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MULTIMYCOTOXIN ANALYSIS

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MULTIMYCOTOXIN ANALYSIS

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The threat of mycotoxin contamination of foodstuffs and feedstuffs destined for human and animal consumption, respectively, has become clearly evident since the discovery of the aflatoxin problem in 1960 (1,2,3). Investigations of field and storage fungi led to the discovery of many mycotoxins which show a broad spectrum of toxicity patterns (4). Several of these toxins, e.g. aflatoxin B_1, aflatoxin M_1, sterigmatocystin, ochratoxin A, α-cyclopiazonic acid, sporidesmin, zearalenone, diacetoxyscirpenol, T_2-toxin, ergot toxins, citrinin, patulin, penicillic acid, PR toxin, deoxynivalenol, and tenuazonic acid occur under natural conditions and have been recognized in the etiology of human and animal diseases. No scientific principle exists for predicting the presence of a particular mycotoxin in foodstuffs, apart from a limited knowledge of fungal ecology - and even then the occurrence of one or more toxigenic fungi on suspected material does not indicate the presence of a mycotoxin. It is furthermore not uncommon for more than one mycotoxin to be isolated from the same mouldered product, as some fungi also possess the ability to simultaneously produce more than one toxin (5).

Determination of the extent of this hazard requires analytical methods for the detection, identification and quantitation of all these important mycotoxins. Excellent methodology is available for the separate analysis of individual mycotoxins (6). A rapid screening for the simultaneous analysis of several mycotoxins would therefore be both time-saving and advantageous. Such a step could be introduced at some intermediate stage in the purification of contaminated samples and only those extracts which show a positive response would then be subjected to methods specifically designed for the quantitative analysis of a particular mycotoxin.

Several reports have appeared on multimycotoxin analysis (7-36). The following general procedures are involved in this methodology, viz. extraction of the toxins from mouldered material; a cleanup procedure (thin layer chromatography, column chromatography, gel filtration chromatography, dialysis or solvent partition); analysis (silica gel thin layer chromatography, high performance liquid chromatography or field desorption mass spectrometry); confirmation of identity; and quantitation.

In a publication of this nature it is virtually impossible to assess the relative scientific and practical merits of the different reports, as only a few of these methods have apparently been extensively used in practice. The method of Eppley (7) was employed by Shotwell *et al.* (37) in a survey of U.S. wheat for ochratoxin and aflatoxin, by Stoloff *et al.* (38) in a survey of corn for aflatoxins and zearalenone, and by Stoloff and Dalrymple (39) in the analysis of aflatoxin and zearalenone in dry-milled corn products. The method of Eppley (7) involved a chloroform-water extraction of the suspected material, combined with a column chromatographic cleanup on silica gel. Zearalenone was eluted from the column with benzene-acetone (95:5 v/v*); the aflatoxins were eluted with chloroform:methanol (97:3) and the ochratoxins with benzene acetic-acid (9:1). The resultant fractions were analysed by silica thin layer chromatography and the concentrations estimated by comparison with standards. Stoloff and Francis (40) recently applied multimycotoxin analytical methodology to a survey of aflatoxins and zearalenone in canned and frozen U.S. sweet corn.

Roberts and Patterson (14) described in 1975 methodology for the detection of twelve mycotoxins (aflatoxins B_1, B_2, G_1, and G_2, citrinin, diacetoxyscirpenol, ochratoxin A, patulin, penitrem A, sterigmatocystin, T_2-toxin and zearalenone) in mixed animal feedstuffs by using a membrane cleanup procedure and the multimycotoxin thin layer chromatographic approach. The method has since then been successfully used at Weybridge and other laboratories in the U.K. for the analysis of mycotoxins in straight and compounded feedstuffs. The method has recently been improved (27) by using two-dimensional instead of one-dimensional TLC and by selecting solvent systems that give good chromatographic windows for a particular toxin (Table 1). The minimum detection levels for aflatoxin B_1, ochratoxin A, sterigmatocystin, T_2-toxin and zearalenone are given in Table 2. A general flow-diagram of the multimycotoxin method of Patterson and

* All solvent systems refer to a volume/volume ratio.

Roberts is given in Figure 1. The improved method of Patterson and Roberts (27) is reputed to be more sensitive than the high performance liquid chromatography methods of Hunt et al. (33,35).

TABLE 1. Optimum Conditions for Two-dimensional Chromatography (27).

Mycotoxin	First solvent[1]	Second solvent[1]	Visualization[2]
Aflatoxin B_1	A	B	a
Ochratoxin A	C	B	a
Sterigmatocystin	D	C	b
Zearalenone	E	F	c
T_2-toxin	G	C	d
General screen	B	C	e

[1] Solvent systems: A, ether:methanol:water (94:4.5:1.5); B, chloroform: acetone (90:10); C, toluene:ethyl acetate:90% formic acid (60:30:10); D, chloroform:methanol (98:2); E, ether:cyclohexane (75:25); F, chloroform:ethanol (95:5); G, chloroform:propan-2-ol:ethyl acetate (95:5:5).

[2] Visualization (Chromato-vue viewing cabinet): a, longwave (360 nm) UV light; b, spray with 20% $AlCl_3$ in ethanol, heat at 100° for 5 min, longwave UV light; c, shortwave (250 nm) UV light; d, spray with 20% H_2SO_4 in CH_3OH, heat at 105° for 5 min, view as blue-green fluorescent spot in longwave UV light; e, for general screening, follow sequence a,c,b,d.

TABLE 2. Multimycotoxin Screening of Animal Feedstuffs:Detection Limits (μg/kg) according to Patterson and Roberts (27).

Feedstuff	Aflatoxin B_1	Ochratoxin A[1]	Sterigmatocystin	Zearalenone	T-2 toxin
Groundnut meal	0.1	-	-	-	-
Barley	0.2	5	10	50	200
Corn (maize)	0.1	5	10	20	200
Wheat	0.2	5	10	20	200
Laboratory rodent diet[2]	0.3	10	20	100	200
Dairy concentrates[2]	0.3	10	20	100	200

[1] Using $NaHCO_3$ modification (see Fig. 1), the standard method gives slightly higher values.

[2] Proprietary feedstuffs.

Van Egmond et al. (28) described two methods for the simultaneous detection of aflatoxin B_1, ochratoxin A, patulin, sterigmatocystin, zearalenone, penicillic acid, citrinin, and α-cyclopiazonic acid in feedstuffs. This method has been in use at Bilthoven for the analysis of feedstuffs since 1975. Both methods involve the same extraction procedure and TLC detection. The basic differences are in the cleanup procedures of the two methods. The more rapid method A employs liquid-liquid partition, whereas method B involves a more effective but time-consuming polyamide column cleanup step. Rabbit feedstuff has been used as test material since this complex mixture contains a lot of fluorescent compounds that might interfere in the interpretation of TLC results.

According to method A (Figure 2) the toxins are extracted from the feedstuff with a mixture of acetonitrile:phosphoric acid (0.1 M) (10:1) and the extract purified by liquid-liquid partition with isooctane to remove lipid material and with a mixture of aqueous sodium hydrogen carbonate and sodium chloride to separate acids from the neutral substances. The final extracts in chloroform are then submitted to two-dimensional TLC. In method B (Figure 3) the toxins are extracted and defatted as for method 1. The extract is further purified by polyamide chromatography. The polyamide column is eluted with an eluotropic

mixture of solvents and the successive fractions are extracted with chloroform and the final extracts are submitted to SiO_2 TLC.

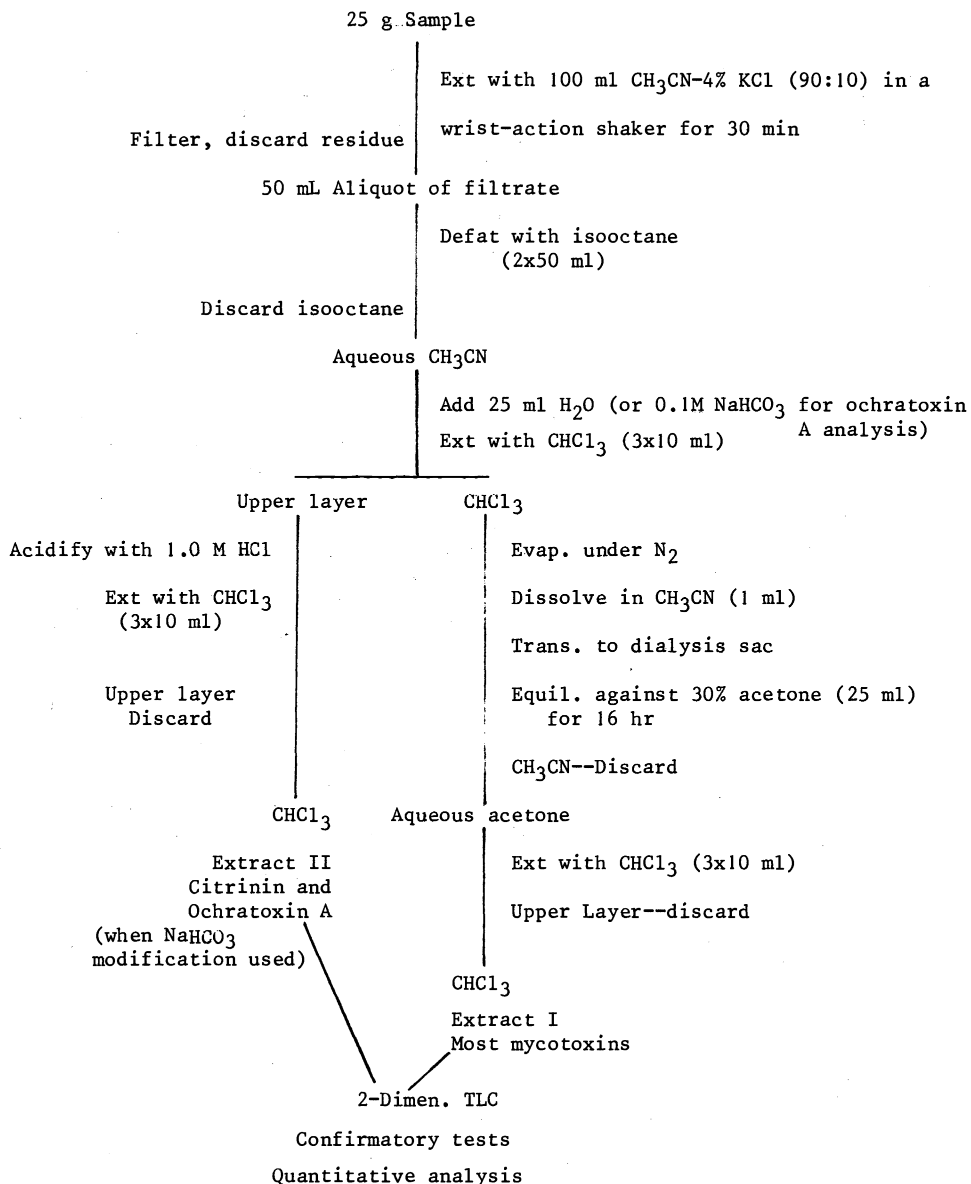
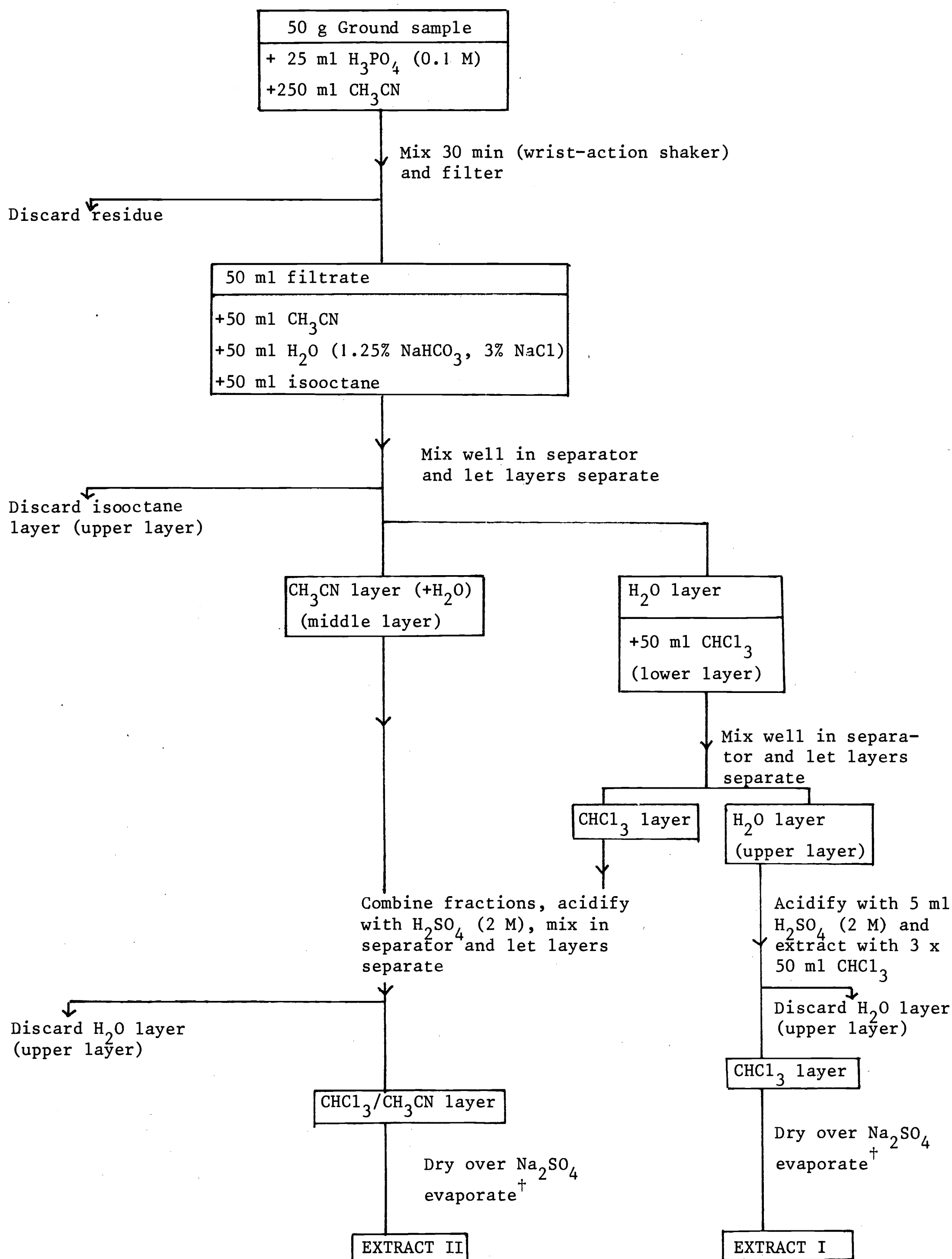


FIGURE 1 Flow diagram of multimycotoxin method of Patterson and Roberts (14,27).

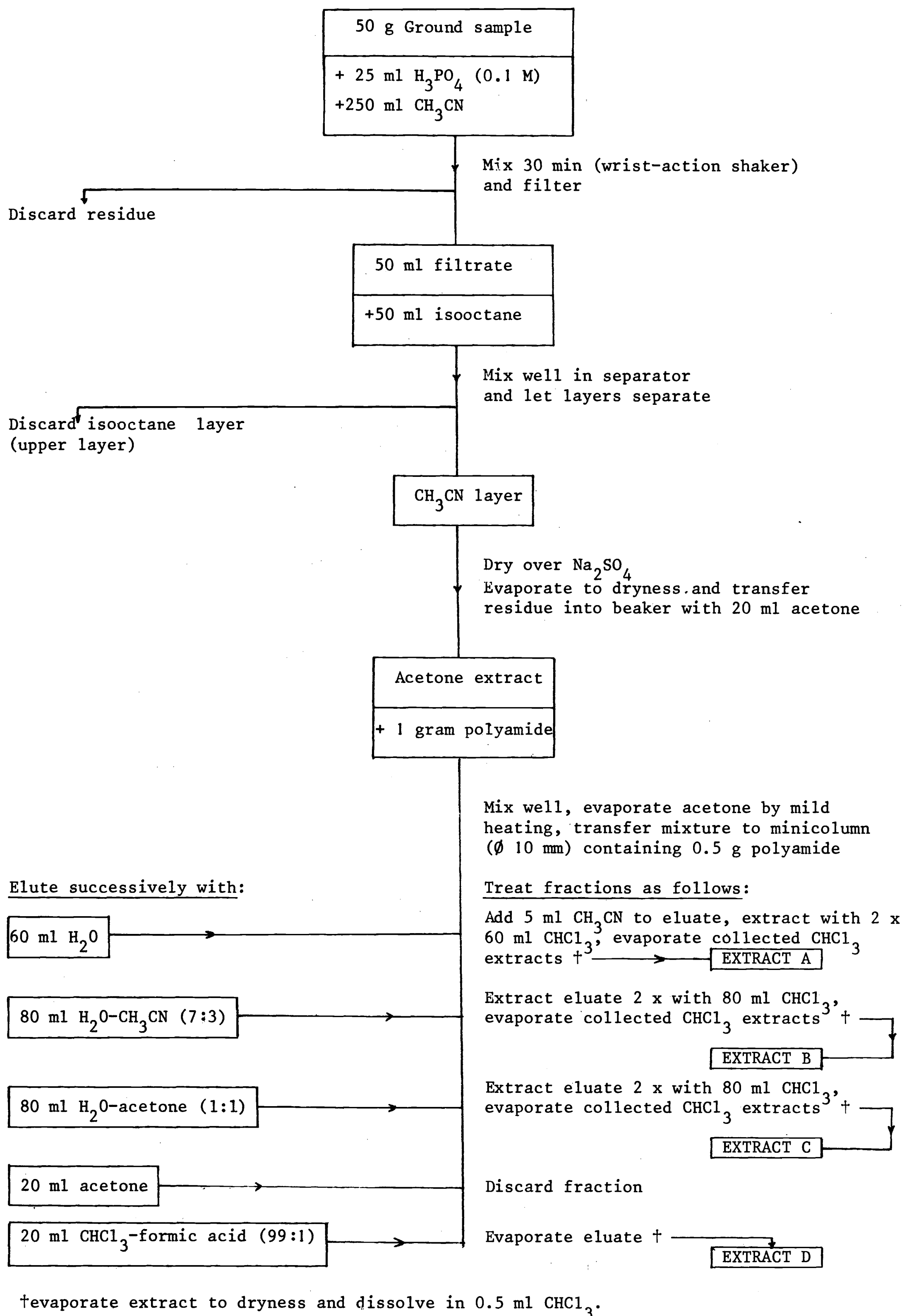
Van Egmond et al. (28) used the extracts obtained by either method A or B for the analysis of each mycotoxin by employing specially selected developing solvents (Table 3).

To obtain an adequate separation between patulin and interfering substances, a predevelopment in ether had to be introduced. The plate was subsequently developed in the first and second developing solvent (Table 3). In case of α-cyclopiazonic acid the plate was developed in ether as the first developing solvent, and then impregnated with oxalic acid by dipping in a 5% solution of oxalic acid in ethanol-water (1:1) and dried for 10 min. at 100°. The plate was subsequently developed in the second direction with chloroform-methyl isobutylketone (4:1). For the analysis of citrinin the extracts and standards were spotted on a plate impregnated with oxalic acid. The plate was developed with toluene:acetone (4:1) in one direction only. Van Egmond used a number of techniques to detect the mycotoxins from feedstuff extracts on TLC plates. In Table 4 the visualization techniques are given which are used for the detection of mycotoxins in feedstuffs (28).



†Evaporate extract to dryness and dissolve in 0.5 ml $CHCl_3$

FIGURE 2 Extraction and Cleanup of Method A according to Van Egmond et al. (28)



†evaporate extract to dryness and dissolve in 0.5 ml $CHCl_3$.

FIGURE 3: Extraction and Cleanup of Method B according to Van Egmond *et al.* (28)

TABLE 3. Developing solvents used for TLC of feedstuff extracts (28).

Mycotoxin	Extract Method A	Extract Method B	1st developing solvent	2nd developing solvent
Aflatoxin B_1	II*	B	Chloroform: Acetone (9:1)	Toluene:Ethylacetate: Formic Acid (6:3:1)
Ochratoxin A	I	C	Chloroform: Acetone (9:1)	Toluene:Ethylacetate: Formic Acid (6:3:1)
Patulin*	II	A	Chloroform: Acetone (8:2)	Chloroform:Ether: Acetic Acid (17:3:1)
Sterigmato-cystin	II	B	Chloroform: Acetone (95:5)	Hexane:Ether: Acetic Acid (75:25:10)
Penicillic acid	I	A	Ether	Chloroform:Acetone (9:1)
Citrinin*	I	-	Toluene: Acetone (8:2)	-
Cyclopiazonic acid*	I + II	D	Ether	Chloroform:Methyl-isobutylketone (4:1)
Zearalenone	II	B	Chloroform: Acetone (95:5)	Hexane:Ether:Acetic Acid (75:25:10)

* For these toxins the TLC procedures deviated from the normal two-dimensional TLC

TABLE 4. Visualization techniques used for detection of mycotoxins in feedstuff extracts (28).

Mycotoxin	Visualization of spots	Interpretation at	Colour of spot
Aflatoxin B_1	-	360 nm	Blue fluor.
Ochratoxin A	Treatment with NH_3 vapour (10 min)	360 nm	Blue fluor.
Patulin	Spraying with MBTH-solution† followed by heating for 15 min at 110°	360 nm	Yellow fluor.
Sterigmato-cystin	Spraying with $AlCl_3$-solution followed by heating for 10 min at 110° in oven (20 g $AlCl_3$ in 100 ml ethanol)	360 nm	Yellow-green fluor.
Zearalenone	Before spraying with $AlCl_3$-solution for sterigmatocystin	254 nm	Blue-green fluor.
Penicillic acid	Treatment with NH_3 vapour (10 min), followed by heating plate for 5 min at 110° in oven	360 nm	Blue fluor.
Citrinin	-	360 nm	Yellow fluor.
Cyclopiazonic acid	Spraying with Ehrlich reagent†	Daylight	Violet

† MBTH-solution:0.5 g 3-methyl-2-benzothiazoline hydrazone hydrochloride in 100 ml H_2O
†† Ehrlich reagent:2 g p-dimethylaminobenzaldehyde in 100 ml 10% HCl.

Takeda et al. (23) reported on the simultaneous extraction, fractionation and thin layer chromatographic determination of fourteen mycotoxins (aflatoxins B_1, B_2, G_1 and G_2, sterigmatocystin, T_2-toxin, diacetoxyscirpenol, neosolaniol, fusarenone-X, zearalenone, ochratoxin A, citrinin, luteoskyrin, and rugulosin) in grains. A number of solvent systems and chromogenic reagents for the verification of the mycotoxins were reported. A densitometric technique was used for the quantitation of the mycotoxins. The method was applied to polished

rice, rough rice, corn, wheat, and peanuts as an analytical procedure.

Gorst-Allman and Steyn (29) reported on screening methods for thirteen common mycotoxins. This methodology can expeditiously be applied in conjuction with the extraction and cleanup methods of Eppley (7), Patterson and Roberts (27), Van Egmond _et al._ (28), and Takeda _et al._ (25). The mycotoxins under investigation were conveniently divided into a group of neutral substances (Table 5) _viz._ aflatoxin B_1, sterigmatocystin, zearalenone, patulin, T_2-toxin, roquefortine, penitrem A, fumitremorgen B and roridin A; and a group of acidic compounds Table 6) _viz._ citrinin, ochratoxin A, α-cyclopiazonic acid, and penicillic acid. The same approach can be applied to other neutral and acidic mycotoxins which were not incorporated in this investigation.

TABLE 5. Mean R_f values of neutral mycotoxins (29).

Mycotoxin	Solvent system					
	Chloroform: methanol (97:3)	Chloroform: acetone: n-hexane (7:2:1)	Chloroform: acetone (9:1)	Ethyl-acetate: n-hexane (1:1)	Chloroform: acetone: propan-2-ol (85:15:20)	Benzene: chloroform: acetone (45:40:15)
Aflatoxin B_1	0.44	0.35	0.27	0.03	0.65	0.24
Sterigmatocystin	0.67	0.53	0.55	0.41	0.74	0.56
Zearalenone	0.40	0.51	0.38	0.41	0.71	0.44
Patulin	0.22	0.27	0.16	0.18	0.56	0.20
T_2-toxin	0.45	0.36	0.22	0.13	0.68	0.22
Roquefortine	0.03	0.01	0.02	0.01	0.13	0.02
Penitrem A	0.40	0.51	0.34	0.49	0.76	0.45
Fumitremorgen B	0.51	0.36	0.28	0.14	0.71	0.30
Roridin A	0.31	0.22	0.13	0.09	0.61	0.14

TABLE 6. Mean R_f values of acidic mycotoxins (29).

Mycotoxin	Solvent system				
	Benzene-acetic acid (4:1)	Ethyl acetate-methanol-ammonia (80:15:10)	Formic acid-diethyl ether (1:19)	Benzene-methanol-acetic acid (90:16:8)	Ethyl acetate-acetone-hydrochloric acid (50:50:20)
Citrinin	-*	-**	-*	0.20	0.34
Ochratoxin A	0.40	0.05	0.89	0.47	0.41
α-Cyclopiazonic acid	-*	0.24	-*	0.45	-*
Penicillic acid	0.18	0.41	0.67	0.41	-*

* Tailing prevents calculation of reliable values

** Decomposes.

The R_f values of the neutral substances in six solvent mixtures using silica precoated chromatoplates are given in Table 5, whereas those of the acidic substances are given in Table 6. Some of the acidic substances failed to appear as clearly defined spots as the compounds frequently tail. This problem was solved by using silica chromatoplates which were impregnated with oxalic acid (8). The impregnation was effected by immersing the plates in

TABLE 7. Mean R_f values of acidic mycotoxins using TLC plates pre-treated with oxalic acid (29).

Mycotoxin	Solvent system	
	Chloroform: methanol (98:2)	Chloroform: acetone (9:1)
Citrinin	0.52	0.51
Ochratoxin A	0.32	0.34
α-Cyclopiazonic acid	0.52	0.44
Penicillic acid	0.16	0.20

a 10% solution of oxalic acid in methanol for 10 min. After heating at 110° for 2 min. and cooling, the plates were immediately spotted and developed in the appropriate solvent sytems. The R_f values obtained using this method are given in Table 7.

The developed plates were examined under UV light at wavelengths of 254 and 366 nm. In the detection the following spray reagents were used: (a) 2,4-dinitrophenylhydrazine (2,4-DNP) (1 g), concentrated sulphuric acid (7.5 ml), ethanol (75 ml), water (170 ml); (b) 2-hydrazono-2,3-dihydro-3-methylbenzothiazole hydrochloride (MBTH) (0.5% aqueous solution); (c) iron(III) chloride (3% solution in ethanol); (d) aluminium chloride (1% solution in chloroform); (e) Ehrlich reagent—solution (1), 4-dimethylaminobenzaldehyde (1% solution in ethanol), solution (2), hydrochloric acid (32%); (f) cerium(IV) sulphate (1% solution in 3 M sulphuric acid; and (g) vanillin (1% solution in 50% phosphoric acid). The plates were sprayed, the immediate effects noted, and then they were heated at 110° for 10 min. After development some of the plates were treated with iodine or ammonia vapour.

The characteristic colours which were obtained under illumination with UV light and with spray reagents are collated in Table 8. Gorst-Allman and Steyn (29) claimed that the correct choice of solvent systems, in combination with absorption and fluorescence properties and the use of chromogenic reagents can lead to the rigorous definition of the mycotoxins involved. The degree of confidence can be improved by direct comparison with standard samples, chemical derivatization and mass spectral analysis of the TLC spots.

Balzer _et al_. (24) reported a thin layer chromatographic method for the determination of aflatoxin B_1, ochratoxin A, and zearalenone in maize. This method was used for the screening of maize and compounded feeds in their laboratory. The mycotoxins are extracted from the samples with acetonitrile:water (9:1), sodium bicarbonate is then added to separate the acidic ochratoxin A from zearalenone and aflatoxin B_1. Zearalenone and aflatoxin B_1 are removed from the aqueous acetonitrile layer by extraction into chloroform. Zearalenone is removed from the chloroform layer by extraction into 1 M NaOH; acidification of the aqueous sodium hydroxide with phosphoric acid then yields zearalenone. The fractions are then analyzed by SiO_2 TLC employing the system toluene:ethyl acetate:formic acid (50:40:10). The recovery for ochratoxin A is about 87%, for zearalenone 85% and for aflatoxin B_1 71%. The high concentration of sodium hydroxide may cause a decomposition of aflatoxin B_1. The sensitivity of the method is approximately 2 ppb for aflatoxin B_1, 40 ppb for ochratoxin A, and 200 ppb for zearalenone.

Pirskorska-Pliszczynska and Juszkiewicz (22) modified the method of Stoloff _et al_. (11) and applied it to the simultaneous analysis of the aflatoxins, ochratoxins A and B, sterigmatocystin, and zearalenone. For the analysis of the aflatoxins the TLC plates were developed with the solvent chloroform:trichloroethylene:isoamyl alcohol:formic acid (80:15:4:1), whereas the other mycotoxins were separated in toluene:ethyl acetate:formic acid (6:3:1). Positive samples are subsequently analysed by suitable methodology for the individual toxins and confirmed chemically. This method was applied with success at the Veterinary Research Institute at Pulaway, Poland; similar methodology is applied at INRA, Paris.

Scott _et al_. (10), Durackova _et al_. (18), and Gimeno (26, 30) reported numerous solvent systems for the TLC analysis of mycotoxins. Lee _et al_. (31) introduced the use of high performance thin-layer chromatography (41) in the simultaneous multimycotoxin analysis. The increased resolution of this technique is related to the uniform particle TLC plates, new sample application devices and micro-optic scanning devices. Lee _et al_. (31) employed 10x10 cm HPTLC plates coated with silica gel 60. Prior to use the plates were impregnated with EDTA by horizontal development in a saturated chamber containing an aqueous solution of EDTA (10% w/v). The wet plates were stored at room temperature for thirty minutes, at 85° for two hours and then allowed to cool to room temperature. Citrinin and luteoskyrin showed streaking with the commercially available plates, this problem was solved by the impregnation with EDTA. Samples were applied to the TLC plate by using a 200 nl Pt-Ir capillary attached

TABLE 8 Characteristic colours with UV light and spray reagents (29).

Mycotoxin	UV light		Cerium(IV) sulphate		2,4-DNP		Iron(III) chloride		Ehrlich reagent		Vanillin		MBTH**	Aluminium chloride	Iodine***	Ammonia†
	254 nm	366 nm	a*	b**	a*	b**	a*	b**	a*	b**	a*	b**				
Aflatoxin B_1	Blue	Blue		Grey		Orange		Light yellow		Light grey		Green	Orange	Yellow	Orange	
Sterigmatocystin	Purple-black		Light red		Grey-green		Grey					Light grey		Light yellow	Light yellow	
Zearalenone	Purple	White		Yellow-brown		Dark orange		Light purple		Light pink		Brown	Light pink		Grey	
Patulin	Purple			Grey		Yellow				White		Grey	Dark orange	Light brown	Light orange	
T_2-toxin				Grey-black		Orange				White		Light grey				
Roquefortine	Purple		Orange	Green-brown		Grey				Grey-green		Purple			Light yellow	
Penitrem A	Light purple		Green	Purple		Purple		Grey		Blue-grey		Light purple	Light grey	Grey	Light yellow	
Fumitremorgen B	Light purple		Yellow-brown	Yellow-brown		Light orange		Orange		Light grey		Yellow	Light orange	Light yellow	Yellow	
Roridin A	Purple			Grey-black		Dark orange				White		Dark brown	Light yellow	Light orange	Dark yellow	
Citrinin	Purple	Yellow	Yellow	Yellow	Yellow	Brown-yellow	Light brown	Light brown	Yellow	Light purple	Yellow	Brown-yellow	Light brown	Light yellow	Orange	Purple
Ochratoxin A	Blue	Blue					Purple-brown									Purple
α-Cyclopiazonic acid	Purple		Red	Black	Light brown	Red-brown	Purple-brown	Brown	Light brown	Dark brown	Light yellow	Purple	Light yellow	Light brown	Orange	
Penicillic acid	Light purple			Light orange								Brown-yellow	Light yellow		Light yellow	Blue

* Immediate colour. ** Colour after 10 min at 110°. *** Plate kept in iodine vapour for 15 min.

† Plate kept in ammonia vapour for 15 min and examined under UV light (254 + 366 nm).

to an EVA-Chrom applicator. The TLC plates were developed in a chamber containing toluene: ethyl acetate:formic acid (30:6:0.5). The plate was developed continuously for 5.0 min and then removed and dried. The continuous development was repeated three further times and the solvent system changed to toluene:ethyl acetate:formic acid (30:14:4.5) and the plate developed a further three times. A total of seven continuous multiple developments with the two solvent systems were used to give a baseline separation of the thirteen mycotoxins: sterigmatocystin, zearalenone, citrinin, ochratoxin A, patulin, penicillic acid, luteoskyrin, and aflatoxins B_1, B_2, G_1, G_2, M_1 and M_2. The in situ scanning of the TLC plate was performed with a micro-optic KM-3 spectrophotometer. About one hour was used for the separation and quantitation of all thirteen mycotoxins from one spot.

High performance liquid chromatography (HPLC) was recently applied to the determination of ochratoxin A and zearalenone in cereals (32), zearalenone, patulin and penicillic acid in food (33); aflatoxin B_1. aflatoxin G_1, ochratoxin A, zearalenone, rubratoxin B, patulin and penicillic acid (34); and aflatoxins and ochratoxin A in food (35). The previously discussed techniques on the cleanup procedures will have to be applied to the preparation of samples prior to HPLC analyses. HPLC (42,43) might become a method of choice in future owing to the advantages of good resoltuion, high degree of precision, reproducibility and sensitivity. The utility of the method is evidenced by the reported detection limit and the range of retention times of nine mycotoxins [patulin (3.6-3.7 min), penicillic acid (3.9-4.0 min), aflatoxin G_1 (4.7-4.8 min), aflatoxin B_1 (5.2-5.3 min), rubratoxin B (6.7-6.8 min), ochratoxin A (7.5-7.6 min), zearalenone (10.1-10.2 min), roseotoxin B (7.2-7.3 min), trichothecin (7.8-7.9 min)] on a μ Bondapak/C_{18} column with the solvent system (acetonitrile:water:acetic acid 55:45:2) and a flow rate of 10 ml/min (34).

It is evident that several methods are available for application of multimycotoxin analyses. Analysts will have to select the methodology most suitable to their particular analytical problem and have to introduce the necessary cleanup and concentration steps.

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