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REVIEW

CHROMATOGRAPHIC METHODS AS TOOLS IN THE FIELD OF MYCOTOXINS

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1. INTRODUCTION

Mycotoxins are secondary metabolites of fungi that have been recognized mostly within the last three decades as a potential threat to animal and human health. Various chromatographic techniques have been used for their separation and in only a relatively few instances have mycotoxins been obtained pure using techniques other than chromatography[1] (*e.g.*, secalonic acid, rubratoxin, patulin and citrinin[2–5]).

In the 1980s, various reviews and book chapters on the chromatography of mycotoxins were published (*e.g.*, refs. 1, 6–13). This review describes the most important achievements of chromatography in the field of mycotoxins. The text is divided into the following sections: (2) paper chromatography (PC), (3) thin-layer chromatography (TLC), (4) liquid column chromatography (LCC), and (5) gas chromatography (GC).

The literature on the chromatography of mycotoxins is immense and it would be virtually impossible to give an exhaustive survey. Whereas references to the PC of mycotoxins cover the 1950s and the early 1960s only (in order to show a historical continuity), the literature on TLC, LCC [high-performance liquid chromatography (HPLC) in particular] and GC covers mostly the early and mid-1980s.

In this review, applications of chromatograpic techniques include so-called multi-mycotoxin analysis, followed by data on structurally related families of mycotoxins and individual compounds. Data concerning recent clean-up methods, qualitative analysis, quantitation and preparative methods are also presented.

2. PAPER CHROMATOGRAPHY

In the 1950s and early 1960s, a variety of fungal secondary metabolites, currently classified as mycotoxins, were studied among antibiotics by means of various PC techniques. These techniques have been successfully used to characterize and classify antibiotics, and have also been helpful in establishing isolation procedures for unknown compounds. A few examples are given below.

In the early stages of studies on the metabolites of the toxigenic fungus *Penicillium islandicum*, PC was adopted for the identification of its hydroxyanthraquinones (islandicin, iridoskyrin, catenarin, chrysophanol, erythroskyrin, luteoskyrin, flavoskyrin, rubroskyrin and oxyskyrin) and applied to the detection of anthraquinone-producing strains[14].

Another mycotoxin, cyclochlorotine or chlorine-containing peptide, isolated from *P. islandicum*, has been characterized by PC in several solvent systems[15].

A classification of antibiotics by means of "salting-out paper chromatograms" using nine solvent systems with increasing concentrations of ammonium chloride in water was developed and the analysed antibiotics were divided into six groups (A–F)[16,17]. The classification was extended to fungal metabolites[18], including the following compounds currently classified as mycotoxins: group A, gliotoxin, kojic acid, patulin, penicillic acid and terreic acid; group C, aspergillic acid, neohydroxyaspergillic acid, citrinin and trichothecin; and group D, alternariol, cyanein (brefeldin A), griseofulvin, mycophenolic acid and rugulosin.

Another principle of classification by means of the so-called "pH chromatograms" has been introduced[19,20]. Using this PC technique, the ionic character of

unknown antibiotics and also the general possibilities of their isolation could be determined when only their crude concentrates from Petri dish cultures were available. The principles of "pH chromatography" have been reviewed elsewhere[21,22]. Several fungal metabolites belonging to mycotoxins, such as citrinin, mycophenolic acid, gliotoxin, rugulosin, neohydroxyaspergillic acid and trichothecin, have also been studied by "pH chromatography"[23,24].

PC studies of compounds in several solvent systems for the purpose of their classification and identification have proved useful in systematic analysis. A combination of the sequential and simultaneous analysis of antibiotics was elaborated by the present author. In addition to a series of antibiotics, the following mycotoxins were characterized by the method: kojic acid, penicillic acid, cyanein (brefeldin A), alternariol, rugulosin, citrinin, trichothecin, patulin, gliotoxin, mycophenolic acid, aspergillic acid and griseofulvin[25].

The three above-mentioned PC techniques were found to be helpful in the isolation and identification of citrinin produced by a penicillium strain[26]. When a crude acetone extract of metabolites from an agar culture was chromatographed in four principal solvent systems of systematic PC analysis[25] and detected with *Bacillus subtilis,* the unknown antibiotic was found to belong to subclass Va. Then a "pH chromatogram", using water-saturated ethyl acetate as the mobile phase, showed the unknown antibiotic to be an acid. According to the course of its S-shaped R_F curve it was decided that the antibiotic should be extracted from the culture filtrate with ethyl acetate at pH 2–3 and then transferred from the organic solvent into a phosphate buffer at pH 8.5. The third step was a re-extraction with chloroform at pH 2.5. (It had been found in earlier studies that the S-shaped R_F curves obtained from the "pH chromatograms" represented the dependence of the R_F values on the following factors: pH of the stationary phase, distribution coefficients between the mobile and stationary phases at these pH values and pK values[23,27]). When the purified antibiotic was again characterized chromatographically, its great similarity to citrinin was apparent. The physical, chemical and biological properties of the isolated compound confirmed its identity as citrinin[26].

In our laboratory, several strains of a collection of fungi have been found to produce substances that inhibit mycobacteria. When the active substances, present in crude extracts from agar cultures of four aspergilli, were analysed by systematic PC analysis[25], all of them belonged to subclass IIa and their "summarized chromatograms" were similar to that of kojic acid of the same subclass. The identity to kojic acid was confirmed after the isolation and purification of the active products[28].

PC was used by Sargeant *et al.*[29] in the first separation of aflatoxins from a crude extract of groundnuts. The toxic component produced a single spot that fluoresced under UV light. PC was also used by Holzapfel *et al.*[30] to isolate aflatoxins M_1 and M_2 from a concentrate from the urine of aflatoxin-fed sheep.

However, TLC using silica gel superseded PC and has resulted in the isolation and purification of almost all the aflatoxins and many related fluorescent metabolites.

3. THIN-LAYER CHROMATOGRAPHY

Historically, TLC has been the method of choice for aflatoxin analysis, following the then-recent rediscovery and development of this technique by Stahl. It was

shown as early as in 1962 by Nesbitt *et al.*[31] and, in 1963, by Hartley *et al.*[32] that the single blue-fluorescing spot of toxin-containing extract, observed by PC, could be split into four main components when the extracts were chromatographed on silica gel TLC plates developed in chloroform–methanol. Two of these components, fluorescing blue under UV light, were designated aflatoxins B_1 and B_2 and the other two components, fluorescing turquoise under UV light, were designated aflatoxins G_1 and G_2. Since then various combinations of silica gel and solvent systems have been proposed for separating aflatoxins by TLC.

Pure aflatoxins for structural determination were obtained by preparative TLC (PLC), and quantitative methods have also been developed. In some instances the aflatoxins served as the model compounds for the development of reliable methods to measure trace levels in foods and feeds. The TLC of aflatoxins has received the most attention over the years; consequently, it is the most refined and generally serves as a model for the other mycotoxins[33].

Of the chromatographic techniques applied to mycotoxins, TLC is by far the most widely used in the detection, analysis and characterization of fungal toxins. With advances in techniques, TLC is becoming the method of choice for some mycotoxins.

In the 1980s, numerous reviews and book chapters on the chromatography of mycotoxins in general and on TLC in particular have been published (*e.g.*, refs. 1, 6–13, 33–39). A recent book devoted to methods for the production, isolation, separation and purification of mycotoxins[11] included sections on the TLC of many mycotoxins.

In a chapter on TLC and high-performance TLC (HPTLC) of mycotoxins, Nesheim and Trucksess[33] described the chief procedures for mycotoxins (including multi-dimensional development, screening methods, quantitation and identification) and presented analytical methods for the best known mycotoxins. In a recent review on the TLC of mycotoxins[12], the literature from 1961 to 1984 was covered. Therefore, in this review, only the major achievements of TLC in mycotoxicology published in the 1980s are presented.

3.1. Multi-mycotoxin TLC

Various multi-mycotoxin methods have been published for the simultaneous detection, in natural products, of a number of mycotoxins, which differ in the extraction solvents, clean-up procedures and final detection methods. Among clean-up techniques, disposable Sep-Pak silica cartridges[38] and gel-permeation colums[39] have been applied.

HPTLC was applied by Lee *et al.*[40] to multi-mycotoxin determination (see below). HPTLC and reversed-phase TLC of ten mycotoxins (ochratoxin A, aflatoxins B_1, B_2, G_1 and G_2, zearalenone, sterigmatocystin, T-2 toxin, diacetoxyscirpenol and vomitoxin) with the use of various normal- and reversed-phase solvents and UV detection were reported by Stahr and Domoto[41].

Golinski and Grabarkiewicz-Szczesna[42] published chemical confirmatory tests for ochratoxin A, citrinin, penicillic acid, sterigmatocystin and zearalenone that are performed directly on TLC plates. Later, Grabarkiewicz-Szczesna *et al.*[43] published a multi-detection procedure for the determination of eleven mycotoxins in cereals.

Coman *et al.*[44] reported a TLC analysis of feed samples in which four aflatox-

ins, ochratoxin A, zearalenone, sterigmatocystin and T-2 toxin were detected. A simultaneous TLC detection of aflatoxin B_1 and zearalenone in mixed feed for pigs was described[45]. Another multi-mycotoxin method involving a membrane clean-up step and two-dimensional TLC was published by Patterson *et al.*[46].

Whidden *et al.*[47] developed a method for the simultaneous extraction, separation and determination of eight mycotoxins in corn. Mycotoxins were extracted with acetonitrile, eluted sequentially from a silica gel mini-column and rendered visible by TLC. A flow chart for the extraction and separation of mycotoxins is presented in Fig. 1. Fractions 2 (containing zearalenone and sterigmatocystin), 3 (containing patulin and penicillic acid) and 4 (containing ochratoxin A, aflatoxin B_1 and diacetoxyscirpenol) were analysed on the same TLC plate using external and internal standards. Fraction 5 (containing rubratoxin B) was applied to a separate TLC plate together with external standards.

Zearalenone, T-2 toxin, HT-2 toxin and neosolaniol were detected in grains of oats, wheat and barley[48]. Nowotny *et al.*[49] detected citrinin, ochratoxin A and sterigmatocystin in commercial cheese using TLC and HPTLC. The same group also published a TLC screening method for the determination, in mouldy foods, of 22 mycotoxins[50].

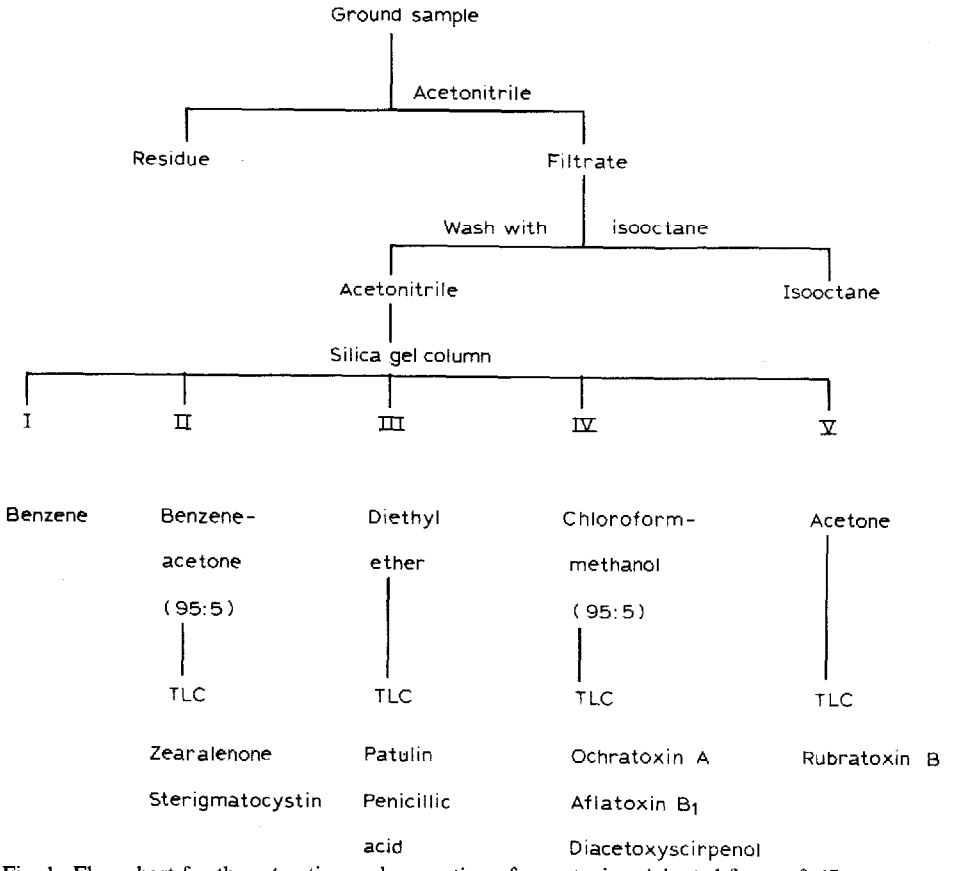

Fig. 1. Flow chart for the extraction and separation of mycotoxins. Adapted from ref. 47.

Gimeno and Martins[51] published a rapid TLC determination of patulin, citrinin and aflatoxin in apples and pears and their juices and jams. The toxins were identified with various developing solvents, spray reagents and chemical reactions and then quantitated by the limit of detection method.

A method for the routine examination of mouldy rice, wheat, bread and other vegetable foodstuffs was described[52]. The mycotoxins were separated by two-dimensional TLC. Aflatoxins were determined fluorimetrically. Ochratoxin A, patulin, penicillic acid and sterigmatocystin were developed on separate plates. Citrinin was chromatographed on an oxalic acid-pretreated plate. Citrinin and ochratoxin A were immediately determined by fluorimetry. The other toxins had to be converted into fluorescent derivatives (penicillic acid using a diphenylboric acid–2-ethanolamine spray, patulin using an N-methylbenzthiazolone-2-hydrazone spray and sterigmatocystin using an aluminium chloride spray reagent) for quantitative determination.

A quantitative TLC method for the analysis of aflatoxins, ochratoxin A, zearalenone, T-2 toxin and sterigmatocystin in foodstuffs was published by Tapia[53]. Detections of *Fusarium moniliforme* toxins[54] and of toxigenic *Fusarium* isolates[55] have been reported. Thrane[55] used griseofulvin as a relative internal standard for *Fusarium* toxins.

Lee *et al.*[40] described a method for the simultaneous determination of thirteen mycotoxins by HPTLC. Using seven continuous multiple developments with two solvent systems of different polarity, a baseline separation of sterigmatocystin, zearalenone, citrinin, ochratoxin A, patulin, penicillic acid, luteoskyrin and aflatoxins (B_1, B_2, G_1, G_2, M_1 and M_2) was achieved. About 1 h was required for the separation and quantitation of all thirteen toxins from one spot. By using *in situ* scanning of the HPTLC plate, detection limits in the low nanogram range were obtained by UV–visible absorption and in the low picogram range by fluorescence, with a relative standard deviation of 0.7–2.2% in the nanogram range. Chromatography was performed on 10 × 10 cm HPTLC plates coated with silica gel 60 and impregnated with EDTA. The development stages and spectroscopic properties used for quantitative determination of the individual mycotoxins are presented in Table 1. The mobile phase migration distance was fixed by arranging for a portion of the plate to protrude through the top of the saturated development chamber, at which point the solvent could evaporate freely. The use of multiple development offered the possibility of quantifying the components as they were separated, the use of more than one solvent system and natural refocusing of the sample spot, which occurred when the plate was dried between developments. At each stage of scanning, the migration distance of the spot to be measured was maintained between 1 and 3 cm. Hence the method is capable of providing good resolution of complex mycotoxin mixtures. However, the authors did not show whether comparable results could be obtained with samples extracted from natural products.

Paterson[56] published standardized one- and two-dimensional TLC methods for the identification of fungal secondary metabolites. A simple screening method for moulds producing the intracellular mycotoxins brevianamide A, citreoviridin, cyclopiazonic acid, luteoskyrin, penitrem A, roquefortine C, sterigmatocystin, verruculogen, viomellein and xanthomegnin was developed by Filtenborg *et al.*[57]. After removing an agar plug from the mould culture, the mycelium on the plug is wetted with a drop of methanol–chloroform (1:2). By this treatment the intracellular mycotoxins

TABLE 1

DEVELOPMENT STAGES AND SPECTROSCOPIC METHODS USED FOR THE DETECTION OF MYCOTOXINS BY HPTLC

Adapted from ref. 40.

Development stage	*Time (min)*	*Mycotoxins separated*	*Spectral characteristic used for detection*
Toluene–ethyl acetate–formic acid (30:6:0.5):			
1st development	5.0	Sterigmatocystin	Reflectance, λ = 324 nm
		Zearalenone	Fluorescence, λ_{ex} = 313 nm, λ_{em} = 460 nm
		Citrinin	
2nd development	5.0	No measurement	
3rd development	6.0	Ochratoxin A	Fluorescence, λ_{ex} = 313 nm, λ_{em} = 460 nm
4th development	6.0	Penicillic acid	Reflectance, λ = 240 nm
		Patulin	Reflectance, λ = 280 nm
		Luteoskyrin	Reflectance λ = 440 nm
Toluene–ethyl acetate–formic acid (30:14:4.5):			
5th development	8.0	No measurement	
6th development	8.0	No measurement	
7th development	8.0	Aflatoxins B_1, B_2, G_1, G_2, M_1 and M_2	Fluorescence, λ_{ex} = 365 nm, λ_{em} = 430 nm

are extracted within a few seconds and transferred directly to a TLC plate by immediately placing the plug on the plate while the mycelium is still wet. After removal of the plug, known TLC procedures are carried out. The same procedure was applied to detect aflatoxins, ochratoxin A, citrinin, patulin and penicillic acid in solid substrates.

Multi-mycotoxin TLC has been used succesfully in chemotaxonomic studies of the *Penicillium viridicatum* group[58,59], common asymmetric penicillia[60,61], triverticillate penicillia[62], *Emericella* spp.[63] and other toxigenic moulds[64].

In most TLC screening procedures, extraction and clean-up techniques are applied prior to the TLC analysis. Krivobok *et al.*[65] published rapid and sensitive methods for detecting toxigenic fungi producing aflatoxins, ochratoxin A, sterigmatocystin, patulin, citrinin, penicillic acid and zearalenone. The toxin-producing moulds tested produced detectable amounts of their respective mycotoxins within 2–4 days of incubation at 24°C in a liquid medium. Sterigmatocystin had to be extracted from the mycelium and the rapid production of zearalenone needed to be temperature programmed (24°C for growth and 10°C for toxin production). Detection of the toxins by means of TLC was possible without extraction of the medium or after extraction without purification. The sensitivity of TLC detection and the recovery after extraction were good (see table 2).

Recently, Frisvad and Thrane[66] published a general standardized method for the analysis of mycotoxins and other fungal metabolites, based on HPLC and combined with TLC in two different eluents using R_F values relative to griseofulvin. Data for 182 metabolites were listed in their paper.

3.2. TLC of aflatoxins and related compounds

Various aspects of the TLC of aflatoxins have been discussed in recent years

TABLE 2

TREATMENTS FOR TLC DETECTION AND MINIMUM TOXIN CONCENTRATION DETECTED

Modified from ref. 65.

Mycotoxin	*Treatment*[a]	*Minimum amount detected (ng)*[b]
Aflatoxins B_1, B_2, G_1, G_2	UV	1
Ochratoxin A	UV	10
Citrinin	UV, NH_3, $AlCl_3$	10
Sterigmatocystin	$AlCl_3$	5
Patulin	MBTH	25
Penicillic acid	ANIS	10
Zearalenone	UV, $AlCl_3$	10

[a] Abbreviations: UV, viewed under UV light at 254 or 366 nm; NH_3, ammonia vapour for 15 s; $AlCl_3$, 20% (v/v) aluminium chloride in 96% ethanol; MBTH, 3-methylbenzothiazol-2-one hydrazone hydrochloride solution; ANIS, 0.5% (v/v) *p*-anisaldehyde in ethanol–acetic acid–concentrated sulphuric acid (17:2:1).

[b] ng deposited on TLC plate.

(*e.g.*, refs. 6, 9 and 67–71). A survey of applications was given in a recent review[12] and by Nesheim and Trucksess[33].

Extraction and clean-up procedures, adsorbents and solvent systems have been reviewed[12]. Modifications and improvements of extraction and clean-up techniques have been published[72–78]. Velasco[79] proposed the replacement of benzene as a solvent for aflatoxin standards. Modern methods of aflatoxin extraction use a water–organic solvent mixture, *e.g.*, chloroform–water (10:1)[9,80]. The water wets the substrate and the small amount taken up in the aqueous phase is immediately removed by the solvent, giving a rapid isolation procedure.

The aflatoxins are highly fluorescent and highly oxygenated heterocyclic compounds. Separation of the four main aflatoxins (B_1, B_2, G_1 and G_2) was compared on six commercial silica gel plates, HPTLC giving more compact spots[81]. Procedures for improving aflatoxin spot size and fluorescence intensity have been published[82]. By using strong eluting solvents, diffuse spots could be reduced in size and poorly resolved chromatograms returned to their original state for redevelopment.

Gulyás[74] reported over-pressurized liquid chromatography on Kieselgel 60 HPTLC plates. Two-dimensional TLC of aflatoxin mixtures has been published[83,84]. Eller *et al.*[83] used acetonitrile–acetone–benzene (9:1:1) for the first and diethyl ether–methanol–water (96:3:1) for the second development. Good separation from impurities was achieved[84] and all four aflatoxins were well resolved using chloroform–acetone–water for the first development and toluene–ethyl acetate–formic acid (30:15:5 or 24:20:6) in the second direction. Silufol plates have been applied in the TLC of aflatoxins[83,85].

Aflatoxins have been detected by TLC in groundnuts[86–88], corn[89], black olives[90], raisins[91], ginger[92], groundnut products, corn and nuts[86], vegetable oils[93], food[85,94] and mixed feeds[95,96].

The determination of aflatoxins in vegetable oils is usually based on partition between two immiscible solvents. Aflatoxins are extracted from the oil into a polar solvent and subsequently partitioned into chloroform. Although the recoveries are acceptable, these methods are time consuming, require large volumes of solvents, and frequently involve troublesome emulsions. Miller *et al.*[97] proposed a simple method for the determination of aflatoxins which was succesfully applied to both crude and degummed oils. The oil sample, dissolved in hexane, was applied to a silica gel column and washed with diethyl ether, toluene and chloroform. Aflatoxins were eluted from the column with chloroform–methanol (97:3). Quantitation was then performed by TLC or HPLC.

Leitao *et al.*[98] used TLC for the identification of aflatoxins in extracts from cultures of *Aspergillus* strains isolated from foodstuffs. The aflatoxins were then quantitated by HPLC.

Quantitative determination of aflatoxins in groundnut products using sequential TLC has been reported[86]. The method involves double development with diethyl ether followed by chloroform–acetone–water (88:12:1.5) and triple development with diethyl ether followed by chloroform–acetone–benzene (90:10:10) and chloroform–acetone–hexane (71:12.5:16.5). The aflatoxins were detected spectrometrically (325 nm) at levels of $\geqslant 0.05$ ng per spot.

In spiked corn samples, greater than 100% recoveries using instrumental HPTLC were observed for aflatoxin analyses[99]. Spots overlying aflatoxins B_1 and B_2 were identified by GC to be C_{16}–C_{18} free fatty acids which enhanced the fluorescence of aflatoxin B_1, resulting in $>100\%$ recoveries. The inclusion of acetic acid in the mobile phase resulted in an increased mobility of the free fatty acids, which eliminated the positive interference on aflatoxin fluorescence.

TLC confirmatory tests were applied in the mini-column chromatography of aflatoxins by Madhyasta and Bhat[100]. Aflatoxins and aflatoxicols in extracts from cereals were detected with and without previous clean-up on a silica gel column. After separation of aflatoxicols from aflatoxins by CC, TLC separated aflatoxicols I and II, aflatoxins M_1 and M_2 being also separated. TLC without previous column separation also separated aflatoxins B, G and M plus aflatoxicols. Fluorodensitometry was used for their quantitation.

Severals papers concerning aflatoxins M have been published. Aflatoxin M_1 has been determined by TLC in milk and various milk products[101–109]. An improved, rapid method for the routine determination of aflatoxin M_1 in milk is based on TLC followed by elution and fluorimetric analysis[110]. TLC data for aflatoxin M_2 have been reported[111]. Koch and Kross[112] described the quantitative determination of harmful aflatoxins in selected cheese samples of food for the military. An assay method for a new hydroxyaflatoxin B_1, aflatoxin M_4, was published by Lafont *et al.*[113].

Reversed-phase HPTLC with fluorimetric detection was used by Blanck *et al.*[114] in their studies of binding of aflatoxin M_1 to milk proteins. A rapid and reproducible method for the extraction and determination of aflatoxin M_1 in milk and dairy products was published[115]. After extraction and clean-up, the aflatoxin was detected by HPLC or TLC. In TLC on silica gel, hexane–acetone (9:1) was used for the first development followed by chloroform–acetone–2-propanol (85:10:5) in the same direction. Fluorescence at 365 nm was observed after spraying the plates with nitric acid and fluorodensitometry was carried out at 440 nm.

Hsieh *et al.*[116] used a sequence of solvent systems for the TLC of aflatoxin B_1 and its metabolites. The plate was first developed in diethyl ether and the separated aflatoxicol was quantitated. The plate was then developed in chloroform–acetone–2-propanol (85:15:15); aflatoxin Q_1 and aflatoxicol H_1 were completely separated after two developments. The final separation of aflatoxins M_1 and B_{2a} was effected by further development in benzene–ethanol (40:4) or chloroform–ethanol (9:1).

Aflatoxins in air samples of refuse-derived fuel were determined by TLC with laser-induced fluorescence spectrometric detection[117]. A rapid determination of aflatoxin together with patulin and citrinin in apples and pears and in products thereof was reported[51]. In a collaborative study, Stubblefield *et al.*[118] carried out the determination and TLC confirmation of the identity of aflatoxins B and M in artificially contaminated beef liver.

TLC was one of the steps leading to the detection of aflatoxin D_1 in ammoniated corn[119]. The most important data on various members of the aflatoxin group have been summarized[67]. They included the hydroxyaflatoxins B_{2a} and G_{2a}, aflatoxins M_1 and M_2, GM_1 and GM_2, M_{2a} and GM_{2a}, parasiticol (aflatoxin B_3) and aflatoxicol (both epimers). Acid dehydration products of aflatoxicol were characterized by TLC[120].

TLC has been applied in studies on aflatoxin biosynthesis[121–123]. Biosynthetically, the aflatoxins are acetate-derived decaketides that are formed via polyhydroxyanthraquinone intermediates. The proposed aflatoxin biosynthetic pathway now consists of the following steps: acetate → norsolorinic acid → averantin → averufanin → averufin → versiconal hemiacetal acetate → versicolorin A → sterigmatocystin → *O*-methylsterigmatocystin → aflatoxin B_1[124].

Townsend *et al.*[125] synthesized labelled averufin, which was subsequently incorporated by mycelial suspensions of *Aspergillus parasiticus*. The intact incorporation of averufin into versiconal acetate, versicolorin A and aflatoxin B_1 was demonstrated. Analytical TLC and preparative TLC (PLC) proved to be extremely useful methods in both the synthesis and incorporation of labelled averufin. When combined medial and mycelial extracts were subjected to PLC on silica gel 60 plates, labelled averufin and aflatoxins were separated using hexane–acetone–diethyl ether (7:6:4). The aflatoxin mixture was further separated on a column of silica gel with chloroform–methanol (97:3). In TLC, chloroform–acetone (9:1) provided much cleaner separation of aflatoxin B_1 from the other aflatoxins, with the following R_F values: with hexane–acetone–diethyl ether (7:3:1), averufin 0.40, all aflatoxins 0.18; with chloroform–methanol (97:3), averufin 0.55, aflatoxins B_1 and B_2 0.52, aflatoxins G_1 and G_2 0.44; and with chloroform–acetone (9:1), aflatoxin B_1 0.54, aflatoxin B_2 and averufin 0.50, aflatoxin G_1 0.40, aflatoxin G_2, 0.35.

Aflatoxins, sterigmatocystin and *O*-methylsterigmatocystin were separated and identified by one-dimensional TLC by Bhatnagar *et al.*[126], who identified *O*-methylsterigmatocystin to be an aflatoxin B_1 and G_1 precursor in *Aspergillus parasiticus*. Identities of the metabolites were established by TLC using six solvent systems as shown in Table 3. The first two of these solvent systems were also used by Cleveland *et al.*[127] in an enzymological study of the conversion of sterigmatocystin to aflatoxin B_1. Analytical TLC and PLC were applied in a study of the biosynthetic origin of aflatoxin G_1[128]. Sterigmatocystin was confirmed to be a precursor of aflatoxins B_1, G_1 and G_2 but no evidence for the conversion of aflatoxin B_1 to aflatoxin G_1 was found.

TABLE 3

TLC SEPARATION OF STERIGMATOCYSTIN (ST), *O*-METHYLSTERIGMATOCYSTIN (OMST), AFLATOXINS B_1 AND G_1 AND THE METABOLITE FROM *ASPERGILLIUS PARASITICUS* (CP461)

Modified from ref. 126.

Solvent system	$R_F \times 100$					*Developing time (min)*[b]
	ST	*OMST*	B_1	G_1	*CP461 product*[a]	
Diethyl ether–methanol–water (96:3:1)	97	44	37	28	44	45
Toluene–ethyl acetate–acetic acid (50:30:4)	75	43	35	24	43	45
Toluene–ethyl acetate–acetone (60:25:15)	86	29	41	30	29	45
Chloroform–acetone (10:0.5)	74	24	22	11	24	40
Chloroform–methanol (10:0.5)	93	69	63	55	69	40
Carbon tetrachloride–methanol (10:2)	81	42	39		41	45

[a] Identical with ST.

[b] The TLC plates were spotted with approximately 50 ng of various compounds and developed for a distance of nearly 14 cm.

Sterigmatocystin and its derivatives have also been included in multi-mycotoxin analyses[40,44,47,52]. TLC determinations of the toxin in cheese have been reported[129,130]. Hu *et al.*[131] reported a two-dimensional TLC determination of sterigmatocystin in cereal grains.

Hence, in the 1980s, TLC has remained a technique of choice in the determination of aflatoxins, their precursors and metabolic products. In addition, PLC has often been used in the isolation and purification of aflatoxins.

3.3. TLC of ochratoxins

Extraction, clean-up procedures and semiquantitative and quantitative methods for ochratoxin A were reviewed by Steyn[132]. Rice starch was recommended as an adsorbent for the TLC of ochratoxin[133]. TLC remains one of the chief methods for the detection, identification and quantitation of ochratoxins.

Stahr *et al.*[134] included TLC among methods of chemical analysis for ochratoxin poisoning. TLC has been applied in the quantitative determination of ochratoxin A in vegetable foods[135].

Problems of streaking of ochratoxin A and B spots in neutral mobile phases accompanied by increasing R_F values with increasing amounts applied and the effects of acidic modifiers on R_F values have been discussed[33].

The fluorescence intensity can change when ochratoxin A is exposed to ammonia–methanol vapour and the magnitude of the change is influenced by the residual mobile phase. This observation was exploited in a method described by Nesheim *et*

al.[136] Samples are spotted on TLC plates in benzene–acetic acid (9:1) and benzene–acetic acid–methanol (90:5:5) is used as the mobile phase. The developed plate is exposed to ammonia–methanol vapour and then is covered with another glass plate to prevent evaporation of the ammonia–methanol. If the ammonia–methanol does escape and the fluorescence intensity drops, it can be restored by re-exposure to fresh ammonia–methanol. The fluorescent spots under these conditions are stable for several days, whereas they occasionally fade in a few minutes on acidic plates. The method is recommended for most commonly contaminated commodities such as corn, barley and pig tissue. The method includes a confirmatory step. Methyl esters are prepared with boron trifluoride as a catalyst. The esters are identified by comparing the R_F values of standard and analyte derivatives.

The heat stability of ochratoxin A in contaminated coffee beans was tested by TLC[137]. Preparative silica gel TLC with benzene–acetic acid (4:1) as the mobile phase was used for the purification of isotopically labelled ochratoxin A[138]. When conversion of ochratoxin C into ochratoxin A in rats was studied, the ochratoxin A-containing fractions from LCC were purified by PLC[139].

Multi-mycotoxin analytical methodology has been applied to ochratoxin A analysis by several workers[44,47,49,52,140]. Lee *et al.*[40] used sequential development on HPTLC plates.

3.4. TLC of patulin and other small lactones

Extraction, clean-up and chromatographic methods for small lactones (patulin, penicillic acid, mycophenolic acid, butenolide and citreoviridin) have been reviewed[12,141].

Several TLC procedures for patulin determination have been published[142–144]. According to one of them[143], quantitation of patulin in fruit and vegetable products is possible. After extraction and clean-up using CC, patulin is chromatographed using toluene–ethyl acetate–85% formic acid (50:40:10) and detected with a fresh 4% solution of *o*-dianisidine in 85% formic acid. Quantitation is based on the yellow fluorescence under longwave UV light (limit 10 ng per spot). Meyer[143] also identified patulin after acetylation. On Kieselgel 60G plates using the above solvent system with 65% formic acid, the R_F values of patulin and of its acetylated product were 0.39 and 0.54, respectively.

Patulin has also been included in multi-mycotoxin TLC[47,51,52].

TLC data for penicillic acid, mycophenolic acid, butenolide and citreoviridin were reviewed[12]. The determination of penicillic acid in extracts from corn, oats, barley and dried beans by TLC was described by Thorpe[145]. TLC of mycophenolic acid in extracts from cheese has been reported[146–148]. Cole *et al.*[149] characterized citreoviridin by TLC on silica gel F_{254} plates using toluene–ethyl acetate–formic acid (5:4:1) as the mobile phase.

3.5. TLC of trichothecenes

About 80 trichothecenes are already known. According to differences in the trichothecene nucleus, the trichothecenes are divided into four types, and of these type A (characterized by a hydrogen atom or a hydroxyl group at the 8-position) and type B (with a ketone group at the 8-position) are the most important in practical analysis. The macrocyclic trichothecenes belong to type C. Group D consist of all the

other trichothecenes which do not fit into the first three categories.

TLC of trichothecenes has been reviewed[12,33]. Romer[150] described the use of small charcoal–alumina clean-up columns in determination of trichothecenes in foods and feeds.

Standard and less frequent detection methods for the TLC of trichothecenes have been described[151–153]. Type A and B trichothecenes have no fluorescence absorption bands under ultraviolet or visible light. Detection requires the TLC plates to be developed with suitable solvents so that the spots can be detected subsequently by colour or fluorescence[154]. Different reagents work best with the different types of trichothecenes. Aluminium chloride is relatively specific for type B trichothecenes whereas type A trichothecenes can be detected with sulphuric acid[33] or chromotrophic acid[152]. Both of these compounds have a poor structural affinity for the 12,13-epoxy group in the trichothecene nucleus[155]. 4-(*p*-Nitrobenzyl)pyridine is reported to interact with the trichothecene nucleus and has been used for the detection of types A, B and D. These reagents react with a wide range of extraneous compounds. Unless the samples are put through several clean-up steps, these reactions can obscure the toxins[155–157]. Some type C compounds fluoresce naturally, but others are detected as fluorescent spots with sulphuric acid and heating. Other detection reagents, such as nicotinamide and 2-acetylpyridine, have been reported[33].

A new, sensitive TLC–HPLC method for detection of trichothecenes was published by Yagen *et al.*[158]. Diphenylindenone sulphonyl (Dis) esters of trichothecenes, when sprayed with sodium methoxide, showed fluorescent spots on silica gel TLC under longwave UV light. The detection limit for trichothecene esters in TLC was 20–25 ng per spot for T-2 toxin, HT-2 toxin, diacetoxyscirpenol, T-2 triol, T-2 tetraol and iso-HT-2 toxin. A quantitative HPLC analysis of Dis trichothecene esters using UV detection at 278 nm was also developed.

Unlike chemical detection, bioautography is based on the biological effects of the substance to be detected[159]. The study of the toxicity of trichothecene mycotoxins has shown that several genera of yeasts are sensitive[160]. A simple method was described[161] for the detection and quantitative determination of T-2 toxin and its separation from HT-2 toxin on silica gel layers based on growth inhibition of *Kluyveromyces fragilis* and *Saccharomyces cerevisiae*. The detection limit for T-2 toxin is 0.2 n*M* per spot. The area of growth inhibition corresponds logarithmically to the toxin concentration. T-2 toxin could be quantitatively detected from 0.2 to 160 n*M* per spot.

Chemical analysis of the culture filtrates of *Fusarium culmorum* CMI 14764 has demonstrated the presence of seven trichothecene mycotoxins[162]. The crude ethyl acetate extract from the culture filtrates was fractionated on a silica gel column by eluting with diethyl ether–acetone (9:1). Fractions were analysed by TLC and combined as appropriate. Merck silica gel F_{254} plates were developed with diethyl ether–acetone (9:1) and the spots were revealed using 20% sulphuric acid or 4-(*p*-nitrobenzyl)pyridine spray reagents. Unfortunately, R_F values of the seven trichothecenes were not included in the paper.

In the early 1980s, tests for trichothecenes were included in various multi-mycotoxin analyses of feedstuffs and foods (e.g., refs. 44, 47 and 48). Many references to TLC can be found in a monograph on trichothecenes[8].

TLC data on trichothecenes have been reported in studies on mycotoxins in

natural products[163–169]. A revised official method of deoxynivalenol analysis in wheat was published[170]. Analytical and preparative TLC have been used in studies of the bioconversion of T-2 toxin into 3'-hydroxy-T-2 toxin and 3'-hydroxy-HT-2 toxin[171]. A rapid method for the determination of trichothecenes was developed[172]. The trichothecenes occurring in purified extracts of food and feed samples were converted into the corresponding free alcohols by transesterification and then analysed by HPTLC or GC. Harrach *et al.*[173] subjected cleaned-up concentrates of satratoxins G and H to PLC on silica gel. The band with R_F values identical with those of standards of satratoxins G and H was collected, extracted with acetone and used for comparison with satratoxin standards by HPTLC.

TLC and other chromatographic techniques were used extensively in the isolation and characterization of thirteen new macrocyclic trichothecenes from the Brasilian plant *Baccharis megapotamica*[174]. A model 7942 Chromatotron was used for PLC with plates prepared as circular glass disks.

Bata *et al.*[175] described an improved three-step (TLC, GC and HPLC) procedure for the determination of the macrocyclic trichothecenes satratoxin G and H and verrucarin J in cereals.

3.6. TLC of tremorgenic mycotoxins

Maes *et al.*[176] devised simple TLC and HPLC systems for the separation, identification and quantitation of penitrems in culture extracts. As the penitrems are unstable in chloroform when exposed directly to light, all contact of the penitrems with chloroform was avoided throughout their investigation. The only system that gave a complete separation of all the penitrems in TLC was benzene–acetone (85:15) and the best results were obtained by developing the chromatograms twice in this solvent system. The order of decreasing R_F values for the penitrems was F, B, A, E, C and D. Penitrems A–F give blue spots immediately after spraying with cerium(IV) sulphate, which become stable, dark purple after heating. Similar procedures were used by De Jesus *et al.*[177].

PLC has been used in the purification of the janthitrems but CC on Mallinckrodt silica AR CC-7 silica gel was more succesful. The janthitrems are high fluorescent under longwave UV light and can also be detected by spraying the TLC plates with Ehrlich's reagent and exposure to hydrogen chloride vapour, resulting in grey–green spots[178].

TLC was used to check paxilline, another indole-isoprenoid tremorgen, in fractions from CC during purification of the toxin from a submerged fermentation. Spraying with Ehrlich's reagent followed by heating revealed paxilline by its colour, yellow becoming green. Complementary detection involves spraying with 50% ethanolic sulphuric acid and heating at 100°C for 5 min[179].

Territrems A and B were separated by means of TLC in three solvent systems, their detection being based on blue fluorescence[180]. Later, territrem C was discovered and characterized by TLC and other methods. PLC was also used to isolate the methylation product of territrem C and its identity with territrem B was proved[81]. More recently, Peng *et al.*[182] succeeded in isolating another related metabolite. As the R_F values of the compound in TLC were between the R_F values of territrems B and C, the compound was designated territrem B'. TLC fluorodensitometric quantitation of territrems A, B and C was also described[183]. The following mobile phases have been

used to characterize territrems A, B and C[180,181]: (a) benzene–ethyl acetate (1:1); (b) toluene–ethyl acetate–65% formic acid (5:4:1); and (c) benzene–ethyl acetate–acetic acid (55:40:5). The fluorescence intensity of territrem C was quenched when the concentration was higher than 20 μg per spot. The fluorescence intensity also gradually faded after development in system (a), but it was enhanced and turned greenish in acidic solvent systems.

TLC of verruculogen has also been reported[184].

3.7. TLC of hydroxyanthraquinones

TLC data for fungal hydroxyanthraquinones have been reviewed[12,15,185]. The hydroxyanthraquinones give yellow, orange or red spots on TLC plates. They are also detected by spraying the plates with a saturated solution of magnesium acetate in methanol or 5% potassium hydroxide in methanol[15]. Detection with methanolic solutions of magnesium acetate and copper acetate was compared[186]. The colour obtained with the latter was more stable, increased for 2 h and then remained stable for 24 h. Spots of hydroxyanthraquinones from *Trichoderma viride* on Silufol plates became intensely orange and violet, respectively, when the plates were exposed to ammonia fumes[187]. Two main anthraquinones from a colour mutant of *T. viride*, 1,3,6,8-tetrahydroxyanthraquinone and 1-acetyl-2,4,5,7-tetrahydroxyanthraquinone, were purified by PLC on Silufol plates using benzene–acetone (75:25) with repeated development[188]. Ueno[189] reported the TLC determination of luteoskyrin in rice grains. PLC was used in studies on the conversion of emodin to physcion by a cell-free preparation of *Aspergillus parasiticus*[190].

In studies of anthraquinones produced by *Aspergillus glaucus* group, Anke *et al.*[191] used PLC and oxalic acid-treated silica gel PF_{245} (Machery, Nagel & Co.) to separate and isolate several pigments. Erythroglaucin, physcion and physcion-9-anthrone were well separated in carbon tetrachloride–chloroform (90:10). Catenarin, rubrocristin and viocristin were separated using benzene–ethyl acetate–acetic acid (45:55:1) as the mobile phase.

3.8. TLC of zearalenone

Techniques for the production, isolation, separation and purification of the strogenic mycotoxin zearalenone have been reviewed[192]. Extraction, clean-up, adsorbents, solvent systems and TLC detection of zearalenone have been summarized elsewhere[12]. Some recent techniques and applications are presented here.

A method was developed for the simultaneous extraction, separation and qualitative analysis of zearalenone and seven other mycotoxins in corn[47]. According to Fig. 1, zearalenone was present in the second fraction from the mini-column.

Another extraction and clean-up procedure was proposed[193]. Samples were extracted with acetonitrile–4% potassium chloride (9:1) in 0.1 *M* hydrochloric acid and the extract was defatted with isooctane. The acetonitrile layer was filtered through anhydrous sodium sulphate, which was washed with chloroform and the washings were added to the filtrate. After evaporation, the residue was dissolved in chloroform and used for TLC analysis. Zearalenone was characterized with nine solvent systems and two spray reagents (an ammonium chloride solution and Fast Violet B salt spray). These were also used in quantitation of the toxin. Solvent systems containing formic acid were not satisfactory when the Fast Violet B salt spray was used.

Overpressurized layer chromatography was also used to determine zearalenone in maize[194].

Swanson *et al.*[195] developed a method for the determination of zearalenone and zearalenol in grains and animal feeds. The method involved extraction with 75% methanol, precipitation of pigments with lead acetate and defatting with light petroleum. The mycotoxins were subsequently partitioned into toluene–ethyl acetate, chromatographed on HPTLC plates and detected after spraying with Fast Violet B salt solution. The sensitivity was > 80 ng/g for zearalenone and 200 ng/g for zearalenol.

Two-dimensional TLC with benzene–acetone (60:35) and toluene–ethyl acetate–formic acid (60:30:10) proved to be insufficient in resolving α- and β-zearalenol, which appeared as a single spot[196]. However, the diastereomeric mixture was resolved into two components by HPLC and GC (see also sections 4.4 and 5.2).

In a study of the biosynthesis of zearalenone, the radiochromatographic homogeneity of the isolated [^{14}C]zearalenone was determined by TLC on silica gel G plates using chloroform–methanol (97:3) as the mobile phase. Among fluorescent bands, only that corresponding to zearalenone was radioactive[197].

In studies of the bioconversion of radioactive α-zearalenol and β-zearalenol into zearalenone, the recovery from CC was ascertained by TLC of eluates[198]. Recently, TLC and PLC were used in studies of the microbial conversion of zearalenone. A strain of *Rhizopus* sp. produced zearalenone 4-β-D-glucopyranoside in addition to α- and β-zearalenol[199].

Bennett *et al.*[200] described a method for the determination of zearalenone and deoxynivalenol in cereal grains. After extraction, clean-up and separation by CC, zearalenone was quantitated by TLC and deoxynivalenol by GC of the trimethylsilyl derivative. Multi-mycotoxin TLC studies in which zearalenone was included have been published[40,44,47,48].

3.9. TLC of citrinin

Chromatographic methods, including TLC, using in studies of this mycotoxin have been reviewed[12,201]. The major problems with the TLC of citrinin have been its weak fluorecence, tailing in normal-phase TLC on silica gel and instability.

More intense fluorescence of citrinin and easier detection were accomplished with an aluminium chloride spray followed by heating, which changes the yellow fluorescence to blue[51]. Another improvement has been the incorporation of an acid in the silica gel to reduce tailing. Oxalic acid was used first[201], but more recently glycolic acid was found to be better because of reduced diffusion of the citrinin spots and hence enhanced detectability[202]. This modification was applied to determine citrinin in corn and barley.

TLC was shown to be of importance in the separation and identification of dihydrocitrinone and ochratoxin A as products of conversion of [^{14}C]citrinin by *Penicillium viridicatum*[203]. The major breakdown product, dihydrocitrinone, appeared on the developed TLC plates as a blue spot under longwave UV light.

3.10. TLC of cyclopiazonic acid

The most important data concerning the production, isolation, separation and purification of cyclopiazonic acid and related toxins were summarized by Cole[204].

Because of its neurotoxicity and possible carcinogenicity, cyclopiazonic acid has been intensely investigated in the 1980s. Analytical methodology for its determination in agricultural products has received considerable attention in the past few years. Most of the TLC methodology relies on the reaction of cyclopiazonic acid with Ehrlich's reagent under acidic conditions. Other detection possibilities have been reviewed[12].

TLC systems including densitometry have been reported[205–207]. According to Lansden[207], samples of peanuts or corn are extracted with methanol–chloroform (20:80); the extract is stripped of most interferences by partitioning with aqueous sodium hydrogencarbonate followed by acidification and repartitioning with chloroform. After TLC and derivatization with Ehrlich's reagent, the toxin is quantitated by reflection densitometry at 540 nm. The recovery of the toxin averages 90% for peanuts and 85% for corn. The absolute detection is 25 ng per spot, which represents a detection limit of 125 μg/kg for a 50-g sample.

A simple determination of the toxin in contaminated food and feeds was described[208]. Trucksess *et al.*[209] examined isolates of *Aspergillus* and *Penicillium* species from dried beans, corn meal, macaroni and pecans for their ability to produce cyclopiazonic acid. The toxin in chloroform extracts was semi-quantitatively determined by TLC. Semi-quantitative TLC of the toxin has also been used in studies of its production by *Penicillium* and *Aspergillus* strains[210,211].

TLC has been succesfully applied to discriminate cyclopiazonic acid-producing (CPA^+) from non-producing (CPA^-) strains of *Aspergillus oryzae*[212].

Crude extracts from culture filtrates were characterized by TLC on silica gel $60F_{254}$ that had been previously impregnated with oxalic acid (0.4 *M*) and dried. Development was carried out in isobutyl methyl ketone–chloroform (1:4) and detection was with Ehrlich's reagent. The toxin from CPA^+ strains revealed a blue-violet spot at R_F 0.75, whereas crude extracts from CPA^- strains never did reveal this spot.

TLC has been applied in studies on the production of cyclopiazonic acid by *Penicillium verrucosum* var. *cyclopium*[213] and for screening the toxin in agricultural commodities[214].

Malik *et al.*[213] performed TLC on silica gel G-1500 LS 254 with ethyl acetate–2-propanol–25% ammonia solution (20:15:10). The toxin was measured quantitatively with a spectrodensitometer with a digital counter and integrator at 282 nm. It was detected as a violet spot under ordinary light after spraying with Ehrlich's reagent diluted with 4 volumes of acetone.

Rao and Husain[214] applied PLC to chloroform extracts from culture filtrates. The standard was spotted at one end of the plate. After development (the same system as in ref. 213), the standard was detected with Ehrlich's reagent (the remainder of the plate being covered with a glass plate). When the standard was detected, the covering plate was removed and the TLC plate exposed to iodine vapour. The area with an R_F value corresponding to the standard spot and coloured with iodine vapour was scrapped off, eluted with methanol and used for colorimetric determination of the toxin using a modification of Ehrlich's reagent.

Determination of cyclopiazonic acid in foods by HPTLC was published recently[215].

3.11. TLC of cytochalasans

The family of cytochalasans include compounds with a common skeleton (cy-

tochalasan) such as cytochalasins, zygosporins and chaetoglobosins[124]. TLC data for this large class of fungal metabolites have been reviewed[12].

Chappuis and Tamm[216] used a variety of solvent systems in the analytical TLC and PLC of derivatives and degradation products of cytochalasin D. Analytical TLC was carried out on Fertigplatten 60 F_{254} and Kieselgel 60 PF_{254} was used as the sorbent in PLC. A TLC method for the determination of cytochalasin H production was reported most recently[217].

Chaetoglobosin K was characterized by means of TLC[218]. Decreasing R_F values of five chaetoglobosins on silica gel 60 TLC plates developed in dichloromethane–ethanol (95:5), showing increasing polarity from left to right, were reported as follows: 19-*O*-acetylchaetoglobosin A, chaetoglobosin C, 19-*O*-acetylchaetoglobosin B, 19-*O*-acetylchaetoglobosin D and chaetoglobosin A[219]. TLC has been used in a study on chaetoglobosins A–J[220] and the isolation and identification of two new cytochalasans from *Phomopsis sojae*[221].

3.12. TLC of miscellaneous mycotoxins

In this section, TLC data on the following compounds are included: PR toxin, xanthomegnin, viomellein, vioxanthin, moniliformin, naphtho-γ-pyrones, wortmannin, echinulin, fusaric acid analogues, viridin and several *Alternaria* toxins. Older data were reviewed elsewhere[12].

The production, isolation and chromatographic techniques for PR toxin were reviewed by Scott[222]. Several solvent systems for the TLC of this toxin have been published[223,224]. The toxin was quantitated *in situ* by fluorodensitometry after spraying the plates with 1% *p*-dimethylaminobenzaldehyde in concentrated hydrochloric acid–acetone (1:10) or in ethanol with subsequent exposure to hydrogen chloride fumes for 10 min, the latter being the preferred method[223].

Methods used for the isolation, separation, purification and detection, including TLC and HPLC, of secalonic acid D were summarized[225]. Ciegler *et al.*[2] quantitated the toxin on pre-coated silica gel F_{254} plates. Secalonic acid D was included in an earlier systematic TLC analysis of 37 mycotoxins[226]. The toxin was detected with *p*-anisaldehyde and iron(III)chloride.

In a screening for toxigenic isolates of *Aspergillus ochraceus* from green coffee beans, Stack *et al.*[227] applied TLC in detecting xanthomegnin, viomellein and vioxanthin in addition to ochratoxins. Standards of xanthomegnin and viomellein were prepared by means of PLC on silica gel plates[228]. Their purity was checked by TLC and HPLC comparisons with reference compounds.

Jansen and Dose[229] described a quantitative TLC determination of moniliformin in vegetable foods and feeds. Crude acetonitrile extracts of *Fusarium moniliforme* cultures were checked for moniliformin[230,231] by spotting, together with a standard, on precoated thin layers of silica gel 60 and developing with chloroform–methanol–formic acid (70:30:0.16). Moniliformin was detected by spraying and heating with 0.5% aqueous 3-methyl-2-benzothiazolinone hydrazone hydrochloride. The limit of detection was approximately 8 μg/g in corn culture.

Monomeric and dimeric naphtho-γ-pyrones, extracted from the mycelium of *Aspergillus niger*, were examined by HPTLC on LHP-KF plates (Whatman) developed with benzene–ethyl acetate–formic acid (10:4:1)[232]. Components were identified by their colour, fluorescence under longwave UV light and colour after spraying with

Gibbs reagent. Data for eight naphtho-γ-pyrones are presented in Table 4.

PLC on silica gel plates developed with chloroform–methanol (97:3) was used to purify a haemorrhagic factor from *Fusarium oxysporum* identical with the antibiotic wortmannin[233].

Echinulin was isolated by means of PLC from acetone extracts of feed refused by swine. The mobile phase was ethyl acetate–hexane (8:2) and the toxin turned blue in the presence of *p*-anisaldehyde reagent at 110°C. The anisaldehyde-reactive material from the PLC was identified with echinulin by its UV and IR spectra[234].

Viridin, a steroid-like antibiotic, is converted by viridin-producing fungi into its dihydro derivative, viridiol, which is ineffective as an antibiotic but is a potent phytotoxin. Both metabolites were isolated from culture extract by means of PLC[235]. TLC was used to characterize two new analogues of fusaric acid from *Fusarium moniliforme*[236].

Alternaria toxins have received much interest in recent years. Production, isolation, clean-up procedures and chromatographic techniques (TLC, GC and HPLC) for the determination of alternariols, altenuene and tenuazonic acid were reviewed[237]. TLC data for alternariol, alternariol monomethyl ether, altertoxin I and II and tenuazonic acid were published[238]. ^{3}H-labelled alternariol and alternariol monomethyl ether were isolated from ethyl acetate extracts of conidia of *A. alternata* by PLC. Two solvent systems were used: (1) toluene–dioxane–acetic acid (95:25:4) and (2) methanol–2*M* HCl (5:1)[239].

Hence, one- and two-dimensional high-performance and preparative TLC have been used extensively in the field of mycotoxins in the 1980s. In addition to comments in this section, achievements and problems with the TLC of mycotoxins are discussed in more detail in the Conclusions.

4. LIQUID COLUMN CHROMATOGRAPHY

Techniques of liquid column chromatography are used in the field of mycotoxins with three main aims: (a) clean-up of mycotoxin-containing extracts for further analysis by other means; (b) large-scale separations and purifications; and (c) qualitative or quantitative analysis of mycotoxins.

TABLE 4

HPTLC DATA FOR NAPHTHOPYRONES

Adapted from ref. 232.

Naphthopyrone	$R_F \times 100^a$	*Gibb's test*	*Fluorescence*
Flavasperone	81	Blue	Violet
Fonsecin monomethyl ether	76	Brown	Violet
Rubrofusarin	72	Blue-green	Orange
Aurasperone A	67	Violet	Yellow
Isoaurasperone A	61	Red-violet	Yellow
Aurasperone B	56	Brown	Yellow
Aurasperone D	53	Violet	Yellow
Aurasperone C	49	Brown	Yellow

[a] With benzene–ethyl acetate–formic acid (100:40:10) on Whatman LHP-KF.

In clean-up procedures, various mini-columns are used. In large-scale separations and purifications, amounts varying between a few miligrams and several grams can be separated on a column. In analytical work, a variety of mini-columns containing different adsorbents are commercially available and may be used as an alternative to TLC. Most analytical work, however, is now performed by means of high-performance liquid chromatography (HPLC).

Almost all large-scale separations and purifications of mycotoxins are performed by column chromatography (CC). The columns used may be normal gravity columns or may run under pressure in the case of the preparative HPLC systems.

In the CC of mycotoxins, four general techniques can be used, *viz.*, adsorption, partition, ion-exchange and gel filtration chromatography. By far the most widely used is adsorption chromatography. Adsorbents such as silica gel (most frequently), alumina, charcoal, cellulose, silicic acid, macroreticular resins, magnesium oxide, magnesium silicate and calcium hydrogenphosphate have been used. Partition chromatography on cellulose impregnated with formamide has given satisfactory results in the purification of some polar toxins such as cyclopiazonic acid. Ion-exchange chromatography has found application in the purification of ochratoxins, cyclopiazonic acid, tentoxin and moniliformin. Gel filtration chromatography has been used to purify verruculotoxin and aflatoxins. The most important techniques of preparative CC of mycotoxins have been described[1] and applications in separations and purifications of the best known mycotoxins have been reviewed[11].

Historically, the gradual introduction of HPLC analysis in the early 1970s for the determination of mycotoxins had to compete with existing widely used TLC-based methods, but the trend has been toward the increased use of HPLC. The rapidly increasing applications of the technique show its inherent advantages over other chromatographic techniques and it seems destined to supplant the other methods in the routine analysis of mycotoxins. Earlier applications of HPLC in the analysis of mycotoxins have been reviewed (*e.g.*, refs. 1, 240 and 241). The most important techniques and applications were summarized by Shepherd[242].

HPLC is applicable to the analysis of nearly all known mycotoxins, in contrast to gas chromatography (GC), which has been used mostly for the trichothecenes, zearalenone, patulin and the anthraquinones[243,244]. In the field of mycotoxins, many of which are highly polar compounds, reversed-phase HPLC has found wide application and most separations or purifications are now performed on reversed-phase columns. The toxins investigated include the aflatoxins, citrinin, the ergot alkaloids, ochratoxin A, patulin, penicillic acid, penitrems A–F, PR toxin, the rubratoxins, sporidesmins, sterigmatocystin and zearalenone.

Among the available detectors, the most frequently used is the variable-wavelength detector, which has a particular application in the mycotoxin field. The main alternative to the UV detector is the spectrofluorimetric detector, which should be inherently more sensitive than UV detectors and has been used in the detection of aflatoxins, ochratoxin A and several other mycotoxins.

Double-beam UV detectors, which can record the UV spectrum of a compound being eluted from the column, have obvious advantages for the characterization of mycotoxins, although in some instances the UV spectrum is not a reliable diagnostic probe. In addition, several mycotoxins exhibit weak absorbance in the UV region and detection at 190 nm is hampered by the background absorption. The introduction of

the recently developed thermospray (TSP) interfacing technique[245] has permitted the measurement of a wider range of organic compounds than previously. This technique was used by Voyksner *et al.*[246] for the HPLC–MS analysis of some *Fusarium* toxins. More recently, Rajakylä *et al.*[247] published a method for the determination of mycotoxins in grain by reversed-phase HPLC and thermospray liquid chromatography–mass spectrometry (TSP–LC–MS), which seems to be a very specific and sensitive method for analysing a wide range of mycotoxins in biological samples.

In the following sub-sections, some applications of HPLC in multi-mycotoxin analysis and in determinations of mycotoxin structural groups or individual toxins are summarized. The data were published in the 1980s.

4.1. HPLC in multi-mycotoxin analysis

Multi-mycotoxin analyses by HPLC have been published[248,249]. Thiel *et al.*[250] studied the natural occurrence of moniliformin together with deoxynivalenol and zearalenone in corn. Grabarkiewicz-Szczesna *et al.*[43] published a simple multi-detection procedure for the determination of eleven mycotoxins in cereals. Griffin *et al.*[251] separated *Alternaria* metabolites. HPLC was used by Scott *et al.*[54] to analyse toxins of *Fusarium moniliforme*.

Hurst *et al.*[252] determined patulin, penicillic acid, zearalenone and sterigmatocystin in artificially contaminated cocoa beans by HPLC. When this method is combined with a method reported earlier for the determination of ochratoxin A[253], it allows the determination of five mycotoxins. Samples are extracted with an acidic acetonitrile solution, partitioned with hexane to remove fat interferences and then partitioned with chloroform to remove the toxins. Interferences are removed by the use of a bonded-phase column followed by the final HPLC determination step. This uses a cyano column with hexane–1-propanol–acetic acid as the mobile phase with dual-channel UV detection at 245 and 280 nm. The method exhibits good linearity, accuracy and precision.

An HPLC method was described[247] for the determination of deoxynivalenol, patulin, diacetoxyscirpenol, HT-2 toxin, T-2 toxin, zearalenone and ochratoxin A using a reversed-phase column and diode-array detector. The combination of the HPLC system together with a modern thermospray (TSP) quadrupole mass spectrometer was shown to be a very specific and sensitive method.

Frisvad[254] developed a general method for most known mycotoxins and other secondary metabolites of fungi based on HPLC, but even using the same type of chromatographic columns the retention times were very variable. The method was also limited by the UV detection wavelength of 254 nm, making the detection of some metabolites with end absorption impossible.

Later, Frisvad and Thrane[66] developed a general standardized method for the analysis of 182 mycotoxins and other fungal metabolites, based on HPLC with an alkylphenone retention index and photodiode-array (PDA) detection combined with TLC in two different solvent systems. Each secondary metabolite was characterized by its bracketed alkylphenone retention time, its UV–VIS absorption maxima and its retardation factor relative to griseofulvin in two TLC systems. The metabolites characterized by this method included aflatoxins B_1, B_2, $G_1$and G_2, ochratoxin A, citrinin, penicillic acid, viomellein, penitrem A, patulin, sterigmatocystin, alternariol, tenuazonic acid, trichothecenes, roquefortines, fusarin C, zearalenone, PR toxin,

citreoviridin, viridicatumtoxin, verruculogen, rugulosin, cyclopiazonic acid and many other alkaloids, polyketides and terpenes. Methods of this type are of great importance also in the chemotaxonomy of fungi.

4.2. HPLC of aflatoxins

Fluorimetry is the detection technique commonly used for the most frequent aflatoxins because of their native fluorescence; λ_{ex} = 360 nm for all four aflatoxins and λ_{em} = 440 nm for B_1 and B_2 and 470 nm for G_1 and G_2[255].

The fluorescence of aflatoxins B_1 and G_1, which is lower than that of B_2 and G_2, can be increased by using strong acids[256] or oxidants such as chloramine T[257], iodine[258–260] or bromine[257,261]. Bromine acts by adding itself to the double bond of the furan ring of aflatoxins B_1 and G_1, thereby increasing their fluorescence by a factor of 20 or more[257]. β-Cyclodextrin also enhances the fluorescence of the two aflatoxins and this effect was utilized in developing a reversed-phase HPLC method for the detection of aflatoxins B_1, B_2, G_1 and G_2 without preparing derivatives of B_1 and G_1[262].

A new approach to the detection of the four aflatoxins involves HPLC with amperometric detection in the differential-pulse mode at a dropping mercury electrode with a 1-s drop time. These aflatoxins can be detected simultaneously with good resolution but with some compromise in sensitivity, The detection limit of underivatized aflatoxin standards is about 5 ng[263].

Although HPLC and flow-injection analysis (FIA) have significant differences (working pressure, presence of interfaces, cost), they involve a number of common components (liquid reservoirs, pumps, injection valves and continuous detectors) and are complementary in nature. Hence attempts to use HPLC and FIA in conjunction are justified[264]. Lázaro *et al.*[257] published a new approach to the joint use of FIA and HPLC in which the flow-injection sub-system allows the total determination of several related compounds. The individual analysis for each analyte is performed by the HPLC sub-system while the flow-injection sub-system acts as a post-column reactor–detector, thereby enhancing the information obtained from the sample. An example of the joint use of these techniques is the analysis of foods for aflatoxins. Bromine was used as the derivatizing agent. The optimum composition of the mobile phase was acetonitrile–methanol–water (23.5:29.5:47), allowing the complete resolution of the four aflatoxins. The concentration and flow-rate of the bromine solution and the flow-rate of the mobile phase were interrelated. The method was used for the determination of aflatoxins in groundnuts and maize.

Other methodological aspects have been studied, such as radial compression separation[265] and post-column derivatization[266–268].

Among many papers published in the 1980s, HPLC has been applied to determine aflatoxins in agricultural products such as heavily contaminated corn[269], corn and groundnuts[270], cotton-seed[271], feedstuffs[266,272,273] and naturally contaminated eggs[274]. The separation of aflatoxin biosynthetic intermediates by HPLC was reported by McCormick *et al.*[275]. A method has been developed for the quantitation of aflatoxins produced by fungal strains in liquid media[98]. HPLC has also been used in toxicological and mutagenic studies of aflatoxins[276–278].

HPLC has been extensively applied in the determination of aflatoxin M_1 in milk and dairy products[105,279–288]. In the 1982 IARC/WHO aflatoxins check sample pro-

gramme[288,289], 21% of the analyses (including 28 out of 115 for aflatoxin M_1 in milk) were completed by means of HPLC. Changes in official methods of analysis of aflatoxins M_1 and M_2 in fluid milk were published[290]. Hsieh *et al.*[291] reported on the production and isolation of aflatoxin M_1 for toxicological studies. An assay method for the contamination of commercial milks by aflatoxin M_4, a new hydroxyaflatoxin B_1, was described by Lafont *et al.*[113].

A recent comparison[292] of a number of published protocols indicated that the best results for aflatoxin M_1 analysis were obtained by direct octadecylsilane solid-phase extraction of liquid milks, followed by reversed-phase HPLC[293]. Recently however, products for the immunological determination of aflatoxins have become available. In the course of evaluating an immunoaffinity column designed for the analysis of aflatoxins B and G in groundnuts and other products, Mortimer *et al.*[294] investigated its suitability for the determination of aflatoxin M_1 in milks. The affinity column clean-up gave excellent results for recovery, sensitivity and sample throughput. The HPLC traces were significantly cleaner than those in alternative methods[292] and the detection limit was as low as 50 ng/l.

Methods for the determination of aflatoxins in human serum[295] and urine[296] have been developed. The determination of aflatoxicol in porcine liver was described by Tyczkowska *et al.*[297] Further HPLC data for aflatoxins have been reviewed[242].

4.3. CC and HPLC of trichothecenes

A variety of preparative and analytical liquid chromatographic methods for trichothecenes have been reported in the 1980s. A review was published[298].

Witt *et al.*[299] purified deoxynivalenol by water-saturated silica gel chromatography. An assessment of extraction procedures for the analysis of naturally contaminated grain products for deoxynivalenol was published[300]. Cohen *et al.*[301] described a preparation of deoxynivalenol using flash chromatography. Isolation and purification of deoxynivalenol and a new trichothecene by HPLC were reported by Bennett *et al.*[302].

The trichothecene fraction produced by a liquid culture of *Fusarium crookwellense* was separated by open-column liquid chromatography on silica gel and by HPLC on a cyano bonded-phase column. The major trichothecene produced was 4,15-diacetoxynivalenol. Other secondary metabolites formed in appreciable amounts were the 7- and 8-hydroxy derivatives of isotrichodermin. Several unknown compounds were isolated and characterized by their mass spectra and ^1H and ^{13}C NMR spectra. Among these compounds were 7,8-dihydroxyisotrichodermin and 4,15-diacetoxy-7-deoxynivalenol[303].

HPLC was used to follow the transmission of [^{14}C]deoxynivalenol to eggs following oral administration to laying hens[304].

Rapid determinations of trichothecenes using small charcoal–alumina clean-up columns have been reported[150,305].

Changes in official methods of analysis of deoxynivalenol in wheat were published[306]. Shepherd and Gilbert[307] described the long-term storage stability of deoxynivalenol standard reference solution. Determination of deoxynivalenol was achieved by HPLC with electrochemical detection[308]. The analysis of several trichothecenes by HPLC was described by Maycock and Utley[309] and the detection of nivalenol, deoxynivalenol, fusarenone X and 3-acetyldeoxynivalenol by HPLC was reported[310].

Analysis of some metabolites of T-2 toxin, diacetoxyscirpenol and deoxynivalenol was achieved by thermospray HPLC–MS[311]. Lauren and Greenhalgh[312] reported on the simultaneous determination of nivalenol and deoxynivalenol in cereals.

The applicability of HPLC with UV detection, based on the absorptivity of the α,β-enone system of type B trichothecenes (such as deoxynivalenol and nivalenol), is often limited by lack of specificity because of the need to use low wavelengths[313]. A pre-column derivatization method using *p*-nitrobenzyl chloride was reported to form a chromophore with an absorption maximum of *ca.* 254 nm[309]. The method has the disadvantages that the reagent gives an interferring peak and its derivatization step is time consuming. HPLC with post-column fluorescence detection should be a suitable method, because it permits efficient separation, selective detection and direct injection of the sample solution after clean-up without further treatment.

Supercritical fluid chromatography with mass spectrometric detection shows interesting possibilities for trichothecenes[314].

A method for the determination of trichothecenes based on the chromogenic or fluorogenic reaction of formaldehyde produced from trichothecenes by an acid decomposition reaction was described by Kato *et al.*[315]. However, application of similar reactions to an HPLC post-column derivatization system is difficult because of the necessity to use viscous concentrated sulphuric acid. Sano *et al.*[313] found that type B trichothecenes give formaldehyde when heated with aqueous alkali. They developed a method based on the HPLC separation of deoxynivalenol, nivalenol and fusarenon-X and a C_{18} column using aqueous acetonitrile, and successive post-column fluorescence derivatization involving an alkaline decomposition to form formaldehyde and a modified Hantzch reaction with methyl acetoacetate and ammonium acetate. With this method, 5–10 ng of the standard trichothecenes could be determined. By employing a clean-up procedure with a Florisil column and a Sep-Pak CN cartridge, 61.4–96.9% recoveries were obtained for deoxynivalenol and nivalenol added to corn, barley and wheat at concentration levels of 0.05–1.0 ppm.

Bata *et al.*[175] published a method for the determination of naturally occurring macrocyclic trichothecene toxins. Certain mycotoxins in this group are either antitumour agents or their precursors. Three trichothecenes produced by the fungus *Myrothecium verrucaria* (roridin J, verrucarin A and roridin K acetate) were separated from a crude extract of the fermentation broth by using a Supelcosil LC-Si column. A step gradient of 20, 30, 40 and 70% ethyl acetate in hexane and 100% ethyl acetate was used as the mobile phase. The ethyl acetate concentration was increased stepwise at 10, 20, 28 and 39 min. Roridin A and 8-β-hydroxyroridin A also have been separated on a Supelcosil LC-Si column[316].

An HPLC analysis of extracts from aerosolized conidia of *Stachybotrys atra* established that conidia contained satratoxins H and G and trichoverrols A and B[317].

The isolation of thirteen new macrocyclic trichothecenes from the Brazilian plant *Baccharis megapotamica* was achieved by Jarvis *et al.*[174]. HPLC and TLC have been used extensively to check the separation and purification procedures. Nine macrocyclic trichothecenes were isolated from *B. coridifolia*. Flash column chromatography was used in their separation and purification[318].

Rood *et al.*[319] developed a rapid screening procedure for the detection of trichothecenes in plasma and urine. HPLC has been applied in studies on the production of deepoxydeoxynivalenol, a metabolite of deoxynivalenol, by *in vitro* rumen

incubation[320] and on the disposition of T-2 toxin in intravascularly dosed swine[321].

Yagen *et al.*[158] described a new, sensitive fluorescence TLC method for the detection of diphenylindenone sulphonyl (Dis) esters of trichothecenes. HPLC of these derivatives using UV detection resulted in a considerable improvement in the detection sensitivity of the trichothecenes diacetoxyscirpenol, T-2 toxin, HT-2 toxin, T-2 triol and T-2 tetraol. The HPLC of Dis trichothecene esters could be used for the quantitation of trichothecenes in plasma and urine.

4.4. HPLC of miscellaneous low-molecular-weight mycotoxins

In addition to the aflatoxins and trichothecenes, the remaining mycotoxins are aranged in this section in an order similar to that used in the TLC sections. Hence, the following toxins are included here: ochratoxin A, citrinin, patulin, citreoviridin, tremorgens, zearalenone, cyclopiazonic acid, secalonic acid D, xanthomegnin, viomellein, moniliformin, anthraquinones, fusaric acid analogues, fusarin C, γ-pyrones, *Alternaria* toxins, PR toxin and rubratoxin B.

HPLC has been applied to determine ochratoxin A in wheat bran[322], feeds, animal tissues and eggs[323]. Abramson[324] measured ochratoxin A in barley extracts by HPLC–MS. Rapid HPLC on a Spherisorb ODS 2 column with fluorescence detection was used for the determination of ochratoxin A in foods[325].

Gareis *et al.*[326] found ochratoxin A in human milk. After extraction and clean-up steps, analysis was performed by reversed-phase HPLC on 5-μm LiChrosorb RP-18 with acetonitrile–water–acetic acid (570:410:20) as the mobile phase and fluorescence detection (λ_{ex} = 330 nm and λ_{em} = 460 nm). Trace amounts of the toxin were found in four out of 36 randomly collected human milk samples.

The occurrence of citrinin in cereal grains was confirmed by HPLC[327]. Citrinin was extracted with chloroform–0.1 *M* phosphoric acid (15:2), passing the chloroform phase through an Extrelut column. The sample was then chromatographed on LiChrospher Si 100, buffered with citrate–phosphate buffer (pH 2.5), with hexane–chloroform (3:2) as the mobile phase and fluorimetric detection (λ_{ex} = 360 nm and λ_{em} = 500 nm).

In the 1980s, several methods have been developed for measuring patulin in food products, including TLC, GC and HPLC. Möller and Josefsson[328] used extraction with ethyl acetate and reversed-phase separation on Spherisorb 50 DS with water as the mobile phase. HPLC was also applied by Ruggieri and Ruggieri[329]. A rapid method was described for the quantitative determination of patulin in ethyl acetate extracts of apple juice[330]. After clean-up with a sodium carbonate solution, patulin was determined by reversed-phase HPLC using a μBondapak C_{18} column and a UV detector at 254 nm.

Citreoviridin levels in *Eupenicillium ochrosalmoneum*-infested maize kernels at harvest were examined by Wicklow *et al.*[331]. The maize was extracted with dichloromethane and the extract was partially purified with silica and amino solid-phase extraction columns. The citreoviridin in the extract was quantitated by using normal-phase HPLC with ethyl acetate–hexane (75:25) as the mobile phase at a flow-rate of 1.5 ml/min. Fluorescence detection with λ_{ex} = 388 nm and λ_{em} = 480 nm gave an optimum response and sufficient densitivity (limit of detection, 2 μg/kg). The main peak, with a retention time of 5.46 min, was citreoviridin. The same method was used to determine citreoviridin in corn and rice[332].

HPLC was applied in combination with TLC to separate and characterize penitrems A–F[176]. The HPLC separation was achieved on an HP 79918A RP-8 reversed-phase column with water–methanol (22:78) as the eluent. The penitrems were detected at 296 nm and it was possible to separate them in one run. For quantitative analysis, penitrem A monoacetate was used as an internal standard.

Lauren and Gallagher[333] developed a method of analysis for the janthitrems using reversed-phase HPLC. The use of both UV and fluorescence detectors allowed the differentiation of the janthitrems from other compounds with similar retention times. A modification of the eluent helped to separate a group of tremorgens as follows: janthitrem B, janthitrem A, verruculogen, penitrem A, fumitremorgin B, janthitrem C and janthitrem D.

Tanaka *et al.*[334] published a method for the rapid and sensitive determination of zearalenone in cereals by HPLC with fluorescence detection. Another method was reported by Cohen and Lapointe[335]. The HPLC determination, with UV and fluorescence detection, of α-zearalenol and zearalenone in corn has been studied collaboratively and the results were published by Bennett *et al.*[336]. Bagneris *et al.*[337] reported the determination of zearalenone and zearalenol in animal feeds and grains, using fluorescence detection. A diastereomeric mixture of α- and β-zearalenol, which could not be resolved by two-dimensional TLC, was resolved into its components by HPLC[196].

Kamimura[199] studied the microbial conversion of zearalenone by various species of fungi and used HPLC, TLC and preparative CC. Joseffson and Möller[338] described an HPLC procedure for the determination of zearalenone and ochratoxin A in cereals.

Another method was reported[339] for the extraction and determination of zearalenone in chicken tissues by HPLC using a reversed-phase radial compression separation system, a UV detector and acetonitrile–water (60:40) as the mobile phase. Recoveries of zearalenone added at levels from 50 to 200 ng/g were in the range 82.6–95.1%.

The HPLC determination of zearalenone and zearalenols in rat urine and liver[340] and in blood plasma and urine[341] has been described. Detection of the fraudulent use of zearalenol and the natural occurrence of zearalenone in cattle urine by HPLC was reported[342].

Zearalenone and α- and β-zearalenol are transmitted into the milk of cows and other animals. They can be extracted with basic acetonitrile and, after acidification, partitioned into dichloromethane and a hydrophilic matrix. After clean-up on an aminopropyl solid-phase extraction column and reversed-phase HPLC, they are detected fluorimetrically. As little as 0.2 ng/ml of zearalenone and α-zearalenol and 2 ng/ml of β-zearalenol can be detected in milk[343].

An HPLC determination of cyclopiazonic acid was reported[344]. A modification of this method based on ligand-exchange HPLC was developed for the determination of cyclopiazonic acid in poultry meat[345]. After extraction and clean-up, the toxin was subjected to ligand-exchange HPLC using a Beckman Ultrasphere ODS column and detection at 284 nm.

According to Goto *et al.*[346], the method reported by Lansden[344] lacks reproducibility, accuracy and sensitivity. To overcome these problems, they developed a sensitive and accurate HPLC method for the analysis of cyclopiazonic acid. The

normal phase with silica gel (Develosil 60-5) gave satisfactory results. The most succesful solvent system was ethyl acetate–2-propanol–25% aqueous ammonia (55:20:5) and the flow-rate was 1.0 ml/min. The detection limit for pure toxin with this system was 0.2 ng, and a linear calibration graph was obtained in the range 0.5 ng–3 μg. This HPLC method was utilized for the analysis of samples contaminated with cyclopiazonic acid. The toxin was extracted from maize, deoiled peanut meal and rice with chloroform–85% phosphoric acid (100:1), purified on Sep-Pak cartridge columns and then analysed by HPLC.

Reddy *et al.*[347] described an HPLC procedure for secalonic acid D and its application to biological fluids. A precolumn before the μBondapack C_{18} column allowed the direct injection of urine and bile without sample clean-up. Rat plasma was acidified and was extracted with ethyl acetate. The extracts were pooled and evaporated under nitrogen. The residue was taken up in the elution solvent system and aliquots were analysed by HPLC. Mixtures of acetonitrile–water–glacial acetic acid–tetrahydrofuran (5:3:0.5:0.5 for system A and 4:3:0.5:0.5 for system B) were used as eluents; system A was used for urine and plasma samples and system B was for bile samples. Detection was effected at 340 nm.

The separation of the 1,4-naphthoquinones xanthomegnin and viomellein by means of HPLC was reported[228]. HPLC has been used for the quantitative determination of xanthomegnin in corn extracts[348], grains and animal feeds[349]. Derivatization of the toxin for fluorimetric determination was reported by Kuan *et al.*[350].

A method was developed for the purification of xanthomegnin produced by *Penicillium viridicatum* on converted rice[351]. The toxin was extracted with dichloromethane and the extract was concentrated to an oil which was partitioned between hexane and methanol–water (9:1). The methanol layer was washed with a second portion of hexane, resulting in deposition of a xanthomegnin-rich precipitate at the interface, which was removed and washed with hexane. Chromatography of the precipitate dissolved in dichloromethane was performed with a Waters Assoc. Prep 500 chromatograph fitted with a Prep Pak 500 silica column and eluted with toluene–methanol–acetic acid (98.5:1:0.5) at a flow-rate of 250 ml/min. The xanthomegnin crystallized in those fractions eluting between 4.5 and 6.5 l. Small amounts of viomellein were eluted between 2.5 and 3.5 l.

A critical analysis of several HPLC systems for separating aflatoxins and their anthraquinone precursors was published recently. An HPLC system with a μBondapak C_{18} column and a solvent system of methanol–tetrahydrofuran acidified with acetic acid was used for the separation of anthraquinone precursors of aflatoxins. The system offers the advantage of a good separation of all the anthraquinones of interest and a running of less than 40 min. It has also been demonstrated that this system can be used as a means of screening fungal strains and mutants for the metabolites which they accumulate, and also for the rapid quantitation of enzymic and non-enzymic interconversions of the metabolites[322].

Monomeric anthraquinones (macrosporin and altersolanol A) and modified anthraquinones (alterporriols A, B and C) from fermentations of *Alternaria porri* were also determined by HPLC[352].

Shepherd and Gilbert[353] developed a method for the determination of moniliformin in maize employing ion-pairing extraction and HPLC. Thiel *et al.*[250] used a paired-ion chromatographic technique in the quantitative determination of monili-

formin together with deoxynivalenol and zearalenone. The same technique and an ion-exchange procedure were used later[354] for the quantitative determinations of moniliformin in corn screenings. Detection was performed at 227 nm. Paired-ion chromatography was carried out on a μBondapak C_{18} column using 0.1 *M* sodium phosphate buffer (pH 7.0)–0.005 *M* tetrabutylammonium hydrogensulphate–8% methanol as the mobile phase. The ion-exchange separations were done on a Partisil 10 SAX column using 0.01 *M* sodium dihydrogenphosphate (pH 5.0) as the mobile phase.

Preparative separations of two fusaric acid analogues from *Fusarium moniliforme* were reported by Burmeister *et al.*[236].

The determination of fusarin C in corn was reported[355]. After extraction and clean-up, fusarin C was quantified in the column eluate by HPLC on an Ultrasphere column using methanol–chloroform (1:19) as the mobile phase and detection at 360 nm. This method was also applied by Thiel *et al.*[354].

Danieli *et al.*[356] employed reversed-phase HPLC with detection at 250 nm for the determination of PR toxin. The retention time and peak shape were found to be critically dependent on the water–acetonitrile ratio in the mobile phase. HPLC of PR toxin and eremofortin C was described by Chang[357].

It was shown[358] that rubratoxin B is heat labile, at least during clean-up. The toxin was detected in mixed-feed extracts using an acidified mobile phase to maintain it in the non-ionized form.

HPLC of alternariol, its monomethyl ether, altenuene and tenuazonic acid was reviewed by Seitz[237]. An HPLC preparation of alternariol, alternariol methyl ether and altenuene was also reported[359]. A method of ligand-exchange HPLC for the determination of tenuazonic acid has been developed by Scott and Kanhere[360].

4.5. HPLC of peptidic mycotoxins

This sub-section deals with cyclosporin A (cyclosporine), its metabolites, phomopsin A, α-amanitin and phalloidin. Cyclosporin A is a lipophilic neutral and cyclic peptide mycotoxin with exceptional immunosuppressive properties.

Edwards and Lillehoj[361] developed techniques for the quantitative assessment of the toxin in rice. The methods include open-bed CC, TLC and HPLC for the separation and quantitation of cyclosporin A from *Trichoderma polysporum*-inoculated rice. The good baseline resolution at ambient temperature demonstrated no necessity for the higher temperature which have been used in most HPLC analyses of the toxin.

Several HPLC methods have been reported for the determination of cyclosporin A in both human serum and urine[362–370]. A temperature of about 70°C is needed for symmetrical cyclosporin peaks[366,367,370]. To meet this requirement, the mobile phase should be heated before it enters the detector.

For some HPLC methods liquid–liquid extraction (*e.g.*, refs. 362–364) and for the others solid-phase extraction is used (*e.g.*, refs. 370–375). Several procedures use a single column (in either a gradient or isocratic mode; *e.g.*, refs. 362, 371, 376–380). Other, more complex, procedures require column switching[381–386] or multi-step sample preparation[387].

Solid-phase extraction and a highly efficient HPLC analysis were reported to provide accurate measurements of cyclosporin A in whole blood, plasma or serum.

There are two steps in this analysis[370]: extraction of the drug from blood by solid-phase extraction, and analysis on a highly efficient 3-μm silica-based HPLC column. Whole blood samples, rather than serum or plasma, provide the most accurate measurements of cyclosporin A. The detector response for cyclosporins A and D (the internal standard) is optimum at 195–215 nm, a range in which many blood components also strongly absorb. Supelclean LC-CN solid-phase extraction tubes efficiently retain the non-polar cyclosporin molecules, then release them for elution with a solvent of moderate strength. Cyclosporin A was well separated from the internal standard on Supelcosil LC-8, LC-18, LC-DP and LC-CN columns. When methanol was included in the mobile phase, blood or other components remaining in the sample were eluted before the cyclosporins.

Cyclosporin A alone or together with its metabolites can be detected by means of HPLC[388,389].

Brossat *et al.*[390] reported a selective and sensitive HPLC method, which involves solid-phase extraction and ion-pair chromatography. It can be used for the assay of cyclosporin A in serum or urine. Samples were cleaned up on a solid-phase extraction system (cyanopropyl column). The system involved a reversed-phase Ultrasphere C_{18} column maintained at 72°C and a linear gradient of acetonitrile (from 65 to 95%) in 0.14% triethylammonium phosphate. Liquid chromatographic analysis of radioimmunoassay standards showed that some samples contained a contaminant peak. Comparison of cyclosporin A levels obtained by radioimmunoassay and HPLC in clinical investigations showed that the former values were generally, but not always, higher than the latter, and that cyclosporin A is very differently metabolized depending on the patient, disease and treatment.

Other HPLC methods have been published that could be used for the measurement of cyclosporin A and two[391], three[392] or four its metabolites[389].

In trying to reproduce several previously published HPLC methods, Christians *et al.*[389] pointed out one or more of the following disadvantages: a short lifespan of the chromatographic column[362,371], low or variable recovery of cyclosporin A[362,387] and laborious and/or time-consuming extraction and sample preparation[362,371,387]. They developed another HPLC method using cyclosporin D as an internal standard for the routine measurement of cyclosporin A and four of its metabolites. Whole-blood samples were purified on refillable solid-phase glass extraction columns. The chromatographic method included gradient elution using acetonitrile and water (pH 3.0) as eluents and an RP-8 analytical column. More than 1000 samples were analysed without any loss. The inter-assay coefficient of variation (C.V.) was 6.3% and the intra-assay C.V. was 4.9%. A linear correlation was found over a range of 0–3000 ng of cyclosporin A per ml of whole blood. The detection limit was 20 ng and the recovery was 80–90%. Metabolites 1, 17, 18 and 21 could be characterized.

Most recently, Gmur *et al.*[391] reported a column-switching HPLC method for measuring metabolite 17 in whole blood and also separating metabolite 1. New methods for the determination of cyclosporin A alone or cyclosporin A and three of its metabolites (17, 1 and 21) were also published recently[392].

An improved HPLC assay of phomopsin A, the principal hexapeptide mycotoxin responsible for lupinosis, was reported[393]. A reversed-phase Cl column, a methanol–water gradient and UV detection were used.

The diagnosis of suspected poisoning by the mushroom *Amanita phalloides* is a

challenge to clinicians. Several radioimmunoassay (RIA) methods have been developed[394]. An alternative method for the determination of the toxic peptides of poisonous mushrooms (α-amanitin in the amatoxin group and phalloidin in the phallotoxin group of substances) is provided by HPLC. Methods in this direction have been published[395–397]. Most recently, a reversed-phase HPLC assay has been developed for the simultaneous determination of α-amanitin and phalloidin in human plasma[394]. The procedure is based on the enrichment of the toxins on a precolumn, followed by the transfer of both compounds in a foreflush mode to the analytical column. α-Amanitin and phalloidin can be quantified down to a minimum concentration of 10 ng/ml in plasma.

5. GAS CHROMATOGRAPHY

Historically, gas chromatography (GC) was introduced into the field of mycotoxins in the early 1970s. If mycotoxins are sufficiently volatile at the column temperature, or if hydroxylated toxins can be converted into volatile derivatives, GC can be used in their analysis.

In GC, mycotoxins or their derivatives are mostly detected with flame ionization or electron-capture detectors. GC can also be effectively coupled to a mass spectrometer (GC–MS) to obtain qualitative data concerning the identity of the components being analysed.

Some pioneer applications of GC in mycotoxin analysis were reviewed by Gorst-Allman and Steyn[1]. The following mycotoxins were included: alternariol, alternariol monomethyl ether, altenuene, patulin, penicillic acid, sterigmatocystin, trichothecenes and zearalenone. An authoritative review on the GC of mycotoxins was written more recently by Beaver[398]. Vesonder and Rohwedder[243] reviewed the GC–MS analysis of mycotoxins.

As GC is most extensively applied in the field of trichothecenes[399], a selection of papers published in the 1980s are referred to in this section. A few applications for the detection of other mycotoxins have also been selected and are referred to below.

5.1. GC of trichothecenes

5.1.1. Development of techniques. The chromatography of trichothecenes has been reviewed[298,400]. The most sensitive and specific analytical techniques are GC with electron-capture detection (GC–ECD) and GC–MS[401–403]. The most common methods of derivatization are silylation, which converts the trichothecenes into trimethylsilyl ethers (TMSE), trifluoroacetylation and heptafluorobutyrylation, which convert the trichothecenes into trifluoroacetyl and heptafluorobutyryl esters, respectively. Silylation is often preferred because it gives much less complicated gas chromatograms[404,405].

Gilbert *et al.*[406] studied the optimization of the conditions for the trimethylsilylation of trichothecenes. Rizzo *et al.*[407] used trimethylsilylimidazole, with and without trimethylchlorosilane, for the silylation with the aim of improving the detectability of six of the most important trichothecenes by performing selective hydrolysis of the reagent, after silylation, in order to eliminate its interferring effect on the products of derivatization during GC.

Scott and Kanhere[408] compared column phases for the separation of deriv-

atized trichothecenes. Decomposition of trifluoroacetyl derivatives of trichothecenes on fused-silica capillary columns was reported[409]. Visconti *et al.*[410] published mass spectrometric evidence for demethylated homologues occurring at trace levels in trichothecene standards. A fast and sensitive GC–MS method for the simultaneous detection and quantification of several simple trichothecenes with good precision was developed[411]. Two semi-synthetic derivatives, 4-deoxyverrucarol and 16-hydroxyverrucarol, were adequate internal standards for both the detection and quantification of trichothecenes. The detection and quantitation of several polar and thermally labile macrocyclic trichothecenes by GC–negative ion chemical ionization mass spectrometry (GC–NICI-MS) was reported[412]. The method is applicable to the detection and quantitation of these compounds in naturally occurring samples.

5.1.2. Trichothecenes in environmental samples. T-2 toxin has been determined in maize samples by GC–MS[413] and in *Fusarium acuminatum* cultures by GC with ^{63}Ni ECD[414]. The latter procedure was also used to detect T-2 toxin and diacetoxyscripenol in corn samples[355,415]. GC–ECD and GC–MS have been applied to the determination of deoxynivalenol[416–418], nivalenol[419] and nivalenol with deoxynivalenol[420] in cereals.

Applications of GC in multi-trichothecene detection have been published[172,398,421–425]. Black *et al.*[424] described methods for the simultaneous detection of a wide range of trichothecenes, including the most polar ones and some macrocyclics, using either GC–MS with selected ion monitoring or GC–ECD. Trichothecenes have been extracted directly from the various matrices, or from Clin-Elut columns, and cleaned up on Florisil Sep-Pak cartridges. Macrocyclics and neosolaniol have been detected after hydrolysis to verrucarol and T-2 tetraol, respectively. For optimum sensitivity over a wide range, trichothecenes have been detected, both before and after hydrolysis of ester groups, as their heptafluorobutyrate derivatives using a quandrupole mass spectrometer and negative ion chemical ionization. The methods have been used to detect the presence of scirpentriol, nivalenol and 15-monoacetoxyscirpenol in sorghum. Trichothecenes in less complex matrices could be detected, after hydrolysis, using GC–ECD.

More recently, another GC screening method for T-2 toxin, diacetoxyscirpenol, deoxynivalenol and related trichothecenes in feed samples was reported[426]. Feeds were extracted with acetonitrile–water and the toxins were purified on charcoal–alumina–Celite, Florisil, and silica mini-columns. Deoxynivalenol, nivalenol, diacetoxyscirpenol, T-2 toxin and their fungal metabolites were hydrolysed to their parent alcohols by alkaline hydrolysis, derivatized to their pentafluoropropionyl analogues and quantitated by capillary GC with ECD. Identity can be confirmed and the sensitivity increased by using negative chemical ionization MS with no additional sample workup.

5.1.3. Trichothecenes in body fluids. Rood *et al.*[319] published a rapid screening procedure for the detection of trichothecenes in plasma and urine. More recently, they described a diagnostic screening method for the determination of trichothecene exposure in animals[427]. Other workers have used either GC–ECD[428–431] or GC–MS[432,433]. Trichothecenes have been detected in human blood[428,433] and urine[432], dog plasma[430], swine plasma and urine[431] and bovine urine and faeces[429]. Individual or several toxins have been determined as follows: deoxynivalenol and its metabolites[429], diacetoxyscirpenol[431], T-2 and HT-2 toxin[430], T-2 toxin, HT-2 toxin, T-2

triol, diacetoxyscirpenol, deoxynivalenol and verrucarol[433] and eleven trichothecenes of widely varying polarity[428]. Some examples are presented here.

A sensitive and selective method was developed for the simultaneous detection of eleven trichothecenes in human blood. The procedure involved precipitation of blood proteins with acetone followed by a clean-up using reversed-phase Sep-Pak C_{18} cartridges. The extracted trichothecenes were derivatized to their pentafluoropropionyl esters, separated using capillary GC and detected using electron-capture negative ion chemical ionization with methane as the reagent gas and selected-ion monitoring. Optimum sensitivity and selectivity were obtained using low source temperatures (60°C) and high source pressures (1 Torr). Detection limits for 1-ml blood samples were in the range 0.1–5 ppb. The method was readily adaptable to the detection of other trichothecenes and was validated in collaborative studies by the successful analysis of 42 blood samples spiked and submitted blind by two independent laboratories for analysis[428].

Capillary column GC–ammonia chemical ionization MS was found to be an excellent technique for the trace detection and identification of underivatized trichothecenes. Abundant $(M + H)^+$ and/or $(M + NH_4)^+$ pseudo-molecular ions were observed for T-2 toxin, HT-2 toxin, T-2 triol, diacetoxyscirpenol, deoxynivalenol and verrucarol under the conditions adopted. This method was succesfully applied to the analysis of human blood samples spiked with mycotoxins in the range 0–500 ng/g during an interlaboratory exercise. T-2 toxin and diacetoxyscirpenol were detected in these samples in the range 2–180 ng/g. Detection limits of 0.7 and 3.6 ng/g for T-2 toxin and diacetoxyscirpenol, respectively, were possible owing to the specificity of the method[433].

5.1.4. Biotransformations of trichothecenes. GC has been used in studies on biotransformations with the following results. The distribution and metabolism of tritium-labelled T-2 toxin was investigated after oral administration to chickens[434] and a lactating cow[435], and after intravascular administration to swine[436]. In all species, T-2 was rapidly biotransformed to a variety of metabolites. Minor metabolites in the cow and chicken were initially identified as simple hydrolysis products including HT-2 toxin, 4-deacetylneosolaniol and neosolaniol. Other metabolites were characterized later[437,438]. In addition to HT-2 toxin, 4-deacetylneosolaniol, T-2 tetraol and neosolaniol were detected in rat liver homogenates[439]. GC–ECD was used to characterize T-2 toxin metabolites by Knupp *et al.*[440]. Rat liver microsomes transformed T-2 toxin *in vitro* to a variety of metabolites including HT-2, neosolaniol, 4-deacetylneosolaniol, T-2 triol and 3′-OH-HT-2, in addition to two unidentified compounds.

An *in vitro* rumen system was used by Swanson *et al.*[441] to compare the metabolism of three trichothecenes by rumen microorganisms: T-2 toxin, diacetoxyscirpenol and deoxynivalenol. GC ECD and GC–MS analyses of extracts indicated that all three toxins were biotransformed to a variety of deepoxy and deacetylated products.

5.2. GC of other mycotoxins

The aflatoxins have been analysed mostly by TLC and less often by HPLC. However, recent reports have described methods for their GC–MS determination. Friedli[442] reported that aflatoxin B_1 could be determined without chemical derivatization by GC using a mass spectrometer as the detector. Trucksess *et al.*[443] were

able to determine aflatoxin B_1 on methylsilicone-coated fused-silica columns. Rosen *et al.*[444] used a fused-silica capillary column coated with a film of bonded 5% phenyl–1% vinylmethylsilicone to chromatograph aflatoxins B_1 and B_2. Dimitrov *et al.*[445] included GC among other methods for the detection of aflatoxins in foods.

Most recently, Goto *et al.*[446] succeeded in determining four major aflatoxins (B_1, B_2, G_1 and G_2) using GC with flame ionization detection (FID) with a capillary column injector and a fused-silica capillary column. A Shimadzu GC-15A gas chromatograph and a Shimadzu GCMS QP1000 mass spectrometer were applied. Two types of stationary phases were tested. The methylsilicone column (DB-1, 10 m) did not separate aflatoxins G_1 and G_2 and barely separated B_1 and B_2. As a result, the shape of the peaks was distorted. In contrast, a 5% phenylmethylsilicone column (DB-5, 10 m) clearly separated aflatoxins B_1 and B_2 and also achieved a 50% separation between aflatoxins G_1 and G_2. A longer column was used to improve the overall separation. Although the four aflatoxins were completely separated on a 25-m DB-5 column, the sensitivity was much lower for aflatoxins G_1 and G_2 than for B_1 and B_2.

In a review chapter on the GC of mycotoxins, Beaver[398] described GC analyses of trichothecenes, zearalenone, patulin, penicillic acid, slaframine, swainsonine, *Alternaria* toxins and aflatoxins. Except for trichothecenes and aflatoxins, most of the methods reviewed were published in the 1970s. Some data published in the 1980s are added below.

Phillips *et al.*[447] described a method for the GC of penicillic acid as its pyrazoline derivative, which was detected by FID. Butenolide was determined by GC–ECD[448]. GC has also been used in the detection of "peptaibols" and other aib-containing peptides of fungal origin, *i.e.*, trichotoxin, alamethicin, suzukacillin, hypelcin and paraselsin[449,450].

Gilbert *et al.*[451] described the derivatization of moniliformin for GC–MS analysis. They found that the reaction of moniliformin with N-methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide containing 1% *tert.*-butyldimethylchlorosilane produced a compound that had a characteristic mass spectrum, was formed quantitatively and in selected ion GC–MS gave a good linear calibration at low levels. The purification of the derivative was achieved by high-performance size-exclusion chromatography.

Bottalico *et al.*[196] extracted zearalenol from *Fusarium*-infected stems of corn. The toxin, which appeared as a single compound in various TLC systems, was resolved into two components by HPLC. A GC–MS examination of a purified fraction confirmed the natural occurrence of zearalenol as a diastereomeric mixture and led to the identification of the α- and β-isomers.

6. CONCLUSIONS

This review was written with the aim of demonstrating the scope of applications of chromatographic techniques in the still developing field of mycotoxins. It is partly arranged with a historical perspective. Although several mycotoxins were recognized before the 1960s (mostly as antibiotic compounds), the origins of the "mycotoxin era" are usually associated with the discovery of aflatoxins in the early 1960s. In that period, the gradual decline of applications of PC was due to the rapid development of TLC. A decade later, the introduction of HPLC and GC had to compete with then-existing methods based on TLC.

Whereas applications of PC in mycotoxicology are now interesting mostly from a historical point of view, it would be impossible to review the mushrooming literature concerning the use of other chromatographic techniques. As earlier contributions of general interest have been repeatedly reviewed, attention is focused here on the achievements of TLC, HPLC and GC published in the selected literature from 1980 onwards.

In most instances, the mycotoxins to be analysed or purified chromatographically are present in contaminated samples. Hence, they must be extracted and cleaned-up prior to TLC, HPLC or GC if reliable results are to be obtained. Extraction procedures include extractions of mycotoxins from feeds and foodstuffs, cultivation media and/or mycelia of toxigenic fungi and body fluids or tissues.

Clean-up procedures include CC, gel-permeation chromatography, liquid–liquid partition and precipitation. In these procedures, contaminating substances have to be removed from the mycotoxin samples. Several recent clean-up methods were included into the sections covering TLC, HPLC and GC.

TLC is by far the most widely used chromatographic technique applied to mycotoxins owing to its relatively simple, fast and inexpensive character. One- and two-dimensional TLC and HPTLC have been used. Preparative TLC is also of great importance in purification procedures. With HPTLC and in quantitations, TLC becomes more expensive owing to the need for densitometers and spectrophotometers.

In addition to its use in clean-up procedures, CC is applied either in large-scale separations and purifications or in some analytical methods using various mini-columns.

HPLC is applied mostly for analytical purposes but preparative columns are also used in mycotoxicology.

It appears that countercurrent chromatography (CCC) has not yet been applied in the field of mycotoxins, although applications of procedures such as droplet CCC, centrifugal droplet CCC, rotation locular CCC and planet coil centrifugal CCC have proved advantageous for the isolation and purification of a diverse array of natural products[452]. For example, CCC greatly facilitated the isolation of a variety of structural types of antibiotics and was more convenient than other chromatographic techniques[453]. Avoidance of contamination with traces from solid chromatographic supports by CCC facilitated instrumental analyses of daunomycin reduction products[454]. The resolution of the actinomycin C complex into its components by CCC has been very successful[455]. Actinomycin C2 contains just one methylene group more than actinomycin C1 and one methylene group less than actinomycin C3. Nonetheless, these components were conveniently resolved utilizing only 600 ml of mobile phase. Hence, one would expect that CCC will find its place among other chromatographic techniques hitherto used in the field of mycotoxins.

The advantages and disadvantages of the use of TLC and HPLC in mycotoxicology were excellently compared as follows[242]:

"The most significant advantage of TLC is that it can be a very inexpensive technique, although in its more sophisticated forms it requires a considerable capital investment in items such as spotters and densitometers. In addition, if one-dimensional TLC gives adequate resolution, a considerable number of samples may be analysed on one plate. Should two-dimensional TLC be found necessary, several plates can be developed simultaneously. Hence results can be obtained rapidly by TLC, even when

TABLE 5

COMPARISON OF VARIOUS FORMS OF AUTOMATED CHROMATOGRAPHIC SYSTEMS

Modified from ref. 456.

Comparisons	*TLC (proposed)*	*LC*	*GC*
Mode	Stepwise, batch	Continuous, flow	Continuous, flow
Limit of detection	pg	pg	pg, fg
Analysis time (per throughput)	Minutes	Minutes	Minutes
Sample per throughput	As many as 72[a]	One	One
Effective analytical time (per sample)	Seconds	Minutes	Minutes[b]
Potential sample capacity (per day)	As many as 10 000[c]	72	72

[a] Per 20-cm HPTLC plate[457].

[b] A high-speed chromatographic gas analyser has been described[458].

[c] Assuming a constant time of 10 min.

taking into account the time required for spotting the plates. The two principal disadvantages of TLC analysis are its lack of potential for automation and the subjective nature of the quantitation step. Use of a densitometer overcomes the latter objection but at a cost equivalent to that of a single HPLC system. Autosamplers permit unattended running of HPLC equipment and allow the sample throughput in this sequential method of analysis to be as great as that for TLC, while the recent development of short, very high-efficiency columns has demonstrated the capability of HPLC to provide extremely rapid results. Because of the general growth in the use of HPLC, many laboratories possess the necessary instrumentation and could therefore perform mycotoxin analysis should it appear to offer definite advantages over the more conventional TLC methods. Comparing HPLC and TLC techniques, a similar high degree of competence is necessary when establishing procedures and validating methods, but it is sometimes not appreciated that although TLC may be carried out using very simple equipment, it then demands greater operator skills and attention to detail in use then does HPLC.

Separation by HPLC may be preferred for other reasons. One factor to consider is safety; liquid chromatography offers greater protection, particularly for preparative work, because toxins are maintained in solution and contaminated silica dust does not arise. Equally, moisture- or oxygen-sensitive samples, such as xanthomegnin, are more readily chromatographed on a column. One potentially important advantage of HPLC lies in its suitability for on-line clean-up of crude extracts, and it is possible that this will ultimately be seen as one of the more compelling reasons for employing HPLC rather than TLC as the analytical technique".

According to Shepherd's comparison of TLC and HPLC, one of the two principal disadvantages of TLC analysis is "its lack of potential for automation"[242]. However, in his "Considerations for automating TLC", Rogers[456] was less pessimistic. He proposed the following operational parameters of automated TLC: (i) plate insertion/retrieval; (ii) sample application; (iii) development, first dimension; (iv) development, second dimension; (v) derivatization, reagent spray; (vi) derivatization, energy imput;

(vii) microdensitometric scanning; and (viii) documentation. His comparison of various forms of automated chromatographic systems are presented in Table 5.

Except for trichothecenes, GC has hitherto been much less used than TLC or HPLC in mycotoxicology. It has been applied in determining some small lactones (patulin, penicillic acid, butenolide), sterigmatocystin, aflatoxins, zearalenone, moniliformin and some *Alternaria* toxins. The derivatives of trichothecenes prepared for GC analysis have usually been trimethylsilyl, heptafluorobutyryl and trifluoroacetyl. However, the ability to detect some mycotoxins in an underivatized state using fused-silica capillary columns has recently been demonstrated (*e.g.*, ref. 433).

In GC, mycotoxins or their derivatives may be detected by FID or ECD. GC can also be effectively coupled with mass spectrometry to obtain qualitative data concerning the identity of compounds being analysed.

7. SUMMARY

Achievements in the applications of chromatographic techniques in mycotoxicology are reviewed. Historically, column chromatography (CC) and paper chromatography (PC) were applied first, followed by thin-layer chromatograhy (TLC), high-performance liquid chromatography (HPLC) and gas chromatography (GC). Although PC techniques are no longer used in the analysis of mycotoxins, selected applications of PC are included to underline historical continuity. The most important achievements published from 1980 onwards are described. They include clean-up methods, TLC, CC, HPLC and GC of mycotoxins in environmental samples, foods, feeds, body fluids and in studies on biosynthesis and biotransformations of mycotoxins. Advantages and disadvantages of chromatographic techniques used in mycotoxicology are also evaluated.

NOTE ADDED IN PROOF

Further possibilities of the use of HPLC techniques in the field of mycotoxins can be documented with the following examples.

A simple LC–MS procedure has been developed recently for the analysis of some of the most toxic (roridins) and few known benevolent (baccharinoids) and isomeric macrocyclic trichothecenes[459]. Roridins and baccharinoids were separated on a reversed-phase HPLC column and effectively ionised under thermospray ionisation conditions. A semisynthetic macrocyclic trichothecene, 8-ketoverrucarin A, was used as the internal standard. Minimum detectable limits were to be 2–5 ng.

In chromatography of cyclosporin A, only reversed-phase HPLC techniques have been developed and the use of normal-phase techniques has not been reported so far (see Section 4.5.). Most recently, Oka *et al.*[460] have developed a normal-phase HPLC method to be conducted in conjunction with rapid flow fractionation for sample pretreatment. The method was used to determine cyclosporin A concentrations in the serum of kidney transplant patients. HPLC rather than the conventional radioimmunoassay (RIA) provided more precise and reliable values for the concentration of cyclosporin A.

The advent of computer-controlled photodiode-array UV detectors in HPLC[461] could even increase the use of HPLC in the field of mycotoxins. For exam-

ple, profiling of mycotoxins in fungal-contaminated foodstuffs by HPLC coupled to detectors that are unable to give retention data corroborated by peak purity and peak identity may result in unreliability owing to the complexity of the matrix. Computer-controlled photodiode-array UV detectors provide considerable help solving such a complex analytical problem by greatly improving peak identification, peak purity assessment and quantitation. The high spectral acquisition rate during elution provides a matrix of absorbance wavelength time data that can be treated by computer-aided techniques for their reduction, manipulation and presentation. The potential of HPLC with diode-array detection for the profiling of mycotoxins in food samples has been demonstrated very recently[462]. A gradient elution reversed-phase chromatographic method was devised that simultaneously separated and detected major *Alternaria* mycotoxins in foodstuffs. According to the authors of this paper, "the multi-signal plotting capability may be an aid for a first identification (through the choice of selective wavelength) and at the same time can optimize the detection sensitivity for compounds (or classes) having different absorption maxima. In addition, three-dimensional spectrochromatograms give UV spectra and the possibility of identification of those toxins having characteristic spectra... Although UV spectra alone can rarely give an absolutely certain identification of a compound, a reasonable degree of confidence may be reached in most instances. Coupling of a diode-array UV detector to a functional group-specific detector, *e.g.*, electrochemical, should provide additional evidence (and sensitivity)".

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