,3-dicarboxaldehyde
OPA	*o*-phthaldialdehyde
PDPID	pulsed-discharge photoionization detector
SAX	strong anion exchange
SFE	supercritical fluid extraction
SPE	solid-phase extraction
TMS	trimethylsilyl
TCA	tricarballylic acid
TFA	trifluoroacetic acid

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