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Review

Thin-layer chromatography of mycotoxins and comparison with other chromatographic methods

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Abstract

This paper highlights the status of thin-layer chromatography (TLC) of mycotoxins in various sample matrices. The outstanding merits of TLC in the field of the qualitative and quantitative determination of mycotoxins have been briefed. A comparison between different TLC methods and TLC with HPLC, enzyme-linked immunosorbent assay and GC methods, etc. is made, in general.

Keywords: Reviews; Thin-layer chromatography; Food analysis; Mycotoxins; Toxins

Contents

1. Introduction

Mycotoxins are fungal metabolites that have been of major concern as the toxic contaminants of foodstuffs and feeds, and have been recognized as a potential threat to human and animal health since the early 1960s resulting in frequent economic losses. Therefore, the development of methods for the analysis of mycotoxins has been constantly in demand. In the last ten years, among the techniques applied in the detection, analysis and characterization of mycotoxins, chromatography has so far been

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widely accepted because there always seems to be a need to separate some primary and secondary fungal metabolites simultaneously produced with mycotoxins. So far numerous work has been done in this field. Information on the techniques and methodologies has been reviewed by some authors such as Betina [1,2], Scott [3,4], Lin Leming [5], Holcomb et al., [6], Kok [7], etc.

Of the chromatographic techniques employed for these purposes, liquid chromatography (LC), high-performance liquid chromatography (HPLC), gas chromatography (GC), thin-layer chromatography (TLC), supercritical fluid chromatography (SFC), capillary zone electrophoresis (CZE), etc. have all played their roles. HPLC especially becomes increasingly the method of choice in the determination of aflatoxins [6]. Cepeda et al., reported measurement of a fluorescence increase after the post-column excitation of aflatoxins using cyclodextrins in HPLC for food analysis [8]. The robotic automated analysis of foods for aflatoxins by reversed-phase LC with fluorescence detection without derivatization after immunoaffinity column clean-up is reported by Carman et al. [9]. Aflatoxins in medicinal herbs and plant extracts have been determined by reversed-phase HPLC with a fluorescence detection limit of 0.05 $\mu g\,kg^{-1}$ and with recoveries in a range of 70–100% [10]. Kussak et al., employed HPLC including post column derivatization with bromine and fluorescence detection to determine aflatoxins in urine [11]. There have also been reports of automated immunoaffinity column clean-up and LC determination of aflatoxins in airborne dust from feed factories [12] and in peanuts and corn [13]. Besides, HPLC with fluorescence detection has also been applied to determine ochratoxin A at a level of 0.001 $\mu g\,l^{-1}$ in human blood, serum, mill and some foodstuffs [14], and to determine the mycotoxin moniliformin in cereals [15]. Furthermore, Dow et al. reported the application of HPLC and matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF-MS) qualitative and quantitative characterization of both isolated toxins and cyanobacterial toxins [16]. Cappiello et al., presented a method for the analysis of aflatoxins in food extracts based on LC–MS with a particle beam interface [17]. Kussak et al., described an LC–electrospray-ionization tandem MS method for the determination of aflatoxins with detection limits of aflatoxin B1=4 pg, B2=4 pg, G1=5 pg and G2=10 pg [18]. Recently, Josephs utilized electrospray-ionization HPLC–MS using both ion trap and triple quadrupole mass spectrometers for the detection and characterization of fumonisin mycotoxin impurities in a purified fumonisin B1 [19].

Matsushima et al., reported the detection of DAS (a list of the abbreviations of the mycotoxin names used in this paper is given in Table 1) and the related toxins by gas–liquid chromatography (GLC) after trimethylsilylation [20]. Song Yang reported the simultaneous analysis of five Fusarium mycotoxins by GC with detection limits of DON=$1\cdot10^{-11}$ g, HT-2 toxin=$5\cdot10^{-11}$ g, T2 toxin, NSL and DAS=$1\cdot10^{-10}$ g [21]. Early in 1984, Trucksess et al., had reported TLC determination of aflatoxins in corn and peanut butter and their confirmation by GC–MS [22]. In recent years, Onji et al., presented an improved method for the qualitative and quantitative determination of Fusarium mycotoxins by GC–MS without the need for chemical derivatization [23]. For the survey of natural occurrence of trichothecene mycotoxins and zearalenone in contaminated grains, Ryu Jae-Chun reported a method using GC–MS-selective ion monitoring mode after trimethylsilyl

Table 1
List of the abbreviations of the names of the mycotoxins mentioned in this article

Abb.	Name of Mycotoxin	Abb.	Name of Mycotoxin
My	Mycotoxin	Ste	Sterigmatocystine
Af	Atlatoxin	Gri	Griseotlulvin
Pa	Patulin	FMN	Fumonisin
Och	Ochratoxin	T-2	T-2 toxin
Tri	Trichothecene	TA	Tanazonic Acid
Ze	Zearalenone	FCN	Fusarochromanone
CA	Cyclopiazonic Acid	Ro	Rodidan
Vo	Vomitoxin	FRN	Fusarenon
FTN	Fumitremorgin	NVL	Nivalenol
Ve	Verruculogen	NSL	Neosolaniol
Te	Temtrem	CSN	Cyclosporin
Pe	Penutrem	MAL	Monoacetoxyscirpenol
Ci	Citrinin	SPL	Scirpentriol
Cy	Cytochalasin	ACN	Ascachalasin
Tre	Tremorgenic	MLN	Mellein
MA	Mycophenolic Acid	CMN	Culmorin
Bu	Butenolide	SBL	Sambucinol
PA	Penicillic Acid	MFN	Moniliformin
Xa	Xanthomegnin	DON	Deoxynivalenol

derivatization [24], and Mossoba et al., described one by employing a coupled GC matrix isolation-Fourier transform infrared spectroscopy (R) system [25]. More recently, Alternaria mycotoxins have also been analyzed using GC–MS by Scott et al. [26].

Besides the above, SFC adds a new dimension to the analysis of mycotoxins. Young et al., applied capillary and packed SFC for the separation of Fusarium mycotoxins of various structure types such as the trichothecene including DON, and its acetylated derivatives and T-2 toxin, as well as Bu CMN, SBL and Ze [27]. Bohrs et al., realized the separation of Och A and B, Ze and MFN by standard CZE and cyclodextrins-modified capillary electrophoresis [28]. Schneider et al., developed a membrane-based visual dipstick enzyme immunoassay for the simultaneous detection of up to five mycotoxins [29]. Sydenham et al., presented a monoclonal antibody-based (MAB) competitive direct enzyme-linked immunosorbent assay (CD–ELISA) method for the determination of FMNs in corn [30].

Despite the fact that many other techniques have been increasingly applied in the determination of certain varieties of mycotoxins, TLC is still the most popular due to its coherent features such as line principle, higher sample throughput, lower operating costs, convenience in identification by comparison of spot color, R_F values and UV–Vis spectrum, and is the method of choice for both semi-quantitative and quantitative purposes.

2. TLC applications

For this article, only selected examples are taken, therefore, the literature cited is not exhaustive. As a matter of fact, in the analysis of mycotoxins, TLC applications are still used in the great majority of cases. Tables 2 and 3 give some examples. It is seen from the Tables that both commonly found individual mycotoxins and miscellaneous mycotoxins in a variety of matrices are analyzed both qualitatively and/or quantitatively. One-dimensional TLC has been widely used, and two-dimensional and bi-directional developments are also employed frequently. The terms TLC and high-performance TLC (HPTLC) are used interchangeably in this article.

Sample preparation is needed for most analytical methods and so it is for TLC analysis of mycotoxins because the sample matrix is usually very complicated. The procedures for extraction and clean-up of given matrices depends on the physico-chemical properties of the mycotoxins. For example, Brandburn et al., reported the use of a pH-bonded cartridge for the clean-up of extracts from cottonseed [51]. Tomlins et al., examined the ability of C_2-, C_8-, C_{18}- and pH-bonded phases in extracting aflatoxins from aqueous methanol extracts of maize [53]. Silica gel columns were also used for the purification of mycotoxins [98]. Chu et al., evaluated by ELISA the clean-up from aflatoxins in corn, peanuts, and peanut butter [54]. Stubblefeld studied the reaction conditions for complete derivative formation of aflatoxin M1 and trifluoroacetic acid before TLC [125]. In recent years, the solid-phase clean-up method [126], immunoaffinity column clean-up [15] and supercritical fluid extraction technique [127] have been well applied in the determination of mycotoxins in groundnut cake, milk, foodstuffs and corn.

Of the layers used, a silica gel layer on a glass plate or on a plastic sheet [99] is the most widely employed, with normal-phase development (see Table 4). However, phenyl non-polar bonded silica gel [48], silanized silica gel [52], octadecyl-bonded silica gel [90,93,102,106–109], silica gel impregnated with a proper organic acid [91,113], and polyamide [122] have also been used as alternative methods to analyze different mycotoxins. Capasso et al., reported the use of reversed-phase TLC for the final purification of a new cytochalasin, ascochalasin [119].

Among a number of solvent systems used in literature, it is difficult to evaluate the relative advantages between them. However, a few solvents like chloroform, ethyl acetate, acetone, methanol, toluene, acetic acid, water and ether have often been employed in the initial systems with various percentages (see Table 5). In the separation of mycotoxins, tank development has been the common configuration. Comparison was made between tank and continuous linear development configurations [47]. Of course, instrumental TLC has been increasingly performed in the mycotoxin analysis. Coker et al., evaluated the instrumentation used in TLC of aflatoxins [55]. Stepwise gradient development was used

Table 2
Examples of TLC applications for individual mycotoxin

Mycotoxin	Matrix	Layer (L)[a]	Solv (S)[a]	Det. (D)[a]	Quant (Q)[a]	Remark	Ref.
Afs		L1 L2				Comparison of the two layers in R_F value, reproducibility, solvent systems,	[31]
Afs	Sorghum	L1				Two-dimensional (2D) HPTLC, Investigation of accuracy and precision of the method. Comparison with AOAC CB method	[32]
AfB1 B2 G1 G2		L1				2D-RPTLC, Study on the influence of temperature gradient for different systems in a tank	[33]
Afs	Chinese rice wine	L1			Q1	Detection limit: 1 μg/kg	[35]
AfB1 B2 G1 G2		L1	S1	D1		The use of the TLC conditions of CB (Contaminants Branch) method gave better results than BF (best Food) and CB–RCS–Mod (Modified CB method-Rapid Modification of the cottonseed Method): less fluorescent interference, better solvent efficiency, lower detection levels.	[36]
AfB1 B2 G1 G2		L1	S2 S3		Q2	Detection limit: 0.04 ng/kg	[37]
AfB1 B2 G1 G2		L1	S4	D1	Q2		[38]
AfB1 B2 G1 G2		L1	S2		Q2	Detection limits: 3.7, 2.5, 3.0, 1.3 μ/kg, respectively.	[39]
AfB1 B2 G1 G2		L1	S9 S2		Q2	2D-HPTLC with S9 and S2	[43]
AfB1 B2 G1		L1			Q2	According to AOAC 26.026-26.031 and AOAC 26.052-26.05844	[44]
AfB1	Cotton seeds	L1	S14	D1	Q2		[45]
AfB1 B2						According to CB-TLC method of AOAC (26.050)	[46]
AfB1 B2 G1 G2	Maize	L1	S9		Q2	Bi-directional TLC on L1 with S9. Comparison of tank development with Continuously linear development. Evaluation of different concentration of aqueous acetone–methanol (1:1) in extraction of Af from maize.	[47]
Afs	Maize	L1 L3				2D-HPTLC; Evaluation of the accuracy and precision of the method for a range of 3.4–901 μg/kg, R.S.D.: 1.7–10.8% and mean recoveries 92–99%; Discussion of the systematic error and the detection limits	[48]
AfB1 B2					Q2	According to AOAC CB method 968.22	[49]

Table 2. Continued

Mycotoxin	Matrix	Layer (L)[a]	Solv (S)[a]	Det. (D)[a]	Quant (Q)[a]	Remark	Ref.
Afs	Cotton seed	L1				Bi-directional TLC; Sample clean-up with a pH-bonded phase cartridge; comparison of the method with the first action AOAC method	[51]
Afs		L4	S9 S2		Q2	2·D-TLC; detection limits: B1 0.8 μg/kg, B2 0.4 μg/kg, G1 1.7 μg/kg, G2 0.4 μg/kg; R.S.D.: B1 1.7%, B2 2.5%, G1 3.1%, G2 3.1%	[52]
Afs		L1	S2		Q2	Two runs with S2; Evaluation of instrumentation used: Optimum sensitivity, accuracy and precision resulted when combination of a full automated TLC sampler, an unsaturated glass developing chamber and a monochromatic densitometer, with benzene–acetone (98:2, *v*/*v*) as the suitable sample application solvent.	[55]
AfB1	Dried figs	L1	S9 S16		Q2	2D-TLC	[57]
Afs		L1	S17 S14	D1 D4 D5		Identification of Afs in various strains of Aspergillus in foodstuffs with D2 and confirmation with D4 or D5	[58]
AfB1 B2	Raisins	L1	S4 S5		Q2	Comparison of TLC methods	[59]
AfB1 B2 G1 G2	Animal Feed Stuffs	L1	S14 S18	D1 D6		1D-TLC with S4; 2D-TLC with S18 and S4; Sensitivity: G1 and B1 0.05–0.1 ng per spot, G2 and B2<0.05 ng per spot	[60]
AfM1	Milk	L1	S17	D1	Q3	Also in milk products at low levels	[62]
AfM1	Cheese	L1	S14 S20	D1	Q2	Detection limit, 10 μg/kg	[63]
AfM1	Milk powder	L1	S14	D1	Q2	Detection limit, 50 ng/kg	[64]
AfM1	Milk	L1	S21	D1		After first elution, heating at 110°C for 10 min., overspotting with D9 and developing with S22. Detection limit, 0.3 ng per spot.	[65] [67] [68]
AfB1 B2 G1 G2	Feeds containing citrus pulp	L1	S23 S24	D1	Q2	Detection limit, 1 ng/kg	[66]
AfB1 B2 G1 G2		L1	S29	D1	Q2	Investigation of the precision, sensitivity, recovery, and linearity of response	[70]
AfB1 B2	Corn	L1	S4	D1	Q2	A rapid sereening method	[71]
Afs	Vegetable oil	L1	S4	D1	Q3	2D-TLC; Detection limits, 1–2 ng per spot	[72]
AfB1 B2 G1 G2 M1		L1	S4 S30 S17		Q2	On L1 with S4or S30 for B1, B2 G1, G2, and with S17 for M1	[73]
AfM1	Milk, Milk powder	L1	S25 S20		Q2	2D-TLC; Detection limits, 0.005 μg kg^{-1} or milk, 0.05 μg kg^{-1} for milk powder	[74]
AfB1 B2 G1 G2	Corn, peanut butter	L1	S4	D1	Q2	Detection limits, at a level of 2 μg/kg	[22]

(*Continued on p. 8*)

Table 2. Continued

Mycotoxin	Matrix	Layer (L)[a]	Solv (S)[a]	Det. (D)[a]	Quant (Q)[a]	Remark	Ref.
AfB1 B2 G1 G2	Feeds, containing citrus pulp	L1	S4 S30	D1		2D-TCL	[76]
AfB1 B2 G1 G2	Foods	L1	S35	D1	Q2	Overpressured TLC	[77]
AfB1 B2 G1 G2	Feed stuffs	L1	S4	D1 D10 D35	Q3	Confirmation with D10 and D35, combined with 2D-TLC	[78]
Ze		L1	S39		Q2 Q6	Comparison of Q2 and Q6 in their sensitivity	[83]
Ze	Cereals	L1	S40	D1 D12		Detection limit, 20–40 μg/kg	[84]
Ze	Pork	L1	S43 S44	D1 D12		Overpressured TLC with S43 and S44; 2D-TLC with S4	[86]
Ze, acetylated		L1	S45 S46	D3		From Fusarium graminearum	[87]
Ze	Ground paprika	L1	S43 S43	D1 D12		Detection limit: 20 ng per spot	[88]
Ze	Soy meal	L1	S42 S47	D12		Study the effect of soy meal of the detection	[89]
Ze		L1 L5	S48 S49 S50	D1 D15 D12		On L1 with S48 or S49, detection with D1 and D15 or D12; On L5 with S50, detection with D1	[90]
Och A	Rice	L1	S19 S53 S54	D1	Q2	On L1 with S53 in the reverse direction for 6 min, then with S19 for 10 min after cutting off the plate close to the origin, then with S54 after cutting off the further 30 mm from the plate bottom	[92]
Och A		L5	S55	D1	Q2	Comparison with LC method	[93]
Och A	Corn, peanuts, beans, rice, cassava	L1	S19	D1	Q3	Screening method	[94]
OchA	Pork kidneys	L1	S56 S57	D16 D1	Q2	Detection limit: 0.3 μg/kg	[95]
Pa	Apple	L1	S19	D1		Screening method	[96]
Tris		L5	S63	D2 D9		18 Tris were investigated	[102]
Ze	Food stuffs Fooders	L1	S4 S64	D1 D12		40–60 ng per spot with D1, 20 ng per spot with D12; recovery, 62.5–84.6%	[103]
Tris		L1	S65	D22		Detection limit: 20–25 ng per spot; Comparison with HPLC	[104]
Tris	Methanol	L1	S66	D10		Tris: T-2, iso-T-2, acetyl-T-2, HT-2, T-2 Triol, T-2 tetraol 3 1/2-OH-T-2, 3′1/2-OH-acetyl T-2	[105]

Table 2. Continued

Mycotoxin	Matrix	Layer (L)[a]	Solv (S)[a]	Det. (D)[a]	Quant (Q)[a]	Remark	Ref.
CSN A	Rice	L1	S69 S70 S71	D23 D24 D25		Identification	[108]
Tris	Fungal fermentation broth	L1	S72 S73	D1 D26		On L1 with S72 for the broth sample and with S73 for Tri derivatives by preparative centrifugal TLC	[109]
Tris	Fusarium	L1	S48	D9 D27		Tris: 8a- and 8b-hydroxysambucoin; Identification	[110]
Tris	Foods, feeds	L1	S74	D27	Q3	Tris: T-2, HT-2, NSL, T-2 tetraol, DAS, MAL, SPL	[111]
Tris	Bovine rumen fluid in vitro	L1	S75 S76	D28	Q8	Identification	[112]
Ci	Corn	L7	S77 S78 S79	D29 D3		Detection limit: 15–20 μg/kg	[113]
Ste	Cheese	L1	S80	D30	Q2	An inter-laboratory study	[114]
CA		L1	S81	D1 D24		Comparison with LC method	[115]
Cys	Plants	L1 L5	S86 S87 S88	D1 D32		For rapid detection	[117] [118]
ACN	Ascochyta heteromorpha	L1 L5	S89 S88	D1 D32		Identification on L1 with S89 and purification on L5 with S88	[119]
Te A B C		L1	S90 S91		Q2	On L1 with S90 for Te A and B, and with S91 for Te	[120]
MLNs	Cultures of fungus Septoria nodorum	L1 L8	S93 S94	D33 D34		On L1 with S93 and on L8 with S94	[122]
Afs Ze Ach A DON	Foods, feeds	L1			Q2	Presentation of a monitoring program for mycotoxin contamination	[123]

[a]The layers, solvent systems, detection procedures and the quantitative techniques used are listed in Tables 4–7, respectively.

for separation of Alternaria mycotoxins [98]. Jaruis et al., realized the isolation of trichothecene derivatives by preparative centrifugal development [109]. Besides these methods, overpressured thin-layer chromatography (OPTLC) has also been applied for mycotoxin analysis [77,86].

To visualize the mycotoxin spots on thin-layer plates, two kinds of techniques have been most frequently applied (see Table 6): examination under UV light of long or short wavelength for naturally fluorescent mycotoxins like aflatoxins, citrinin, ochratoxin A; spraying the plates with a chemical reagent that reacts with the mycotoxins to produce a colored or a fluorescent product. Other techniques such as fluorescence quenching [83], autoradiography [101], exposing into iodine [108,115] or ammonia vapors [91,113], and exposing to X-ray film [112] etc., have sometimes been used for the confirmation of some mycotoxins.

For the quantitation of mycotoxins (see Table 7), in situ evaluation has been dominant in the past decade, with fluorescence densitometry as the method of choice, including the fluorescence quenching technique [83] and absorbency densitometry. Semi-

Table 3
Examples of TLC applications for miscellaneous mycotoxins

Mycotoxin	Matrix	Layer (L)[a]	Solv (S)[a]	Det. (D)[a]	Quant (Q)[a]	Remark	Ref.
AfM1 M2 B2 G1 G2 Tri F T-2 T-2		L1				Identification of individual components by FAB-MS	[34]
AfB1 B2 G1 Ze Och A T-2 DAS DON		L1	S7 S8	D1 D2 D3		D1 for Afs, Ze, Och A; D2 for T-2 and DAS and S8, D3 for DON	[41]
AfB1 B2 G1 G2 Ze Tri DON DAS	Corn	L1	S9 S10 S11 S12 S13	D1 D3 D4		On L1 with S9 and S10 or S11 for AfB1, B2, G1,G2; on Ll with S12for Ze,Tri and detected of Ze with D1, and then developed with S13, detected with D3 and D1 for DON, with D4 for T-2 and DAS	[42]
Afs AchA Ze Ste	Brazil foods	L1	S4 S9 S15 S19 S32 S33 S34	D1 D3		Detection of Afs, OchA with D1 and of Ze, Ste with D3; detection limits: Afs 2 μg/kg, Och A 5 μg/kg, Ste 15 μg/kg, 55 μg/kg	[79]
CA Afs Pa Gri	Beans, corn, macaroni, pecans	L1	S18 S14 S19	D7 D8		On L1 with S18 for CA, detection with D7; On L1 with S14 for Afs; On L1 with S19 for Pa and Gri, detection with D1 and D8	[61]
Afs Och A Ze Vo SA	Brain dust	L1	S14 S31		Q2	Detection limits: Afs<0.5 μg/kg, Och A 10 μg/kg	[75]
Ze Pa	Maize	L1	S37 S38	D1	Q2 Q5	Twice development with S37 and S38; Q2 at 313 nm for Ze, Q5 at 275 nm for Pa	[82]
Ze Tri T-2 HT-2		L1	S41 S42	D1 D3 D13 D14		On L1 with S41, D1 and D3 for Ze; On L1 with S42, D13 and D14 for Tri	[85]
Och A Ci PA Ze Ste		L1 L6	S51 S52	D3 D29 D1		On L6 with S52 for Ste and on L1 with S51 for others. Chemical confirmation test with D29, D3 and D1	[91]

Table 3. Continued

Mycotoxin	Matrix	Layer (L)[a]	Solv (S)[a]	Det. (D)[a]	Quant (Q)[a]	Remark	Ref.
Pa PA Ste CA Tri TA	Cereals, feedstuffs	L1	S95	D1 D3 D10 D17		2-D TLC	[97]
AOH AME	Cereals, fruits, vegetables	L1	S58	D1	Q5	Using stepwise gradient development; the sample extracts purified on silica gel column; detection limits: ca. 60 mg/kg	[98]
T-2 HT-2 T-2 triol T-2 tetraol	Rat organs	L1	S61 S62	D13 D21		D13 for unlabelled toxins and D21 for radio-labelled ones	[101]
T-2 DAS Ro Vo		L5 L1	S67 S7	D2 D9	Q5	On L5 with S67 and on L1 with S7	[106]
Afs		L1			Q5	Detection limits: ca. in pg per spot level	[124]

[a]The layers, solvent systems, detection procedures and the quantitative techniques are given in Table 4, Table 5, Table 6 and Table 7, respectively.

quantitative assessment by visual comparison of the fluorescencing zone with the standards on the plates has long been widely employed for mycotoxin screening. Evaluation of the mycotoxin zone after elution from the plate is still used by some authors [35,69]. Liquid scintillation counting was used for quantification of Tris after scraping and elution [112]. Recently a scientifically operated charge-coupled device (CCD) detector has been developed for the quantitative analysis of aflatoxins [124]. Up to now, some quantitative procedures for mycotoxin assay have been validated [92].

3. The principal advantages of TLC in mycotoxin analysis

3.1. Crude extract analysis

One of the important features of TLC is its off line operating principle. A TLC plate can sometimes be conveniently used both for the isolation of impurities from a mixture of interest and for the separation of the compounds of interest. In practice, TLC is more suitable for crude extract analysis. For instance, Lin Leming et al., [82] employed a two development

Table 4
The layers employed for the separation of the mycotoxins

Symbol	Layer
L1	Silica gel
L2	Empore sheets
L3	Phenyl non-polar bonded-silica gel
L4	Silanized silica gel
L5	Octadecyl-bonded silica gel
L6	Silica gel impregnated with 0.3 *M* sulfuric acid or 10% oxalic acid
L7	Silica gel impregnated with 10% glycolic acid in ethanol
L8	Polyamide

Table 5
The solvent systems employed for the separation of the mycotoxins

Symbol	Solvent system
S1	Chloroform–acetone (22:3, v/v)
S2	Chloroform–xylene–acetone (6:3:1, v/v)
S3	Xylene–ethyl acetate–acetic acid (6:3:1, v/v)
S4	Chloroform–acetone (9:1, v/v)
S5	Diethyl ether–methanol–water (188:9:3, v/v)
S6	Chloroform–acetone (17:3, v/v)
S7	Toluene–ethyl aeetate–acetone (3:2:1, v/v), containing 2% formic acid
S8	Toluene–acetone (1:1, v/v)
S9	Anhydrous diethyl ether
S10	Chloroform–acetone (22:3, v/v)
S11	Chloroform–diethyl ether–acetic acid (17:3:1, v/v)
S12	Hexane–diethyl ether–acetic acid (70:30:2, v/v)
S13	Hexane–ethyl acetate (1:3, v/v)
S14	Diethyl ether–methanol–water (96:3:1, v/v)
S15	Toluene–ethyl acetate–formic acid (60:40:0.5, v/v)
S16	Chloroform–acetone–water (440:60:1, v/v)
S17	Chloroform–acetone–isopropanol (85:10:5, v/v)
S18	Benzene–methanol–acetic acid (90:7:5, v/v)
S19	Toluene–ethyl acetate–90% formic acid (5:4:1, v/v)
S20	Chloroform–acetone (70:30, v/v)
S21	Toluene–ethyl acetate–diethyl ether–formic acid (25:35:40:5, v/v)
S22	Hexane–acetone–chloroform (15:50:35, v/v)
S23	Water–saturated chloroform–acetone (88:22, v/v)
S24	Toluene–ethyl acetate–formic acid (48:40:12, v/v)
S25	Diethyl ether–methanol–water (95:4:1, v/v)
S26	Chloroform–acetone–isopropanol (85:15:2.5, v/v)
S27	Benzene–hexane (3:1, v/v)
S28	Toluene–ethyl acetate–formic acid (60:30:15, v/v)
S29	Chloroform–acetone (88:12, v/v)
S30	Chloroform–isopropanol–acetone (94:4.5:1.5, v/v)
S31	Toluene–acetic acid (95:5, v/v)
S32	Toluene–ethyl acetate–chloroform–formic acid (35:25:25:10, v/v)
S33	Hexane–ethyl acetate–acetic acid (18:2:1, v/v)
S34	Benzene–methanol–acetic acid (90:5:5, v/v)
S35	Ethyl acetate–chloroform–tetrahydrofuran (15:10:1, v/v)
S36	Methyl chloride–methanol–isopropanol (97:1:2, v/v)
S37	Isooctane
S38	Chlorofonn–acetone (18:1, v/v)
S39	Toluene–ethyl acetate–water (6:3:1, v/v)
S40	Chloroform–methanol (96.5:3.5, v/v)
S41	Chloroform–acetone (60:40, v/v)
S42	Chloroform–ethanol (97:3, v/v)
S43	Light petroleum–diethyl ether (1:1, v/v)
S44	Chloroform–ethanol (95:5, v/v)
S45	Hexane–butanol–acetic acid (35:15:1, v/v)
S46	Hexane–ethyl acetate (1:1, v/v) or (1:3, v/v)
S47	Toluene–ethyl acetate–chloroform (95:55:50, v/v)
S48	Benzene–acetone (3:2, v/v)
S49	Chloroform–methanol (93:3, v/v)
S50	Acetonitrile–7.5% sodium chloride–methanol (6:4:1, v/v)
S51	Toluene–ethyl acetate–90% formic acid (6:3:1, v/v)

Table 5. Continued

Symbol	Solvent system
S52	Methanol
S53	Diethyl ether–methanol (49:1, v/v)
S54	Hexane–ethyl acetate–acetic acid (18:3:1, v/v)
S55	Methanol–water (7:3, v/v)
S56	Diethyl ether–hexane–formic acid (70:30:1, v/v)
S57	Chloroform–methanol–water–concentrated ammonia (70:30:2:2, v/v)
S58	Mixtures of chloroform and ethyl acetate in proportions for stepwise gradient
S59	Chloroform–methanol–concentrated ammonia (40:10:1, v/v)
S60	Chloroform–methanol–acetic acid (6:3:1, v/v)
S61	Chloroform–ethyl acetate–ethanol (50:25:25, v/v)
S62	Chloroform–ethyl acetate–ethanol (80:10:10, v/v)
S63	Ethanol–water–acetic acid (65:35:1, v/v), containing 0.5% sodium chloride
S64	Toluene–ethyl acetate–formic acid (30:14:2, v/v)
S65	Toluene–ethyl acetate–acetone (7:2:1, v/v)
S66	Toluene–ethyl acetate (1:3, v/v)
S67	Methanol–water–acetic acid (65:35:1, v/v), containing 0.5% sodium chloride
S68	Ethyl acetate–methanol (20:1, v/v)
S69	Butanol–acetic acid–water (4:1:1, v/v)
S70	Chloroform–acetic acid–methanol (85:10:5, v/v)
S71	Ethyl acetate–hexane–acetone (2:1:1, v/v)
S72	Dichloromethane–methanol (92:8, v/v)
S73	Ethyl acetate–hexane (4:1, v/v)
S74	Benzene–acetone (12:7, v/v)
S75	Ethyl acetate–toluene (3:1, v/v)
S76	Diethyl ether–acetone (1:1, v/v)
S77	Diethyl ether–hexane–ethyl acetate–90% formic acid (70:90:40:2, v/v)
S78	Toluene–ethyl acetate–chloroform–90% formic acid (7:5:5:2, v/v)
S79	Diethyl ether–hexane–ethyl acetate (5:10:5, v/v)
S80	Hexane–methanol (95:5, v/v)
S81	Ethyl acetate–methanol–concentrated ammonia (60:35:5, v/v)
S82	Ethyl acetate–2–propanol–concentrated ammonia (50:15:10, v/v)
S83	Chloroform–isopropanol (32:1, v/v)
S84	Dichloromethane–isopropanol (19:1, v/v)
S85	Water–ethanol (10:23, v/v)
S86	Chloroform–methanol (92:8, v/v)
S87	Chloroform–isopropanol (9:1, v/v)
S88	Acetonitrile–water (60:40, v/v)
S89	Chloroform–isopropanol (93:7, v/v)
S90	Benzene–ethyl acetate (65:35, v/v)
S91	Benzene–ethyl acetate–acetic acid (55:40:5, v/v)
S92	Chloroform–acetone (93:7, v/v)
S93	Light petroleum (b.p. 40–70°C)–ethyl acetate (6:4, v/v)
S94	Ethanol–water (4:6, v/v)
S95	Methanol–acetonitrile–water (1:1:3, v/v)

system for the determination of zearalenone and patulin in maize: first development was carried out with isooctane in a longer distance for the isolation of lipids in the sample extract; the second with chloroform–acetone (18:1, v/v) in a shorter distance for the separation of the two mycotoxins. Another example is the determination of Och A in rice reported by Dawlatana et al., [92] on a silica gel plate developed with ethanol (49:1, v/v) in the reverse direction for 6 min, and then with toluene–ethyl acetate–formic acid (5:4:1, v/v) for 10 min after cutting off the end of the plate closest to the spot origin, afterwards, having the cut edge down and developed with hexane–ethyl acetate–acetic acid

Table 6
The procedures used for the detection of the mycotoxins

Symbol	Detection method
D1	Under UV light (365 nm or 254 nm)
D2	Spraying with chromotropic acid and heating for 4–5 min at 110°C, evaluation under normal light
D3	Spraying with 20% methanolic aluminum chloride solution followed by heating at 110°C for 4–5 min and under UV light
D4	Spraying with Sulfuic acid–methanol (1:4, v/v) and heating
D5	Spraying with 5% nitric acid and heating
D6	Spraying with 25% aqueous nitric acid
D7	Spraying with 1% *p*-dimethylaminobenzaldehyde in ethanol–hydrochloric acid (3:1, v/v)
D8	Spraying with 0.5% 3-methylbenzothiazolinone hydrazone and heating at 130°C
D9	*p*-Anisaldehyde solution (0.25 ml anisaldehyde in methanol–acetic acid–sulfuric acid (85:15:5, v/v)
D10	Spraying with sulfuric acid (1:3, v/v) and heating
D11	Exposure to iodine vapor and spraying with 1% starch solution
D12	Spraying with a solution of 4-methoxybenzene-diazonium fluoroborate and heating at 110°C for 10 min
D13	Spraying with 4-(*p*-nitrobenzyl)pyridine and heating at 150°C for 30 min
D14	Spraying with tetraethylenepentamine solution
D15	Spraying with Fast Violet B
D16	Exposure to vapor phase over methanol–concentrated ammonia (8:2, v/v)
D17	Spraying with 10% *p*-dimethylaminobenzaldehyde in hydrochloric acid–acetone (1:4, v/v)
D18	Spraying with 3-methyl-2-benzothiazoline hydrazone hydrochloride solution
D19	Spraying with 5% acetic anhydride in acetonitrile solution–4-dimethylaminopyridine solution (1:1, v/v). Five min after spraying the sheet was cut into strips (ca. 1 cm wide) for each sample applied; the each strip was further cut into 0.5 cm sections and assayed by ELISA
D20	Spraying with a solution of 0.5% anisaldehyde in methanol–acetic acid–sulfuric acid (85:10:5, v/v) and heating for 5 min at 110°C
D21	Autoradiography
D22	Spraying with sodium methoxide or butoxide and examining under UV light while the plate is wet
D23	Exposing into iodine vapors
D24	Spraying with 6 *M* hydrochloric acid
D25	Spraying with 0.1% ninhydrin in butanol
D26	Spraying with vanillin reagent
D27	Spraying with 1% 4-(*p*-nitrobenzyl)-pyridine in carbon tetrachloride–chloroform (3:2, v/v), followed with 10% tetraethylenepentamine in carbon tetrachloride–chloroform (3:2, v/v) after heating for 30 min at 150°C
D28	Exposing to X-ray film
D29	Exposing into ammonia vapors
D30	Spraying with 15% aluminum chloride 3 in ethanol and silicane–diethyl ether (18:82, v/v)
D31	Spraying with 1 g 4–dimethylaminobenzaldehyde in 75 ml ethanol and 25 ml concentrated hydrochloric acid
D32	Spraying with 3% phosphomolybdic acid–10% sulfuric acid in methanol and heating at 105°C for 5 min
D33	Spraying with 1 *M* sodium hydroxide
D34	Spraying with 1% ferric chloride in water
D35	Spraying with trifluoroacetic acid solution

Table 7
The techniques used for the quantitation of the mycotoxins

Symbol	Techniques
Q1	Colorimetry
Q2	Fluorescence densitometry at 365 nm or other wavelength
Q3	Semi-quantitative assessment by visual comparison of fluorescing zone with standards
Q4	Spectrophotometry
Q5	Densitometry
Q6	Fluorescence quenching at 254 nm or 366 nm
Q7	Scientifically operated charge-coupled device detector
Q8	Liquid scintillation counting

(18:3:1, v/v) after cutting off the further 30 mm from the plate bottom.

3.2. Multiple-alternative detection

Both physical and chemical, pre- and post-chromatographic visualization techniques are used for the TLC detection and confirmation of mycotoxins (refer to Tables 2 and 3 and 6). If the spot colors and R_F values are combined with the in situ absorbency or visual spectra comparison [128], the TLC identification of mycotoxins will be extremely reliable. In practice, there have been numerous successful examples. Here only a few are taken: Martin et al., detected 18 trichothecenes successfully on octadecyl-bonded silica gel layer [102]. The secondary metabolites from Fusarium, two new modified trichothecenes, 8α- and 8β-hydroxysambucoin were identified by Coriey et al., using several derivatization techniques [110]. T-2, acetyl T-2, HT-2 and acetyl HT-2 toxins were also identified and confirmed by Munger et al., using chemical visualization techniques combined with exposing the plate to X-ray film [112]. In a few laboratories, some sophisticated coupling techniques have been employed in the identification of mycotoxins. An example is the separation and identification of aflatoxin M1, M2, B2, G1, G2 and trichothecenes by Tripathi et al., employing two-dimensional TLC and fast atom bombardment (FAB) MS [34].

3.3. Easier to lower the limits of detection and quantification

Mycotoxins occurring in a matrix are usually in very low quantity levels. This means the analysis method employed must be sensitive enough even though an enriching procedure may be performed before measurement Table 7. In the first place, the techniques used for detection of mycotoxins are often very sensitive. In the next place, TLC has the possibility to lower the operating detection limits through applying bigger volume of sample solution. Therefore, it is easy to accomplish a detection range down to ng per spot levels without the need to concentrate the samples of interest in the application solution for a number of mycotoxins, which fulfil most practical requirements. For example, in a survey of several mycotoxins by spraying with aluminum chloride solution followed by heating at 100°C for 5 min, the detection limits of aflatoxins, ochratoxin A, zearalenone and sterigmatocystine in some Brazilian foods were ca. 2 $\mu g\ kg^{-1}$, 5 $\mu g\ kg^{-1}$, 15 $\mu g\ kg^{-1}$ and 55 $\mu g\ kg^{-1}$, respectively [50]. In an application of simultaneous determination of aflatoxins B1, B2, G1, G2 in animal feedstuffs, the sensitivities were 0.05–0.1 ng per spot for B1 and G1, <0.05 ng per spot for B2 and G2 by detecting under W light and confirming through derivatization by spraying the plate with 25% aqueous nitric acid [60]. In another application of TLC confirmation of Af M1 extracted from milk [65], between two developments the plate was treated by heating at 110°C for 10 min and overspotting the located M1 spot with a *p*-anisaldehyde solution, and finally, Af M1 was examined under UV light with a detection limit as low as 0.3 ng per spot.

Most quantitative evaluation techniques available at present give a reasonable sensitivity to the corresponding mycotoxins. The accurate quantification of

mycotoxins are popularly performed by fluorescence or absorbency densitometry with quantification limits ranging from 10 $\mu g\,kg^{-1}$ down to 0.005 $\mu g\,kg^{-1}$ levels for various mycotoxins [22,37–40,43–45, 47, 49, 52, 55, 57,59,63,64,66,70,71,73–75,77,82,83, 92,93,95,98,100,106,114,116,120,121]. Semi-quantitative determination of some mycotoxins by visual comparison of the fluorescencing zone with standards has been carried out with quantification limits of low ng levels [72,78,81,94,111]. Even some off line quantitative methods like colorimetry [35], spectrophotometry [69] have still reached quantification limits of 1–7 $\mu g\,kg^{-1}$ for aflatoxins and ca. 20 $\mu g\,kg^{-1}$ for ochratoxin A.

3.4. Screening routine analysis

The higher sample throughput of TLC suggests that it is one of the quickest, simplest and most effective analytical techniques and is most suitable for the screening routine which is usually important in the duration of the storage and exchange of commodities such as foodstuffs and feedstuffs etc. In fact, TLC has long been used as the rapid screening method of aflatoxins in corn [71], zearalenone and its metabolites in chicken muscle tissues [80], ochratoxin A in corn, peanuts, beans, rice [94], patulin in apples with mouldy cores [96], cytochalasins in infected plants [118], aflatoxins, sterigmatocystine, citrinin, ochratoxin A and zearalenone etc., in a total of 120 different samples belonging to 24 kinds of spices collected from different places in Egypt [129], aflatoxins in 4818 samples of animal feedstuffs comprising cereals, oilseed cakes, compound feeds, and other ingredients in India [78]. Recently, Pineiro et al., conducted a pilot study for monitoring mycotoxin contamination in foodstuffs and feedstuffs by employing TLC/densitometry and proposed a monitoring program for the purpose [123].

4. A general comparison with other techniques

The challenges in mycotoxin analysis include sampling, sample preparation procedures and the choice of analytical methods. Apparently, the three points are equally important to all analytical methods and related closely to each other for reaching satisfactory results.

4.1. Among TLC methods

There are still differences found by comparative studies in performance and effectiveness, even among existing TLC procedures [73]. Diprossimo et al., have made a comparison of three methods for determining aflatoxins in melon seeds [36]. They found that the use of TLC conditions of the CB (Contaminants Branch) method gave better results than BF (Best Food) and CB–RCS–Mod (Modified CB method–Rapid Modification of the Cottonseed Method): less fluorescence interference, better solvent efficiency, and lower detection levels. Koeltzow et al., [46] concluded after comparative evaluation of commercially available aflatoxin test that good agreement existed between CB method and AOAC (26.050). Bradburn et al., performed a comparative study of phenyl-bonded phase, CB and Romer clean-up procedures for TLC determinination of aflatoxin levels in maize and discussed the systematic errors and the detection limits of them [48]. Boyacioglu et al., found after a similar comparison that the Romer method was more accurate and precise than other TLC methods [59]. Rosario et al., concluded that for the determination of aflatoxins in copra meal the methods of the Association of Official Analytical Chemists (AOAC), the American Oil Chemists' Society (AOCS) and the European Community (EC) were all comparable in precision, accuracy and practicality [56].

4.2. TLC and enzyme-linked immunosorbent assay methods

Immunoassay has been an important tool for aflatoxin testing since ELISA kits for recognizing different mycotoxins were commercially available. The ELISA format for aflatoxins contains typically three specific reagents: the mono- or poly-clonal antibiotics which recognize and bind with a specific aflatoxin, an aflatoxin-enzyme conjugate and an enzyme substrate. The bonded enzyme catalyzes the oxidation of a substrate to form a colored complex for further qualitative or quantitative evaluation [130,131]. The comparison of ELISA with TLC

methods made by Dell et al., indicated that the latter gave more consistent data with lower levels of relative standard deviation (R.S.D.) [43]. Whitaker et al., studied the variability of the methods used to measure aflatoxin in agricultural commodities and showed that ELISA was less precise than TLC [132]. Wolf Hall et al., had different results in a comparison of TLC and ELISA for the detection and quantification of DON [133]: the methods varied considerably in the amounts of DON detected in 51 different grain samples, with the ELISA not only giving higher recovery rates and higher concentrations, but also being faster and less laborious than the TLC method. However, in a report on the determination of Fumonisins in corn by the monoclonal antibody-based competitive direct ELISA method, Sydenham et al., concluded [30] that constructurally related fumonisin-like compounds present in contaminated corn may contribute to the differences recorded.

4.3. TLC and HPLC methods

Both normal- and reversed-phase HPLC with fluorescence detection has become an accepted method for the determination of aflatoxins. From the view points of the techniques themselves, the most important advantage of HPLC lies in its potential for automation [6]. Dell et al., reported [43] that for the determination of aflatoxins in peanut butter, TLC method was more precise than HPLC. In the applications of quantification of mycotoxins in different samples, Tosch et al., [70] performed a comparison of TLC with LC method and showed that with respect to precision, accuracy, sensitivity, recovery and linearity of response, TLC appeared to be equivalent to LC method. Lee et al., reported TLC results in good agreement with HPLC for the determination of aflatoxin B1 in cotton balls [45].

A similar conclusion was conducted by Beaver et al., [49,60,79,93,104,122,132,135]. Horwitz et al., have recalculated the precision parameters of method performance for mycotoxins published in the literature through 1991 following the IUPAC protocol and concluded that the precision of TLC and LC methods was about the same, and that of ELISA was somewhat poorer [134]. HPLC requires usually derivatization to add in the chromatography, because the commonly used UV or fluorescence detectors require the presence of UV or fluorescence chromophores in the molecules. It is apparent that TLC has greater convenience than HPLC for the sensitive detection of underivatized mycotoxins [102]. Of course, HPLC–MS is more universally applicable although the type of interface used may place limits on the chromatographic solvents that can be chosen. Besides, the technique also has disadvantages in its cost versatility.

4.4. TLC and GC methods

GC method has rarely been used for determination of some mycotoxins like T-2 toxin in grains and animal feeds [136]. GC–MS was used for identifying aflatoxins in corn and peanut butter as early in 1984 [45]. However, in comparison with TLC, GC method requires that the compounds be volatile enough and relatively non-polar. Those mycotoxins which are not sufficiently volatile require reaction such as silylation or polyfluoroacylation to obtain a volatile derivative. Apparently, such steps add to the complexity of the procedure. In this respect, SFC reveals advantage to GC [27].

5. Conclusion and future trends

TLC is a well developed, facile, fast, routine, cost effective and suitable method for the analysis of a wide range of mycotoxins, owing to its coherent features: crude extract analysis, the versatility in improving the solvent system, multiple-alternative detection, as well as the feasibility of performing semi-quantitative and quantitative analyses with a precision and accuracy as good as necessary, etc.

As the worldwide requirement for the control analysis of mycotoxins in various commodities is increasing, TLC will continue to play an important role in the field. However, TLC method can still be further advanced by improving the sample preparation, optimizing the separation efficiency and developing more sensitive, specific detection techniques, as well as completing the instrumentation to make it more efficient in performance and more friendly in operation. Of course, TLC method will frequently be confirmed and compensated by combining with other techniques, especially, with HPLC,

in many cases. Automation for the whole procedure does not seem a reality in the foreseeable future for such purposes. Besides the analytical step itself, to improve the sampling and subsampling is most critical in order to reduce overall variations and to make the final results more accurate.

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