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CHREV. 199

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC THEORY FOR THE PRACTITIONER

VERONIKA R. MEYER
Institute of Organic Chemistry, University of Bern, Freiestrasse 3, CH-3012 Bern (Switzerland)
(First received May 24th, 1985; revised manuscript received June 26th, 1985)

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1. INTRODUCTION

The theory of high-performance liquid chromatography (HPLC) was developed before and during the development of the method. Although concise descriptions of this theory can be found in all HPLC books, *e.g.*, refs. 1–4, it seems that laboratory technicians and in some cases even analytical chemists have little knowledge of it other than the formula for calculation of the plate number. This is very regrettable because careful application of the practical aspects of HPLC theory in routine laboratory work could be fruitful and satisfying. As will be shown in this text, the calculations needed are quite simple.

The question to be answered in this paper is: What kind of useful information can be calculated beginning with the geometrical properties of the HPLC column at our disposal? As the basis of the calculations, no more than three parameters, namely the length of the column, its inner diameter and the particle diameter of the stationary phase, will be employed. To illustrate the formulae given, values for these parameters were chosen to be 25 cm, 4.6 mm and 5 μm, respectively, corresponding to a very common type of HPLC column. The key to the calculations is the concept of reduced (dimensionless) parameters as introduced by Bristow and Knox in 1977[5]. Two of the four reduced parameters are of fundamental importance in this connection. They are the reduced plate height and the reduced velocity.

The reduced plate height is defined as $h = H/d_p$ where H is the height of a theoretical plate and d_p is the particle diameter of the stationary phase. The value of h indicates how many layers of particles of the stationary phase are needed to yield one theoretical plate. The smaller this value the better is the column. For excellently packed columns the reduced plate height reaches a value of 2. Smaller values seem not to be possible. On the other hand, liquid chromatography columns with $h = 10$ do not merit the term "high performance".

The reduced velocity is defined as $\nu = ud_p/D_m$, where u is the linear flow velocity of the mobile phase, d_p the particle diameter and D_m the diffusion coefficient of the solute of interest in the mobile phase. The great importance of ν lies in the fact that the optimum velocity of the mobile phase in liquid chromatography is known to occur at $\nu = 3$. If the molecular mass and the density of the solute are known or estimable, its diffusion coefficient in a given mobile phase can be calculated by use of the Wilke–Chang equation[6]. Therefore, with all necessary parameters known, the optimum velocity of the eluent can easily be calculated. (Of course, the optimum velocity can also be determined experimentally.) At this velocity a liquid chromatography column performs at its best, *i.e.*, the plate height has the minimum value possible with the given stationary phase.

Any discussion on chromatographic performance gains clarity and simplicity if reduced parameters are used because the considerations are set free from external factors as there are the dimensions of the column and stationary phase. On the other hand, only with reduced parameters is it possible to predict the performance of a chromatographic column and to estimate such interesting parameters as the maximum allowed injection volume and many others. This paper will show how to do this.

In the course of the calculations we have to choose other parameters in addition to those mentioned above. They are the porosity of the column packing, the

diffusion coefficient of the solute, the capacity factor of the solute, the fraction of the allowed increase in peak width, the inner diameter of the connecting capillaries, the concentration of the solute injected, the molar mass and the molar absorption coefficient of the solute, the path length of the detector, the viscosity of the mobile phase and the flow resistance of the column. All these parameters will be accorded values typically found in liquid chromatography.

All the equations are given without derivations, which can be found in the literature cited. However, the reader should be familiar with the basic theory of liquid chromatography.

2. CALCULATIONS

2.1. Plate number

Consider an HPLC column of length, l_c = 25 cm, inner diameter, d_c = 4.6 mm and particle diameter of the stationary phase, d_p = 5 μm. The number of theoretical plates, N, can be calculated by assuming that the column is very well (not excellently) packed, *i.e.*, h = 2.5:

$$N = \frac{l_c}{hd_p} = \frac{250\,000}{2.5 \times 5} = 20\,000 \tag{1}$$

2.2. Dead volume

The total porosity, ε_{tot}, is about 0.8 for all column packings of totally porous particles. Thus, the dead volume, V_0, can be obtained from:

$$V_0 = \frac{d_c^2\pi}{4} \cdot l_c\varepsilon_{tot} = \frac{4.6^2\pi}{4} \cdot 250 \times 0.8 \text{ mm}^3 = 3320 \text{ mm}^3 = 3.3 \text{ ml} \tag{2}$$

2.3. Flow velocity and flow-rate

The column is regarded as being used at its optimum flow which corresponds to ν_{opt} = 3. For the calculation of the matching flow velocity of the mobile phase, a mean diffusion coefficient, D_m, of the solute molecules in the mobile phase of 1×10^{-9} m^2 s^{-1} is assumed. Thus the linear flow velocity, u, and the flow-rate, v', of the mobile phase are given by:

$$u = \frac{\nu D_m}{d_p} = \frac{3 \times 1 \times 10^{-9}}{5 \times 10^{-6}} \text{ m s}^{-1} = 0.6 \times 10^{-3} \text{ m s}^{-1} = 0.6 \text{ mm s}^{-1} \tag{3}$$

$$v' = \frac{ud_c^2\pi\varepsilon_{tot}}{4} = \frac{0.6 \times 4.6^2 \times \pi \times 0.8}{4} \text{ mm}^3 \text{ s}^{-1} = 8.0 \text{ mm}^3 \text{ s}^{-1} = 0.48 \text{ ml min}^{-1} \tag{4}$$

2.4. Retention time

The retention time of an unretained solute, t_0, is:

$$t_0 = \frac{l_c}{u} = \frac{250}{0.6} \text{ s} = 417 \text{ s} \approx 7 \text{ min} \tag{5}$$

For two retained solutes having capacity factors, $k' = 1$ and $k' = 10$:

$$t_R = k' \times t_0 + t_0 = 1 \times 7 + 7 \text{ min} = 14 \text{ min}$$
$$t_R = 10 \times 7 + 7 \text{ min} = 77 \text{ min} \quad (6)$$

2.5. Retention volume

The volumes of mobile phase, V_R, necessary to elute peaks of either $k' = 1$ or $k' = 10$ are:

$$V_R = V' \times t_R = 0.48 \times 14 \text{ ml} = 6.7 \text{ ml}$$
$$V_R = 0.48 \times 77 \text{ ml} = 37 \text{ ml} \quad (7)$$

2.6. Peak capacity

The peak capacity, n, of the column if $k' = 10$ is not exceeded is:

$$n = 1 + \frac{\sqrt{N}}{4} \cdot \ln (1 + k'_{max}) = 1 + \frac{\sqrt{20\,000}}{4} \cdot \ln (1 + 10) = 86 \quad (8)$$

2.7. Peak width and peak volume

If the first peak has $k' = 1$, its base width, 4σ, and its volume, V_p, are:

$$4\sigma = 4 \frac{t_R}{\sqrt{N}} = 4 \frac{14 \times 60}{\sqrt{20\,000}} \text{ s} = 23.8 \text{ s} \quad (9)$$

$$V_p = 4\sigma \times V' = 23.8 \times 8 \ \mu\text{l} = 190 \ \mu\text{l} \quad (9a)$$

2.8. Injection volume

The maximum allowed injection volume, V_i, to avoid an excessive broadening of the first peak is defined as:

$$V_i = \theta V_R \cdot \frac{K}{\sqrt{N}} \quad (10)$$

where K is a parameter characteristic of the quality of injection and is assumed to be equal to 2 and θ^2 defines the fraction of peak broadening. Thus at 1% peak broadening, *i.e.*, $\theta^2 = 0.01$ and $\theta = 0.1$:

$$V_i = 0.1 \times 6700 \cdot \frac{2}{\sqrt{20\,000}} \ \mu\text{l} = 9.5 \ \mu\text{l}$$

At 9% peak broadening, *i.e.*, $\theta^2 = 0.09$ and $\theta = 0.3$:

$$V_i = 0.3 \times 6700 \cdot \frac{2}{\sqrt{20\,000}} \ \mu\text{l} = 28 \ \mu\text{l}$$

2.9. Detector volume

The maximum allowed volume of the detector cell, V_d, is defined as:

$$V_d = \frac{\theta V_R}{\sqrt{N}} = \frac{V_i}{2} \tag{11}$$

Thus, at 1% peak broadening, $V_d = 9.5/2\ \mu l \approx 5\ \mu l$ and at 9% peak broadening, $V_d = 28/2\ \mu l = 14\ \mu l$.

2.10. Detector time constant

The maximum allowed time constant of the detector, τ, is defined as:

$$\tau = \theta \cdot \frac{t_R}{\sqrt{N}} \tag{12}$$

At 1% peak broadening:

$$\tau = 0.1 \cdot \frac{14 \times 60}{\sqrt{20\,000}}\ s = 0.6\ s$$

At 9% peak broadening:

$$\tau = 0.3 \cdot \frac{14 \times 60}{\sqrt{20\,000}}\ s = 1.8\ s$$

2.11. Capillary tube length

In most HPLC instruments, capillary tubing is used to connect the injector, column and detector. An inner diameter of the capillary, d_{cap}, of 0.25 mm is usual. The maximum allowed length, l_{cap}, of such a capillary is defined as:

$$l_{cap} = \frac{384\ \theta^2 D_m V_R^2}{\pi N V' d_{cap}^4} \tag{13}$$

At 1% peak broadening:

$$l_{cap} = \frac{384 \times 0.01 \times 10^{-5} \times 6.7^2}{\pi \times 20\,000 \times 8 \times 10^{-3} \times 0.025^4}\ cm = 8.8\ cm$$

At 9% peak broadening:

$$l_{cap} = \frac{384 \times 0.09 \times 10^{-5} \times 6.7^2}{\pi \times 20\,000 \times 8 \times 10^{-3} \times 0.025^4}\ cm = 79\ cm$$

2.12. Dilution factor

Due to the chromatographic process, the injected solutes become diluted, *i.e.*,

the concentration of the solute in the peak maximum, c_p, is lower than that in the injected solution, c_i. The dilution factor is defined as:

$$\frac{c_i}{c_p} = \frac{V_R}{V_i}\sqrt{\frac{2\pi}{N}} \tag{14}$$

Thus, if 9% peak broadening is allowed, for $k' = 1$:

$$\frac{c_i}{c_p} = \frac{6700}{28}\sqrt{\frac{2\pi}{20\,000}} = 4.2$$

and for $k' = 10$:

$$\frac{c_i}{c_p} = \frac{37\,000}{28}\sqrt{\frac{2\pi}{20\,000}} = 23$$

2.13. Concentration at peak maximum

If the concentration of each of the solutes in the sample is 1 ppm (10^{-6} g ml^{-1}), their concentrations at the respective peak maxima are, for $k' = 1$:

$$c_p = c_i \cdot \frac{c_p}{c_i} = 10^{-6} \cdot \frac{1}{4.2}\ \text{g ml}^{-1} = 0.24 \times 10^{-6}\ \text{g ml}^{-1} \tag{15}$$

and for $k' = 10$:

$$c_p = 10^{-6} \cdot \frac{1}{23}\ \text{g ml}^{-1} = 0.043 \times 10^{-6}\ \text{g ml}^{-1}$$

2.14. UV detector signal

The second component is nitrobenzene with a molar absorption coefficient, $\varepsilon = 10^4$ (254 nm), and a molar mass of 123 g mol^{-1}. The signal, E, which will result in the UV detector can be calculated with the Lambert–Beer law, $E = \varepsilon cd$, where c is the concentration in mol l^{-1} and d the path length in cm. Thus, if $c_p = 0.043 \times 10^{-6}$ g $ml^{-1} = 0.35 \times 10^{-6}$ mol l^{-1} and $d = 1$ cm (as is the case for many UV detectors), we obtain:

$$E = 10^4 \times 0.35 \times 10^{-6} \times 1 = 3.5 \times 10^{-3}\ \text{absorption units (a.u.)} \tag{16}$$

2.15. Detection limit

A common definition of the detection limit is a signal of four times (sometimes also twice) the noise level of the detector. The typical noise level of a UV detector is

10^{-5} a.u. This means a detection limit of 4×10^{-4} a.u. which is about one order of magnitude lower than the signal calculated in eqn. 16. Since the injected solution had a concentration of 1 ppm, the detection limit is about 0.1 ppm or, when related to the injection volume of 28 μl, 2.8 ng.

2.16. Pressure drop

What pressure drop, Δp, will result if the given column is used at v_{opt} which corresponds to a mobile phase flow-rate of $V' = 0.48$ ml min^{-1}? Of course, the pressure depends on the viscosity, η, of the mobile phase used. Let us assume $\eta = 1$ mPa s, the value for water or mixtures of water and acetonitrile. The pressure drop can be calculated by use of the dimensionless flow resistance parameter, φ:

$$\Delta p = \frac{\varphi l_c \eta u}{d_p^2} = \frac{\varphi \times l_c\,(\text{mm}) \times \eta\,(\text{mPa s}) \times u\,(\text{mm s}^{-1})}{100 \times d_p^2\,(\mu\text{m}^2)}\ \text{bar} \qquad (17)$$

Assuming $\varphi = 500$ for slurry-packed spherical totally porous stationary phases used in HPLC:

$$\Delta p = \frac{500 \times 250 \times 1 \times 0.6}{100 \times 5^2}\ \text{bar} = 30\ \text{bar}$$

3. DISCUSSION

3.1. Eqn. 1

The number of theoretical plates is the first parameter out of three in the calculations described which results from the application of reduced parameters[5]. The other two are the linear flow velocity in eqn. 3 and the pressure drop in eqn. 17. The value for N of 20 000 is rather high and in fact is seldom reached by 25-cm HPLC columns ($d_p = 5$ μm) used routinely. A value of 10 000 or 15 000 is, unfortunately, more realistic.

3.2. Eqn. 2

The so-called dead volume is the elution volume of an unretained solute. It should be mentioned that the value of V_0 would be reduced by half if a column of 3.2 mm I.D. were to be used instead of one of 4.6 mm I.D. since V_0 is proportional to d_c^2. From this point of view it is not clear why the 4.6 column is so popular; one of 3.2 mm would enable a saving in solvent consumption of 50%!

3.3 Eqns. 3 and 4

The linear flow velocity is obtained by rearrangement of the equation which defines the reduced velocity[5]. It allows the calculation of the flow-rate by simple geometrical considerations. However, in all calculations of flow-rates from velocities or *vice versa* it must not be forgotten that the chromatographic column is not empty but a rather small fraction of it is occupied by the framework of the stationary phase; this fact is represented in eqn. 4 (as well as in eqn. 2) by the term ε_{tot}.

The diffusion coefficient, which needs to be known for the calculation of the linear flow velocity, can be obtained from the Wilke–Chang equation[6]

$$D_m\ (m^2\ s^{-1}) = \frac{7.4 \times 10^{-12} \sqrt{\psi MT}}{\eta \times V_s^{0.6}} \tag{18}$$

where ψ = a solvent constant (2.6 for water, 1.9 for methanol, 1.5 for ethanol, 1.0 for other solvents), M = the molecular mass of the solvent, T = the absolute temperature, η = the solvent viscosity in mPa s and V_S = the molar volume of the solute in $cm^3\ mol^{-1}$. In eqn. 3 the diffusion coefficient was assumed to be $1 \times 10^{-9}\ m^2\ s^{-1}$. The value is often higher in adsorption chromatography due to the use of non-viscous organic solvents, *i.e.*, the optimum flow velocity is higher. On the other hand, the diffusion coefficients of solutes in the methanol–water mixtures often used for reversed-phase chromatography are smaller due to the rather high viscosity of these eluents[4,5]. Although this effect is of minor practical relevance it should be kept in mind. Moreover, it is of great importance in the chromatography of macromolecules which have very small diffusion coefficients which result from their large molar volumes. This means that large molecules must be chromatographed at low flow velocities[4].

Chromatography at higher flow-rates than the optimum one results in a decrease in the separation ability of the column. For well packed microparticulate HPLC columns this effect is nearly negligible if the reduced velocity is less than 10.

3.4. Eqns. 5 and 6

The retention times which result from chromatography at the optimum flow-rate are rather high. Note that all retention times are independent of the inner diameter of the column if the analysis is performed at a given reduced velocity. At a higher flow-rate the peak widths of all peaks, as calculated in eqn. 9, will diminish, a fact which influences the value obtained from eqn. 12: if the flow-rate of the mobile phase is increased, the time constant of the detector has to be decreased. All values obtained by use of the following equations, except 9 and 12, are independent of the flow-rate and the retention time.

3.5. Eqn. 7

As already mentioned in the discussion of eqn. 2, the retention volume, V_R, of a peak is proportional to the square of the column diameter! If the first eluted peak has $k' = 0$, *i.e.*, it is a non-retained solute, its retention volume is half the value calculated for $k' = 1$: only 3.3 ml (the dead volume). In this case the values obtained from eqns. 10, 11, 13 and 14 would be decreased!

3.6. Eqn. 8

The peak capacity indicates the number of peaks, all eluted with the same plate number, *i.e.*, by isocratic elution, which could be placed in the chromatogram side by side (with resolution 1) from $k' = 1$ to the given capacity factor; in the example shown, to $k' = 10$. The equation was first published in this form by Grushka[7].

3.7. Eqns. 9 and 9a

Eqn. 9 is a rearrangement of the well known relationship which allows calculation of the plate number of a column if the peak width and retention time are known: $N = 16(t_R/4\sigma)^2$. As already mentioned above, the peak width in seconds (eqn. 9) is influenced by the mobile phase flow-rate, whilst the peak width in microlitres (the peak volume, eqn. 9a) is not if we assume that the decrease in plate number by a flow-rate other than the optimum one is negligible.

3.8. Eqn. 10

This relationship was first derived by Martin *et al.*[8] in 1975. Whilst the retention volume, V_R, and the plate number, N, are known and the fraction of peak broadening, θ, can be assumed, the quality parameter of the injection, K, depends on the particular circumstances. It was claimed to be about 2 by Karger *et al.*[9] who also gave a method for its determination. Clearly, the better the injection, the higher is K and the allowed injection volume. Moreover, the maximum injection volume depends on the retention volume of the solute; therefore, for capillary columns with their extremely small retention volumes (since V_R decreases with the square of the capillary diameter), the allowed injection volume has values in the nanolitre range, making trace analysis impossible[10].

There is one way to avoid the limitations in injection volume. If the sample solvent is markedly weaker than the mobile phase for the HPLC analysis, *i.e.*, if its elution strength is low the solutes are concentrated at the top of the column. In this case the injection volume may be unusually high, in the range of millilitres or even litres[11,12]. However, a prerequisite is that the amount of solute is small enough to prevent the adsorption isotherm from becoming non-linear.

For the calculation of the injection volume, a rule of thumb was proposed by Huber[13]: the maximum injection volume should be equal to the volume standard deviation, σ, of the peak of interest, *i.e.*, to 1/4 of its volume as calculated by eqn. 9a. Since V_p was calculated to be 190 μl, the injection volume should be 47 μl. This is markedly higher than the 28 μl calculated with eqn. 10 and a peak broadening fraction of 9%. Thus Huber's recommendation has to be applied with caution.

At this point the attention of the reader is drawn to a fact whose importance will be apparent later in the discussion of the detection limit (eqns. 14–16). If for the calculation of the injection volume in eqn. 10 the retention volume (37 ml) of the second peak with $k' = 10$ is used, V_i is found to be much higher: 157 μl may be injected without broadening of the second peak by more than 9%. Of course the first peak would be broadened dramatically but one can imagine situations where the early eluted peaks are not of interest.

3.9. Eqn. 11

This equation was also reported by Martin *et al.*[8]. The relationship $V_d = V_i/2$ is only true if K is assumed to be 2. It is obvious that a standard 8-μl detector cell causes a peak broadening of more than 1% for the early eluted peaks on a 25 cm × 4.6 mm column.

3.10. Eqn. 12

This equation was also reported by Martin *et al.*[8]. The time constant of a

detector should not exceed 0.5 s if used with columns of 25 cm in length, a requirement which is satisfied by all modern HPLC detectors. However, if the columns are as short as 10 or 5 cm, a time constant of 0.5 s is too high. As already mentioned at the discussion of eqns. 5 and 6, the time constant must be decreased if the reduced velocity of the mobile phase is increased.

3.11. Eqn. 13

The maximum allowed capillary length may be calculated by this equation of Martin *et al.*[8] or by use of a similar equation of Scott and Kucera[14]. It is important to realize that the capillary length depends on the square of the retention volume of the peak of interest and on the inverse fourth power of the inner diameter of the capillary. In practice, the inner diameter of connecting tubes should not exceed 0.25 mm; they should be as short as possible.

3.12. Eqns. 14 and 15

The important eqn. 14 was derived by Karger *et al.*[9]. Of course the later eluted peak is diluted more than the early eluted one. However, if only the peak with $k' = 10$ is important, the injection volume can be increased to 157 μl as shown in the discussion of eqn. 10. In this case the dilution factor of this second peak is as small as that for the first peak with an injection volume of 28 μl, namely 4.2. Therefore, the peak maximum concentrations will be the same in both cases, 0.24×10^{-6} g ml^{-1}. Note that this is only true if the respective maximum allowed injection volume is used for both peaks.

The validity of these conclusions can also be shown by combination of eqns. 10 and 14:

$$c_p = c_i \cdot \frac{\theta K}{\sqrt{2\pi}} \tag{19}$$

Eqn. 19 can also be found in the paper by Karger *et al.*[9]. It means that, contrary to what is often claimed, the peak maximum concentration (and thus the minimum detectable concentration) does not depend on the column inner diameter, the capacity factor of the peak, *i.e.*, its retention volume or the plate number of the column if, as already mentioned, the injection volume is as high as allowed by the peak of interest. Besides the two concentrations c_p and c_i, eqn. 19 only involves constants. Therefore, if $\theta = 0.1$ (1% peak broadening), $c_p = 0.08\ c_i$, and if $\theta = 0.3$ (9% peak broadening), $c_p = 0.24\ c_i$ (assuming $K = 2$).

3.13. Eqn. 16 and the detection limit

In the case of the detection limit we have to distinguish between the minimum detectable concentration and the minimum detectable mass. The minimum detectable concentration at the peak maximum of a given solute only depends on the properties of the detector (for concentration-dependent detectors such as UV detectors) as can easily be shown. Rearrangement of eqn. 16, the Lambert–Beer law, gives:

$$c_p = E/\varepsilon d$$

With a typical UV detector noise of 1×10^{-4} a.u., the minimum detectable signal,

E, becomes 4×10^{-4} a.u. For nitrobenzene, ε is 10^4 (at 254 nm) and d is 1 cm for many UV detectors. Therefore c_p becomes 4×10^{-8} mol l^{-1} or 4.9×10^{-6} g l^{-1}. This is the lowest peak concentration of nitrobenzene which can be detected, independent of the retention volume.

What is the minimum concentration of nitrobenzene in the sample solution in order to obtain a detectable peak? If we inject the maximum allowed sample volume as calculated from eqn. 10, eqn. 19 is valid and we can rearrange it:

$$c_i = \frac{c_p \sqrt{2\pi}}{\theta K} \tag{19a}$$

With $\theta = 0.3$ (9% peak broadening) and $K = 2$, $c_i = 4.2\, c_p$. For nitrobenzene, the minimum detectable peak concentration was calculated above to be 4.9×10^{-6} g l^{-1}. Therefore the minimum detectable concentration of nitrobenzene in the sample is 21×10^{-6} g l^{-1} (0.021 ppm). This is only true if the maximum allowed sample volume is injected and is independent of the capacity factor of the peak.

It may be recalled that a detection limit for nitrobenzene of 0.1 ppm was calculated (page 203). This is five times higher than possible because the injection volume of 28 μl was adjusted to the first peak with $k' = 1$ and not to the nitrobenzene peak with $k' = 10$. As already mentioned, the maximum injection volume of the second peak is 157 μl, about five times higher than that of the first peak.

To summarize, the minimum detectable concentration of a solute in the sample solution depends only on the detector properties and on the optical properties of the solute, *i.e.*, its absorbance, if the maximum tolerable sample volume with respect to the retention volume of this solute is injected. The minimum detectable concentration is independent of the column dimensions, plate number or capacity factor.

In contrast to the concentration, the minimum detectable mass depends on the capacity factor of the solute. The earlier a peak is eluted, the smaller is the maximum injection volume and, with the concentration of the sample solution being constant, the smaller is the absolute mass of solute injected. The same is true if the column inner diameter is reduced.

For trace analysis, this means that small bore columns should be used and low capacity factors are advantageous if the sample volume is limited, as is often the case in clinical or forensic chemistry. If enough sample is available, *e.g.*, in food analysis, it is not necessary to use small bore columns and low capacity factors. However, the analysis time, solvent consumption and column overload by accompanying substances (not discussed here) need to be kept in mind.

3.14. Eqn. 17

The calculation of the pressure drop expected is possible by use of the reduced flow resistance parameter, φ, as defined by Bristow and Knox[5]; φ is about 500 for spherical and less than 1000 for irregular, slurry-packed HPLC materials. The pressure drop is markedly lower if organic solvents of low viscosity, *e.g.*, hexane, are used and it is higher for mixtures of methanol and water, the viscosities of which can reach 1.8 mPa s[4,5].

It may be of interest that the fourth reduced parameter according to Bristow

and Knox[5], besides the reduced plate height, h, the reduced velocity, v, and the flow resistance, φ, is the so-called separation impedance, E, which is defined as $E = h^2\varphi$. For the column discussed in this paper, E has the excellent value of 3125 ($2.5^2 \times 500$). The higher is E the poorer is the column.

4. CONCLUSIONS

It is obvious that the calculations described are interesting and moreover are useful for routine laboratory work. They allow one to recognize some of the instrumental and chromatographic limitations and benefits of HPLC. However, the basic problem of chromatography is not solved with these considerations: What physico-chemical conditions are needed to make possible the separation of the different types of molecules present in the sample mixture?

The different parameters which allow such a separation are to be found in the resolution equation, which is the basic equation of liquid chromatography:

$$R_S = \frac{1}{4}\left(\frac{\alpha - 1}{\alpha}\right)\sqrt{N}\left(\frac{k'}{1 + k'}\right) \tag{20}$$

In this equation there are only three parameters. Two of them, the plate number, N, and the capacity factor, k', have been met in the calculations described. In principle, they can be altered at will. The plate number can always be adjusted by using a shorter column or by coupling two or more columns; the capacity factor can be adjusted by using a mobile phase of greater or lower elution strength. In contrast, the relative retention, α, which represents the selectivity parameter, depends only on the physico-chemical properties of the separation system used, such as adsorption, hydrophobic or ion-exchange equilibria. Often these phenomena are not easy to understand and the chemist has to search for the best separation system by trial and error. Finding systems of high selectivity is the fine art of chromatography and the use of calculations such as those presented here is no more than an aid to this end.

5. SUMMARY

Starting from the basic geometrical properties of a column used in high-performance liquid chromatography, it is shown how many data of practical interest can be calculated using the well known theory of liquid chromatography. The basic geometrical properties employed are the column length, column diameter and particle diameter of the stationary phase. Among the calculable data are the plate number of the column, the volume of a chromatographic peak, the maximum allowed injection volume and the dilution of the sample by the chromatographic process. All calculations are illustrated with numerical examples. Special attention is given to the problem of the injection volume and detection limit in trace analysis.

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THIN-LAYER CHROMATOGRAPHY OF MYCOTOXINS

VLADIMÍR BETINA

Department of Environmental Chemistry and Technology, Faculty of Chemistry, Slovak Polytechnical University, Jánska 1, CS-812 37 Bratislava (Czechoslovakia)

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CONTENTS

1. INTRODUCTION

Mycotoxins are fungal metabolites that have been recognized within the last two decades as a potential threat to human and animal health. The effects of mycotoxins result in economical losses to various extents (*e.g.*, due to aflatoxins, trichothecenes and ochratoxin A). Increasing awareness of the hazards posed to both animal and human health by mycotoxins in feeds and foodstuffs has led to the development of methods for their purification and analysis. The simultaneous production of many non-toxic secondary and primary fungal metabolites with the mycotoxins has resulted in the extensive use of chromatographic techniques for their separation. According to Gorst-Allman and Steyn[1], in only a relative few instances have mycotoxins been obtained pure using techniques other than chromatography.

Of the chromatographic techniques applied to mycotoxins, thin-layer chromatography (TLC) is by far the most widely used in the detection, analysis and characterization of fungal toxins. At the end of the 1970s and in the early 1980s, reviews and book chapters on the chromatography of mycotoxins in general and on TLC in particular have been published (*e.g.*, refs. 1–9). A recent book devoted to methods for the production, isolation, separation and purification of mycotoxins[8] included sections on the TLC of the following mycotoxins: aflatoxins, trichothecenes, ochratoxins, citrinin, zearalenone, cytochalasans, patulin, penicillic acid, citreoviridin and other small lactones, rubratoxins, hydroxyanthraquinones, epipolythiodioxopiperazines, aspergillic acid and related pyrazine derivatives, cyclopiazonic acid and related toxins, indole-derived tremorgenic toxins, *Alternaria* toxins, roquefortine, PR toxin, cyclochlorotine and secalonic acid D.

The present review is based partly on some chapters of this book but the information on TLC is enlarged and brought up to date because TLC data in the book are mostly from the literature before 1981. The literature in this review covers

the period from 1961 to 1984 with the following percentages for the years in the parentheses: 4 (1961–1965), 14 (1966–1970), 23 (1971–1975), 30 (1976–1980), and 27 (1981–1984). A few citations from 1985 are also included.

The literature on the TLC of mycotoxins is immense and it would be virtually impossible to give a complete survey. Instead, areas of mycotoxin research in which TLC has found the greatest application will be covered. In the general part of this review, the chief techniques for the preparation of samples and clean-up methods, adsorbents, solvent systems, detection, two-dimensional development, high-performance TLC (HPTLC), quantitative TLC and preparative TLC (PLC) are described. In the second part, the so-called multi-toxin TLC and systematic analysis are discussed, followed by applications of TLC to the best known structurally related classes of mycotoxins and to miscellaneous toxins produced by major fungal genera such as *Aspergillus*, *Penicillium* and *Fusarium*. Applications of TLC techniques to structurally related and individual mycotoxins include data concerning extraction and clean-up methods, adsorbents, solvent systems, detection, qualitative analysis, quantitation and PLC.

2. TECHNIQUES

It has been stressed[1] that the successful application of TLC in the analysis and purification of mycotoxins requires the manipulation of three variables: (a) the adsorbent, (b) the solvent system and (c) the method of detection. In most instances, however, the toxins have to be isolated from samples that are contaminated. In addition, it is often necessary to use a clean-up step before TLC investigation in order to remove interfering substances.

2.1. Extraction and clean-up procedures

When mycotoxins are not available as pure substances for TLC they are obtained either from contaminated samples (feeds or foodstuffs) or from laboratory and large-scale fermentations.

The isolation of the toxin(s) from a contaminated sample is accomplished by continuous extraction in a Soxhlet apparatus or brief extraction in a Waring blender. The latter method is used if the toxins are heat sensitive or if a rapid extraction is necessary. Most common mycotoxins are amenable to extraction with chloroform–methanol (1:1), although some toxins (*e.g.*, moniliformins) are not soluble in chloroform and different systems [*e.g.*, acetone–water (1:1)] are recommended. Many workers use acetonitrile, methylene chloride or other solvents. After the extraction, the solid residue is removed by filtration.

When mycotoxins are produced in liquid cultivation media they are extracted from the culture filtrates and/or from mycelia of the toxigenic fungi. Procedures of this type are covered in the sections dealing with particular toxins but typical examples are given also here.

After extraction of a sample containing mycotoxins, it is often necessary to apply a clean-up procedure to the crude extract. Defatting may be achieved with hexane or isooctane or by developing the TLC plate spotted with the crude extract in benzene–hexane (3:1); in the latter instance many of the contaminants move with the solvent front, whereas the mycotoxins remain on the baseline and can be developed using another solvent system.

Clean-up procedures are often used in multi-toxin screening of mycotoxins in foods and feedstuffs. At least some examples can be mentioned here. When screening aflatoxins, ochratoxin and zearalenone, Howell and Taylor[10] extracted mixed feeds with chloroform–water and the extract was cleaned up by using a disposable Sep-Pak silica cartridge. The duration of the procedure was 15 min from sample extraction to extract preparation, and less solvent was necessary than in conventional methods.

Another procedure was used by Tonsager *et al.*[11]. Feed or grain containing aflatoxins, diacetoxyscirpenol, T-2 toxin, deoxynivalenol and zearalenone were extracted with methanol–water (1:1), the aqueous filtrate was partitioned with chloroform, the chloroform fraction dried over Na_2SO_4, evaporated, dissolved in toluene–methylene chloride (85:15), applied to a gel-permeation column (Bio-Beads S-X3) and individual mycotoxin fractions were eluted with toluene–methylene chloride (85:15). The purified fractions were then applied to TLC or HPTLC plates. Gel-permeation chromatography provided a simple and effective procedure for the purification of aflatoxins and trichothecenes but was not amenable for ochratoxin separation.

A method for screening a large number of *Fusarium* isolates for toxin production, described by Richardson *et al.*[12], consisted in extraction of rice and vermiculite cultures with acetonitrile, removal of lipids with hexane and drying before TLC analysis.

Another simple and rapid technique for screening large numbers of fungal strains for the presence of toxins was used by Barr and Downey[13], who extracted mycotoxins from an agar medium with chloroform, followed by direct evaluation by TLC.

In screening *Chaetomium* spp. cultures for chaetoglobosin production, mouldy rice grains were extracted first with methylene chloride and then with ethyl acetate. The extracts were examined by TLC and cytotoxicity tests[14]. Heathcote and Hibbert[15] found that freeze-drying of extracts before TLC improved considerably the subsequent separation of aflatoxins.

Procedures used for the extraction and clean-up techniques for ochratoxins were surveyed recently by Steyn[16].

The production of citrinin in the course of submerged fermentation of a strain of *Penicillium* was monitored by Betina *et al.*[17] as follows. The filtrate was extracted with butyl acetate after acidification and the extract was dried over sodium sulphate and evaporated to dryness. The metabolites in the residue were separated into an acidic and a neutral fraction by dissolution in benzene and extraction with an aqueous solution of potassium hydrogen carbonate. The acidic compounds were re-extracted from the aqueous phase with benzene after adjusting the pH to 3.8. The benzene extrract was washed with deionized water, dried over anhydrous sodium sulphate and evaporated to dryness *in vacuo*. The residue was dissolved in methanol and used for TLC.

Citrinin and other acidic mycotoxins such as ochratoxin A, cyclopiazonic acid, penicillic acid and secalonic acid may be extracted from the crude extract with sodium hydrogen carbonate solution. Consequent acidification and extraction into chloroform constitutes a useful clean-up procedure[18].

2.2. TLC adsorbents

Various adsorbents possessing different properties are commercially available that may be slurried alone or admixed with reagents for specific applications and spread on glass, aluminium foil or plastic supports. The most commonly used adsorbent for mycotoxins is silica gel, alumina being only rarely used. TLC on plates of rice starch and $CaSO_4$ were also used by Mišković[19], who separated twelve aflatoxins, ochratoxins, zearalenone and zearalenol using three solvent systems.

Pre-coated TLC plates are also available without or with a binder or an indicator for solute detection. The thickness of the layer is often 0.1–0.3 mm for analytical applications and 0.5–2.0 mm for preparative use. The particle size also varies.

Silica gel may be treated with different reagents to enhance its separating capacity. For example, the peak tailing of acidic mycotoxins such as citrinin, ochratoxin A, cyclopiazonic acid and penicillic acid can be prevented by using silica gel TLC plates that have been pre-treated with oxalic acid by immersion in a 10% solution of oxalic acid in methanol for 2 min. After heating at 110°C for 2 min and cooling, the plates were immediately spotted and developed in appropriate solvent systems[18]. In this laboratory, pre-coated silica gel plates (Silufol; Kavalier, Czechoslovakia) were impregnated with 0.25 *M* oxalic acid in methanol by developing the plates in the solution. They were dried in air and immediately used for TLC of citrinin and other acidic metabolites of a *Penicillium* sp.[17]. Similar effects on separating capacity can be achieved by treating plates with tartaric acid[20] or EDTA[21].

Heathcote and Hibbert[15] found that lack of reproducibility, a recurrent problem in the analysis of mixtures containing aflatoxins, could be overcome by using Bio-Sil A of particle size 2–10 μm to coat the TLC plates. When a number of silica gel preparations were tested by these workers for resolution of aflatoxin mixtures, neutral SilicAR TLC-7G was found to be the most satisfactory with the three solvent systems tested. Pre-coated Silufol sheats were found to be satisfactory for a wide variety of mycotoxins and other fungal metabolites[22].

Chemically bonded reversed-phase layers have also been used for the separation and detection of mycotoxins[23]. Other data concerning adsorbents may be found under Applications.

2.3. Solvent systems

It is virtually impossible to evaluate the relative merits of the enormous variety of solvent systems developed for the analysis and characterization of various mycotoxins by means of TLC. The initial systems consisted of various percentages of methanol in chloroform. Later acetone was substituted for methanol owing to the great sensitivity of the chloroform–methanol systems to humidity and other environmental changes. Examples of similar and other systems are given under Applications.

2.4. Detection

The following standard techniques exist that can be used to locate the mycotoxins on the developed thin-layer chromatograms: (a) examination under visible light for coloured substances (*e.g.*, hydroxyanthraquinones and secalonic acids); (b) examination of compounds that fluoresce under UV light of a certain wavelength (*e.g.*, aflatoxins, citrinin, citreoviridin, ochratoxin A, and sterigmatocystin); (c) compounds that absorb UV radiation strongly at a certain wavelength may be chro-

matographed on TLC plates pre-treated with a fluorescent indicator; they appear as dark spots on a uniformly fluorescing background (*e.g.*, moniliformins, patulin and rubratoxins); (d) spraying the plate with a reagent that will react with the mycotoxin to produce a coloured product. In addition, bioautographic detection may be used, based on the toxicity of mycotoxins to model organisms.

Scott *et al.*[24] described a TLC screening procedure for the detection of 18 mycotoxins, which were revealed on the TLC plates under UV and visible light before and after the plates were sprayed with freshly prepared *p*-anisaldehyde reagent and heated. In addition, Ďuračková *et al.*[22] detected 37 mycotoxins and other fungal metabolites with a solution of iron(III) chloride. Characteristic colours revealed under UV light and with spray reagents were reported.

Steyn[25] reported a TLC system in which the mycotoxins were detected on TLC plates by examination under long-wavelength UV light and spraying with 1% cerium(IV) sulphate in concentrated sulphuric acid, concentrated sulphuric acid or 1% ethanolic iron(III) chloride. Later, Gorst-Allman and Steyn[18] used the following spray reagents: (a) 2,4-dinitrophenylhydrazine (1 g)–concentrated sulphuric acid (7.5 ml)–ethanol (75 ml)–water (170 ml); (b) hydrazono-2,3-dihydro-3-methylbenzothiazole hydrochloride (0.5% aqueous solution); (c) iron(III) chloride (3% solution in ethanol); (d) aluminium chloride (1% solution in chloroform); (e) Ehrlich reagent; (f) cerium(IV) sulphate (1% solution in 3 *M* sulphuric acid); and (g) vanillin (1% in 50% phosphoric acid). The plates were sprayed, the immediate effects noted, and they were then heated at 110°C for 10 min. Iodine and ammonia fumes were also used for some plates. Characteristic colours were reported.

Sterigmatocystin forms a yellow fluorescent complex with aluminium chloride, which can be used in both qualitative and quantitative detection[26]. Patulin forms a yellowish brown fluorescent derivative with N-methylbenzthiazolone-2-hydrazone[27] and penicillic acid gives a blue fluorescent derivative with diphenylboric acid–2-ethanolamine[28]. Chemical confirmatory tests for ochratoxin A, citrinin, penicillic acid, sterigmatocystin and zearalenone performed directly on TLC plates were published recently[29]. In many instances the colours produced by reaction of a mycotoxin with different chromogenic reagents are characteristic and can be used as a means of identification. A list of spray reagents for various classes of compounds has been published[30].

Some mycotoxins are known to possess antimicrobial properties and bioautographic detection with sensitive microbial cultures is also possible using procedures for the bioautography of antibiotics[31,32]. For example, Téren and Ferenczy[33] proposed a semi-quantitative bioautographic test for trichothecene mycotoxins using the colourless alga *Prototheca wickerhamii* as the test organism. The developed TLC plate is overlayered with a suspension of the test organism in an agar medium, and after appropriate incubation the contours of the inhibition zones are revealed with bromocresol purple indicator.

A special bioautographic detection of mycotoxins, based on their toxicity to *Artemia salina* larvae, has been reported[34]. The method was tested first with standard samples of mycotoxins (aflatoxin B_1, kojic acid and sterigmatocystin) in combination with UV detection *in situ*, and with crude extracts from toxigenic fungi. The procedure was also applied to the preparative TLC of crude extracts.

2.5. *Two-dimensional TLC*

Two-dimensional chromatography, or bi-directional chromatography as named by Zakaria *et al.*[35] in a recent review of its applications in TLC, is a technique of increasing importance in the separation of mixtures of varying complexity.

Two-dimensional TLC has been aplied to aflatoxins, citrinin, cyclopiazonic acid, ochratoxin A, patulin, penicillic acid and sterigmatocystin. The most important data concerning the stationary phases and solvent systems are presented in Table 1.

TABLE 1

TWO-DIMENSIONAL TLC OF MYCOTOXINS

Toxins	*Stationary phase*	*Solvent 1**	*Solvent 2**	*Ref.*
Aflatoxins	Silica gel 60	Tol–EtOAc–90% FA (5:4:1)	Me_2CO–$CHCl_3$ (1:9)	36
	SILG-HR-25	$CHCl_3$–Me_2CO (88:12)	95% denatured EtOH	37
	Silica gel 60	$CHCl_3$–Me_2CO–iPrOH (80:15:15)	Tol–EtOAc–95% FA (60:30:10)	38
	Silica gel	$CHCl_3$–Me_2CO (9:1)	CH_2Cl_2–CH_3CN (8:2)	26
	Silica gel	$CHCl_3$–Me_2CO–H_2O (46:6:free)	Tol–EtOAc–FA (30:15:15 or 24:20:6)	39
	Chemically bonded C_8 reversed-phase	MeOH–H_2O (20:1)	MeOH–H_2O (4:1)	23
Citrinin	Silica gel pre-treated with oxalic acid	Tol–EtOAc–FA (6:3:1)	C_6H_6–HOAc (8:2)	26
Cyclopiazonic acid	Silica gel	EtOAc–iPrOH–conc. ammonia soln. (20:15:10)	$CHCl_3$–HOAc (10:1)	40
Ochratoxin A	Silica gel	Tol–EtOAc–90% FA (6:3:1)	$CHCl_3$–Me_2CO (9:1)	41
	Silica gel	Tol–EtOAc–FA (6:3:1)	C_6H_6–HOAc (8:2)	26
Patulin	Silica gel	Tol–EtOAc–FA (6:3:1)	C_6H_6–HOAc (8:2)	26
	Silica gel	Any suitable pair of the following solvents: Tol–EtOAc–90% FA (6:3:1) C_6H_6–MeOH–HOAc (24:2:1) C_6H_6–EtOH (95:5) $CHCl_3$–MeOH (4:1) $CHCl_3$–MIBK (4:1) $CHCl_3$–Me_2CO (9:1) $CHCl_3$–HOAc–Et_2O (17:3:1) nBuOH–HOAc–H_2O (4:1:4)		42
Penicillic acid	Silica gel	Tol–EtOAc–FA (6:3:1)	C_6H_6–HOAc (8:2)	26
Sterigmatocystin	Silica gel	Tol–EtOAc–FA (6:3:1)	C_6H_6–HOAc (8:2)	26
	Silica gel	C_6H_6–MeOH–HOAc (90:5:5)	Tol–EtOAc–FA (7:7:1)	43

* Abbreviations: Tol, toluene; iPrOH, 2-propanol; EtOAc, ethyl acetate; Me_2CO, acetone; FA, formic acid; EtOH, ethanol; C_6H_6, benzene; $CHCl_3$, chloroform; CH_2Cl_2, methylene chloride; HOAc, acetic acid; MeOH, methanol; MIBK, methyl isobutyl ketone; Et_2O, diethyl ether; nBuOH, *n*-butanol.

2.6. High-performance TLC (HPTLC)

The ideal assay of mycotoxins should be precise, accurate, sensitive, rapid and specific with regard to accompanying substances. HPTLC has advantages over classical TLC in terms of higher speed, better sensitivity and efficiency, use of less solvent and a large number of samples per plate. The last factor gives HPTLC advantages over high-performance liquid chromatography (HPLC) and gas–liquid chromatography (GLC). However, it requires an expensive densitometer. HPTLC has not yet found extensive applications in mycotoxin monitoring. It was recently applied by Lee *et al.*[21] to multi-mycotoxin determinations. The increased resolution due to uniform particle plates, new sample application apparatus and multi-optic scanning devices combined with continuous multiple developments with two solvent systems of different polarity gave a baseline separation and quantitation of thirteen mycotoxins. Some of their results are presented in Section 3.1.

More recently, HPTLC and reversed-phase TLC of ten mycotoxins (ochratoxin A, aflatoxin 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 was reported by Stahr and Domoto[44]. Use of both methods allowed more sensitive analyses to be made of mycotoxins in food and feedstuffs. Further development of the applications of HPTLC in the screening of feedstuffs and foods to control the presence and the level of mycotoxins should be expected. More examples are given under Applications.

2.7. Quantitation

Both types of evaluation, *in situ* and after extraction from the plate, have been used for the quantitation of mycotoxins in TLC. UV spectroscopy is the most common quantitative method for procedures in which mycotoxins are extracted from the adsorbent. The absorbance obtained from the sample is used in conjunction with the molar absorptivity of the pure toxin to calculate the concentrations[1]. Procedures of this type have been described, *e.g.*, for the determination of zearalenone[45]. Another method of quantitation was used to determine [^{14}C]zearalenone by Wolf and Mirocha[46]. After detection under UV light, the zearalenone-containing area was scraped off the plate and the radioactivity was counted directly or after purification by gas chromatography.

Semi-quantitative measurements *in situ* are also possible by comparing spot areas with the spots of known concentrations or with a grey scale commonly used in photographic laboratories. The latter evaluation has been applied to sterigmatocystin[47] and aflatoxins[48].

In the mid-1960s, chromatogram spectrophotometers were introduced that showed great technical improvements, and with their aid it became possible to carry out quantitative determinations on thin-layer plates of substances that absorb light at wavelengths approximately between 200 and 800 nm in the UV and visible spectral ranges or that emit visible light on excitation with UV light. Fluorescence densitometry can be used to measure mycotoxins with greater accuracy and precision than by using visual comparison and, in fact, TLC with densitometry is the primary procedure for these analyses. Densitometric determinations have been reported for citrinin in feeds at the 0.5 μg/g level[49], citrinin on EDTA-impregnated plates at a sensitivity of 10 ng per spot[3], fumitoxins A–D in contaminated plant food and

wastes[50], aflatoxin B_1 in eggs[51], patulin in apple juice by preparation and TLC of the aniline imine, HCl hydrolysis of the imine and fluorophore formation from the liberated aniline with fluorescamine[52], sterigmatocystin after two-dimensional TLC[43] and other mycotoxins in food extracts[53].

Ripphahn and Halpaap[54], who optimized quantitation in HPTLC, were among the first to apply HPTLC to the quantitative determination of aflatoxins. Aflatoxins B_1, B_2, G_1 and G_2 were separated on the HPTLC plate after two-fold development with chloroform–acetone (9:1). Alternating amounts of 200, 500 and 1000 pg were applied for the determination of the calibration graph. Twenty-four bands could be accommodated on a 10-cm wide plate, which made it possible to perform an eight-fold determination at each concentration. The standard deviation of the individual values for 1000 pg was 1.5–3.6%, for 500 pg 3.0–4.5% and for 200 pg 3.5–12.5%. The regression coefficients of the regression lines were better than 0.9987 in all instances. In the range 100–1000 pg, there was a linear relationship between the fluorescence signal and the concentration. The limit of detection for aflatoxins B_1, G_1 and G_2 was about 10 pg and that for B_2 was even lower. Application, development, measurement and calculation required about 1 h.

In multi-toxin analysis using HPTLC, Lee *et al.*[21] used the reflectance mode for the determination of patulin, sterigmatocystin, aflatoxin B_2, zearalenone, luteoskyrin and penicillic acid and fluorescence measurements for aflatoxin B_1, B_2 and M_2, citrinin, zearalenone and ochratoxin A. The detection limits with UV–visible scanning were 0.2–2.0 ng and in the fluorescence mode 2–50 pg.

Quantitative determinations using the fluorescence mode have been reported for aflatoxins[55–62], patulin[52], ochratoxin A[63] and trichothecenes[64]. Laser-induced fluorescence has also been applied to the determination of aflatoxins[55]. In the quantitation of moniliformin, which occurs naturally as the Na^+ or K^+ salt, flame photometry was used to determine the Na^+ or K^+ content[65].

A review on mycotoxin analysis by densitometry with many references was published by Stubblefield[66].

2.8. Preparative TLC (PLC)

The principles of the PLC of mycotoxins were summarized by Gorst-Allman and Steyn[1]. In addition to thicker layers (in comparison with analytical TLC), special techniques are used to apply larger volumes uniformly and in scraping off the separated zones. Mobilities are lower in PLC in the corresponding solvent systems suitable for TLC, so a more polar solvent system or multiple development is recommended. A small column packed with about 3 cm of coarse adsorbent (as a slurry) is prepared, to which the scraped sorbent with the toxin is added. After an initial elution with a less polar system [*e.g.*, benzene–hexane (3:1)] to remove possible lipid contaminants, the same solvent system as in the PLC development is used to elute the toxin. PLC has often been used in the initial preparations of various mycotoxins, such as cytochalasins H and J[67], chaetoglobosins K and L[68], proxiphomin and protophomin[69], citreoviridin[70], paspalitrem A[71] and tetrahydroxyanthraquinone from a mutant of *Trichoderma viride*[72]. Pathre *et al.*[73] used PLC to provide *trans*-zearalenone free from *cis*-zearalenone and other detectable impurities.

3. APPLICATIONS

3.1. Multi-toxin TLC and systematic analysis

As no principle exists for predicting the presence of a particular mycotoxin in natural products, various multi-toxin (multi-mycotoxin) methods have been published for the simultaneous detection of a number of mycotoxins, which differ in the extraction solvents, clean-up procedure and final detection procedure (mostly involving TLC). In clean-up techniques, mini-column chromatography has been used by several workers (*e.g.*, refs. 18, 26, 53 and 74–78). Patterson *et al.*[79] used a dialysis clean-up procedure. A final TLC analysis has been adopted in the following instances.

Originally, Eppley[75] described a screening method for zearalenone, aflatoxin and ochratoxin and his techniques were subsequently used or adapted by various workers. Steyn[25] reported a TLC system for the simultaneous separation and detection of eleven mycotoxins, in which extensive purification of acidic mycotoxins was achieved by removal of the neutral material. The procedure used silica gel G TLC plates impregnated with oxalic acid, with development in chloroform–methyl isobutyl ketone (4:1). His detection methods were mentioned in Section 2.4. The mobility of the neutral mycotoxins was essentially unaffected when oxalic acid was omitted, whereas the acidic mycotoxins, *e.g.*, cyclopiazonic acid and secalonic acid D, and the ochratoxins remained at the origin.

Scott *et al.*[24] described a TLC screening procedure for the detection of 18 mycotoxins commonly produced by species of *Aspergillus*, *Penicillium* and *Fusarium*. They employed Adsorbosil 5 silica gel plates, with development in toluene–ethyl acetate–90% formic acid (6:3:1) and benzene–methanol–acetic acid (24:2:1). Toxins were rendered visible on the plates under UV or visible light before and after spraying with freshly prepared *p*-anisaldehyde reagent (a mixture of 0.5 ml of *p*-anisaldehyde in 85 ml of methanol containing 10 ml of glacial acetic acid and 5 ml of concentrated sulphuric acid) and heating at 130°C for 8–20 min. All the toxins analysed migrated in the acidic solvent systems but citrinin and luteoskyrin streaked.

Fishbein and Falk[80] developed TLC procedures for five types of mycotoxins (aflatoxins, ochratoxins, aspertoxin, O-methylsterigmatocystin and sterigmatocystin) and some other fungal metabolites.

Stoloff *et al.*[81] described a multi-mycotoxin TLC method for aflatoxins, ochratoxins, zearalenone, sterigmatocystin and patulin in a number of agricultural products. They used silica gel plates with internal fluorophores and benzene–methanol–acetic acid (18:1:1) or hexane–acetone–acetic acid (18:2:1) as the solvent system. The developed plates were viewed under both short- and long-wave UV light. The limits of detection ranged from 20 (aflatoxin) to 450 μg/kg (patulin). Joseffson and Möller[76] reported detection limits of aflatoxin 5, ochratoxin 10, patulin 50, sterigmatocystin 10 and zearalenone 35 μg/kg by using gel filtration on Sephadex LH-20 as a clean-up procedure prior to TLC.

A simple and rapid method for screening large numbers of fungi for the presence of toxigenic strains and identifying the mycotoxins produced under different environmental conditions was developed by Barr and Downey[13]. Wilson *et al.*[82] published a method for the detection of aflatoxins, ochratoxins, zearalenone, citrinin and penicillic acid. Mycotoxins in chloroform extracts were isolated by column chromatography (CC) and then separated by TLC on Adsorbosil-1 pre-coated plates.

Moubasher *et al.*[83] evaluated the toxin-producing potential of fungi isolated from blue-veined cheese. The fungal metabolites tested for were aflatoxins, patulin, versicolorin, sterigmatocystin, ochratoxin A, kojic acid and penicillic acid by the method of Scott *et al.*[24]. Coman *et al.*[84] reported a TLC analysis of feed samples in which four aflatoxins, ochratoxin A, zearalenone, sterigmatocystin and T-2 toxin, were detected. A multi-toxin method involving a membrane clean-up step and two-dimensional TLC was published by Patterson *et al.*[79].

Whidden *et al.*[78] developed a method for simultaneous extraction, separation and qualitative analysis of rubratoxin B, aflatoxin B_1, diacetoxyscirpenol, ochratoxin A, patulin, penicillic acid, sterigmatocystin and zearalenone in corn. Mycotoxins were extracted with acetonitrile, sequentially eluted from a silica gel mini-column and rendered visible by TLC. A flow chart for the extraction and separation of the eight mycotoxins is presented in Fig. 1. Fractions 2–4 were analysed on the same TLC plate using external and internal standards and the solvent system toluene–ethyl acetate–formic acid (6:3:1). Fraction 5 (containing rubratoxin B) was applied to a separate TLC plate together with external standards (five concentrations of the toxin) and developed in acetonitrile–acetic acid (100:2).

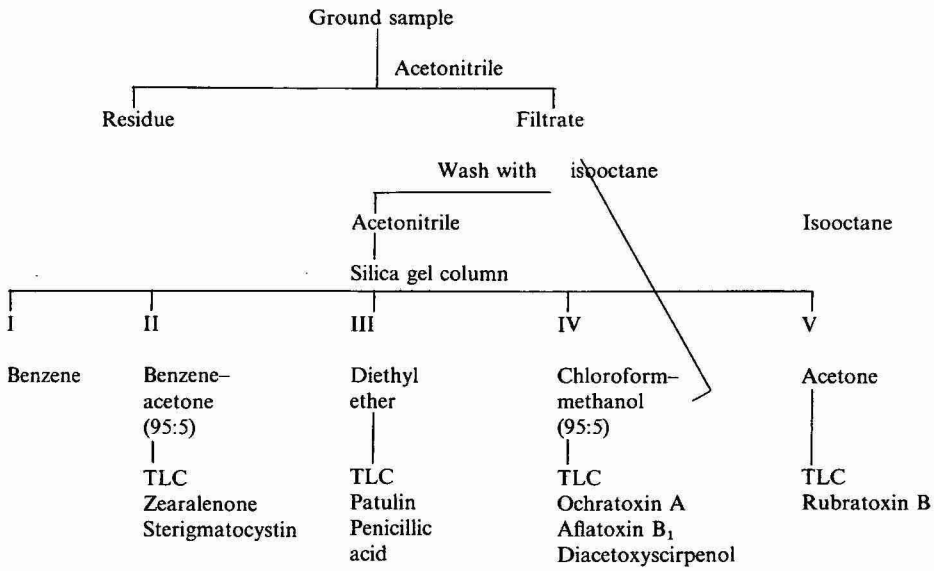

Fig. 1. Flow chart for the extraction and separation of mycotoxins. Adapted from Whidden *et al.*[78].

Zearalenone, T-2 toxin, neosolaniol and HT-2 toxin were detected in grains of barley, wheat and oats by Ilus *et al.*[85] as follows. Toxins were extracted with ethyl acetate, purified on a Kieselgel TLC plate and analysed by TLC using acetone–hexane as the solvent with detection at 366 nm or with *p*-anisaldehyde reagent. Nowotny *et al.*[86] detected citrinin, ochratoxin A and sterigmatocystin in samples of commercial cheese using HPLC and TLC.

Gimeno and Martins[87] described a rapid TLC determination of patulin, citrinin and aflatoxin in apples and pears and their juices and jams. The mycotoxins are extracted with a mixture of acetonitrile and 4% aqueous KCl (9:1). The extract is cleaned up with water and then acidified, and the toxins are recovered with chloroform and separated by TLC. Toxin identity is confirmed with various developing solvents, spray reagents and chemical reactions and then quantitated by the limit of detection method. The minimal detectable concentrations were: patulin 120–130, citrinin 30–40, aflatoxin B_1 and G_1 2–2.8 and aflatoxin B_2 and G_2 2 μg/kg.

Johann and Dose[26] described a method for the routine examination of mouldy rice, wheat bread and other vegetable foodstuffs. The mycotoxins are first extracted with acetonitrile–4% KCl and cyclohexane and then transferred from acetonitrile into a methylene chloride phase and separated by two-dimensional TLC. Aflatoxins are determined fluorimetrically after development in chloroform–acetone (9:1) and methylene chloride–acetonitrile (8:2). Other mycotoxins (ochratoxin A, patulin, penicillic acid and sterigmatocystin) are separated on separate plates with toluene–ethyl acetate–acetic acid (6:3:1) and benzene–acetic acid (8:2). Citrinin is chromatographed on a plate pre-treated with oxalic acid. Citrinin and ochratoxin A, like the aflatoxins, can be immediately determined by fluorimetry, whereas the other toxins have to be converted into fluorescent derivatives using spray reagents (penicillic acid using diphenylboric acid–2-ethanolamine, patulin using N-methylbenzthiazolone-2-hydrazone and sterigmatocystin using aluminium chloride) for quantitative determination.

Ďuračková *et al.*[22] presented a novel TLC systematic analysis for 37 mycotoxins and 6 other fungal metabolites in which "chromatographic spectra" were generated for each toxin from their R_F values in eight different solvent systems. The detection methods used were mentioned in Section 2.4. The advantage of this system lies in the comparisons of relative rather than absolute R_F values, as the latter show greater variations than the former with changes in the conditions of the environment. This method was developed for the identification of known mycotoxin. The chromatographic spectrum of an unknown substance provides a preliminary identification by comparison with known chromatographic spectra or eliminates the known metabolites from the unknown. The method was extended to the detection of unknown mycotoxins by combining it with a bioassay to yield a bioautographic detection method[34]. The bioautographic detection of mycotoxins on thin-layer chromatograms is especially suitable at the stage where the unknown mycotoxins are available only in the form of crude extracts.

Gorst-Allman and Steyn[18] published the results of a study of screening methods for thirteen mycotoxins, which showed that they can be separated as neutral (aflatoxin B_1, sterigmatocystin, zearalenone, patulin, T-2 toxin, roquefortine, penitrem A, fumitremorgin B and roridin A) and acidic (citrinin, ochratoxin A, α-cyclopiazonic acid and penicillic acid) metabolites. R_F values were determined in several solvent systems, and the reactions of the toxins with well known spray reagents and their detection limits were established. Mean R_F values of the neutral mycotoxins are presented in Table 2 and those of the acidic mycotoxins in Table 3. The acidic mycotoxins were well separated on silica gel TLC plates pre-treated with oxalic acid. The authors also described a general procedure for the extraction of mycotoxins from contaminated samples that enables one to obtain a fraction of neutral mycotoxins and another fraction of acidic mycotoxins.

TABLE 2

MEAN R_F × 100 VALUES OF NEUTRAL MYCOTOXINS

Adapted from ref. 18.

Mycotoxin	*Solvent system**					
	A	*B*	*C*	*D*	*E*	*F*
Aflatoxin B_1	44	35	27	3	65	24
Sterigmatocystin	67	53	55	41	74	56
Zearalenone	40	51	38	41	71	44
Patulin	22	27	16	18	56	20
T2-toxin	45	36	22	13	68	22
Roquefortine	3	1	2	1	13	2
Penitrem A	40	51	34	49	76	45
Fumitremorgin B	51	36	28	14	71	30
Roridin A	31	22	13	9	61	14

* Solvent systems: A, chloroform–methanol (97:3); B, chloroform–acetone–*n*-hexane (7:2:1); C, chloroform–acetone (9:1); D, ethyl acetate–*n*-hexane (1:1); E, chloroform–acetone–2-propanol (85:15:20); F, benzene–chloroform–acetone (45:40:15).

TABLE 3

MEAN R_F × 100 VALUES OF ACIDIC MYCOTOXINS USING TLC PLATES PRE-TREATED WITH OXALIC ACID

Adapted from ref. 18.

Mycotoxin	R_F × *100*	
	Chloroform–methanol (98:2)	*Chloroform–acetone (9:1)*
Citrinin	52	51
Ochratoxin A	32	34
α-Cyclopiazonic acid	52	44
Penicillic acid	16	20

The multi-toxin procedures, mentioned above, are based on conventional TLC systems, which are often slow, require the use of several solvent systems and have been developed to the stage of providing mainly qualitative information useful for identification purposes or for semi-quantitative analysis. Lee *et al.*[21] described a method for the simultaneous determination of thirteen mycotoxins by HPTLC. With 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 obtained. About 1 h was required for the separation and quantitation of all thirteen mycotoxins 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 stage and spectroscopic properties used for quantitative determination of the individual mycotoxins are given in Table 4.

TABLE 4

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

Adapted from ref. 21.

Development stage	*Time (min)*	*Mycotoxin 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,
		Citrinin	λ_{em} = 460 nm
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

In the method of Lee *et al.*[21], the mobile phase migration distance was 4 cm and 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 freely evaporate. For the very complex sample of thirteen mycotoxins, the use of continuous multiple development offered certain advantages, such as 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. The resolution of sterigmatocystin, zearalenone and citrinin was obtained in the first continuous development. The plate was removed from the chamber and air-dried prior to making the quantitative measurement of the three separated toxins. The other toxins remained close to the origin. After a second and third development, ochratoxin A was separated sufficiently to be determined. A fourth development enabled penicillic acid, patulin and luteoskyrin to be determined. For the separation of the aflatoxins, still remaining close to the origin, a second, more polar, solvent system was used. After three continuous developments with this mobile phase, the six aflatoxins were completely separated. At each scanning stage, the migration distance of the spot to be measured was maintained between 1 and 3 cm. Only patulin and luteoskyrin slightly overlapped each other, but as patulin does not show any absorption at the absorption maximum for luteoskyrin (440 nm), this

was no problem. Hence the method described is capable of providing good resolution of complex mycotoxin mixtures. However, the authors used standard mycotoxin solutions and did not show whether comparable results could be obtained with samples extracted from natural products.

3.2. Aflatoxins and related compounds

The four naturally occurring aflatoxins, B_1, B_2, G_1 and G_2, are acutely toxic and carcinogenic metabolites produced by *Aspergillus flavus* and the closely related species *A. parasiticus*. Other members of the group are derived from these four as metabolic products of microbial and animal systems (such as M_1, M_2, P_1, Q_1 and aflatoxicol) or are produced spontaneously in response to the chemical environment (such as B_{2a}, G_{2a} and D_1). The aflatoxins are highly fluorescent, highly oxygenated, heterocyclic compounds characterized by dihydrodifurano or tetrahydrofurano moieties fused to a substituted coumarin moiety. Aflatoxin B_1 is the most prevalent naturally occurring member of the group that has had the most profound impact on the development of the science of mycotoxicology.

The sterigmatocystins and the versicolorins are also characterized by the presence of the dihydrodifurano and tetrahydrodifurano moieties. In addition to these moieties, sterigmatocystins are characterized by a xanthone moiety, whereas the versicolorins contain anthraquinone moieties. Members of both groups are biosynthetic precursors of aflatoxins. Several sterigmatocystins are also toxic and carcinogenic like aflatoxins.

Various aspects of the TLC of aflatoxins has been discussed in recent years in several books and reviews (*e.g.*, refs. 2, 4, 9 and 88–92). There are numerous TLC techniques for the separation of these highly toxic and sometimes potent carcinogens. A survey of applications is given below on extraction and clean-up techniques, stationary phases and solvent systems, detection, qualitative and quantitative analyses and PLC.

3.2.1. Extraction and clean-up

As many food products contain large amounts of natural lipids which contaminate primary aflatoxin extracts, these components must be removed as completely as possible in order to prevent interference with the final purification and assay.

In addition to lipids, some products contain other components that interfere with subsequent analyses by TLC and should be removed from the primary extracts. Primary extracts in mixtures of acetone with water contain proteins that can be removed by precipitation with lead acetate. Extracts from cotton-seed contain interfering gossypol pigments; extracts from cereals contain fluorescing substances with TLC properties similar to those of aflatoxins. These pigments need to be removed by column chromatography (CC). It was noticed by Heathcote and Hibbert[15] that one of the difficulties encountered in the analysis of extracts from mycelia of *Aspergillus flavus* was the occurrence of a dark pigment, which remained immobile on the TLC plate and interfered seriously with the chromatography. This pigment was formed during air-drying of the mycelium. By using freeze-drying and Soxhlet extraction at a temperature below 35°C, a considerably cleaner aflatoxin extract was obtained with a consequent improvement in the quality of the chromatogram. In coffee beans it is caffeine and other metabolites whereas in cocoa beans it is chiefly

TABLE 5

EXTRACTION AND CLEAN-UP PROCEDURES FOR AFLATOXINS

Material analysed	*Extraction solvent(s)**	*Clean-up**	*Ref.*
Culture filtrate	$CHCl_3$ after neutralization	–	96
	$CHCl_3$	Wash extract with PE or Hex	97
Mycelium	$CHCl_3$	–	15
Agar medium	$CHCl_3$	–	98
Mycelium plus culture filtrate	$CHCl_3$	Evaporate extract, dissolve in C_6H_6–CH_3CN	99
Groundnuts and products thereof	Hex–MeOH–H_2O	CC of MeOH–H_2O phase; elute lipids with Hex and toxins with Hex–$CHCl_3$ (1:1)	100
	Hex–H_2O–Me_2CO (48.5:1.5:50)	Partition extraction with NaCl solution	101
	Hex–H_2O–Me_2CO	CC on Florisil; elute contaminants with THF and toxins with Me_2CO	102
	H_2O–Me_2CO (30:70)	Precipitate impurities with $Pb(OAc)_2$; extract with $CHCl_3$	59, 103, 104
	$CHCl_3$ after defatting with PE and slurry with H_2O	CC on silica gel; elute lipids with Hex and Et_2O, toxins with $CHCl_3$–MeOH	105
	Hex–MeOH–H_2O (with 4% NaCl)	Extract MeOH phase with $CHCl_3$, evaporate, dissolve in C_6H_6–CH_3CN (98:2)	106, 107
	Me_2CO–H_2O (85:15)	$Fe(OH)_3$ gel, dilute with H_2O, extract with $CHCl_3$, use mini-CC	108
	$CHCl_3$–MeOH–Hex (8:2:1)	CC silica gel; elute lipids with PE–Et_2O (75:25), toxins with $CHCl_3$–MeOH (97:3)	109
	Me_2CO	$Fe(OH)_3$ purification	110
	H_2O–$CHCl_3$ (1:10)	CC: Na_2SO_4 + silica gel + Na_2SO_4; elute lipids with Hex and Et_2O, toxins with $CHCl_3$–MeOH (97:3)	111
Cotton seed	H_2O–Me_2CO	Precipitate with $Pb(OAc)_2$, extract with $CHCl_3$ and CC on silica gel; elute pigments with Hex–Et_2O (1:3), toxins with $CHCl_3$–Me_2CO (8:2)	59, 112
	H_2O–Me_2CO	CC on cellulose, elute pigments with Hex, toxins with $CHCl_3$–Hex (1:1)	113
	Me_2CO–H_2O–HOAc ((85:15:8)	Precipitate with $Pb(OAc)_2$, concentrate, add H_2O + diatomaceous earth, extract filtrate with $CHCl_3$	114
	Me_2CO–H_2O (85:15)	Precipitate with $Pb(OAc)_2$, extract filtrate with CH_2Cl_2; CC silica gel + alumina, elute with CH_2Cl_2 and CH_2Cl_2–Me_2CO (9:1), combine	115
	Me_2CO–H_2O (85:15)	$Fe(OH)_3$ gel, extract filtrate with $CHCl_3$	108

Cereals and flours	$CHCl_3$–MeOH–Hex (8:2:1)	CC silica gel, elute lipids with PE–Et_2O, toxins with $CHCl_3$–MeOH (97:3)	109
	Me_2CO–H_2O (85:15)	$Fe(OH)_3$ gel, extract filtrate with $CHCl_3$	116
	Me_2CO–H_2O (85:15)	Dilute filtrate with H_2O, add sat. $(NH_4)_2SO_4$, extract filtrate with C_6H_6, clarify with Na_2SO_4, mini-CC	117
	MeOH–1% NaCl soln. (85:15)	(a) Wash with Hex (lipids), re-extract with $CHCl_3$. (b) To separate aflatoxicols prior to TLC: submit extract from (a) to CC silica gel, elute aflatoxicols with Et_2O, aflatoxins with $CHCl_3$–MeOH (97:3), evaporate, dissolve in $CHCl_3$	118
	CH_3CN–4% KCl soln. (9:1)	–	119
	$CHCl_3$	CC Kieselgel, elute lipids with Hex–Et_2O (1:1), toxins with $CHCl_3$–MeOH (97:3), evaporate, dissolve in C_6H_6–CH_3CN (98:2)	120
Coffee beans	H_2O–$CHCl_3$ (1:10)	CC Florisil, wash with $CHCl_3$ and THF, elute with Me_2CO–MeOH (99:1), evaporate, dissolve in $CHCl_3$	111
Cocoa beans	$CHCl_3$ after defatting with Hex and slurry with 25% $AgNO_3$ soln.	Shake with $AgNO_3$ soln., separate, CC silica gel: elute lipids with Hex and Et_2O, toxins with $CHCl_3$–MeOH (97:3)	111
Plant products	25% NaCl soln.–Me_2CO	Precipitate with $Pb(OAc)_2$, extract filtrate with Hex (lipids) and $CHCl_3$ (toxins)	121
Meats	$CHCl_3$	CC silica gel, elute with $CHCl_3$–MeOH (97:3)	122
Mixed feeds	20% CA soln.–CH_2Cl_2 (1:10)	CC silica gel, wash with glacial HOAc–Tol (1:9), THF–Hex (1:3) and CH_3CN–Et_2O–Hex (1:3:6); elute with Me_2CO–CH_2Cl_2 (1:4)	123
Milk (powdered)	Me_2CO–$CHCl_3$–H_2O	Precipitate with $Pb(OAc)_2$ soln., defat with Hex, extract with $CHCl_3$	124
	MeOH–H_2O (1:1)	Extract filtrate with $CHCl_3$, wash extract with H_2O, evaporate	125
	Me_2CO	CC cellulose with $Pb(OAc)_2$ soln., evaporate, dissolve in $CHCl_3$	120
Milk (liquid)	Mix with Me_2CO, dialyse against aq. Me_2CO	Extract with $CHCl_3$ and dry	126
Urine	$CHCl_3$ after precipitation with $Pb(OAc)_2$ soln. and $Fe(OH)_3$	Wash extract with NaCl soln. and evaporate	127

* Abbreviations: CC, column chromatography; $CHCl_3$, chloroform; PE, light pretroleum; Hex, *n*-hexane; C_6H_6, benzene; CH_3CN, acetonitrile; MeOH, methanol; Me_2CO, acetone; NaCl, sodium chloride; THF, tetrahydrofuran; $Pb(OAc)_2$, lead acetate; $Fe(OH)_3$, iron(III) hydroxide; Et_2O, diethyl ether; HOAc, acetic acid; $(NH_4)_2SO_4$, ammonium sulphate; CA, citric acid; Tol, toluene.

theobromine that must be removed. Scott[93] showed that a coffee-bean extract could be purified by passage through a Florisil column and the unwanted contaminants eluted with tetrahydrofuran. It was also shown by Scott[94] that theobromine could be removed from crude cocoa-bean extracts by treatment with silver nitrate solution.

Schuller *et al.*[95] reviewed sampling procedures and collaboratively studied methods for the analysis of aflatoxins. An exhaustive review of extraction and clean-up procedures for aflatoxins present in cultivation media and various natural commodities (groundnuts, cotton-seed, cereals, milk, meats, coffee, cocoa beans and others was given by Heathcote and Hibbert[2] or also in a more condensed form in a book devoted to production, isolation, separation and purification techniques for mycotoxins[88]. The best known extraction and clean-up techniques are summarized in Table 5.

Lovelace *et al.*[127] published a screening method for the detection of aflatoxins and metabolites in human urine. In the clean-up, Celite 545 filter aid was added to the samples, followed by acetone and filtration. To the stirred filtrate was added water followed by 20% lead acetate solution. After coagulation, a saturated solution of NaCl was added with stirring, followed by Celite. The mixture was filtered, the filtrate stirred and freshly prepared iron(III) hydroxide slurry added, followed by Celite 545. After filtration, 0.1% sulphuric acid was added and the filtrate was extracted twice with chloroform. The combined extracts were washed with 5% NaCl solution and evaporated to dryness under nitrogen. The residue was dissolved in chloroform and again evaporated. The final residue was re-dissolved in chloroform and analysed by TLC.

3.2.2. Adsorbents and solvent systems

The first successful separation of aflatoxins into the main components, B_1, B_2, G_1 and G_2, was carried out on silica gel plates using chloroform–methanol (98:2)[128]. Numerous combinations of silica gel and solvent systems have been proposed in efforts to improve the separation and to obtain more reproducible R_F values. Most of the solvent systems were based on chloroform plus 2–7% of methanol. Subsequently, methanol was replaced with 10–15% of acetone. The use of non-chloroform-based solvents has been suggested by several workers. Examples are given in Table 6.

In an attempt to overcome the lack of reproducibility of TLC resolution, Heathcote and Hibbert[15] investigated a number of silica gel preparations and solvent systems. They found that the neutral SilicAR TLC-7G (Mallinckrodt) gave an excellent resolution of aflatoxins in the solvent systems chloroform–methanol (97:3), toluene–ethyl acetate (8:1) and benzene–ethanol–water (46:35:19).

Problems concerning the solvent systems, adsorbents and environmental effects (especially relative humidity) were discussed by Heathcote and Hibbert[2] and summarized more recently by Heathcote[88].

Kozloski[138] described procedures for improving aflatoxin spot size and fluorescence intensity. By using strong eluting solvents, diffuse spots could be reduced in size and poorly resolved chromatograms returned to their original state for re-development.

Issaq and Cutchin[136] compared the separation of four aflatoxins on six commercial silica gel plates in twelve solvent systems for aflatoxins frequently mentioned

TABLE 6

SOLVENTS SYSTEMS FOR TLC OF AFLATOXINS

*System**	*Notes*	*Ref.*
$CHCl_3$–MeOH (98:2)	Separation of B_1, B_2, G_1 and G_2	88
$CHCl_3$–MeOH (94:6 or 49:1) C_6H_6–EtOH–H_2O (46:35:19) Tol–EtOAc (8:1)	Aflatoxins plus sterigmatocystin and versicolorin group on SilicAR TLC-7G	15
$CHCl_3$–Me_2CO–Hex (85:15:20)	On SilicAR AGF	129
$CHCl_3$–Me_2CO–iPrOH (825:150:25)		103
$CHCl_3$–Me_2CO (85:15)	PLC of B_1	130
Et_2O–MeOH–H_2O (96:3:1)		131
C_6H_6–EtOH–H_2O (46:35:19, upper layer)	Sensitive to changes in humidity	132
H_2O–MeOH–Et_2O (1:3:96)	Separation of B_1, B_2, G_1 and G_2	131
iPrOH–Me_2CO–H_2O (5:10:85)	M_1 in milk and dairy products	133
$CHCl_3$–MeOH (9:1)	B_1 in extracts from *A. flavus* cultures	99
CH_2Cl_2–MeOH (95:5)	D_1 in ammoniated corn extracts	134
Et_2O followed by $CHCl_3$–Me_2CO–H_2O (88:12:1.5)	Quantitation of B_1, B_2 and G_2 in groundnuts	135
Et_2O followed by $CHCl_3$–Me_2CO–C_6H_6 (9:1:1) and by $CHCl_3$–Me_2CO–Hex (71:12.5:16.5)	As above	135
$CHCl_3$–EtOAc–THF (8:12:0.6)	Over-pressured liquid chromatography on HPTLC Kieselgel 60	120
$CHCl_3$–Me_2CO–NH_3 (90:10:0.25)	Separation of B_1, B_2, G_1 and G_2 on six commercial silica gel plates	136
$CHCl_3$–Me_2CO–Hex (85:15:20)	As above	136
1st: CH_3CN–Me_2CO–C_6H_6 (9:1:1) 2nd: Et_2O–MeOH–H_2O (96:3:1)	Two-dimensional on Silufol	137
1st: Tol–EtOAc–90% FA (5:4:1) 2nd: $CHCl_3$–Me_2CO (9:1)	B_1, B_2, G_1 and G_2 in figs on activated silica gel 60	36
1st: $CHCl_3$–Me_2CO (88:12) 2nd: 95% denatured EtOH	Aflatoxins in corn, on SILG-HR-25 plates	37
1st: $CHCl_3$–Me_2CO–iPrOH (80:15:15) 2nd: Tol–EtOAc–90% FA (60:30:10)	M_1 on silica gel 60	38

* Abbreviations: FA, formic acid; NH_3, ammonia solution; iPrOH, 2-propanol; others as in Table 5.

in the literature. Two of the solvent systems resolved the four aflatoxins on all the tested plates (see Table 7). Their results showed that the use of ammonia solution as a solvent modifier at a concentration of 0.5% played an important role in achieving good resolution of the four aflatoxins on silica gel TLC plates, that HPTLC plates gave more compact spots than the other plates and that separation can be achieved when the optimum solvent system is selected.

3.2.3. Selected recent applications

Two-dimensional TLC of four aflatoxins in feed extracts was conducted by Jain and Hatch[39] on pre-coated silica gel plates. Excellent separation of aflatoxins from impurities was achieved and all four aflatoxins were well resolved using chloroform–acetone–water for the first development and with toluene–ethyl acetate–formic acid (30:15:5, or 24:20:6 for samples containing citrus pulp) in the

TABLE 7

COMPARATIVE SEPARATION OF AFLATOXINS ON COMMERCIAL SILICA GEL PLATES

Data from ref. 136.

Plates	$R_F \times 100$							
	*Solvent F**				*Solvent G***			
	B_1	B_2	G_1	G_2	B_1	B_2	G_1	G_2
Silica gel 60	33	29	23	20	48	43	36	30
K5F	87	84	76	71	59	54	46	43***
HPTLC	48	44	37	33§	48	41	36	30
Sil G 25 HR	87	82	72	65	54	46	41	36
Adsorbosil-1	44	37	31	25	80	71	63	54
Silica gel IBF	63	57	52	46***	68	61	52	45

* Chloroform–acetone–ammonia solution (90:10:0.25).
** Chloroform–acetone–hexane (85:15:20).
*** Diffuse spots.
§ Compact spots.

second direction and far-UV detection. Two-dimensional TLC on Silufol plates with acetonitrile–acetone–benzene (9:1:1) and diethyl ether–methanol–water (96:3:1) as the solvent systems, visualization with nitric acid–water (1:2) and quantitative detection by fluorescence under long-wave light was reported by Eller *et al.*[137]. Aflatoxins were detected by TLC in corn[139], black olives[140] and milk[141]. Madhyasta and Bhat[142] applied TLC confirmatory tests in the mini-column chromatography of aflatoxins.

Hsieh *et al.*[143] employed a sequence of solvent systems for the TLC of aflatoxin B_1 and its metabolites. The silica gel plate was first developed in diethyl ether, thereby mobilizing aflatoxicol and completely separating it from other metabolites. After quantitation for aflatoxicol, the same TLC plate was developed in chloroform–acetone–2-propanol (85:15:15). Aflatoxin Q_1 and aflatoxicol H_1 were completely separated. Final separation of aflatoxins M_1 and B_{2a} was effected by a third development in benzene–ethanol (40:4) or chloroform–methanol (9:1).

Kostyukovskii and Melamed[144] determined aflatoxins B_1, B_2, G_1 and G_2 on Silufol and silica gel L 5/40 plates using benzene–diethyl ether–hexane (1:1:1), chloroform–benzene–acetone (9:1:1) or chloroform–benzene–ethyl acetate–acetone (10:4:4:3) as the solvent systems and detecting the separated spots under UV light at 365 nm. The detection limits were 0.2–0.5 ng per spot or 2–5 μg/kg.

Lovelace *et al.*[127] published a screening method for the detection of aflatoxins and metabolites in human urine. The extraction and clean-up procedures were described in Section 3.2.1. The final residue was redissolved in chloroform and analysed by TLC on activated silica gel G-HR plates using acetone–chloroform–2-propanol (10:85:5) with equilibration. The recoveries and R_F values are given in Table 8.

Quantitative determination of aflatoxins in groudnut products using sequential TLC was reported by Klemm[135]. The method involves double development with diethyl ether followed by chloroform–acetone–water (88:12:1.5) and triple develop-

TABLE 8

RECOVERY OF AFLATOXIN METABOLITES FROM HUMAN URINE AND R_F VALUES

Adapted from ref. 127.

Metabolite	*Recovery (%)*	R_F
Aflatoxin B_1	70 ± 9	0.73 ± 0.08
Aflatoxin G_1	76 ± 2	0.59 ± 0.06
Aflatoxin M_1	75 ± 15	0.44 ± 0.08
Aflatoxin B_{2a}	16 ± 6	0.39 ± 0.08
Aflatoxin G_{2a}	55 ± 5	0.29 ± 0.07
Aflatoxicol I (natural isomer)	35 ± 3	0.69 ± 0.07
Aflatoxicol II (unnatural isomer)	48 ± 3	0.62 ± 0.06
Tetrahydrodeoxyaflatoxin B_1	60 ± 0	0.79 ± 0.06

ment with diethyl ether followed by chloroform–acetone–benzene (90:10:10) and chloroform–acetone–*n*-hexane (71:12.5:16.5). The aflatoxins could be detected spectrometrically (325 nm) at levels $\geq$0.05 ng per spot.

In a study of aflatoxin biosynthesis, Kachholz and Demain[145] extracted the whole broth with chloroform. The extract was dried, evaporated to dryness and resuspended in chloroform. An aliquot was used for PLC on a silica gel plate with development in chloroform–methanol (9:1). Aflatoxin spots (fluorescence under UV light) were scraped off the plate, eluted with methanol and quantitated spectrometrically at 363 nm.

Greater than 100% recoveries using instrumental HPTLC were observed by Zemnie[146] for aflatoxin analyses in spiked corn samples. Spots overlying aflatoxins B_1 and B_2 were identified by GLC as C_{16}–C_{18} free fatty acids. These fatty acids enhanced the fluorescence of aflatoxin B_1 from 13.7 to 35.7% greater than controls, 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.

Saito *et al.*[118] detected aflatoxins and aflatoxicols in extracts from cereals with and without previous clean-up on a silica gel column. TLC using diethyl ether as the solvent system after separation of aflatoxicols from aflatoxins by CC separated aflatoxicols I and II, aflatoxins M_1 and M_2 being separated with chloroform–acetone–acetic acid (80:10:8). In TLC without previous column separation, the solvent chloroform–diethyl ether–methanol (70:10:7) separated aflatoxins B, G and M plus aflatoxicols. Fluorodensitometry was used for quantitation.

A new method was developed by Shannon *et al.*[117] for the determination of aflatoxin B_1 in commercially prepared mixed feeds. Eluates from CC (see Table 5, ref. 117) were evaporated, dissolved in benzene–acetonitrile (98:2) and applied on to Sil G-25 HR plates. Two solvent systems were used for TLC. The routine solvent system was chloroform–acetone–water (90:10:1.5) and in the second system methylene chloride was used instead of chloroform, which resulted in a less polar system that left interferences in a lower R_F range than aflatoxin B_1. Methylene chloride could not be used if aflatoxins G_1 and G_2 were present because this system changed the order of resolution to B_1, G_1, B_2, then G_2. Visual and densitometric quantitation was used.

TLC was one of the steps leading to the detection of aflatoxin D_1 in ammoniated corn[134]. Corn cultured with *Aspergillus flavus* to produce a high level of aflatoxin was ammoniated to reduce the high level. An extract of the ammoniated corn was separated by TLC on silica gel 60 plates in methylene chloride–methanol (95:5) and detected under short-wave UV light (R_F = 0.4), followed by reversed-phase HPLC. Examination of the fractions by tandem mass spectrometry led to the detection of aflatoxin D_1 as a product of the ammoniation process.

Bicking *et al.*[55] determined aflatoxins in air samples of refuse-derived fuel by TLC with laser-induced fluorescence spectrometric detection. Gimeno and Martins[87] described the rapid TLC determination of aflatoxin together with patulin and citrinin in apples and pears and in the products thereof. In a collaborative study, Stubblefield *et al.*[147] reported the determination and TLC confirmation of the identity of aflatoxins B_1 and M_1 in artificially contaminated beef livers.

Reversed-phase HPTLC with fluorimetric detection was used in studies of binding of aflatoxin M_1 to milk proteins[148]. Cirilli[149] published a rapid and reproducible method for the extraction and determination of aflatoxin M_1 in milk and dairy products. After extraction and clean-up, the aflatoxin was detected by TLC or HPLC. In TLC on silica gel, hexane–acetone (9:1) mixture was used for the first migration 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.

Most recently, Gulyás[120] reported a rapid separation and quantitation of aflatoxins with very good reproducibility using the technique of over-pressured liquid chromatography (OPLC) on HPTLC $Kieselgel_{60}$ plates.

TLC data for members of the aflatoxin group (B_1, B_2, G_1, G_2, M_1, M_2, B_{2a}, G_{2a}, D_1, Q_1, aflatoxicol, aflatoxicol O-ethyl ether A and aflatoxicol O-ethyl ether B) were compiled by Cole and Cox[150].

The most important TLC data on various members of the aflatoxin group, concerning their original characterization, have been summarized by Heathcote[88]. They include the hydroxyaflatoxins B_{2a} and G_{2a}, aflatoxins M_1 and M_2, GM_1 and GM_2, M_{2a} and GM_{2a}, aflatoxin B_3 (parasiticol) and aflatoxicol (both epimers). Heathcote also summarized and critically evaluated the most important visual and instrumental determinations of aflatoxins by TLC as one of the means of detection and assay of these toxins in natural products.

3.2.4. Sterigmatocystins and versicolorin group

Older TLC data for sterigmatocystin and related substances were compiled by Cole and Cox[150]. Extraction and clean-up procedures have been described in several papers (*e.g.*, refs. 78, 111, 151 and 152).

Sterigmatocystin appears as a red spot when viewed under long-wave UV light[151]. Other detection methods include *p*-anisaldehyde and iron(III) chloride spray[22], aluminium(III) chloride spray, giving yellow fluorescence[150], iodine vapour[153] and cerium(IV) sulphate[18]. Sterigmatocystin and its derivatives have often been included in multi-toxin TLC analyses (*e.g.*, refs. 21, 26, 76, 78, 80, 81, 83 and 84). Recently, Hu *et al.*[154] reported the two-dimensional TLC determination of sterigmatocystin in cereal grains. A variety of solvent systems has been recommended for the TLC of sterigmatocystins. Some of them, together with the reported R_F values, are compiled in Table 9.

TABLE 9

TLC OF STERIGMATOCYSTINS

Adsorbent	*Solvent system**	*Detection**	$R_F \times 100$			*Ref.*
			*S**	*MS**	*OMS**	
Silica gel G	C_6H_6–Me_2CO (10:0.2)	Iodine	26	18	1	153
	C_6H_6–MeOH (10:0.2)		48	12	25	
	CCl_4–Me_2CO (10:0.2)		60	17	32	
	$CHCl_3$–Me_2CO (10:0.5)		58	24	23	
	CCl_4–MeOH (10:2)		71	44	38	
	CH_2Cl_2–MeOH (10:0.5)		85	52	19	
	$CHCl_3$–MeOH (10:0.5)		92	65	23	
Silica gel	Tol–MeOH (99:1)		43	34		152
Silufol	C_6H_6–MeOH–HOAc (24:2:1)	UV, pAA, $FeCl_3$	51			22
	Tol–EtOAc–FA (6:3:1)		49			
	C_6H_6–EtOH (95:5)		46			
	$CHCl_3$–MeOH		81			
	$CHCl_3$–MIBK (4:1)		80			
	$CHCl_3$–Me_2CO (9:1)		56			
	$CHCl_3$–HOAc–Et_2O (17:1:3)		73			
	nBuOH–HOAc–H_2O (4:1:4, upper layer)		83			
Silica gel	$CHCl_3$–MeOH (97:3)	UV (254 nm)	67	UV		18
	$CHCl_3$–Me_2CO–Hex (7:2:1)		35			
	$CHCl_3$–Me_2CO (9:1)		27			
	C_6H_6–$CHCl_3$–Me_2CO (9:8:3)		24			
Kieselgel G	C_6H_6–EtOH (95:5)	$AlCl_3$, heat, UV				155

* Abbreviations: S, sterigmatocystin; MS, 5-methoxysterigmatocystin; OMS, O-methylsterigmatocystin; FA, 90% formic acid; EtOH, ethanol; MIBK, methyl isobutyl ketone; nBuOH, *n*-butanol; pAA, *p*-anisaldehyde; $FeCl_3$, iron(III) chloride; $AlCl_3$, aluminium chloride; others as in Table 5.

The significance of the versicolorin metabolites is based on the fact that representative members of the group are biosynthetic precursors of the aflatoxins and often accompany aflatoxins in extracts for analysis. Most of them can be detected under visible light. Selected TLC data for representatives of the group are given in Table 10.

3.3. Ochratoxins

The ochratoxins are composed of a 3,4-dihydro-3-methylisocoumarin moiety linked via the 7-carboxy group to L-β-phenylalanine by an amine bond. The group consists of ochratoxin A and its methyl and ethyl (ochratoxin C) esters, ochratoxin B, its methyl and ethyl esters, and 4-hydroxyochratoxin A. Ochratoxin A and its esters are the toxic members of the group.

Extraction and clean-up procedures for ochratoxins were reviewed recently by Steyn[16]. The extraction of mouldy material is effected with various solvents and their combinations (*e.g.*, methanol–water, acetonitrile–aqueous KCl, chloroform–methanol, mixtures of organic solvents with dilute phosphoric acid). Clean-up procedures include CC, gel filtration chromatography, solvent partition or dialysis (see ref. 16 for details and references).

TABLE 10

TLC OF VERSICOLORINS

Metabolite	*Adsorbent*	*Solvent system**	*Detection in visible light*	$R_F \times 100$	*Ref.*
Versicolorin A	Adsorbosil-1	C_6H_6–HOAc (95:5)	Yellow-orange	32	156
Versicolorin B	Silica gel	C_6H_6–HOAc (95:5)	Yellow	23	157
Versicolorin C	Silica gel	C_6H_6–HOAc (95:5)	Yellow	23	
Averufin	Silica gel	$CHCl_3$–Me_2CO–HOAc (97:2:1)	Red	50	158
Norsolorinic acid	Adsorbosil-1	$CHCl_3$–Me_2CO–Hex (85:15:20)	Orange-red	69	159
Versiconal hemi-acetal acetate	SilicAR TLC-7G	Tol–EtOAc (27:12)	Orange-red	32	160
		$CHCl_3$–Me_2CO (85:15)		33	

* Abbreviations as in Tables 5 and 6.

TABLE 11

TLC DATA FOR OCHRATOXINS

Adsorbent	*Solvent system**	$R_F \times 100$				*Ref.*
		*A**	*B**	*C**	*HA**	
Silica gel	C_6H_6–HOAc (3:1)	50	35			161
Silica gel	Tol–EtOAc–HOAc (5:4:1)	70				162
	C_6H_6–HOAc (4:1)	40				
	Tol–TCE–AmOH–HOAc (80:15:4:1)	60				
Rice starch	Tol–HOAc (20:0.15)	43	30			19
Silica gel G	C_6H_6–MeOH–HOAc (24:2:1)	52	41	80		22
	Tol–EtOAc–FA (6:3:1)	59	46	72		
	C_6H_6–EtOH (95:5)	34**	12**	75		
	$CHCl_3$–MeOH (4:1)	79	65	91		
	$CHCl_3$–MIBK (4:1)	11***	0	53**		
	$CHCl_3$–Me_2CO (9:1)	23***	2	73		
	$CHCl_3$–HOAc–Et_2O (17:1:3)	56	33	86		
	nBuOH–HOAc–H_2O (4:1:4, upper layer)	95	79	87		
Oxalic acid-treated silica gel	$CHCl_3$–MeOH (98:2)	32				18
	$CHCl_3$–Me_2CO (9:1)	34				
Silica gel	C_6H_6–HOAc (3:1)	50				161
	C_6H_6–HOAc (4:1)		35		25	161, 163
	C_6H_6–HOAc (25:1)			55		164

* Abbreviations: A, ochratoxin A; B, ochratoxin B; C, ochratoxin C; HA, 4-hydroxyochratoxin A; C_6H_6, benzene; HOAc, acetic acid; Tol, toluene; EtOAc, ethyl acetate; TCE, trichloroethylene; AmOH, amyl alcohol; MeOH, methanol; FA, 90% formic acid; EtOH, ethanol; $CHCl_3$, chloroform; MIBK, methyl isobutyl ketone; Me_2CO, acetone; Et_2O, diethyl ether; nBuOH, *n*-butanol.

** Tailing.

*** Elongated spot.

TLC is one of the chief methods for the detection, identification and quantitation of ochratoxin. Selected TLC data for ochratoxins are given in Table 11.

Several methods are available for the detection of ochratoxins on TLC plates. A generally used technique is to view the plate under long-wave (366 nm) UV illumination; ochratoxin A appears as a green fluorescent spot (blue-green on acidic plates) and ochratoxin B has blue fluorescence. The fluorescence of the ochratoxins changes to purple-blue on exposure to ammonia fumes or spraying with aqueous $NaHCO_3$ or NaOH[161,162].

In their report on the TLC analysis of 37 fungal metabolites in eight solvent systems, Ďuračková *et al.*[22] included data for ochratoxins A, B and C.

A very efficient separation of ochratoxins A and B was achieved by impregnation of the silica gel with oxalic acid. The TLC plates were then developed with the neutral solvent systems chloroform–methyl isobutyl ketone (4:1), chloroform–methanol (98:2) or chloroform–acetone (9:1)[18].

Semi-quantitative and quantitative methods for the determination of low levels of ochratoxin A have been developed (*e.g.*, refs. 165–167) and have been reviewed[16,80,168]. Patterson and Roberts[41] applied two-dimensional TLC to the analysis of feedstuffs, the chromatogram being developed with toluene–ethyl acetate–90%

formic acid (6:3:1) (first direction) and chloroform–acetone (9:1) (second direction) and then examined at 366 nm. Czerwiecki[63] described optimal parameters for the TLC of ochratoxin A, including extraction from cereals and spectrofluorimetric determination. Quantitation of ochratoxin A was described by Meyer[92] and Johann and Dose[26].

The method developed by Nesheim *et al.*[169] for the determination of ochratoxins A and B in barley is very sensitive and specific for ochratoxin A. The method was adopted by the Association of Official Analytical Chemists as an official, first action method[170], is frequently applied in screening programmes and was used by Pleština *et al.*[171] in the analysis of food samples from areas in Yugoslavia where Balkan endemic nephropathy is a major problem.

Preparative silica gel TLC with benzene–acetic acid (4:1) as the solvent system was used for the purification of isotopically labelled ochratoxin A by De Jesus *et al.*[172]. When conversion of ochratoxin C into ochratoxin A in rats was studied by Fuchs *et al.*[173], the ochratoxin A-containing fractions from a silica gel column were purified by PLC in toluene–dioxane–acetic acid (95:35:4).

A recent development in mycotoxin analysis is the application of multi-mycotoxin analytical methodology[18,174]. This technique has been successfully applied to ochratoxin A analysis.

Multi-mycotoxin analyses, in which ochratoxins have been included, have been described by Scott *et al.*[24], Fishbein and Falk[80], Stoloff *et al.*[81], Wilson *et al.*[82], Moubasher *et al.*[83], Joseffson and Möller[76], Coman *et al.*[84], Whidden *et al.*[78], Nowotny *et al.*[86], Johann and Dose[26] and Lee *et al.*[21], who used sequential development of HPTLC plates (see also Section 3.1).

3.4. Rubratoxins

Rubratoxins A and B, produced by *Penicillium rubrum* and *P. purpurogenum*, are structurally related toxins. Rubratoxin has a central nonadiene ring structure with two anhydride rings, a lactone ring, a six-carbon aliphatic side-chain and three hydroxy groups. The less toxic rubratoxin A has one of the anhydride groups reduced to the lactol. Their physical, chemical and biological properties were recently summarized by Davis and Richard[175].

Rubratoxin B can be extracted after concentrating the culture filtrate and mycelial washings, the concentrate being acidified with HCl and extracted with diethyl ether. The ether extract is evaporated and the residue is dissolved in acetone and analysed by TLC[176].

For corn, extraction with ethanol, acetone and ethyl acetate yields the maximum amount of rubratoxin A, whereas refluxing with diethyl ether yields the maximum amount of rubratoxin B. For rice, extraction with ethyl acetate in benzene yields the maximum amount of rubratoxin A, whereas extraction with ethyl acetate–benzene and diethyl ether yields the maximum amount of rubratoxin B[175].

Hayes and McCain[177] reported that acetonitrile was satisfactory for extracting rubratoxin B from corn. Extraction with acetonitrile was also used by Whidden *et al.*[78] as a first step of isolation, followed by the procedures shown in Fig. 1. Rubratoxin B was present in fraction 5 of the isolation scheme.

TLC of rubratoxin can be accomplished according to the procedure of Cottral[178] as follows. Spotting of the silica gel plates should be carried out under nitrogen

to prevent oxidation and internal and external standards should be included on the plates. The solvent system is chloroform–methanol–glacial acetic acid–water (80:20:1:1). Rubratoxin adopts a greenish fluorescence after heating the plate at 200°C for 10 min. The intensity of the fluorescence can be increased by subsequently spraying the plate with 2′,7′-dichlorofluorescein; however, the background will also have a yellow-green fluorescence.

Whidden *et al.*[178] quantitated rubratoxin B according to Hayes and McCain[177] and described the following confirmatory tests. The fluorescent derivatives, which were formed from rubratoxin B on a TLC plate after heating at 200°C for 10 min, were exposed to ammonia vapour for 10 min. Examination under long-wave UV light revealed a change in the intensity and colour of the fluorescence. Rubratoxin was then more easily observed as a light blue spot, although the detection limit remained the same. Further, the fluorescence intensity of fluorescent compounds near rubratoxin B was greatly reduced, which considerably improved the contrast and thereby the ease of detecting rubratoxin B. Also, after prolonged heating of the TLC plates at 100°C for 2–10 h with ammonium hydrogen carbonate, rubratoxin B became visible under UV light. The reactions of ammonia and ammonium hydrogen carbonate with rubratoxin B both produced very similar fluorescent derivatives on the TLC plates. The ammonium ion apparently combined with the anhydride derivative of rubratoxin B to produce an amide or imide, which reacted with chlorine fumes and a spray reagent to produce a colour reaction. The spray reagent was prepared by mixing equal volumes of a 0.2 *M* pyridine solution of 1-phenyl-3-methyl-2-pyrazolin-5-one and 1 *M* aqueous potassium cyanide. Subsequently, rubratoxin B first turned pink under visible light, then quickly changed to blue and subsequently brown. The detection limit was 10 μg.

Emeh and Marth[176] used PLC on freshly activated plates prepared with silica gel $HF_{254+366}$ and developed the plates with ethyl acetate–acetic acid (85:15).

TLC data for rubratoxins reported by Hayes and Wilson[179] were as follows: on silica gel HF_{254} plates with glacial acetic acid–methanol–chloroform (2:20:80) the R_F values for rubratoxin A and B were 70 and 56, respectively. With six of the eight solvent systems used by Ďuračková *et al.*[22] no migration of rubratoxin B was observed on Silufol plates. With chloroform–methanol (4:1) and *n*-butanol–acetic acid–water (4:1:4, upper layer) its R_F values were 28 and 88, respectively.

3.5. Patulin and other small lactones

The mycotoxins patulin, penicillic acid, ascladiol, mycophenolic acid and butenolide contain a five-membered cyclic lactone ring. Citreoviridin has a six-membered cyclic lactone in its structure. TLC and other chromatographic techniques for patulin and other lactones have been reviewed by Scott[180] and Engel and Teuber[181].

3.5.1. Patulin

This mycotoxin is a natural contaminant of apple juice. According to Scott and Kennedy[182], the samples are extracted with ethyl acetate and the extract is concentrated, diluted with a four-fold volume of benzene and transferred on to a silica gel column. After elution with benzene, patulin is eluted from the column with ethyl acetate–benzene (25:75). The eluate is evaporated nearly to dryness and the residue is taken up in ethyl acetate and evaporated to dryness under nitrogen. For TLC

evaluation, the residue is dissolved in ethyl acetate. Siriwardana and Lafont[183] extracted patulin from apple juice with isopropanol–ethyl acetate, cleaned up the extract on a silica gel column, eluted with ethyl acetate–benzene, evaporated the eluate to dryness and dissolved the residue in chloroform. Leuenberger *et al.*[184] described an apparatus with which apple juice is directly applied on to a column of diatomaceous earth, eluted with toluene–ethyl acetate (3:1), the eluate is transferred on to a Kieselgel 60 F column and patulin is eluted with the same solvent and evaporated under nitrogen.

Several solvent systems have been used in the TLC of patulin, as shown in Table 12. Scott and Sommers[189] detected patulin using phenylhydrazinium chloride as a spray reagent (detection limit 100–300 μg/l of juice). Later, the sensitivity was improved using 0.5% 3-methyl-2-benzothiazolinone hydrazone (MBTH)–hydro-

TABLE 12

TLC DATA FOR PATULIN

Adsorbent	*Solvent system**	$R_F \times 100$	*Ref.*
Silica gel G-HR	Tol–EtOAc–FA (5:4:1)	58	180
Silica gel	Tol–EtOAc–90% FA (6:3:1)	41	24
	C_6H_6–MeOH–HOAc (24:2:1)	21	
Silufol	C_6H_6–MeOH–HOAc (24:2:1)	24	22
	Tol–EtOAc–90% FA (6:3:1)	27	
	C_6H_6–EtOH (95:5)	14	
	$CHCl_3$–MeOH (4:1)	61	
	$CHCl_3$–MIBK (4:1)	19	
	$CHCl_3$–Me_2CO (9:1)	17	
	$CHCl_3$–HOAc–Et_2O (17:1:3)	24	
	nBuOH–HOAc–H_2O (4:1:4, upper layer)	70	
Silica gel F_{254}	$CHCl_3$–MeOH (97:3)	22	18
	$CHCl_3$–Me_2CO–Hex (7:2:1)	27	
	$CHCl_3$–Me_2CO (9:1)	16	
	EtOAc–Hex (1:1)	18	
	$CHCl_3$–Me_2CO–PrOH (85:15:20)	56	
	C_6H_6–$CHCl_3$–Me_2CO (45:40:15)	20	
Silica gel	EtOH–H_2O (4:1)	71	185
	Tol–EtOAc–90% FA (6:3:1)	37	
	C_6H_6–MeOH–HOAc (24:2:1)	13	
	C_6H_6–HOPr–H_2O (2:2:1)	64	
	$CHCl_3$	4	
	$CHCl_3$–MeOH (1:1)	71	
	MeOH	66	
Silica gel AR-7GF	C_6H_6–MeOH–HOAc (18:1:1)	25	186
Kieselgel 60 F	CH_2Cl_2–EtOAc (95:45)	15	184
Silica gel 60	iPr_2O–Pen–EtOH–Pyr (84:12:4:0.8)	32	183
	Tol–EtOAc–90% FA (50:40:10)	39	
	$CHCl_3$–Me_2CO (90:10)	42	
	$CHCl_3$–MeOH (95:5)	35	
	Pen–EtOAc (96:4)	0	
Kieselgel 60 G	Tol–EtOAc–85% FA (50:40:10)	39	187
Silica gel K5	Tol–EtOAc–95% FA (5:4:1)	60	188

* Abbreviations: FA, formic acid; EtOH, ethanol; MIBK, methyl isobutyl ketone; nBuOH, *n*-butanol; PrOH, propanol; HOPr, propionic acid; iPr_2O, diisopropyl ether; Pen, pentane; others as in Tables 5 and 6.

chloric acid solution, permitting the detection of 20–25 μg/l or 10 ng of patulin per spot[182,190]. This method of detection has also been used by other workers[27,183,184]. After spraying with MBTH, patulin appears as a yellow fluorescent spot and can be detected by fluorodensitometry. Young[52] detected patulin by TLC of its aniline imine and quantitated it by measurement of fluorescence.

Meyer[187] published a TLC method for the quantitation of patulin in fruit and vegetable products. After extraction and clean-up using CC, patulin was 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 was based on the yellow fluorescence under long-wave UV light (limit 10 ng per spot). Meyer also identified patulin after acetylation. On Kieselgel 60 G plates and using toluene–ethyl acetate–65% formic acid (50:40:10), the R_F values of patulin and of the acetylated product were 0.39 and 0.54, respectively. Leuenberger *et al.*[184] reported R_F values of 0.15 for patulin and 0.75 for the acetylated product on Kieselgel 60 F with methylene chloride–ethyl acetate (95:5) as the solvent system.

Patulin has been included in multi-mycotoxin TLC by several workers (*e.g.*, refs. 18, 26, 76, 78, 81, 83 and 87) (see also Section 3.1).

TLC of patulin and intermediates in its biosynthesis by *Penicillium urticae* has been successfully applied by Bu'Lock *et al.*[191], and Gaucher and co-workers[192–196].

3.5.2. Penicillic acid

Extraction, clean-up and chromatographic methods for penicillic acid were reviewed recently by Engel and Teuber[42]. Extraction from culture filtrates *Penicillium cyclopium* and clean-up techniques prior to TLC have been described[197,198]. A variety of procedures for extraction from foods and feedstuffs have been published. Penicillic acid was extracted with chloroform–methanol (9:1) from corn[199], with methylene chloride–methanol (1:1) from peas, rice, oats and crushed coconut[28] and with acetonitrile–4% KCl (9:1) from cheese[200] and from raw sausages[201]. Thorpe and Johnson[202] used extraction with ethyl acetate from corn, dried beans and apple juice. Penicillic acid was then extracted with 3% aqueous $NaHCO_3$ and, after acidification to pH 3 with HCl, re-extracted with ethyl acetate, dried and evaporated under nitrogen. Further purification was achieved on a silica gel column using hexane–ethyl acetate–formic acid (750:250:1) as the eluent. The eluate was concentrated and evaporated to dryness under nitrogen. For chromatography, the residue was dissolved in chloroform.

The solvent systems listed in Table 13 have been used for the TLC detection and assay of penicillic acid.

After TLC, penicillic acid can be rendered visible by several methods: (a) at 254 nm as a light purple spot[18]; (b) after spraying with *p*-anisaldehyde it develops a green colour under visible light and a blue fluorescence under long-wave UV light[24]; (c) spraying with diphenylboric acid–2-ethanolamine[28] yields a blue fluorescence with an excitation maximum at 365–370 nm and an emission maximum at 440 nm, with a detection limit of 5 ng; (d) application of ammonia fumes[198,203] induces a blue fluorescence with an excitation peak at 350 nm and an emission maximum at 440 nm; (e) with cerium(IV) sulphate, penicillic acid gives a light orange spot[18]; (f) it may be detected by UV densitometry at 234 nm[198]; (g) spraying with 3-methyl-2-benzthiazolinone hydrazone hydrochloride solution and heating produces a yellow fluo-

TABLE 13

TLC DATA FOR PENICILLIC ACID

Adsorbent	*Solvent system**	$R_F \times 100$	*Ref.*
Silica gel 60	iPr_2O–Pen–EtOH–Pyr (84:12:4:0.8)	23	183
	Tol–EtOAc–90% FA (50:40:10)	41	
	$CHCl_3$–Me_2CO (90:10)	40	
	$CHCl_3$–MeOH (95:5)	35	
Kieselgel G	C_6H_6–MeOH–HOAc (18:1:1)	25	186
Silica gel	Tol–EtOAc–90% FA (6:3:1)	47	24
	C_6H_6–MeOH–HOAc (24:2:1)	22	
Silufol	C_6H_6–MeOH–HOAc (24:2:1)	27	22
	Tol–EtOAc–90% FA (6:3:1)	31	
	C_6H_6–EtOH (95:5)	14	
	$CHCl_3$–MeOH (4:1)	66	
	$CHCl_3$–MIBK (4:1)	17	
	$CHCl_3$–Me_2CO (9:1)	15	
	$CHCl_3$–HOAc–Et_2O (17:1:3)	26	
	nBuOH–HOAc–H_2O (4:1:4, upper layer)	76	
Silica gel + oxalic acid	$CHCl_3$–MeOH (98:2)	16	18
	$CHCl_3$–Me_2CO (9:1)	20	
Silica gel	$CHCl_3$–MeOH–H_2O–FA (250:24:25:1)		197
	$CHCl_3$–EtOAc–90% FA (60:40:1)		198

* Abbreviations: iPr_2O, diisopropyl ether; EtOH, ethanol; Pyr, pyridine; Tol, toluene; EtOAc, ethyl acetate; $CHCl_3$, chloroform; Me_2CO, acetone; C_6H_6, benzene; HOAc, acetic acid; FA, formic acid; MIBK, methyl isobutyl ketone; Et_2O, diethyl ether; nBuOH, *n*-butanol.

rescence under long-wave UV light[82]. Penicillic acid has also been included in several multi-mycotoxin analyses (*e.g.*, refs. 18, 21, 22, 26, 78, 82 and 83) (see also Section 3.1).

3.5.3. Mycophenolic acid

Mycophenolic acid can be extracted from acidified culture filtrates with chloroform, the extract being dried and evaporated to dryness. The residue is dissolved in hot acetone and filtered. Crystallization is achieved on addition of cold *n*-hexane[42]. Mycophenolic acid has been reported in blue cheese and starter cultures of *Penicillium roqueforti*[204].

The compound is extracted from cheese samples with methanol–acetone at pH 6. After filtration and precipitation of casein, the supernatant is concentrated, defatted with hexane and extracted with chloroform, chloroform–ethyl acetate (1:1) and ethyl acetate. The combined extracts are dried and evaporated to dryness and the residue is dissolved in chloroform and used for TLC[183,205].

Suitable solvent systems are given in Table 14. Mycophenolic acid can be detected with *p*-anisaldehyde[22,24], giving a grey spot under visible light and pale blue fluorescence at 366 nm, with ethanolic iron(III) chloride, giving a grey-brown spot under visible light[22], with fumes of ammonia or diethyl amine, giving immediately an unstable bright sky-blue spot under long-wave UV light[183,208,209], or by fluorescence quenching of a fluorescence indicator incorporated into the TLC plates (excitation at 254 nm)[42]. Jones *et al.*[207] described a preparative TLC method for mycophenolic acid and its transformation products.

TABLE 14

TLC DATA FOR MYCOPHENOLIC ACID

Adsorbent	*Solvent system**	$R_F \times 100$	*Ref.*
Silica gel	AmOAc–PrOH–HOAc–H_2O (4:3:2:1)	65	206
	C_6H_6–EtOAc–FA (66:33:1)	–	207
Silufol	C_6H_6–MeOH–HOAc (24:2:1)	57	22
	Tol–EtOAc–90% FA (6:3:1)	67	
	C_6H_6–EtOH (95:5)	30	
	$CHCl_3$–MeOH (4:1)	82	
	$CHCl_3$–MIBK (4:1)	90	
	$CHCl_3$–Me_2CO (9:1)	43	
	$CHCl_3$–HOAc–Et_2O (17:1:3)	72	
	nBuOH–HOAc–H_2O (4:1:4, upper layer)	87	
Silica gel	Et_2O–Hex–90% FA (60:20:0.4)	39	183
	iPr_2O–Pen–EtOH–Pyr (42:6:2:0.4)	15	
	$CHCl_3$–Me_2CO–H_2O (93:7:1)	22	

* Abbreviations: AmOAc, amyl acetate; PrOH, *n*-propanol; HOAc, acetic acid; C_6H_6, benzene; EtOAc, ethyl acetate; FA, formic acid; MeOH, methanol; Tol, toluene; EtOH, ethanol; $CHCl_3$, chloroform; MIBK, methyl isobutyl ketone; Me_2CO, acetone; Et_2O, diethyl ether; nBuOH, *n*-butanol; Hex, *n*-hexane; iPr_2O, diisopropyl ether.

3.5.4. Butenolide .

Agar cultures of *Fusarium nivale* are extracted with diethyl ether, ethanol–water (80:20) or methylene chloride. From liquid media or mouldy grain, butenolide is extracted with ethyl acetate[210]. R_F values of the toxin on silica gel plates in various solvent systems are listed in Table 15. The toxin is detected by spraying with *p*-anisaldehyde, showing a grey reaction product under visible light[22]. Spraying with 2,4-dinitrophenylhydrazine and heating to 100°C produce a yellow spot[211].

TABLE 15

TLC DATA FOR BUTENOLIDE

Adsorbent	*Solvent system**	$R_F \times 100$	*Ref.*
Silica gel	Tol–EtOAc–FA (6:3:1)	10	24
	$CHCl_3$–iPrOH–EtOAc (40:5:5)	–	211
	$CHCl_3$–MeOH (93:7)	–	
Silufol	Tol–EtOAc–FA (6:3:1)	10	22
	$CHCl_3$–MeOH (4:1)	41	
	nBuOH–HOAc–H_2O (4:1:4, upper layer)	43	

* Abbreviations: Tol, toluene; FA, 90% formic acid; $CHCl_3$, chloroform; iPrOH, 2-propanol; EtOAc, ethyl acetate; MeOH, methanol; nBuOH, *n*-butanol; HOAc, acetic acid.

3.5.5. Citreoviridin

Extraction and clean-up techniques have been summarized by Engel and Teuber[42]. Mouldy rice is extracted with ethanol, the extract is evaporated to dryness and the residue is dissolved in benzene and precipitated with *n*-hexane. The precipitate is applied to a silica gel column, which is eluted with *n*-hexane–acetone (2:1). The citreoviridin-containing fraction is evaporated to dryness and the residue dissolved in methanol to obtain yellow needles of citreoviridin.

The separation of citreoviridin from natural extracts has been achieved only with TLC. Suitable solvent systems and R_F values are given in Table 16. Citreoviridin appears as a yellow spot under visible light and shows yellow fluorescence under long-wave UV light. UV densitometric and fluorodensitometric evaluations on TLC plates were described by Engel[214]. On TLC plates, the UV maximum and the emission maximum were at 360 and 525 nm, respectively. The former evaluation was found to be the more reliable. More recently, citreoviridin was characterized by Cole *et al.*[215] by TLC on silica gel 60 F_{254} plates with toluene–ethyl acetate–formic acid (5:4:1) as the solvent system.

TABLE 16

TLC DATA FOR CITREOVIRIDIN

Adsorbent	*Solvent system**	$R_F \times 100$	*Ref.*
Silica gel	Me_2CO–Hex (1:1)	55, 45	70, 212
	EtOAc–Tol (1:1)	40, 50	
	$CHCl_3$–MeOH (9:1)	35, 85	
	$CHCl_3$–MeOH–Me_2CO (45:3:2)	74	213
Silufol	C_6H_6–MeOH–HOAc (24:2:1)	23	22
	$CHCl_3$–MeOH (4:1)	60	
	$CHCl_3$–HOAc–Et_2O (17:1:3)	10	
	nBuOH–HOAc–H_2O (4:1:4, upper layer)	79	
Kieselgel G 1500	EtOAc–Tol (3:1)	–	214

* Abbreviations: Me_2CO, acetone; Hex, *n*-hexane; EtOAc, ethyl acetate; Tol, toluene; $CHCl_3$, chloroform; MeOH, methanol; HOAc, acetic acid; Et_2O, diethyl ether; nBuOH, *n*-butanol.

3.6. Trichothecenes

The trichothecenes represent a family of structurally related natural substances produced mostly by fungi and, in a few instances by higher plants (*e.g.*, baccharin). Recently, 58 natural members of this family were reviewed by Tamm and Tori[216]. The trichothecenes can be divided into two groups, one consisting of the alcoholic derivatives of the trichothecene nucleus and their simple esters and the other of the more complex macrocyclic di- and triesters. According to differences in the trichothecene nucleus, the trichothecenes are further divided into four types, and of these type A (characterized by a hydrogen atom or a hydroxy group at the 8-position) and type B (with a ketone group at the 8-position) are the most important in practical analysis. The macrocylic trichothecenes belong to type C. (For more details on the chemistry of the trichothecenes see, *e.g.*, refs. 216 and 217.)

TLC together with GC have been widely applied in the qualitative and quantitative analysis of trichothecenes and have been described in several reviews and books[4,6,216–224].

Most TLC studies have used silica gel as the adsorbent. Usually, before the samples are submitted to TLC, clean-up techniques are used. Except for type C trichothecenes, trichothecene mycotoxins show almost no absorption bands or fluorescence under UV or visible light and various detection reagents have been reported for use on thin-layer plates. In the following sub-sections, extraction and clean-up procedures, detection methods and some practical applications are reviewed.

3.6.1. Extraction and clean-up

Clean-up methods for the TLC of trichothecenes were reviewed by Takitani and Asabe[6], who summarized the general procedures as follows. The mycotoxins are extracted with methanol or acetonitrile (or their mixtures with water) from food or feed samples and the lipids are removed from the extracts with *n*-hexane or isooctane. In many instances, the mycotoxins are re-extracted from the original extracts with chloroform. After washing with water, the solutions are applied to a column of silica gel or Florisil and the eluates containing the toxins are submitted to TLC. Other methods have also been used (see Section 2.1).

Harrach *et al.*[225] recently isolated satratoxins from a sample of straw shown to be responsible for a serious outbreak of stachybotryotoxicosis in sheep. Unground straw was extracted with methanol, the methanol was evaporated and the residue was partitioned between light petroleum and water. The water layer was extracted with methylene chloride and the residue from the organic layer was placed on a silica gel column. Elution with ethyl acetate gave a residue that was subjected to PLC.

3.6.2. Detection

It has already been mentioned that chromogenic reagents are necessary to render visible type A and B trichothecenes on TLC plates. Current procedures employ reagents such as sulphuric acid[226], aluminium chloride[77,227], *p*-anisaldehyde[22,24], 4(*p*-nitrobenzyl)pyridine[228], cerium(IV) sulphate and nicotinamid–2-acetylpyridine[64]. Ehrlich and Lillehoj[229] monitored triacetyldeoxynivalenol in CC eluates by TLC. As the latter compound is not stained with an aluminium chloride spray, portions of the fractions from CC were treated with 0.5 *M* NaOH in 90% aqueous ethanol to regenerate deoxynivalenol. The samples were then submitted to TLC and deoxynivalenol was detected by spraying with 20% aqueous aluminium chloride and heating to produce a characteristic blue fluorescence.

Baxter *et al.*[230] developed a procedure employing chromotropic acid (disodium 4,5-dihydroxynaphthalene-2,7-disulphonate dihydrate) as a sensitive and specific spray reagent to detect trichothecenes on TLC plates. They found that aluminium chloride was relatively specific for type B trichothecenes such as vomitoxin (deoxynivalenol) and that the type A trichothecenes, which do not react with aluminium chloride, can be rendered visible with chromotropic acid. In their procedure, following TLC development, the dried plate was sprayed with aluminium chloride reagent, heated and then viewed at 365 nm to determine the presence of vomitoxin (bright blue colour). The same plate was then sprayed with chromotropic acid reagent [1

TABLE 17

VISUALIZATION OF TRICHOTHECENES ON TLC PLATES

*Reagent**	*Trichothecene*	*Visible light*		*Long-wave UV light*		*Ref.*
		Colour	*Detection limit (ng per spot)*	*Colour*	*Detection limit (ng per spot)*	
Sulphuric acid	Type A	Greyish black	250	Blue	50	6, 77
	Type B	Brown	250			
p-Anisaldehyde	Type A	Pinkish violet	250	Blue		6, 24
	Type B	Yellowish brown-greenish	250			
	Diacetoxyscirpenol	Violet		Yellow-orange		22
	Nivalenol	Pale grey		Grey		
	T-2 toxin	Violet		Yellow		
	Trichothecin	Beige		Blue		
	Deoxynivalenol	Yellow				
Aluminium chloride	Fusarenon-X			Blue	50	231, 232
	Type B					230, 233
	Deoxynivalenol			Bright blue	50	230, 233
NBP	All types	Blue-violet	20–200			228
NAP	Type A and type B			Blue	20–50	229
Chromotropic acid	T-2 toxin	Purple	100	Bright blue	50	230
	Diacetoxyscirpenol	Brown	200	Blue-white	100	
	HT-2 toxin	Purple	100	Bright blue	50	
	Deoxynivalenol	Grey	100	Intense black	100	
	T-2 triol	Purple	100	Bright blue	50	
	T-2 tetraol	Purple	100	Bright blue	50	
Cerrium(IV) sulphate	T-2 toxin	Grey-black	100			18
	Roridin A	Grey-black				

* Abbreviations: NBP, 4-(*p*-nitrobenzyl)pyridine; NAP, nicotinamide-2-pyridine.

part of a 10% aqueous solution of chromotropic acid mixed with 5 parts of concentrated sulphuric acid–water (5:3)] and heated at 110°C for 5–15 min until all the reference standards appeared as dark spots against a light mauve background. After cooling, the plate was re-examined at 365 nm. The above-mentioned and less frequently used detection reagents are summarized in Table 17. Some type C trichothecenes can be detected by absorption or fluorescence under UV light[18,234].

Standard procedures using sulphuric acid, *p*-anisaldehyde, aluminium chloride, 4-(*p*-nitrobenzyl)pyridine and nicotinamide-2-acetylpyridine have been described by Takitani and Asabe[6]. They also reviewed other, less frequent detection methods.

3.6.3. TLC data for trichothecenes

R_F values of type A and B trichothecenes are given in Table 18 and those of type C (macrocyclic) trichothecenes in Table 19.

3.6.4. Recent applications

Trichothecenes of the type A and B are mostly metabolites of various species of *Fusarium*. Some fusaria also produce zearalenone (see Section 3.11). In the late 1970s and early 1980s, tests for the two types of trichothecenes have been included in various multi-mycotoxin analyses of feedstuffs and foods. Coman *et al.*[84] detected T-2 toxin, zearalenone and other toxins by TLC analysis of samples of feeds. Diacetoxyscirpenol and zearalenone were included in TLC analyses of corn by Whidden *et al.*[78]. T-2 toxin, HT-2 toxin and neosolaniol were detected in grains of barley together with zearalenone by Ilus *et al.*[85]. Trichothecenes were also included in multi-toxin analyses by Gorst-Allman and Steyn[18]. PLC was applied in studies of microbial transformations of 4,15-diacetoxyscirpenol[240]. Many references to TLC and GC analyses of trichothecenes can be found in a recent monograph on these toxins[217], in the section devoted to the natural occurrence of toxigenic fungi in Asia, Europe, North America and South Africa.

Recently, TLC data on trichothecenes have been reported in studies of mycotoxins in natural products by Eppley *et al.*[241], Schultz *et al.*[242], Trucksess *et al.*[243], Richardson *et al.*[12] and Hagler *et al.*[244]. Analytical and preparative TLC has been used in studies of the bioconversion of T-2 toxin into 3′-hydroxy-T-2 toxin and 3′-hydroxy-HT-2 toxin[245]. A rapid method for the determination of trichothecenes was developed by Bata *et al.*[246]. The trichothecenes occurring in purified extracts of food and feed samples are converted into the corresponding free alcohols by transesterification and then analysed by HPTLC or GC. Harrach *et al.*[225] subjected cleaned-up concentrates of satratoxins G and H to PLC on silica gel using 5% methanol in methylene chloride for development. 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.

3.7. Cytochalasans

The cytochalasans are secondary metabolites of fungi that have peculiar effects on mammalian cells in tissue cultures. Their trivial names are derived either from their biological effects (*e.g.*, cytochalasins) or from their producing organisms (*e.g.*, zygosporins and chaetoglobosins). Their systematic nomenclature as a family of com-

TABLE 18

$R_F \times 100$ VALUES OF TYPE A AND TYPE B TRICHOTHECENES ON SILICA GEL PLATES

Type	*Mycotoxin*	*Solvent system**						
		A	*B*	*C*	*D*	*E*	*F*	*G*
Type A	Trichodermol					17		
	Trichodermin					51		
	Verrucarol					3		
	Scirpentriol	4					7	
	Monoacetoxyscirpentriol	7					12	
	Diacetoxyscirpenol	47		52	50	14	37	47
	7α-Hydroxydiacetoxyscirpenol							
	7α,8α-Dihydroxydiacetoxyscirpenol			35	24			
	T-2 tetraol	0				0	2	
	Neosolaniol	19		38	29		15	15
	HT-2 toxin	10		30	21	3	13	10
	T-2 toxin	53		55	52	16	41	61
	Acetyl T-2 toxin							
	T-2 triol							
	T-2 tetraol							
Type B	Trichothecolone					13		
	Trichothecin					53		
	Deoxynivalenol	7	47	31	20		16	
	Nivalenol		22	9	3			2
	Fusarenon-X	17	64	41	29		25	31
	Nivalenol diacetate			51	44			47

pounds with a common skeleton (cytochalasan) was proposed by Binder *et al.*[247] and its rules were summarized by Tamm[248]. More recently procedures used for the production, isolation, separation and purification of 37 known cytochalasans were reviewed[249]. So far, the following four types of natural cytochalasans are known: (a) the [11]cytochalasans cytochalasin C, cytochalasin D (= zygosporin A), cytochalasin G, cytochalasin H (= paspalin P-1 = kodo-cytochalasin-1), cytochalasin J (= paspalin P-2 = kodo-cytochalasin-2), zygosporins D, E, F and G, aspochalasins A, B, C and D and engleromycin; (b) the [13]cytochalasans deoxaphomin, proxiphomin, protophomin, chaetoglobosins A, B, C, D, E, F, G, J and K, 19-O-acetylchaetoglobosins A, B and D and cytochalasins K, L and M; (c) the 24-oxa-[14]cytochalasans cytochalasin A (= dehydrophomin), cytochalasin B (= phomin) and cytochalasin F; and (d) the 21,23-dioxa-[13]cytochalasan cytochalasin E.

H	*I*	*J*	*K*	*L*	*M*	*N*	*O*	*P*	*Q*	*R*	*S*	*T*	*U*	*V*	*W*	*X*	*Y*	*Z*
			36															
			67															
			6															
												37						
46	68	82	86	39	30	19	90	14	34	27		37	39					
											50							
											38							
													37		52			
25	32																	
17	23		9										28		60	56	55	
68	78	81	40	43	32	16	92	16	38	38			43					
														74				59
													15					
													9					
		81	66	68	55	43	92	63	73	69								
													18					
1	4	67		7	5	0	50	0	0	0								
21	32																	
43	54																	

* Solvent systems: A[224], chloroform–methanol (95:5); B[229], chloroform–methanol (7:1); C[228], benzene–acetone (1:1); D[228], chloroform–acetone (3:2); E[222], benzene–tetrahydrofuran (85:15); F[224], ethyl acetate–toluene (3:1); G[226], ethyl acetate–*n*-hexane (3:1); H[226], chloroform–2-propanol–ethyl acetate (95:5:5); I[226], chloroform–ethanol–ethyl acetate (90:5:5); J[22], *n*-butanol–acetic acid–water (4:1:4, upper layer); K[218], chloroform–methanol (98:2); L[22], benzene–methanol–acetic acid (24:2:1); M[22], toluene–ethyl acetate–90% formic acid (6:3:1); N[22], benzene–ethanol (95:5); O[22], chloroform–methanol (4:1); P[22], chloroform–methyl isobutyl ketone (4:1); Q[22], chloroform–acetone (9:1); R[22], chloroform–acetic acid–diethyl ether (17:1:3); S[235], acetone–*n*-hexane (1:1); T[236], chloroform–methanol (90:10); U[230], toluene–ethyl acetate–90% formic acid (5:4:1); V[237], ethanol–ethyl acetate–acetone (1:4:4); W[216], ethanol–benzene–acetone (1:3:3); X[216], ethanol–chloroform–acetone (1:4:4); Y[238], ethyl acetate–ethanol (6:1); Z[238], ethyl acetate.

For practical reasons, the cytochalasans discussed here are grouped according to their trivial names.

3.7.1. Cytochalasins

Analytical and preparative TLC of cytochalasins has been carried out on silica gel by most workers. Examples of solvent systems are given below. Detection was effected with iodine vapour[250], ethanolic sulphuric acid, vanillin–phosphoric acid and Dragendorff reagent[251] or *p*-anisaldehyde followed by examination under long-wave UV light[22].

Padhye *et al.*[25] carried out TLC separations of seven cytochalasins on silica gel G plates in eleven solvent systems and good separations were obtained in systems A, C and E (Table 20). In these systems cytochalasin A, which is a ketone, migrated

TABLE 19

R_F × 100 VALUES FOR MACROCYCLIC TRICHOTHECENES

Trichothecene	*Adsorbent*				
	Alumina	*Silica gel*			
	*A**	*A**	*B**	*C**	*D**
Verrucarin A	70	28	59	47	
2'-Dehydroverrucarin A	82	58	68		
Verrucarin B	83	47	69	63	37
Verrucarin C	74	28	52		
Verrucarin D	70	28	55		
Verrucarin E	0	0	9		
Verrucarin F		54			
Verrucarin G		49			
Vrrucarin H		59		72	51
Verrucarin J		59		64	42
Roridin A	18	70		20	14
Roridin D		35		29	18
Roridin E		40		35	24
Roridin H		59		72	24

* Solvent systems: $A^{234,239}$, chloroform–methanol (98:2); $B^{234,239}$, chloroform–methanol (97:3); C^{239}, benzene–tetrahydrofuran (85:15); D^{239}, diethyl ether (twice).

TABLE 20

R_F × 100 VALUES OF CYTOCHALASINS ON SILICA GEL G PLATES

Adapted from ref. 251.

Cytochalasin	*Solvent system**										
	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*	*K*
A	63	53	62	39	36	0	93	67	79	95	56
B	58	38	53	36	30	0	92	63	78	95	47
C	46	34	44	23	26	0	90	59	78	92	30
D	45	32	42	19**	20**	0	89	58	76	93	34
H	63	55	64	46	50	0	87	50	80	92	60
J	44	38	56	34	35	0	75	55	72	90	46
E	79	57	63	48	40	0	90	62	82	96	71**

* Solvent systems: A, chloroform–methanol (95:5); B, chloroform–methanol–formic acid (95:5:5); C, chloroform–diethylamine (90:10); D, diisopropyl ether–ethyl acetate (90:10); E, cyclohexane–ethyl acetate–diethylamine (60:30:10); F, benzene–chloroform (50:50); G, *n*-butanol–formic acid–water (80:10:10); H, benzene–methanol (70:30); I, 2-propanol; J, acetone; K, benzene–acetone (70:30).

** Tailing.

faster than the corresponding alcohol (cytochalasin B). Cytochalasins C and D, which are double-bond isomers, had almost identical R_F values in most of the solvent systems used. They could, however, be well distinguished from each other by spraying with ethanolic sulphuric acid and observing their fluorescence under UV light, cytochalasin C giving a dull orange and cytochalasin D a weak yellow colour.

Ďuračková *et al.*[22] compared the mobilities of cytochalasins A, B and C on silica gel G plates and pre-coated Silufol sheets in eight solvent systems (Table 21). In most systems, the mobilities of the three cytochalasins decreased in the order A > B > C.

TABLE 21

R_F × 100 VALUES OF CYTOCHALASINS ON SILICA GEL PLATES AND SILUFOL SHEETS

Adapted from ref. 22.

Cyto-chalasin	*Adsor-bent*	R_F × *100**								*Detection***	
		A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*1*	*2*
A	Silical gel	41	58	25	85	17	33	46	99	Pale beige	Blue
	Silufol	34	42	23	83	20	31	44	95		
B	Silica gel	28	46	13	78	8	16	24	98	Violet	Blue
	Silufol	34	34	13	74	7	14	26	94		
D	Silica gel	19	24	10	75	0	8	14	90	Beige	Orange
	Silufol	32	27	10	74	4	9	20	85		

* Solvent systems: A, benzene–methanol–acetic acid (24:2:1); B, toluene–ethyl acetate–90% formic acid (6:3:1); C, benzene–ethanol (95:5); D, chloroform–methanol (4:1); E, chloroform–methyl isobutyl ketone (4:1); F, chloroform–acetone (9:1); G, chloroform–acetic acid–diethyl ether (17:1:3); H, *n*-butanol–acetic acid–water (4:1:4, upper layer).

** Detection: 1, *p*-anisaldehyde; 2, UV at 366 nm after *p*-anisaldehyde.

TLC on silica gel G of several derivatives of cytochalasin B was carried out by Rothweiler and Tamm[250]. Chloroform containing 0.5–20% of methanol was used as the solvent system. TLC was used by Lees and Lin[252] to purify 7,20-diacetylcytochalasin B on silica gel GF plates. In the system chloroform–ethyl acetate (1:1), the R_F values of cytochalasin B, dihydrocytochalasin B and diacetylcytochalasin B were 0.4, 0.5 and 0.6, respectively.

Aldridge and Turner[253] separated cytochalasins C and D on silica gel G plates using chloroform–methanol–formic acid (90:5:5). The plates were sprayed with 5% ethanolic sulphuric acid and heated at *ca.* 110°C for a few minutes. Cytochalasin G gave an orange and cytochalasin D a yellow fluorescence under UV light.

TLC of cytochalasin D was carried out on silica gel GF_{254} with benzene–ethyl acetate (7:3) or chloroform–methanol (9:1) (or both) and the spots were rendered visible by spraying with concentrated sulphuric acid followed by heating at *ca.* 180°C[254].

A variety of solvent systems have been used in the analytical TLC and PLC of derivatives and degradation products of cytochalasin D[255]. In PLC, Kieselgel 60 PF_{254} was used as the sorbent and analytical TLC was carried out on Fertigplatten 60 F_{254}.

TLC and PLC were carried out on silica gel layers containing 15% of gypsum, cytochalasins H and J being isolated from *Phomopsis paspalli*[67]. PLC of crude diethyl ether extracts in chloroform–methanol (9:1) and spraying with concentrated sulphuric acid showed four spots with R_F 0.56 (yellow, minor), 0.49 (red, major), 0.36 (yellow, minor) and 0.32 (red, major). The red spots corresponded to cytochalasin

H (kodo-cytochalasin-1) and cytochalasin J (kodo-cytochalasin-2). In a typical experiment, 337 mg of the ether extract on PLC gave 132 mg of cytochalasin H and 28 mg of cytochalasin J.

3.7.2. Zygosporins

PLC was used in the isolation and purification of zygosporins from a culture of *Zygosporium masonii*[256]. The culture filtrate was extracted with ethyl acetate, the washed and dried extract was evaporated to about one-third of its volume and the separated product was filtered off. The filtrate was evaporated *in vacuo* to give crude cytochalasin D (zygosporin A) and a paste (A). Recrystallization of the crude cytochalasin D from acetone gave the pure compound and a residue (B). The residues A and B were combined and used for isolation of zygosporins using CC and PLC.

The residues A and B were combined and chromatographed on silica gel. Fraction 2, eluted with chloroform, was crystallized from ethyl acetate to give a crystalline product (C) and a paste (D). The latter was chromatographed on alumina to give an oil and a paste (E), eluted with chloroform–methanol.

Fraction 3, eluted from the silica gel column with chloroform–methanol (9:1), was dissolved in light petroleum and the precipitate (F) was collected.

The crystalline product C was separated into cytochalasin D (R_F 0.40) and zygosporin E (R_F 0.48) by PLC with ethyl acetate as the solvent.

The paste E was re-chromatographed on silica gel to give an amorphous powder, which was separated into zygosporin G (R_F 0.35) and zygosporin F (R_F 0.28) by PLC using toluene–methanol (10:1) as the solvent system.

The precipitate F was separated into cytochalasin D (R_F 0.50) and zygosporin D (R_F 0.40) by PLC using chloroform–methanol (10:1) as the solvent system.

In addition to the use of PLC in the isolation of zygosporins, TLC has been used to characterize degradation products and derivatives of the four zygosporins[256,257].

Minato *et al.*[258] reported the following R_F values of zygosporins on silica gel TLC plates after development with chloroform–methanol (9:1): 0.50 for zygosporin A (cytochalasin D), 0.40 for zygosporin D, 0.55 for zygosporin E and 0.57 for zygosporins F and G.

3.7.3. Aspochalasins

Aspochalasins A, B, C and D were isolated from *Aspergillus microcysticus*. TLC of the aspochalasins was performed on Kieselgel 60–Fertigplatten F_{254} and the spots were rendered visible by spraying with 50% sulphuric acid and heating at 200°C, with iodine vapour or fluorescence under UV light[259]. R_F values of 0.35, 0.27 and 0.54 were obtained for aspochalasins C, D and B, respectively (in ethyl acetate, blue fluorescence). In chloroform–methyl acetate (4:1), aspochalasins A and B had R_F values of 0.53 and 0.26, respectively. TLC data for derivatives and degradation products of aspochalasins were also given[259].

3.7.4. Deoxaphomin, proxiphomin and protophomin

Deoxaphomin was isolated by PLC from mother liquors after crystallization of phomin (cytochalasin B) as follows[260]. The mother liquors were combined and deoxaphomin was separated on preparative plates with chloroform–acetone (3:1).

The crude product was further purified using four preparative separations (chloroform–acetone, 3:1; twice with chloroform–acetone–formic acid, 90:5:5; chloroform–acetone, 3:1). The substance was extracted with chloroform–acetone (1:1) and the extracts were checked for their purity by means of TLC (chloroform–acetone, 3:1; chloroform–acetone–formic acid, 90:5:5).

The following procedure was used to isolate proxiphomin and protophomin[69]. The residue after isolation of phomin and deoxaphomin was chromatographed on a Kieselgel column. The fractions eluted with methylene chloride–methanol (9:1) containing several non-polar components. The fractions were combined and chromatographed again on Kieselgel. From the eluate in methylene chloride crude proxiphomin was obtained and the methylene chloride–methanol (98:2) fractions contained protophomin.

The crude preparation of proxiphomin was purified using PLC in methylene chloride–methanol. Extraction of the main zone with chloroform–acetone (4:1) resulted in 55 mg of chromatographically pure proxiphomin. TLC was carried out with methylene chloride–methanol (98:2) and methylene chloride–ethyl acetate (9:1).

The protophomin-containing fractions were chromatographed on PLC layers, yielding crude protophomin, which was submitted to further purification by means of PLC: twice with chloroform–acetone–formic acid (96:2:2) and once with methylene chloride–methanol (98:2). Extraction of the zones with chloroform–acetone (3:1) yielded almost pure protophomin from which, after crystallization from acetone, pure protophomin was obtained.

3.7.5. Chaetoglobosins

TLC was used to check the presence of compounds with a positive reaction to phenols and indoles [spraying with 5% solutions of ammonium cerium(IV) nitrate in acetone and of hydroxylammonium chloride in 80% aqueous acetone] in extracts from *Diplodia macrospora* cultures[68]. The extracts with positive reactions were cleaned up on a silica gel column. The fractions containing chaetoglobosins K and L were purified by PLC on silica gel plates using toluene–ethyl acetate–formic acid (5:4:1) as the solvent system.

Chaetoglobosins A, B, C, D and E were analysed on silica gel F_{254} using benzene–ethyl acetate (1:1) and benzene–chloroform–methanol (10:10:3) as the solvents. Metabolites were detected by UV irradiation at 254 and 365 nm and by spraying with Ehrlich's reagent and coloration after heating[14].

The R_F value of chaetoglobosin K was 0.53–0.56 on silica gel 60 TLC plates developed with toluene–ethyl acetate–formic acid (5:4:1) and it was observed as a dark spot under short-wave UV light[261]

Decreasing R_F values of five chaetoglobosins on the same sorbent and with methylene chloride–methanol (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 > chaetoglobosin A[262].

TLC was used recently by Sekita *et al.*[263] in their work on chaetoglobosins A–J.

3.8. Tremorgenic mycotoxins

Except for the territrems, the known tremorgenic mycotoxins have in common an indole moiety and can be placed into four groups: the penitrem group, the fumitremorgin–verruculogen group, the paspalitrem group and the tryptoquivaline group. TLC has been used in monitoring the CC separation and purification of most of the tremorgens, and also in preparative and qualitative separations. The janthitrems could be added as a fifth group.

Silica gel has been used in most TLC studies of the tremorgens. Selected solvent systems are mentioned below. Penitrems A–F are unstable in chloroform when exposed directly to light, presumably as a result of acid formation in the solvent. Hence, the use of chloroform must be avoided in work with these toxins[264].

3.8.1. Detection of indole-derived tremorgens

Several detection methods have been used in the TLC analysis of indole-derived tremorgens. They include short- and long-wave UV light and the following spray reagents: 50% sulphuric acid in ethanol without and with heating, cerium(IV) sulphate in sulphuric or phosphoric acid, phosphomolybdic acid, iron(III) chloride, aluminium chloride, *m*-dinitrobenzene, 2,4-dinitrophenylhydrazine and Van Urk reagent. The following results have been obtained.

3.8.1.1. Paspalitrem group. Aflatrem appeared as a dark spot under long-wave UV light; spraying with *m*-dinitrobenzene caused the spots to turn a non-specific brown colour, but spraying with phosphomolybdic acid with applied heat turned the spots an orchid to violet colour[265,266]. Paspaline and paspalicine were detected as pale green spoots with Van Urk reagent[267]. Paspaline and paspalitrem A were revealed as grey-blue spots in visible light after spraying with 50% ethanolic sulphuric acid and heating for 5 min at 150°C and were fluorescent under long- and short-wave UV light. Under the same conditions, paspalitrem B was visible as a green spot immediately after spraying[71]. Paxilline was detected after spraying TLC plates with 50% ethanolic sulphuric acid or 3% phosphomolybdic acid and heating for 5 min at 100°C. With the latter treatment paxilline gave a dark blue spot and with the former a greenish grey spot. It was also revealed under long-wave UV light as a blue-grey fluorescent spot after the former but not the latter treatment[268]. Cockrum *et al.*[269] detected paxilline as spots showing a characteristic colour (purple-blue fading through yellow with a blue border to salmon pink) when sprayed with a 10% solution of cerium(IV) sulphate in concentrated phosphoric acid, diluted immediately before use with acetone (1:4).

3.8.1.2. Fumitremorgin–verruculogen group. Fumitremorgin A develops a slate grey-blue spot under visible light or a mustard-coloured spot under UV light immediately after spraying with 50% ethanolic sulphuric acid[150]. Fumitremorgin C develops a bright orange spot immediately after spraying with the same reagent and minimal heating[270]. Fumitremorgin B was detected under UV light and with the following spray reagents: (a) cerium(IV) sulphate (1% solution in 3 *M* sulphuric acid); (b) 2,4-dinitrophenylhydrazine (1 g), concentrated sulphuric acid (7.5 ml), ethanol (75 ml) and water (170 ml); (c) iron(III) chloride (3% solution in ethanol). Characteristic colours of fumitremorgin B were light purple at 254 nm, yellow-brown with reagent (a) immediately and also after heating for 10 min at 110°C, light orange with reagent (b) after heating and orange with reagent (c) after heating. The most sensitive

detection was at 254 nm with reagent (a). The lowest detectable amount of fumitremorgin B was 1 μg[18].

Verruculogen[271] and 15-acetoxyverruculogen[150] become visible immediately after spraying with 50% ethanolic sulphuric acid as slate-grey spots under visible light. When sprayed with a 10% solution of cerium(IV) sulphate in concentrated phosphoric acid, diluted immediately before use with acetone (1:4), verruculogen produced pinkish blue spots, fading to yellow-green[269]. Mycotoxin TR-2 produced a light-brown fluorescent spot after spraying with 50% ethanolic sulphuric acid and heating for 5 min at 100°C[150].

3.8.1.3. Penitrem group. Penitrem A was revealed as a blue spot after spraying with 50% ethanolic sulphuric acid and heating[272]. Penitrems A and B produce stable green spots after spraying with 1–2% iron(III) chloride in butanol and gentle heating[273]. Penitrems A–F give blue spots immediately after spraying with cerium(IV) sulphate, which become stable dark purple after heating[264].

3.8.1.4. Janthitrems. Unlike all previously discovered *Penicillium* tremorgenic toxins, the janthitrems are high fluorescent under long-wave UV light. The intense blue fluorescence in reminiscent of that of the aflatoxins. They can be also detected by spraying the TLC plates with Ehrlich reagent and exposure to HCl vapour for 5–10 min, ressulting in grey-green spots[274].

3.8.2. Applications

Aflatrem on silica gel G plates developed in chloroform–methanol (95:5) was characterized by an R_F value of about 0.8[265]. TLC was applied in monitoring the CC purification of paspalicine[267] and paxilline[268]. TLC of paspaline and paspalicine carried out on Kieselgel HF plates using chloroform as the solvent gave R_F values of 0.35 and 0.7, respectively. PLC was used to isolate and to purify paspalinine, paspalitrem A and paspalitrem B. The three tremorgens appeared on silica gel GH-R plates, developed in chloroform–acetone (93:7), at R_F 0.60 (paspalitrem A), 0.52 (paspalinine) and 0.20 (paspalitrem B)[71]. The R_F values of paxilline on silica gel GH-R[268] and on silica gel 60 F_{254}[269] were 0.75 and 0.52, respectively, when developed in toluene–ethyl acetate–formic acid (5:4:1).

The R_F values of fumitremorgin A on silica gel GH-R plates in chloroform–acetone (97:3) and toluene–ethyl acetate–formic acid (5:4:1) were 0.30 and 0.65, respectively[150]. In the latter system, the R_F value of fumitremorgin C was 0.55[270]. Using the same adsorbent, fumitremorgin B had an R_F value of 0.67 in diethyl ether and 0.38 in acetone–methylene chloride (5:95) as the solvent systems[275]. Mean R_F values of fumotremorgin B on Merck pre-coated silica gel F_{254} plates in six solvent systems were reported[18] as follows: 0.51 in chloroform–methanol (97:3); 0.36 in chloroform–acetone–*n*-hexane (7:2:1); 0.28 in chloroform–acetone (9:1); 0.14 in ethyl acetate–*n*-hexane (1:1); 0.71 in chloroform–acetone–2-propanol (85:15:20); and 0.30 in benzene–chloroform–acetone (45:40:15).

For verruculogen chromatographed on either MN-Kieselgel GH-R[271] and silica gel 60 F_{254}[269] plates developed in toluene–ethyl acetate–formic acid (5:4:1), R_F values of 0.65 and 0.48, respectively, have been reported.

PLC has been applied as a purification step for penitrem A[272]. TLC data for penitrem A have been reported by Wilson *et al.*[276], Ciegler[272] and Gorst-Allman and Steyn[18]. A procedure for the quantitative detection of penitrems (then called

tremortins) in agricultural products involved extraction with chloroform–methanol (2:1) followed by TLC and colorimetric assay[277]. Richard and Arp[278], using extraction and TLC analysis, reported on the occurrence of penitrem A in mouldy cream cheese.

Maes *et al.*[264] devised simple HPLC and TLC systems for the separation, identification and quantitation of the various 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 most efficient solvent systems for the TLC separation of the penitrems were found to be (a) *n*-hexane–ethyl acetate (70:30), (b) dichloromethane–acetone (85:15) and (c) benzene–acetone (85:15). In solvent system (a) penitrems B and F and penitrems C and D still overlapped, whereas penitrems C and E overlapped in system (b). The only system that gave a complete separation of all the penitrems was (c). The best results were obtained by developing the chromatogram twice in this solvent system. The order of decreasing R_F values for the penitrems was F, B, A, E, C and D[264]. R_F values of penitrems in these and other solvent systems are given in Table 22.

PLC has been used in the purification of the janthitrems but CC on Mallinckrodt Silica AR CC-7 silica gel was more successful[274]. The three major tremorgens have the following R_F values on silica gel 60 F_{254} pre-coated plates, developed in toluene–ethyl acetate–acetone (3:2:1): janthitrem A 0.61, janthitrem B 0.54 and janthitrem C 0.74.

TABLE 22

$R_F \times 100$ VALUES OF PENITREMS A–F

Data from refs. 264 and 279.

Penitrem	$R_F \times 100$*				
	A	*B*	*C*	*D*	*E*
A	16	49	37	32	
B	18	53	39	36	46
C	9	39	28	22	32
D	9	37	26	22	29
E	13	46	33	28	
F	18	55	42	36	50

* Solvent systems: A, *n*-hexane–ethyl acetate (70:30); B, dichloromethane–acetone (85:15); C, benzene–acetone (85:15); D, *n*-hexane–ethyl acetate (6:4); E, methylene chloride–ethyl acetate (9:1).

3.8.3. Territrems

Territrems A, B and C are tremorgenic metabolites of *Aspergillus terreus*[280,281]. The following solvent systems were used for the isolation and separation of territrems A and B[282]: (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). Detection is based on blue fluorescence of the territrems[280]. Territrem C exhibited light-blue fluorescence on silica gel 60 F_{254} pre-coated plates at R_F values of 0.25 in system (a), 0.43 in system (b) and 0.42 in system (c). The fluorescence intensity was quenched

when the concentration was higher than 20 μg per spot. The fluorescence intensity also gradually faded after development in system (a), but was enhanced and turned greenish in acidic solvent systems. PLC was also used to isolate the methylation product of territrem C [solvent system (a)] and its identity with territrem B was proved[281].

3.9. Epipolythiopiperazine-3,6-diones

This class of fungal metabolites consists of more than 50 known compounds with interesting biological activities. At present, economically the most important mycotoxins are the sporidesmins, which have a profound toxic effect on grazing animals. In addition, they also possess antimicrobial properties. Other members of the class are known as antibacterial, antifungal, antiviral or cytotoxic compounds. The isolation, separation, purification and chemical and biological properties of all members of the class known up to 1981 have been summarized recently[283].

PLC has been used in the preparation of sporidesmin H and J[283]. Hodges *et al.*[284] characterized sporidesmin A on silica gel F_{254} plates with benzene–ethyl acetate (4:1) and chloroform–methanol (19:1) as the solvent system, resulting in R_F values of 0.38 and 0.57, respectively. Detection involved spraying with 5% aqueous silver nitrate or viewing under reflected short-wave UV light. The melinacidin factors were differentiated from each other by paper chromatography (PC) using benzene–methanol–water (1:1:2) as the solvent system. In TLC, silica gel G plates were used with the solvent systems toluene–ethyl acetate (1:1 or 3:2) and methylene chloride–ethyl acetate (7:3). In PC and TLC, bioautography with *Bacillus subtilis* was employed[285].

Repeated PLC of fractions from a silica gel column afforded sirodesmins A, C and G[286]. Analytical TLC was performed on silica gel GF_{254} with toluene–ethyl acetate (1:2), chloroform–methanol–formic acid (95:4:1) and chloroform–methanol (95:5) as the solvent systems. The plates were sprayed with chromic acid and heated.

Elution of hyalodendrin from a silica gel column was monitored by TLC on silica gel GF_{254} and detection under UV light, giving an R_F value of 0.60. Hyalodendron tetrasulphide was obtained from an enriched CC fraction by PLC[287]. The latter compound also gave an R_F value of 0.5 on Kieselgel plates developed in benzene–acetone (9:1)[288].

Several epipolythiopiperazine-3,6-diones possess antibiotic activity. Silica gel plates were used in the TLC of the antibiotic A30641 and developed in benzene–ethyl acetate (1:1) or chloroform–acetone (3:2). Biological activity coincided with the spots located under UV light and with an iodine–azide spray reagent. Bioactivity was detected by bioautography employing *Neurospora crassa*[289]. Chromatographic separations of A26771A, A26771C and A26771E were monitored by TLC using a number of solvent systems. *Sarcina lutea* was used as the detection organism for A26771A and A26771C. These two metabolites could also be detected using silver nitrate spray reagent. A26771E was detected with phosphomolybdic acid spray reagent[290]. TLC has been used in monitoring the CC fractionation of aranotin and its derivatives[291]. Epicorazines A and B were purified by means of PLC[292].

3.10. Hydroxyanthraquinones

Many derivatives of anthraquinone are known as fungal or plant metabolites.

The important mycotoxins are luteoskyrin, rugulosin and emodin. Both analytical TLC and PLC have been used in studies of hydroxyanthraquinones. Silica gel is usually used as the adsorbent, sometimes impregnated with oxalic acid.

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[293]. Varna *et al.*[294] compared detection with methanolic solutions of magnesium acetate and copper acetate. The colour obtained with 0.2% copper acetate was more stable than that with magnesium acetate. The colour obtained with copper acetate increased for 2 h and then remained stable for 24 h. Ďuračková *et al.*[22] detected luteoskytin and rugulosin with *p*-anisaldehyde reagent. Spots of two hydroxyanthraquinones from *Trichoderma viride* on Silufol plates became intensely orange and violet, respectively, when the plate was exposed to ammonia fumes[295].

Analytical TLC was used to characterize emodin on silica-7GF plates developed in (a) toluene–ethyl acetate–formic acid (5:4:1) and (b) chloroform–acetone (83:7). Orange-red spots in visible light had R_F values of 0.80 in the former system and 0.45 in the latter[296]. Rugulosin on silica gel G plates impregnated with 0.5 *M* oxalic acid and developed in benzene–hexane (1:1) gave an R_F value of 0.25[297]. An R_F value of 0.40 was reported[298] for luteoskyrin chromatographed on silica gel G plates impregnated with 0.5 *M* oxalic acid using acetone–*n*-hexane–water (6:3:1.5) as the solvent system. TLC data for hydroxyanthraquinones from *Penicillium islandicum* are presented in Table 23.

The separation of skyrin, rugulosin and 2,2-dimethoxy-4a,4a-dehydrorugulosin (rugulin), a minor metabolite from *Penicillium rugulosum*, obtained by CC was monitored by TLC on Silufol plates developed in chloroform–ethyl acetate (2:1). Detection was carried out at 366 nm and by bioautography using *Bacillus subtilis*[299].

Two main anthraquinone pigments from a colour mutant of *Trichoderma viride*, 1,3,6,8-tetrahydroxyanthraquinone and 1-acetyl-2,4,5,7-tetrahydroxy-9,10-anthracenedione, were purified by PLC on Silufol plates using benzene–acetone (75:25) for repeated development[72]. The same PLC technique was used to isolate a minor pigment, the structure of which is under investigation[300].

3.11. Zearalenone

Zearalenone is an estrogenic mycotoxin produced by various species of *Fusarium* colonizing maize, oats, barley, wheat and sorghum. It often occurs with trichothecene toxins produced by fusaria. Analytical, quantitative and preparative TLC of zearalenone have been employed by a variety of workers. An excellent review of assay procedures for zearalenone was given by Shotwell[301]. Methods for the production, isolation, separation and purification of zearalenone, including chromatographic methods, have been reviewed recently[302]. Extraction, clean-up and TLC techniques for zearalenone are summarized here.

3.11.1. Extraction and clean-up

A versatile method for the isolation, detection and quantitation of zearalenone in maize and barley was developed by Mirocha *et al.*[303]. The method employs either TLC, GLC or GLC–mass spectrometry or their combinations. Two extractions and two clean-up procedures were used. Either extraction was carried out in a Soxhlet

TABLE 23

TLC DATA FOR HYDROXYANTHRAQUINONES OF *PENICILLIUM ISLANDICUM*

Adapted from ref. 293.

Pigment	$R_F \times 100$*			
	A	*B*	*C*	*D*
Islandicin	82		85	95
Chrysophanol	65(Y)**		84	
Iridoskyrin	58			90
Roseoskyrin	36			
Dianhydrorugulosin	25(Y)			
Catenarin		67	70	
Punicoskyrin		85		
Rhodoislandin A		75		
Rhodoislandin B		80		
Auroskyrin		75(Y)		
Emodin		65(Y)	64(Y)	28(Y)
Skyrin			51	25
Aurantioskyrin			54	
Dicatenarin			51	
Luteoskyrin			49(Y)	40(Y)
Deoxyluteoskyrin			43(Y)	
4α-Oxyluteoskyrin			38	
Rubroskyrin			26	18
Deoxyrubroskyrin			21	

* Solvent systems: A, benzene–hexane (1:1); B, benzene–acetone (20:1); C, benzene–acetone (4:1); D, acetone–*n*-hexane–water (5:5:3.5, upper layer).

** Y, yellow on spraying with magnesium sulphate reagent. The remaining pigments red or purple using the same detection.

apparatus or batch extraction was used, in both instances with ethyl acetate as the solvent. The extracts were concentrated nearly to dryness and re-dissolved in chloroform. In the first clean-up procedure, zearalenone was extracted from chloroform with 1 *M* NaOH and, after adjusting the pH of the aqueous phase to 9.5 with phosphoric acid, the toxin was re-extracted with chloroform. The extract was dried with sodium sulphate and concentrated nearly to dryness. The residue was dissolved in acetone and used for TLC or GLC analysis.

Several workers have used Eppley's extraction procedure[75], which proved to be efficient for zearalenone, aflatoxins and ochratoxins with chloroform–water (10:1) as the extraction mixture. The clean-up procedure consists in chromatography on a sodium sulphate–silica gel–sodium sulphate column with sequential elution with *n*-hexane followed by benzene, both washes being discarded. Zearalenone is eluted with benzene–acetone (95:5), aflatoxins with chloroform–ethanol (97:3) and ochratoxins with benzene–glacial acetic acid (9:1). A modification of Eppley's procedure was published by Ishii *et al.*[45].

A method was developed for the simultaneous extraction, separation and qualitative analysis of zearalenone and seven other mycotoxins in corn[78]. A flow chart of the extraction and separation procedure is depicted in Fig. 1. Zearalenone (to-

gether with sterigmatocystin) was present in the second fraction from the mini-column.

Gimeno[304] proposed another extraction and clean-up procedure. Ground samples are extracted with acetonitrile–4% KCl (9:1) in 0.1 *M* HCl and the extract is defatted with isooctane. The acetonitrile layer is filtered through anhydrous sodium sulphate and the sodium sulphate is washed repeatedly with chloroform, which is added to the filtrate already collected. After evaporation under vacuum, the residue is dissolved in chloroform and used for TLC analysis.

3.11.2. Adsorbents and solvent systems

Silica gel is mostly used as the adsorbent. A selection of solvent systems is presented in Table 24. It was found by Gimeno[304] that solvent systems containing formic acid were not satisfactory when Fast Violet B salt spray detection was used.

TABLE 24

SOLVENT SYSTEMS FOR TLC OF ZEARALENONE

Solvent system	*Sorbent*	$R_F \times 100$	*Ref.*
Chloroform–methanol (97:3)	Silica gel	40	18
Chloroform–acetone–*n*-hexane (7:2:1)		51	
Chloroform–acetone (9:1)		38	
Ethyl acetate–*n*-hexane (1:1)		41	
Chloroform–acetone–2-propanol (85:15:20)		71	
Benzene–chloroform–acetone (45:40:15)		44	
Benzene–methanol–acetic acid (24:2:1)	Silica gel G	57	22
Toluene–ethyl acetate–90% formic acid (6:3:1)		58	
Benzene–ethanol (95:5)		40	
Chloroform–methanol (4:1)		88	
Chloroform–methyl isobutyl ketone (4:1)		61	
Chloroform–acetone (9:1)		61	
Chloroform–acetic acid–diethyl ether (17:1:3)		64	
n-Butanol–acetic acid–water (4:1:4, upper layer)		84	
Toluene–ethyl acetate–88% formic acid (6:3:1)	Silica gel		78
Toluene–ethyl acetate–chloroform (2:1:1)	Silica gel	64	305
Toluene–ethyl acetate–90% formic acid (6:3:1)		66	
Diethyl ether–cyclohexane (3:1)		52	
Toluene–chloroform–acetone (3:15:2)	Silica gel	52	306

3.11.3. Detection

Zearalenone appears as a greenish blue fluorescent spot under short-wave (254 nm) UV light, but the fluorescence is less intense under long-wave UV light[75]. Fluorescence provides satisfactory detection but additional methods have been recommended. Two of them were used by Mirocha *et al.*[303] as follows. (a) The plate is sprayed with fresh 50% sulphuric acid in methanol and then heated for 10–20 min at 120°C. Zearalenone turns yellow and then brown. (b) The freshly developed and dried plate is sprayed with a freshly prepared solution of 1% aqueous $K_3Fe(CN)_6$–2% aqueous iron(III) chloride (1:1), followed by 2 *M* HCl. Zearalenone appears as an intense blue spot.

Pathre *et al.*[73] sprayed TLC plates with concentrated H_2SO_4 and heated for 10 min at 110°C, giving charred spots.

Gorst-Allman and Steyn[18] examined the developed plates under UV light at wavelengths of 254 and 366 nm and the following spray reagents gave characteristic colours with zearalenone after heating for 10 min at 110°C: (a) cerium(IV) sulphate (1% solution in 3 *M* sulphuric acid); (b) 2,4-dinitrophenylhydrazine (1 g), concentrated sulphuric acid (7.5 ml), ethanol (75 ml) and water (170 ml); and (c) iron(III) chloride (3% solution in ethanol). The characteristic colours of zearalenone were purple at 254 nm, white at 366 nm, yellow-brown with reagent (a), dark orange with reagent (b) and light purple with reagent (c). The most sensitive detection was at 254 nm and the detection limit was 1 μg.

Malayandi *et al.*[307] described the use of a bis-diazotized benzidine spray reagent, which forms a brick-red derivative with zearalenone on TLC plates (detection limit 2 ng).

Eppley *et al.*[308] and later Martin and Keen[309] used an aluminium chloride spray reagent to enhance the fluorescence of zearalenone.

Gimeno[304] sprayed plates with a 20% ethanolic solution of aluminium chloride and observed them under 366 and 254 nm UV light; zearalenone showed a bright blue spot fluorescence, especially under 366 nm UV light. The plate was then heated for 10 min at 105°C, cooled and sprayed again with aluminium chloride solution (not heated) and observed under 366 and 254 nm UV light; zearalenone showed a bright blue-violet fluorescence spot with improved contrast with respect to the background after this second spraying.

Scott *et al.*[310] sprayed plates with 0.7% aqueous Fast Violet B salt solution followed by pH 9.0 buffer solution (a mixture of 50 ml of 0.025 *M* sodium borate and 4.6 ml of 0.1 *M* HCl) until the silica gel layer appeared wet. After drying in an air current, zearalenone gave pink spots under visible light. The plates were then sprayed with 50% sulphuric acid and heated for 5 min at 120°C; zearalenone gave mauve spots under visible light. With this spray reagent, 5 ng of zearalenone on a TLC plate could be detected, compared with 2 ng of the toxin claimed by Malayandi *et al.*[307].

3.11.4. Applications

Ishii *et al.*[45] detected zearalenone in samples from CC using Kieselgel G TLC plates and the solvent systems benzene–acetic acid (9:1), benzene–acetone (9:1) and chloroform–ethanol (95:5) with detection at 254 nm.

A rapid and inexpensive method was developed by Swanson *et al.*[311] for the analysis 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 of the method was >80 ng/g for zearalenone and 200 ng/g for zearalenol. The analysis can be completed in less than 2 h.

A two-dimensional TLC method for the detection of zearalenone in animal feeds was developoed by Jemmali[312].

In a study of the biosynthesis of [^{14}C]zearalenone from the [1-^{14}C]acetate, the

radiochromatographic homogeneity of the isolated zearalenone was determined by TLC on silica gel G plates developed in chloroform–methanol (97:3). The plates were examined under long- and short-wave UV light for the presence of other fluorescent substances and then scraped off into scintillation vials. Only one band corresponding to zearalenone was radioactive[313].

In studies of the bioconversion of α-[^{14}C]zearalenol and β-[^{14}C]zearalenol into [^{14}C]zearalenone by Richardson *et al.*[314], the recovery from the silica gel columns was ascertained by TLC of acetone and methanol eluates.

In most instances, TLC of zearalenone was included in multi-toxin screening methods. Eppley[75] applied TLC in procedures developed for detecting zearalenone, aflatoxins and ochratoxin. Multi-mycotoxin TLC studies in which zearalenone was included have been published by Stoloff *et al.*[81], Wilson *et al.*[82], Joseffson and Möller[76], Coman *et al.*[84], Whidden *et al.*[78], Ilus *et al.*[85], Ďuračková *et al.*[22], Gorst-Allman and Steyn[18] and Lee *et al.*[21].

Lee *et al.*[21] also performed quantitations (see Section 3.1). Recently, Bennett *et al.*[315] described a method for the analysis of zearalenone and deoxynivalenol from cereal grains. After extraction, clean-up and separation by CC, zearalenone was quantitated by TLC and deoxynivalenol by GLC of the trimethylsilyl derivative.

A micro-method was used to extract [^{14}C]zearalenone and to separate it by means of TLC on silica gel using chloroform–ethanol (97:3). After detection under UV light, the zearalenone-containing area was scraped from the plate and used to count the radioactivity directly or after purification by GLC[46].

Jemmali[316] devised a quantitative TLC method for zearalenone, aflatoxins and ochratoxins based on flurodensitometry. The maximum sensitivity for zearalenone was 25–150 ng.

A rapid TLC quantitation of zearalenone in corn, sorghum and wheat was described by Gimeno[304]. After extraction and clean-up (see Section 3.11.1), zearalenone was separated by TLC and its identity was confirmed with nine solvent systems and two spray reagents. Zearalenone was then quantitated by the limit of detection method. The minimal detectable concentration was 140–160 μg/kg with aluminium chloride solution as the spray reagent and 85–110 μg/kg with Fast Violet B salt as the spray reagent.

Pathre *et al.*[73] used PLC with light petroleum–diethyl ether–glacial acetic acid (70:30:2) as the solvent system to provide *trans*-zearalenone free from *cis*-zearalenone and other detectable impurities. To determine the chromatographic purity, zearalenone was dissolved in toluene to give a *ca.* 4 μg/μl solution of which 5 μl were spotted and developed in chloroform–methanol (97:3).

PLC was also used by Thouvenot and Morfin[317] to obtain zearalenone and zearalanone (internal standard) for GLC.

3.12. Citrinin

TLC has been used by many workers to characterize, identify and quantitate citrinin in various materials and also in preparative work. Chromatographic methods, including TLC, used in studies of this mycotoxin and antibiotic were reviewed recently[318].

3.12.1. Extraction and clean-up

Extraction solvents and clean-up techniques for citrinin from various materials are given in Table 25. Chloroform, ethyl or butyl acetate and methanol are the most commonly used solvents for extraction. Originally, precipitation from culture filtrates with concentrated hydrochloric acid was successfully applied[319]. In clean-up procedures, silica gel CC and partition at different pH values between aqueous and organic phases have been employed.

TABLE 25

EXTRACTION AND CLEAN-UP OF CITRININ

Material analysed	*Extraction* solvent(s)*	*Clean-up procedure**	*Ref.*
Culture filtrate	Precipitation with conc. HCl (12.5 ml/l filtrate)	Crude CIT dissolved in $CHCl_3$, evaporation, crystallization from EtOH	319
Culture filtrate	EtOAc at pH 2.5	Partition into buffer pH 8.5, re-extraction with $CHCl_3$ at pH 2.5, evaporation, partition between CCl_4 and $(CH_2OH)_2$, CCl_4 phase evaporated, crystallization from Me_2CO	320
Culture filtrate	$CHCl_3$ followed by EtOAc from conc. filtrate	CC silica gel, elution with $CHCl_3$, partition into 0.2 *M* $NaHCO_3$, acidification, crystallization of precipitate from EtOH	321
Culture filtrate	BuOAc at pH 2.5	Evaporation, dissolution in C_6H_6, partition into sat. aq. $KHCO_3$, re-extraction with C_6H_6 at pH 3.8, evaporation, dissolution in EtOH	17, 322
Corn	$CHCl_3$	Extract rinsed with dil. HCl and H_2O, partition into 0.1 *M* $NaHCO_3$, re-extraction with $CHCl_3$ at pH 2.5 and concentration, partition into 0.1 *M* $NaHCO_3$, precipitation at pH 2.5	323
Culture filtrate	$CHCl_3$	Concentration and TLC	324
Culture filtrate	$CHCl_3$ at pH 1.5	Evaporation, dissolution in $CHCl_3$ or 0.1 *M* buffer (pH 10)	325
Static culture	EtOAc	Concentration	321
Culture filtrates and mycelia	EtOAc (filtrate) Hot EtOAc (mycelium)	Extract passed through Na_2SO_4 and concentrated under N_2	326
Tomatoes	MeOH and Hex	Centrifugation, 5 *M* H_2SO_4 added, partition into $CHCl_3$, evaporation, dissolution in $CHCl_3$	326
Maize	MeOH–$CHCl_3$ (1:1)	Filtration, evaporation, partition, Hex–90% MeOH (1:1), MeOH layer evaporated, partition $CHCl_3$–H_2O (1:1), $CHCl_3$ layer extracted with sat. $NaHCO_3$, re-extraction with $CHCl_3$ at pH 2, concentration	18

* Abbreviations: CIT, citrinin; EtOAc, ethyl acetate; $CHCl_3$, chloroform; CCl_4, carbon tetrachloride; $(CH_2OH)_2$, ethylene glycol; Me_2CO, acetone; BuOAc, butyl acetate; C_6H_6, benzene; EtOH, ethanol; MeOH, methanol; Hex, *n*-hexane.

3.12.2. Adsorbents and solvent systems

Silica gel is the most often used adsorbent in the TLC of citrinin. Better results are obtained on oxalic acid pre-treated plates. Silufol plates were impregnated with

0.25 *M* oxalic acid in methanol by developing the plates in the solution. The plates were then dried in air and spotted[17]. Marti *et al.*[327] dipped inactivated silica gel 60 in 10% oxalic acid. Another technique used[18] was as follows. The plates were immersed in a 10% solution of oxalic acid in methanol for 2 min. After heating at 110°C for 2 min and cooling, the plates were immediately spotted and developed. Stubblefield[3] used TLC plates impregnated with Na_2EDTA.

A variety of solvent systems have been used by various workers; some of them are mentioned under Applications.

3.12.3. Detection

Citrinin can be observed on chromatograms under UV light owing to its yellow fluorescence. In addition, several spray reagents have been employed. Curtis *et al.*[328] used a freshly prepared solution of a stabilized diazonium salt of *o*-dianisidine [0.05 g in 40 ml of methanol–water (1:1)], followed by methanol–aqueous ammonia (sp.gr. 0.88) (1:1) to promote the coupling reaction. Citrinin produced a pale pink colour. Improved colour resolution was obtained if the TLC plates were allowed to dry overnight before spraying. After spraying with a 3% solution of iron(III) chloride in methanol, citrinin is detected as a brown spot[17]. Citrinin was also detected with *p*-anisaldehyde spray reagent[22,24]. Gorst-Allman and Steyn[18] detected citrinin and other acidic mycotoxins under short- and long-wave UV light or by spraying with cerium(IV) sulphate, 2,4-dinitrophenylhydrazine and iron(III) chloride reagents. Marti *et al.*[327] obtained a detection limit of 20 ng per spot of citrinin by measuring the yellow-green fluorescence under UV light. Stubblefield[3] detected 10 ng per spot on Na_2EDTA-impregnated plates.

3.12.4. Applications

Various analytical uses of TLC of citrinin have been published. Curtis *et al.*[328] examined phenolic metabolites (including citrinin) from mutants of *Aspergillus flavus* using Kieselgel G plates and benzene–methanol–acetic acid (10.2:1) as the solvent system. Betina and Bínovská[322] monitored the production of citrinin in the course of submerged fermentation of *Penicillium janthinellum* (originally believed to be *P. notatum*). The cleaned-up samples (see Table 25, ref. 322) were spotted on oxalic acid-impregnated Silufol plates. The most suitable solvent systems were benzene–methanol–acetic acid (10.2:1) and benzene–methanol (95:5).

Phillips *et al.*[329], in producing [^{14}C]citrinin by *P. citrinum*, isolated and purified the toxin by the method of Davis *et al.*[319]. The identity and purity of the compound were established by TLC using diethyl ether–hexane–formic acid (75:25:1) and ethyl acetate–acetone–0.1 *M* (40:40:20) as the solvent systems. A single peak of radioactivity appeared, which co-chromatographed with authentic, chemically pure citrinin.

The production of citrinin by *P. citrinum* in corn was monitored by TLC on silica gel F_{254} using the solvent system chloroform–methanol (75:25) and detection under 366 nm UV light[323].

Harwig *et al.*[326] detected citrinin and other mycotoxins in extracts from *Penicillium* spp. cultures isolated from decaying tomato fruits and also in tomato extracts using silica gel plates and the solvent systems toluene–ethyl acetate–formic acid (5:4:1) and ethyl acetate–acetone–water (5:5:2). The rapid TLC determination of citrinin, patulin and aflatoxin in apples and pears and their juices and jams was reported by Gimeno and Martins[87].

Citrinin has been included in multi-mycotoxin analyses by Scott and co-workers[330,331], Wilson *et al.*[82], Gimeno and Martins[87], Ďuračková *et al.*[22], Gorst-Allman and Steyn[18] and Lee *et al.*[21].

Several TLC quantitations of citrinin have been published. Wu *et al.*[324] separated citrinin-containing extracts by TLC on Adsorbosil-1 using toluene–ethyl acetate–formic acid (6:3:1) as the developing solvent and fluorodensitometry. Damodaran *et al.*[325] reported a procedure for the isolation and quantitation of citrinin in culture filtrates. Cleaned-up samples were spotted on to silica gel plates and developed in toluene–ethyl acetate–formic acid (5:4:1). The fluorescent portions were scraped off, citrinin was extracted with carbonate–hydrogen carbonate buffer (pH 10) and the determinations were carried out using Folin's reagent. Another quantitation of citrinin was reported by Ciegler *et al.*[321].

Scott *et al.*[330] separated citrinin and ochratoxin A produced by *Penicillium viridicatum* by PLC of concentrated chloroform extracts on acidic alumina (Woelm) developed in chloroform–acetone (4:1). PLC of citrinin from extracts of cultivation media after growth of citrinin-producing penicillia was carried out on silica gel plates and the isolated product was identified by mass spectrometry.

3.13. Miscellaneous mycotoxins

In this section, the TLC of secalonic acids, cyclopiazonic acid, PR toxin, roquefortine, xanthomegnin, viomellein and naphthopyrones is reviewed.

3.13.1. Secalonic acids

The secalonic acids are a group of closely related fungal metabolites, xanthone dimers with identical molecular weights and molecular formulae differing in their stereochemistry. They have been isolated from the sclerotia of *Claviceps purpurea* and more recently from *Aspergillus ochraceus* (secalonic acid A), *A. aculeatus* (secalonic acids D and F), *Pyrenochaeta terrestris* (secalonic acids A, B and G) and *Penicillium oxalicum* (secalonic acid D)[150]. Secalonic acid D is the most studied member of this group of ergochromes.

On TLC plates, secalonic acids can be detected by quenching fluorescence[332] or by spraying with cerium(IV) sulphate reagent[333], iron(III) chloride[20,22] or *p*-anisaldehyde reagent[22]. R_F values of secalonic acids in a variety of solvent systems are given in Table 26. Secalonic acid D was included in a TLC separation and detection of eleven mycotoxins[25] and in a systematic analysis of 37 mycotoxins[22]. Ciegler *et al.*[332] quantitated the toxin on pre-coated silica gel 60 F_{254} plates using benzene–ethyl acetate–formic acid (100:40:10) as the solvent system.

Methods used for the production, isolation, separation, purification and detection of secalonic acid D have been summarized elsewhere[335]. TLC and HPLC techniques were also included.

3.13.2. α-Cyclopiazonic acid

Of the known tetramic acids, cyclopiazonic acid is the most studied.

Extraction of *Penicillium cyclopium*-contaminated maize was carried out as follows[18]. The mouldy meal was extracted with methanol–chloroform (1:1), the mixture filtered and the filtrate evaporated to dryness. The residue was partitioned between *n*-hexane and 90% methanol (1:1) and the methanol layer was evaporated to

TABLE 26

$R_F \times 100$ VALUES FOR SECALONIC ACIDS

*Sorbent**	*Solvent system**	*Secalonic acid*				*Ref.*
		A	*B*	*D*	*F*	
OA-treated silica gel	$CHCl_3$–MP (9:1)	23	46	23		333, 334
TA-treated silica gel	$CHCl_3$–Pen			17	29	20
Silufol	C_6H_6–MeOH–HOAc (24:2:1)			28		22
	Tol–EtOAc–FA (6:3:1)			32		
	$CHCl_3$–MeOH (4:1)			68		

* Abbreviations: OA, oxalic acid; TA, tartaric acid; $CHCl_3$, chloroform; MP, 4-methyl-2-pentanone; C_6H_6, benzene; MeOH, methanol; HOAc, acetic acid; Tol, toluene; EtOAc, ethyl acetate; FA, 90% formic acid; Pen, 2-pentanone.

dryness. The solid was partitioned between chloroform and water (1:1) and the chloroform layer was extracted with saturated sodium hydrogen carbonate solution. The aqeous layer was acidified to pH 2 and extracted with chloroform. The chloroform extract was concentrated and contained cyclopiazonic acid.

LeBars[336] extracted cheese samples with azeotropic chloroform–methanol. The filtered and evaporated extract was dissolved in acetone–water–lead acetate solution. A saturated solution of sodium sulphate and Celite were added and the suspension was filtered. The filtrate was defatted by partition against hexane, acidified to pH 3 and extracted with chloroform. The centrifuged and dried extract was evaporated to dryness and dissolved in the minimum volume of chloroform for TLC.

Silica gel TLC plates have been impregnated with oxalic acid or tartaric acid[18,25,336]. A variety of solvent systems can be used, *e.g.*, (a) chloroform–methyl isobutyl ketone (4:1)[25], (b) chloroform–methanol (98:2)[18], (c) chloroform–acetone (9:1)[18], (d) ethyl acetate–2-propanol–ammonia solution (20:15:10)[336], (e) chloroform–acetone (95:5)[336], or (f) toluene–ethyl acetate–formic acid (5:4:1)[336]. Systems (b) and (c) are recommended for the separation of acidic mycotoxins (see Table 3). Systems (a), (d) and (f) were used by LeBars[336] for the quantitation of cyclopiazonic acid from commercial cheese samples. Quantitation was accomplished on TLC plates after spraying with Ehrlich reagent by comparison with a standard range of concentrations of cyclopiazonic acid (10, 20, 30 and 40 ng per 10 μl spots) in chloroform. The detection limit of cyclopiazonic acid was 0.02 μg/g.

Ohmomo *et al.*[40] used two-dimensional TLC on silica gel with ethyl acetate–2-propanol–concentrated ammonia solution (20:15:10) and chloroform–concentrated acetic acid (10:1) as solvent systems. The indole derivatives were quantitated on TLC plates with a chromatogram scanner after treating the plates with Ehrlich reagent.

In addition to Ehrlich reagent, cyclopiazonic acid can be detected with either iron(III) chloride or concentrated sulphuric acid and heating[25]. Other detection methods were reported by Gorst-Allman and Steyn[18].

The most important data concerning the production, isolation, separation and purification of cyclopiazonic acid and related toxins were summarized by Cole[337].

3.13.3. PR toxin and roquefortine

PR toxin and roquefortine are metabolites of *Penicillium roqueforti* and have been isolated from isolates from blue cheese and other sources. The production, isolation and chromatographic techniques for PR toxin were reviewed recently by Scott[338]. Extraction and clean-up procedures for PR toxin from culture filtrates have been reported. Still[339] extracted the toxin with chloroform and Scott *et al.*[340] used ethyl acetate. Two basic procedures and their modifications for extraction and clean-up from blue cheese were published by Scott and Kanhere[341]. In the first procedure, the sample was extracted with a mixture of methanol–water and hexane and centrifuged. After filtration, the methanol–water layer was extracted with chloroform, the extract was evaporated and the residue was dissolved in chloroform and immediately analysed by TLC for PR toxin and/or PR imine. In the second procedure, cheese was blended with ethyl acetate and centrifuged. The extract was evaporated and partitioned between hexane and acetonitrile, the acetonitrile layer was evaporated and the residue was dissolved in chloroform for immediate TLC analysis.

Solvent systems for the TLC of PR toxin on silica gel include chloroform–methanol (96:4), chloroform–2-propanol (10:1 or 4:1), toluene–ethyl acetate–formic acid (5:4:1 or 6:3:1) and toluene–ethyl acetate (30:70) saturated with water[341–343].

PR toxin can be detected by its green fluorescence under long-wave UV light following exposure of the chromatogram to short-wave UV light for about 0.5 min[341,342]. After spraying the chromatograms with 50% sulphuric acid, the toxin appears as a yellow spot[342]. The toxin was quantitated *in situ* by fluorodensitometry after spraying the plates with 1% *p*-dimethylaminobenzaldehyde in concentrated HCl–acetone (1:10) or in ethanol with subsequent exposure to HCl fumes for 10 min; the latter is the preferred method[343].

PLC on silica gel 7GF developed with toluene–ethyl acetate–formic acid (5:4:1 or 6:3:1) was effective in the purification of PR toxin[340].

Roquefortine is present mainly in the mycelium of *P. roqueforti*. Extraction and clean-up procedures were summarized by Scott[344]. CC procedures for the separation of roquefortine from other alkaloids isolated from *P. roqueforti* or other penicillia have also been described. It has been found that roquefortine could be eluted from silica gels with chloroform–methanol-25% ammonia solution (70:10:0.5)[345], from basic alumina with chloroform–ethanol (95:5)[346] and from activity grade III–IV alumina with chloroform–ethanol (100:1)[345]. Column fractions were monitored by TLC[340].

Solvent systems that have been used with silica gel TLC plates include chloroform–methanol–28% ammonia solution (90:10:1), chloroform–methanol (9:1), chloroform–re-distilled diethylamine (8:2), chloroform–ethanol (10:1.5), acetone–chloroform (3:2) and benzene–methanol (93:7)[347,348].

Roquefortine on TLC plates can be detected as a blue-grey spot after spraying with 50% sulphuric acid and heating at 110°C for 10 min[182]. Other spray reagents are Pauli reagent[349], Van Urk reagent[345] and Ehrlich reagent[346,348].

3.13.4. Xanthomegnin and viomellein

Xanthomegnin and viomellein are toxic metabolites of a number of fungi that include *Aspergillus* and *Penicillium* species; these microbes are of particular interest because they are routinely implicated in toxin contamination of foods and feeds.

Wall and Lillehoj[350] used the following extraction and clean-up procedure. A strain of *A. ochraceus* was cultivated on rice for 10 days. The mouldy rice was extracted by suspension in methylene chloride and grinding. The extract was filtered and the solvent removed by vacuum evaporation. The crude oil was sequentially extracted three times with acetonitrile and the acetonitrile solutions were used for chromatography.

TLC methods for the detection of xanthomegnin and viomellein utilize silica gel plates and benzene–methanol–acetic acid (18:1:1) or toluene–ethyl acetate–formic acid (6:3:1) as the solvents[351].

After standing for 6 h, the spots of xanthomegnin turns from yellow to orange and that of viomellein turns from yellowish green to yellowish brown. Exposure to ammonia fumes turns the pigments from yellow to purple[351]. The detection limits were 0.1 μg for xanthomegnin and 0.3 μg for viomellein.

Wall and Lillehoj[350] prepared standards of the two toxins by PLC on silica gel plates that were developed in benzene–methanol–acetic acid (18:1:1). Appropriate bands were scraped off the plates and the compounds were eluted with methylene chloride. The solvent was removed under a stream of nitrogen and standards were stored as dry films in a freezer. Purity was determined by TLC and HPLC comparisons with reference compounds.

3.13.5. Naphtho-γ-pyrones

Monomeric and dimeric naphtho-γ-pyrones have been isolated from the mycelium of *Aspergillus niger* by several groups of workers. Ehrlich *et al.*[352] subcultured an *A. niger* isolate on rice, corn, cottonseed and two liquid media. After incubation, the *A. niger* culture (in the case of culture on liquid media, the mycelial mat) was extracted with methylene chloride. The solvent was evaporated and the residual red paste was treated with 9 volumes of cold hexane and kept at 5°C overnight. The red precipitate was collected, dissolved in methylene chloride and filtered. Samples were examined by HPTLC.

HPTLC was carried out on LHP-KF plates (Whatman) and developed with benzene–ethyl acetate–formic acid (10:4:1). Components were identified by their colour, fluorescence under long-wave UV light and colour after spraying with Gibbs reagent. HPTLC showed that the mixture contained more than 18 components, but only the material migrating at R_F 0.5–0.8 contained naphtho-γ-pyrones. The results are presented in Table 27.

4. CONCLUSION

TLC is by far the most widely used chromatographic technique applied to mycotoxins owing to its relative simple, fast and inexpensive character.

As in most instances the mycotoxins to be analysed (or purified) by means of TLC are present in contaminated samples, they must be extracted and cleaned up prior to TLC if reliable results are to be obtained. Extraction procedures, reviewed

TABLE 27

HPTLC DATA FOR NAPHTHOPYRONES

Adapted from ref. 352.

Naphthopyrone	$R_F \times 100$*	*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

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

here, include extractions of mycotoxins from feeds and foodstuffs, cultivation media and/or mycelia of toxigenic fungi. Extraction solvents include chloroform, methylene chloride, ethyl acetate, acetone, acetonitrile, methanol and their combinations.

Clean-up procedures include CC (mostly on silica gel columns), gel-permeation chromatography, liquid–liquid partition and, in a few instances, precipitation techniques. In these procedures, contaminating lipids, fatty acids, proteins and various pigments have to be removed from the mycotoxin samples.

Silica gel is the most commonly used adsorbent in the TLC of mycotoxins. With acidic mycotoxins, better results have been obtained when the silica gel plates were pre-treated with oxalic acid, tartaric acid or EDTA. Chemically bonded reversed-phase layers have been used in special applications.

The variety of solvent systems used is enormous. The most often used solvents combined in various ratios include chloroform, benzene, toluene, ethyl acetate, methylene chloride, acetone, methanol, formic acid and acetic acid.

The detection techniques vary with the mycotoxins to be detected. Coloured substances are examined under visible light, fluorescent toxins are revealed under short- and/or long-wave UV light and colourless and non-fluorescent compounds can be detected by means of appropriate spray reagents producing colours or fluorescence. Bioautographic detection has also been described, using microbial cultures or *Artemia salina* larvae as sensitive test organisms. The detection reagents vary according to the structures of the mycotoxins.

In addition to the classical one-dimensional TLC, two-dimensional chromatography and HPTLC have been used by various workers. With HPTLC and in quantitations, TLC becomes more expensive owing to the need for densitometers and spectrophotometers. Comparisons of sample spots with spots of known concentrations of standards give only semi-quantitative results. Flame photometry has been used in the quantitation of moniliformin, which occurs naturally as the Na^+ or K^+ salt[65].

PLC has been used in the initial preparation of several mycotoxins belonging to the cytochalasans, hydroxyanthraquinones, indole-derived tremorgens, zearalenone, etc.

Applications of TLC in multi-mycotoxin analyses have been reviewed. The reasons for the simultaneous detection of a number of mycotoxins in natural samples have been explained and documented by the most important results.

The reviewed applications of the TLC of aflatoxins, ochratoxins, rubratoxins, small lactones, trichothecenes, cytochalasans, tremorgenic mycotoxins, epipolythiopiperazine-3,6-diones, hydroxyanthraquinones, zearalenone, citrinin, secalonic acids, cyclopiazonic acid, PR toxin, roquefortine, xanthomegnin, viomellein and naphtho-γ-pyrones emphasize the importance of TLC in the relatively young field of mycotoxicology.

5. SUMMARY

TLC has become an extremely powerful, rapid and in most instances inexpensive separation technique in mycotoxicology. This review presents achievements of its applications in this field. General technical aspects of the TLC of mycotoxins that are discussed include extraction and clean-up procedures, adsorbents and solvent systems, detection methods, two-dimensional TLC, high-performance TLC (HPTLC), quantitation and preparative TLC (PLC). Special applications of TLC deal with multi-mycotoxin analyses and with structurally related or individual mycotoxins (aflatoxins, sterigmatocystins, versicolorins, ochratoxins, rubratoxins, patulin, penicillic acid, mycophenolic acid, butenolide, citreoviridin, trichothecenes, cytochalasans, tremorgenic toxins, epipolythiopiperazine-3,6-diones, hydroxyanthraquinones, zearalenone, citrinin, secalonic acids, cyclopiazonic acid, PR toxin, roquefortine, xanthomegnin, viomellein and naphtho-γ-pyrones).

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RECENT ADVANCES IN THE ANALYSIS OF POLYCHLORINATED BIPHENYLS IN ENVIRONMENTAL AND BIOLOGICAL MEDIA

E. D. PELLIZZARI*, M. A. MOSELEY and S. D. COOPER
Analytical and Chemical Sciences, Research Triangle Institute, Research Triangle Park, NC 27709 (U.S.A.)
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CONTENTS

1. INTRODUCTION

Polychlorinated biphenyls (PCBs), terphenyls (PCTs) and quadphenyls (PCQs) had been manufactured as commercial mixtures [Aroclors (U.S.A.), Clophen (F.R.G.) and Kanoclor (Japan)] for more than four decades before they were banned in the U.S.A. in 1976. Because of their long-term heavy usage as dielectric fluids, in transformers and capacitors, in hydraulic fluids, fire retardants, etc., and their persistence, PCBs have permeated into practically every environmental medium throughout the world[1]. Because of their ubiquity, PCBs have received considerable attention in recent years in the area of analytical measurements and toxicology.

Since environmental pollution by PCBs first became apparent, many purification procedures, gas chromatographic (GC) systems, detectors and methods for quantifying GC responses have been reported for determining the contamination of environmental and biological samples[1–11]. However, until recent advances in analytical instrumentation, most of the conventional methodology yielded only semi-

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quantitative data with virtually no qualitative or quantitative information on the PCB isomer composition in environmental or biological samples. The analytical problems are complicated by the fact that there are 209 individual chlorinated biphenyl isomers[12] spanning 10 homologous series (1–10 chlorine atoms per biphenyl). A commercial mixture itself (*e.g.*, Aroclor) may contain as many as 60 chlorobiphenyl isomers[12].

The conventional quantification method is based on packed column GC and reports the PCB content in environmental and biological samples by referring it to a particular Aroclor mixture (*e.g.*, Aroclor 1242, 1254 or 1260). The GC detector is first calibrated using commercial Aroclor mixtures, and then the appropriate commercial Aroclor profile is matched to the sample profile. Using the Webb and McCall technique or a variation of it the total PCB content is calculated[13]. This approach, however, is potentially subject to error.

Environmental contamination may be derived from Aroclor mixtures or from incidentally generated chlorobiphenyls whose profiles do not resemble Aroclor patterns[14]. In either instance, the conventional quantification method is inadequate. As time passes, the "Aroclor patterns" undergo alteration in the environment, as selective weathering and biotransformation and bioaccumulation in living organisms perturb these patterns. The problem of Aroclor pattern dissimilarity will be further aggravated as PCB monitoring continues into the future.

The quantification problem is further exacerbated by the production of chlorobiphenyl isomers in chemical process streams, incineration, etc., via chemical or pyrolysis reactions that are not the same as the chemical reactions once used for the manufacture of Aroclors[14]. As incidental generation does not necessarily produce any fixed pattern of chlorobiphenyl isomers, the analyst cannot identify and quantify chlorobiphenyls based on pattern recognition from the packed column gas chromatogram. Hence the qualitative and quantitative analysis of PCBs in environmental and biological samples and in samples from process streams involves the difficult issue of having to detect, identify and quantify each individual isomer.

The accuracy of PCB determination in environmental, biological and process stream samples is, in addition to the reasons given above, also related to the degree of variability in the analytical response of each chlorobiphenyl isomer to the detector employed[1,15–18].

Recent theoretical and experimental studies have indicated that the biological properties of chlorobiphenyl isomers are significantly influenced by the number and biphenyl ring position of the chlorine atoms[19–23]. Because the toxicological properties vary considerably among isomers[19,20,23], more sophisticated methods capable of yielding information about the chemical composition at the isomeric level are required in order to be able precisely to investigate and assess the toxicological consequences of PCB pollution. Obviously, the ideal analytical procedure is one that identifies and measures each individual chlorobiphenyl isomer.

This review examines the most recent advances during the period from 1971 to the present that strive to meet the objective of individual chlorobiphenyl isomer identification and quantification. The areas reviewed are: (a) development of high-resolution GC, (b) improvement in detection systems; (c) availability of chlorobiphenyl isomer standards; and (d) application of state-of-the-art methods to the analysis of environmental and biological samples. It is beyond the scope of this review to include sampling techniques and isolation and purification methods for PCBs.

2. HIGH-RESOLUTION GAS CHROMATOGRAPHIC TECHNOLOGY

In order to analyze for 209 individual chlorobiphenyl isomers, the use of high-resolution chromatographic techniques is mandatory. In this respect, GC currently is far superior to high-resolution thin-layer and high-resolution liquid chromatography for PCB analysis. The relative merits of packed column (low resolution) *versus* capillary column (high resolution) GC analysis of PCBs have been succinctly reported by Mullin and Filkins[23]. Their work provided the impetus for further research into the determination of an optimum capillary column(s) for the analysis of individual isomers of PCBs, polybrominated biphenyls, pesticides and other halogenated hydrocarbons in biological and environmental samples.

2.1. Development of capillary columns

In addition to early research using metal capillaries[24], Mullin *et al.*[25], Bush, *et al.*[26], Nygren and Mattson[27] and Moseley and co-workers[28–30] have more recently reported on extensive investigations into five variables that affect glass and fused-silica capillary performance for the analysis of PCBs[28–30]. The variables studied were: (a) material of construction; (b) pre-treatment/deactivation procedures; (c) stationary phase type; (d) stationary phase film thickness; and (e) capillary dimensions. Evaluation criteria employed were: (a) separation number (Trennzahl, *TZ*) between 2,2′,4′,5-tetrachlorobiphenyl and 2,2′,4,4′,6,6′-hexachlorobiphenyl; (b) resolution between 2,2′,5,5′-tetrachlorobiphenyl and 2,2′,4′,5-tetrachlorobiphenyl; (c) height equivalent to an effective theoretical plate (HEETP) for 2,2′,4′, 5-tetrachlorobiphenyl; (d) adsorption characteristics; (e) thermal stability; and (f) general performance on an Aroclor 1242–1260 mixture (1:1, w/w).

Capillaries were made from Pyrex and soft glass, quartz, vitreous silica and fused-silica materials[28–31]. The objectives of these studies were to determine the most suitable material for construction, pre-treatment/deactivation and amenability to coating of a thin, uniform, stable film of stationary phase. It was recognized that these variables were not independent of each other. Except for the hydrocarbon $C_{87}H_{176}$ (C_{87}), all stationary phases evaluated could be successfully coated on the flexible silica capillaries[30]. Preference for the silica capillaries was attributed to their flexible nature, facilitating their assembly into gas chromatographs even by the novice. Thus, a major impediment to the use of capillaries by the analytical community had been removed.

Many pre-treatment/deactivation procedures were investigated as the raw construction material was not suitable for coating directly with stationary phase[28–30]. The procedures studied were: (a) barium carbonate treatment; (b) Carbowax 20M; (c) Superox-4; (d) HCl etching; (e) persilylation; and (f) thermally induced polysiloxane (SE-52 or OV-101) bonding. The preferred methods were polysiloxane deactivation on silica and Pyrex and persilylation on Pyrex[28–30].

Although glass capillaries coated with C_{87} stationary phase provide excellent resolution of individual chlorobiphenyl isomers, use of C_{87} is limited by two factors —its temperature stability (*ca.* 220°C) and the inability to coat successfully a uniform film on silica. Because of the upper temperature limit, the C_{87}-coated capillaries have been inadequate for the analysis of PCTs, PCQ, PBBs and sample extracts that contain many impurities (*e.g.*,from fish). For these reasons, alternative stationary phases were sought.

There have been several reports on different stationary phases used in capillary GC for the analysis of PCBs[16,25,31–34]; however, until recently there was no concerted effort to evaluate stationary phases systematically[28–30]. Using the previously mentioned criteria, a matrix study design was performed in concert and while making a comparison of phase selectivity (McReynolds constants) to guide the overall investigation toward the "optimum" phase. Among the phases evaluated were C_{87}, SE-54, SP-2100, QF-1, SE-52, OV-101, Dexsil 410, Apiezon M and Apiezon L. Early results predicted (from McReynolds constants) that Apiezon M would most closely mimic the excellent separation pattern of C_{87}[30]. In fact, this correspondence was demonstrated experimentally[28]. Several advantages of Apiezon M will be discussed later. Finally, a complimentary stationary phase to Apiezon M was sought so that one phase could serve as a primary analytical column and the other as a reference column. SE-54-coated capillaries provided a significantly different resolution pattern to Apiezon M[28,29].

It has long been recognized that the thinner the stationary film the higher is the mass transfer coefficient. The sample capacity, however, decreases. Research has been performed to determine a film thickness that possessed a very high mass transfer coefficient (as measured by HEETP), adequate sample capacity for the detection system to be employed and stability to long periods of usage[28–30]. Stability (to solvent and thermal shock) was imparted by immobilizing the film on the silica surface by a cross-linking/surface bonding reaction[29].

Two methods were reported that immobilized phases to silica[29]. One utilized a thermal technique (using SE-52), the other a dicumyl peroxide reaction (using SE-54). The first method easily produced the desired 0.025 μm film capillaries for use with electron-capture and negative ion chemical ionization (NICI) mass spectrometric (MS) detection of PCBs. These capillaries exhibited a bleed of 0.7 pA (flame-ionization detection) at 320°C[29]. The other method yielded thicker films (0.1 μm) for higher capacity for use with electron-impact MS.

An Apiezon M phase immobilized to silica has not been reported.

Investigations on the resolution of PCBs indicated that a silica capillary, polysiloxane deactivated, 50 m × 0.2 mm I.D. in length, coated with Apiezon M (0.025 μm film) or SE-54 (0.025 μm film) was preferable[28–30]. An Apiezon M-coated silica capillary allowed the elution of PCBs to occur at temperatures approximately 30–40°C lower than other stationary phases[28–30] and was thermally stable to up to *ca.* 285°C.

2.2. Comparison of packed and capillary column profiles

The inherent differences between low- and high-resolution GC columns are exemplified by Figs. 1 and 2. Fig. 1 compares the profiles for a standard mixture of Aroclor 1242 and 1260. Fig. 2 depicts the profiles for a stack (stationary source) sample[35]. Both high-resolution analyses were performed on SE-54 fused-silica capillaries.

It is evident in Fig. 2 that the low-resolution profile does not readily resemble a commercial Aroclor mixture. Therefore, it would be highly inaccurate to quantify the low-resolution profile using a commercial Aroclor standard for instrument calibration and the Webb and McCall method[13].

Several examples demonstrating the performance of Apiezon M silica capillaries will be discussed later.

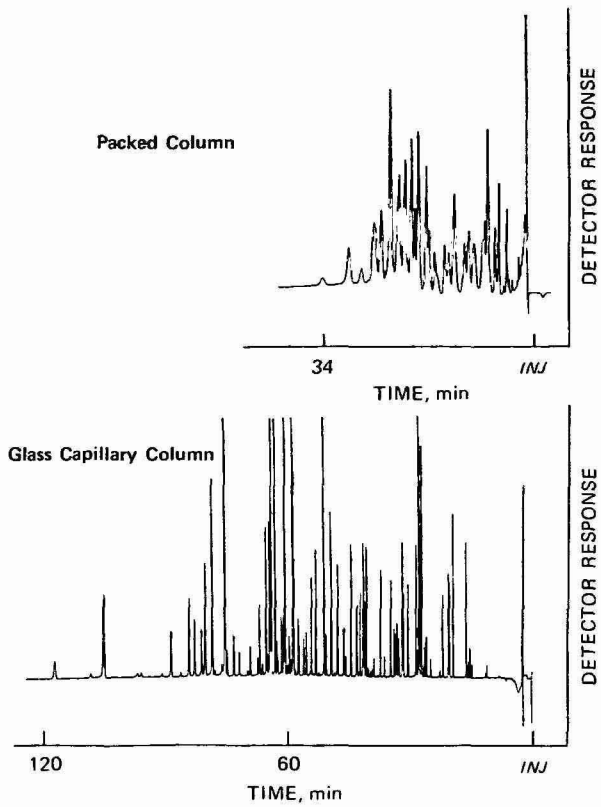

Fig. 1. Chromatograms of Aroclor 1242–1260. Top, packed column; bottom, capillary.

3. DETECTION SYSTEMS

3.1. Modes of detection

Use of several types of detectors in the analysis of PCBs has been reported over the years. A few of the more significant recent advances are noteworthy. These are electron capture, negative ion chemical ionization mass spectrometry (NICI-MS) and selected ion monitoring (SIM), a variant of electron-impact mass spectrometry.

3.2. Electron-capture detection

The electron-capture detector continues to be one of the most sensitive and hence valuable selective detectors for PCB detection. It is only recently, however, that its full potential has been realized and incorporated into commercial systems.

From a quantitative standpoint, one of the earlier limitations of the electron-capture detector was the non-linearity of response. Until the mid-1970s the electron-capture detector had a linear dynamic range of approximately 50–100. Several papers have dealt with the determination of the proper function that would yield a linear relationship with concentration[36,37]. It has been suggested that the response was logarithmic by analogy to light absorption[37]. However, when the electron-cap-

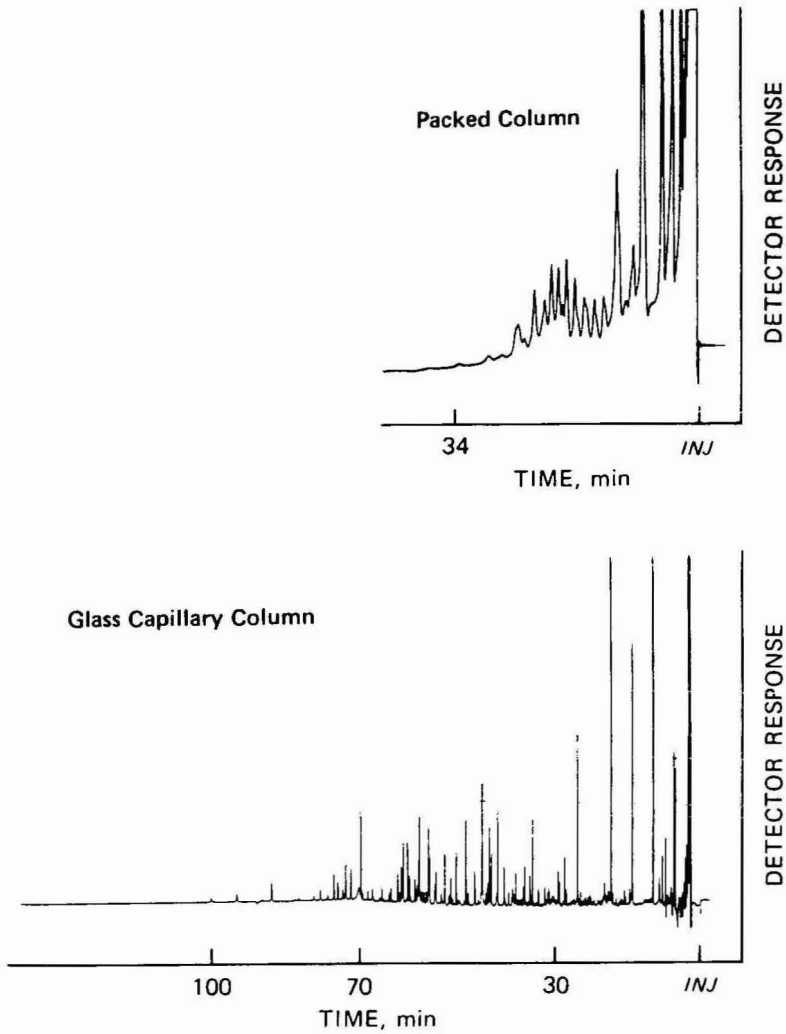

Fig. 2. Chromatograms of stack sample. Top, packed column; bottom, capillary.

ture detector was operated in the pulse sampling mode the reactions occurred primarily in the field-free period, so that the analogy was not considered very appropriate[37].

Once a valid kinetic model for the electron-capture processes for thermal reactions had been derived by Wentworth and Chen[37] and research in atmospheric pressure ionization mass spectrometry gave direct evidence on negative and positive ion formation (under electron-capture conditions), the solution to electron-capture detector non-linearity was in hand. Attention was focused on the electron-capture detector's concentration dependence, which had been of great concern in analytical chemistry. Analysis of the kinetic model was carried out by numerical solution of differential equations, which disproved some of the early mathematical assumptions[37].

As a result of the experimental work on electron-capture mechanisms, subsequent reports appeared describing an alternative method of linearization of response[38,39]. The fundamental principle was based on modulating the pulse frequency on the detector electrode so that the plasma current was maintained constant. This function is given by

$$K_a = \frac{F - F_0}{F_0}$$

where F_0 is the frequency giving the base current in the absence of capturing species and F is the frequency giving the base current in the presence of a capturing species. Initially there were reports of a "break" in linearity, but with improved instrument design parameters this problem was eliminated[39]. Thus, the modern electron-capture detector utilizes modulated pulsed frequency to achieve a dynamic range of approximately four orders of magnitude, an improvement that is necessary for the analysis of PCBs in environmental and biological samples.

A second limitation was the cell volume of the electron-capture detector. This problem was not apparent until analysts began to investigate the use of high-resolution capillary columns[40]. The original cell volumes of 2–4 ml were adequate when used with packed columns with high flow-rates where the chromatographic peak shape and efficiency were preserved. A few isolated reports appeared that considered improved cell design, in particular low-volume cells, for use with capillaries. Electron-capture detector cell volumes between 250 and 500 μl utilizing a coaxial design still required a scavenger gas after the capillary column to reduce the residence time in the cell and preserve the ultra-high numbers of theoretical plates that were attainable by capillaries[40]. However, it is only recently that commercial GC systems have become routinely available with electron-capture detectors compatible with capillary flow-rates.

With these two parallel developments it became feasible to perform elegant quantitative electron-capture detection of PCBs.

3.3. Negative ion chemical ionization mass spectrometry

This detection method is a variant of positive ion chemical ionization mass spectrometry[41]. The associated electronics to regulate, focus and pass negative ions to an appropriately polarized electron multiplier were developed in the mid-1970s; however, it was not until recent years that ion source and reagent gas conditions were investigated for optimizing PCB analysis[30].

NICI-MS is uniquely suited to measuring trace amounts of polyhalogenated chemicals in environmental samples because of its high sensitivity for these chemicals and its virtual transparency to otherwise potentially interfering molecules[42]. It also provides, in addition to sensitivity, molecular ion information and thus a verification of the structural entity being measured, a highly desirable feature when examining complex environmental and biological samples.

NICI-MS is very closely analogous to electron-capture detection in that the ion-forming reactions are common to both. The ion-forming reactions that are important for polychlorinated molecules have been described[42]. They include: (a) the resident capture of thermal electrons; (b) chloride attachment; (c) deprotonation; and

(d) oxygen exchange. Because NICI-MS is a novel technique, the operating parameters that optimize the technique preferentially to one of the above mechanisms for PCB analysis have only recently been studied.

Investigations have been performed on the use of high-resolution GC in combination with NICI-MS while elucidating and characterizing instrumental parameters suitable for PCB analyses[30]. These investigations have included: (a) examination of the performance of two different ion-source designs; (b) the effect of source pressure on sensitivity and spectral signature; and (c) the effect of various reagent gases on sensitivity and fragmentation of PCB isomers. Compared with conventional electron-impact MS, less information about the structure of the compound is obtained. Therefore, the specific aim of the investigation was to study several moderating and reagent gases to enhance the formation of molecular anions of the individual chlorobiphenyl isomers or their dissociation to yield chloride-35 and -37 isotopic anions[30].

Reagent gas studies were conducted with methane, oxygen–nitrogen, nitrous oxide–nitrogen, nitrous oxide–methane, difluorodichloromethane and tetrafluoromethane. This variety of reagent gases was necessary because a major problem encountered in the NICI-MS analysis of PCBs had been the lack of molecular weight information obtained for the lower molecular weight PCBs (C_1–C_6) under methane-moderated electron-capture conditions. As the lower molecular weight PCBs undergo dissociative electron capture to form Cl^- ions under these conditions, the use of a reagent gas that reacts chemically with individual chlorobiphenyl isomers rather than just moderating the electron energy led to the observation of useful molecular weight information in the spectral signature.

Field and co-workers[43,44] had observed that a mixture of nitrous oxide and methane produced abundant OH^- ions under negative ion conditions. These ions were observed to react with a wide variety of compounds by proton abstraction to form $(M-H)^-$ ions. Therefore, this reagent gas mixture was studied in our laboratory as a likely candidate to provide molecular weight information for individual chlorobiphenyl isomers.

Experiments have been conducted with an LKB 2091 magnetic sector instrument with a relatively open source design. Figs. 3–7 depict mass spectra of monochlorobiphenyl, trichlorobiphenyl, hexachlorobiphenyl, octachlorobiphenyl and decachlorobiphenyl obtained under nitrous oxide–methane NICI conditions. Nitrous oxide was introduced through a reagent gas inlet ($5 \cdot 10^{-5}$ Torr as measured at the Penning gauge) and methane ($3 \cdot 10^{-15}$ Torr) was passed into the ion source via a GC make-up line and separator. Based on calculations made for positive ion methane CI, the actual source pressure exerted by both reagent gases was 0.2–0.3 Torr. The MS system was optimized for m/z 17 (OH^-). A 2-μl injection (*ca.* 10 pg) of a standard solution of PCBs was made with a 5:1 splitting ratio. As indicated in Fig. 3, the spectrum of 2-chlorobiphenyl exhibited $(M-H)$ ions. The M^- peak at m/z 188 is no larger than expected from ^{13}C isotope abundance. In addition, very low intensity ions were found for the $(M-H+O)^-$ ion at m/z 203, $(M-H+N_2O)^-$ ion at m/z 231 and $(M-H+NO)^-$ ion at m/z 217.

The high-mass region for trichlorobiphenyl ($> m/z$ 40, Fig. 4) mass spectrum was dominated by the $(M-H)^-$ ions at m/z 255, 257 and 259. The M^- intensity was greater than predicted on the basis of ^{13}C isotope abundance (24% *vs.* 13%), sug-

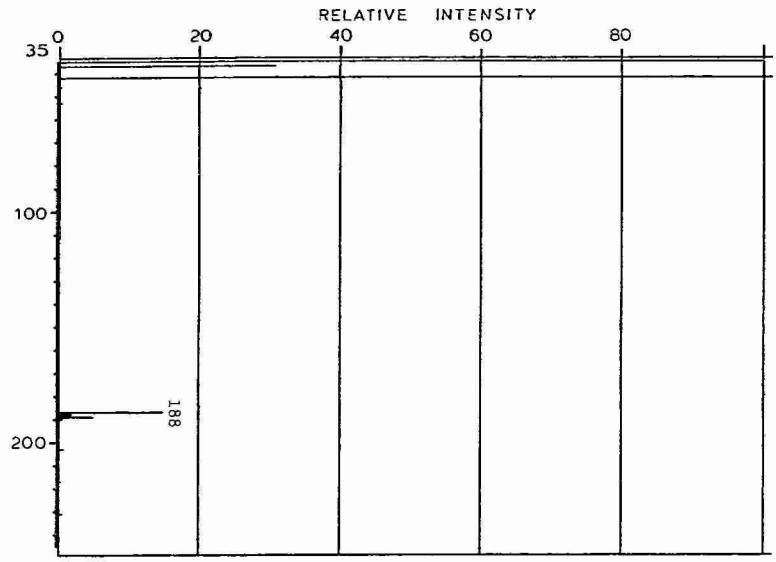

Fig. 3. N_2O–CH_4 NICI mass spectrum of monochlorobiphenyl.

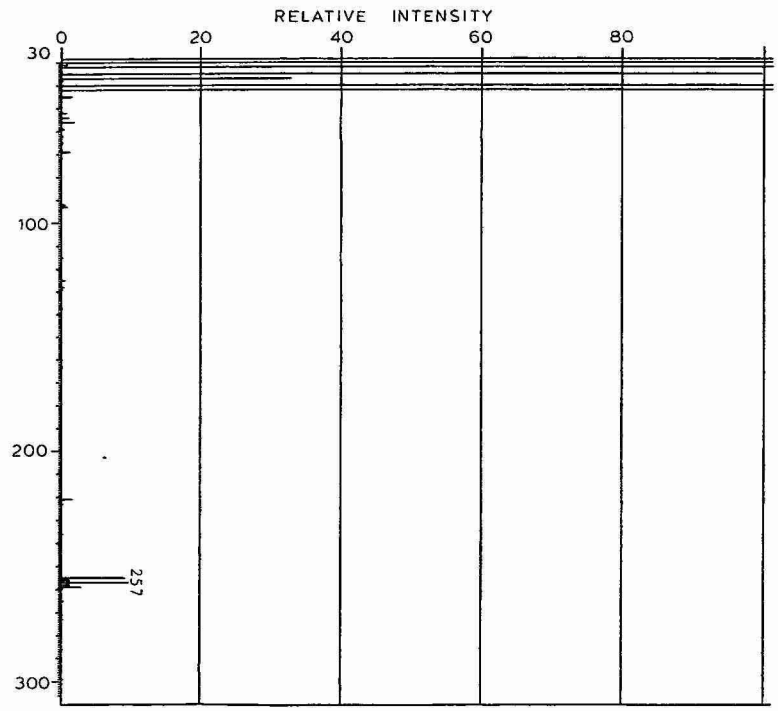

Fig. 4. N_2O–CH_4 NICI mass spectrum of trichlorobiphenyl.

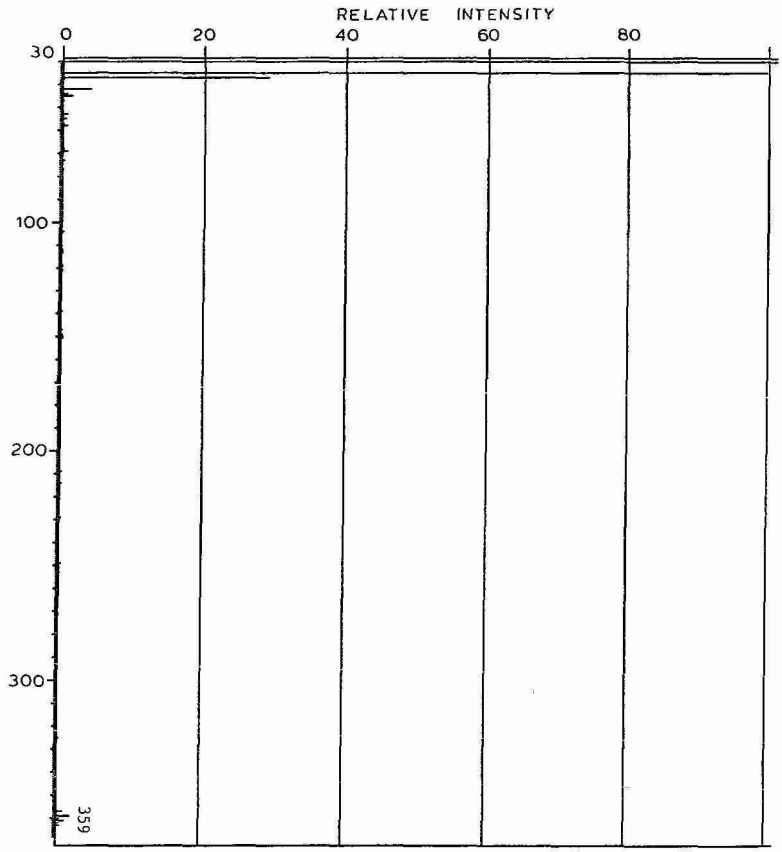

Fig. 5. N_2O–CH_4 NICI mass spectrum of hexachlorobiphenyl.

gesting that some stabilization of M^- was occurring. Also, $(M-Cl)^-$ ions were evident at *m/z* 221 and 223.

For hexachlorobiphenyl (Fig. 5) the $(M-H)^-$ ions at *m/z* 357, 359, 361 and 363 were present. Also, a substantial abundance of M ions was observed (53% observed *vs.* 13% calculated as ^{13}C). Ion clusters for the loss of one chlorine (*m/z* 323) and two chlorine atoms (*m/z* 288) from the parent molecule were detected. An interesting ion cluster formed by the loss of chlorine and the addition of oxygen, $(M-19)^-$, was observed (*m/z* 338).

The mass spectrum of octachlorobiphenyl (Fig. 6) exhibited negligible hydrogen abstraction but was certainly dominated by M^- ions. As there are only two hydrogens available for abstraction on this PCB homolog, this result was unexpected.

Decachlorobiphenyl (Fig. 7) with no hydrogens available for abstraction yields a spectrum that is due to electron capture (formation of M^-) and Cl^-.

The above observations for nitrous oxide–methane reagent gas indicated that verification of the molecular weight of the chlorobiphenyl isomer could be achieved in high-resolution GC–NICI-MS. Further, the homogeneity of the GC peaks in complex mixture analysis could be established, as non-PCB substances or non-homolo-

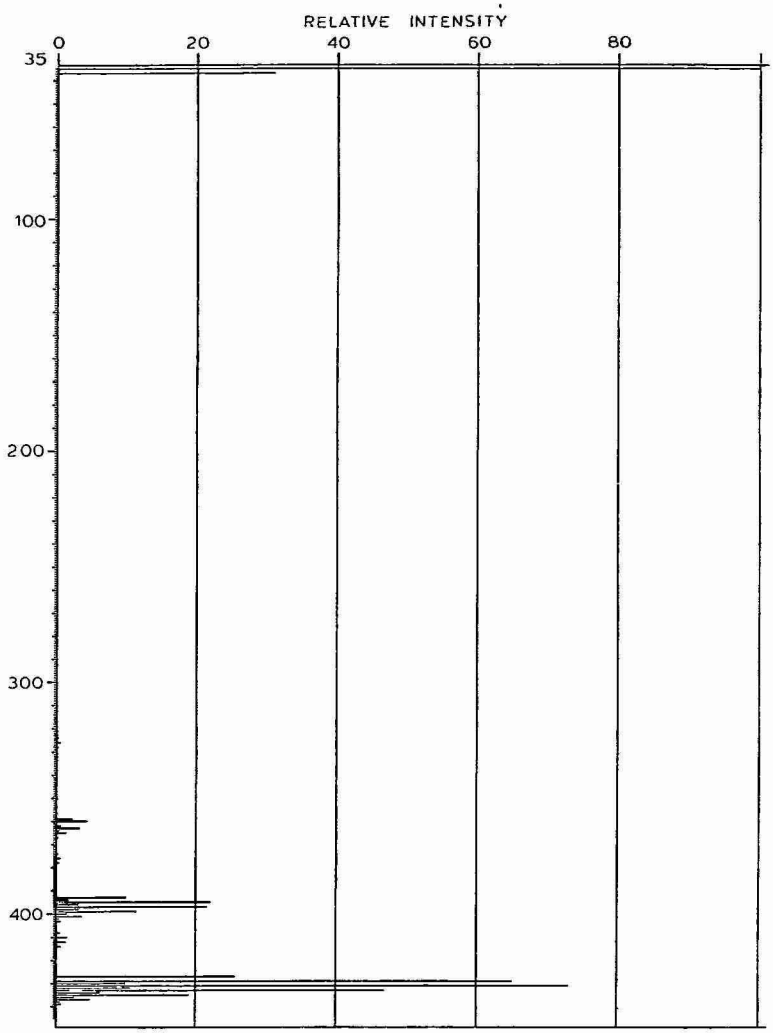

Fig. 6. N_2O–CH_4 NICI mass spectrum of octachlorobiphenyl.

gous PCB isomers can be distinguished. NICI-MS can thus provide molecular weight information, which has been one of the advantages claimed for EI-MS.

Another moderating gas typically used in NICI-MS has been methane[41]. Experiments on the effects of source pressure on sensitivity indicated that as the methane pressure increased so also did sensitivity[30]. As a compromise between maximum sensitivity and excessive pressure, a reagent gas pressure of $4 \cdot 10^{-5}$ Torr is normally employed (measured at the diffusion pump throat; the actual ion source pressure might be higher).

Of the reagent and moderating gases studied, methane provided one of the more sensitive modes of operation. However, the principal mechanism of reaction was dissociative electron capture, leading to *m/z* 35 and 37.

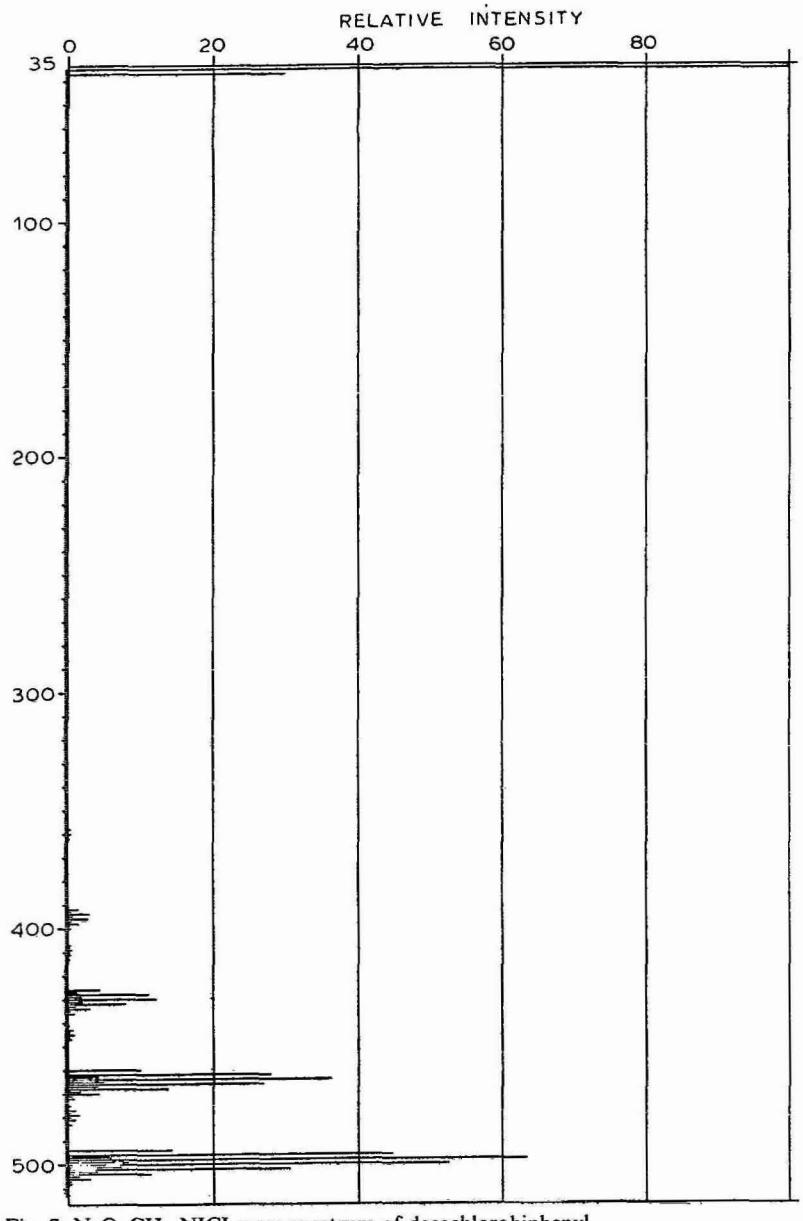

Fig. 7. N_2O–CH_4 NICI mass spectrum of decachlorobiphenyl.

The chemical thermodynamics in NICI-MS were also found to be affected by the physical design of the GC–MS source where the CI takes place. The experiments conducted with a relatively open and closed source designs gave parallel results. The principal feature of the open source was that it was less subject to filament carbonization and its sensitivity was maintained stable for a few weeks. Considerable absolute differences in the limits of detection were observed between two different in-

strument makes. One system was clearly capable of detecting in the high femtogram region, whereas the other required two orders of magnitude more PCB material.

3.4. Selected ion monitoring utilizing EI-MS

Electron-impact mass spectrometry has been a very popular analytical tool, in addition to the electron-capture detector, as a GC detector for PCBs. The use of the conventional scanning mode suffers from an inadequate limit of detection for chlorobiphenyl isomers. The limits of detection between EI-MS and ECD may differ by as much as three orders of magnitude[30]. For this reason, EI-MS *per se* has not been as widely used unless there are large amounts of PCBs present in environmental and biological samples[45–56]. There have been reports, however, of a specialized application of EI-MS. Selected ion monitoring (SIM) had been primarily developed for drug analysis, but recently it has been applied to verifying and quantifying PCBs[45–48]. Improved limits of detection were achieved.

Another variant of EI-MS has been limited mass scanning (LMS). The use of SIM (programmed mode) and LMS permits the spectrometer to spend more time transmitting through the analyzer to the electron multiplier ions of interest to yield lower limits of detection[46]. Both of these techniques were under computer control and were also available to the analyst when operating in the NICI-MS mode.

3.5. Pulsed positive ion–negative ion chemical ionization (PPINICI) mass spectrometry

During the past few years PPINICI has been available; rapid switching between positive and negative CI (12 kHz) allows simultaneous information to be acquired[30]. Little research has been performed on its optimization and application to PCB analysis.

3.6. Combination of high-resolution gas chromatography and ultrasensitive detection

Figs. 1 and 2 depict the combination of state-of-the-art high-resolution chromatography and electron-capture detection. It is clearly evident from these profiles that modern electron-capture detectors are designed to preserve the high resolution that capillary columns are now capable of delivering.

The direct coupling of high-resolution GC capillaries with the ion source of mass spectrometers has also been successfully accomplished. The flexible nature of silica capillaries has greatly facilitated this accomplishment, as most MS systems are not well engineered to accept the rigid glass capillaries.

Fig. 8 depicts an example of analysis of an Aroclor 1016–1254–1260 mixture (2.5:2.0:1.0, w/w) utilizing an Apiezon M coating (0.025 μm film), a PSD silica capillary and NICI-MS detection. Table 1 lists the operating conditions, which were optimized for dissociative electron capture (*i.e.*, enhancement of m/z 35 and 37). The mass chromatogram (m/z 35) exemplifies the close similarity between the proportional response for individual chlorobiphenyl isomers under methane NICI conditions and those obtained with electron-capture detection. An expanded version of this chromatogram is given in Figs. 9–11. Chromatographic peaks 3, 35 and 103 are the internal standards, 1,2-dichloronaphthalene, 1,2,3,4-tetrachloronaphthalene and octachloronaphthalene, respectively. The chromatographic peaks depicted in Figs. 9–11 indicate ideal symmetry and thus the chromatography was preserved. The peak

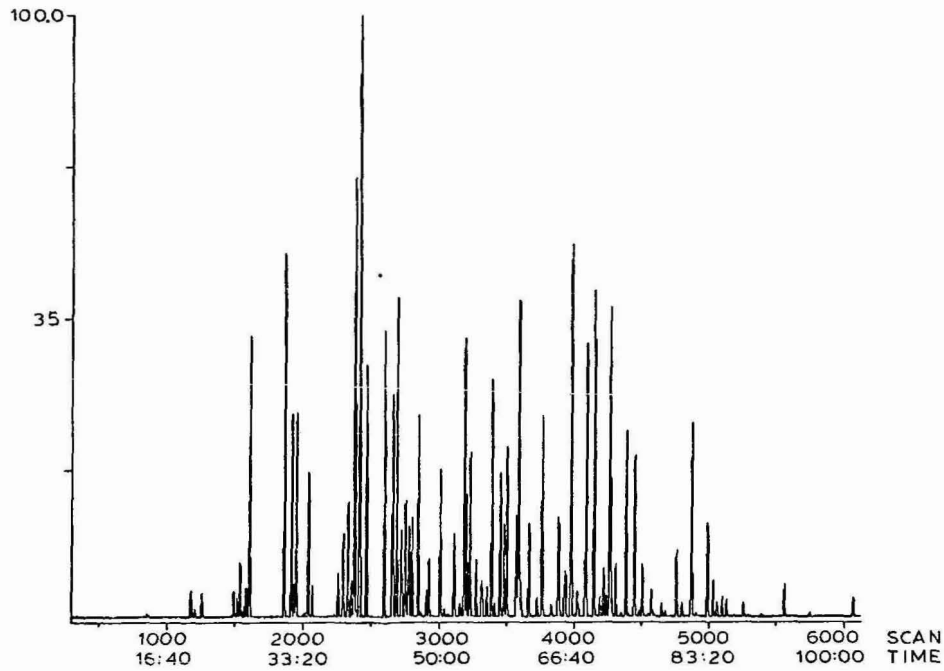

Fig. 8. Mass chromatogram (m/z 35) of Aroclor 1016–1254–1260 obtained by CH_4 NICI-MS.

TABLE 1

CAPILLARY GC–NICI-MS OPERATING CONDITIONS (FINNIGAN 4021)

Mode	*Parameter*	*Condition*
GC	Capillary:	Fused silica
	I.D.	0.23 mm
	Length	45 m
	Deactivation	Polysiloxane
	Stationary phase	Apiezon M
	Film thickness	0.025 μm
	He carrier gas	0.6 ml/min (32 cm/sec)
	Splitless/split	40 sec; 10:1
	Temperature	100°C (0.1 min) to 260°C at 1.5°C/min
MS	Reagent gas:	Methane
	Fore-pressure	0.2 Torr
	High-vacuum pressure	$4.2 \cdot 10^{-5}$ Torr
	Manifold temperature	120°C
	Ionizer temperature	260°C
	Emission current	0.5 mA
	Electron energy	70 eV
	Scan cycle	1.0 sec
	Scan range	30–700 daltons

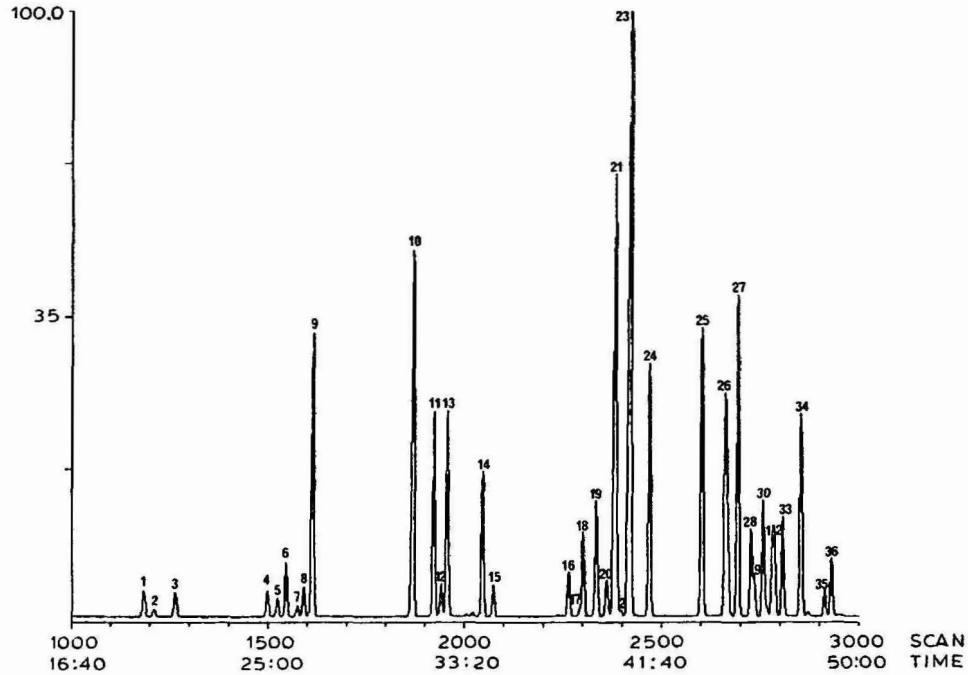

Fig. 9. Mass chromatogram (m/z 35) of Aroclor 1016–1254–1260 obtained by CH_4 NICI-MS (Fig. 8 expanded). Peak No. 3 is the internal standard, 1,2-dichloronaphthalene.

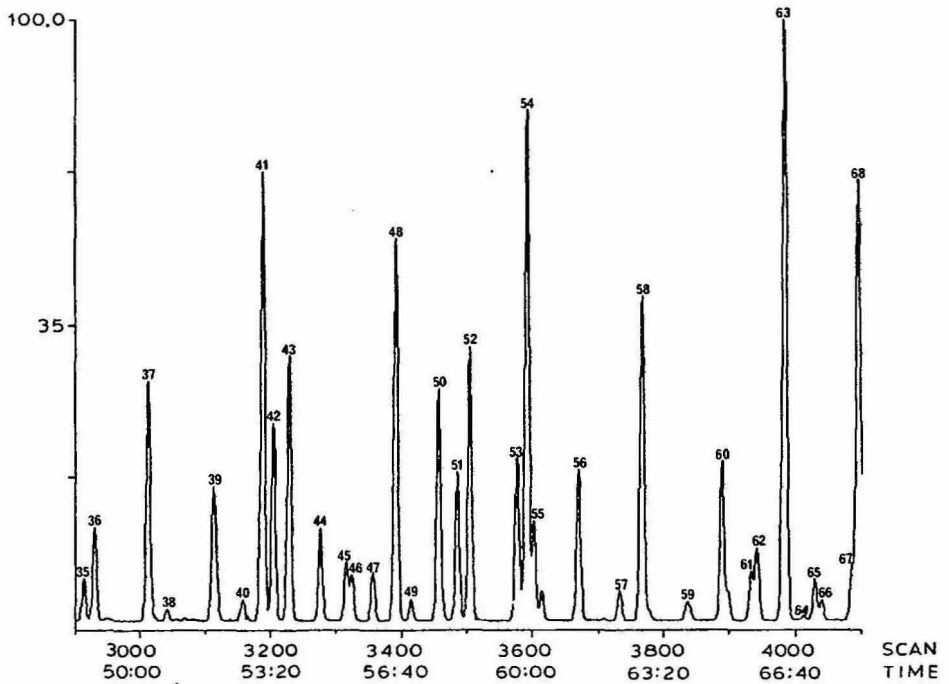

Fig. 10. Mass chromatogram (m/z 35) of Aroclor 1016–1254–1260 obtained by CH_4 NICI-MS (Fig. 8 expanded, middle portion). Peak No. 35 is the internal standard, 1,2,3,4-tetrachloronaphthalene.

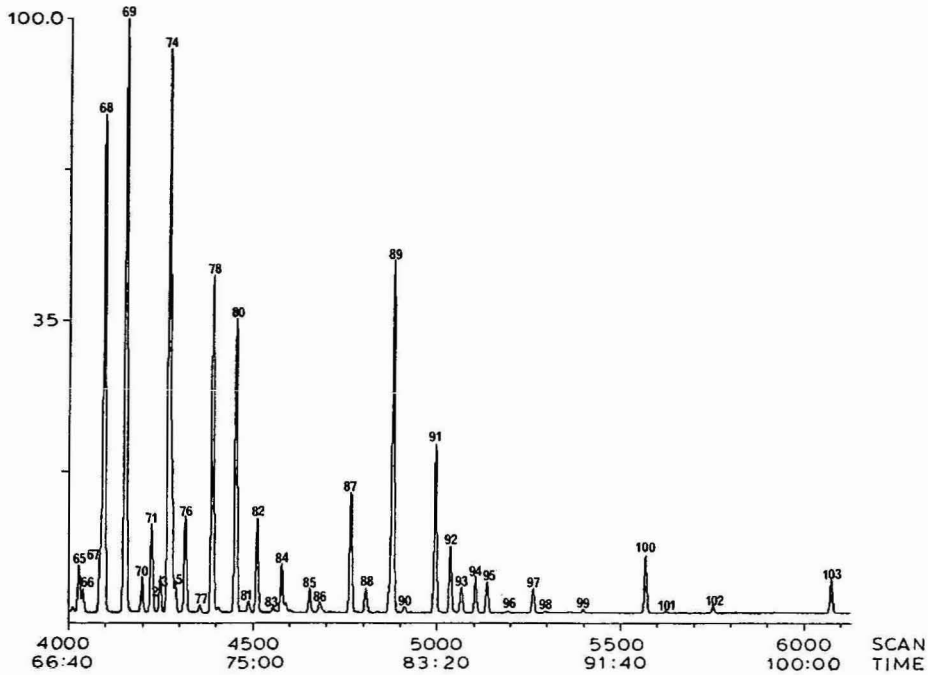

Fig. 11. Mass chromatogram (*m/z* 35) of Aroclor 1016–1254–1260 obtained by CH_4 NICI-MS (Fig. 8 expanded, later portion). Peak No. 103 is the internal standard, octachloronaphthalene.

residence times are generally 7–8 sec. Because the electron-capture detector and NICI-MS profiles are superimposable, the numbering scheme shown in Figs. 9–11 was standardized for our characterization research and for our cross-referencing and verification efforts.

Another means of representing NICI-MS information is depicted in Figs. 12 and 13. The upper trace is the one shown in Fig. 8 but it is considerably reduced. The remaining profiles in Fig. 12 are mass chromatograms for *m/z* 188 (Cl_1), 222 (Cl_2), 256 (Cl_3), 292 (Cl_4) and 326 (Cl_5) representing the five homologous series. It is important to note that under methane NICI conditions the intensity of molecular anions for Cl_1–Cl_5 was very weak to non-existent. It is more appropriate to use nitrous oxide–methane for detecting which homolog is represented by a chromatographic peak. Fig. 13 shows mass chromatograms for *m/z* 360 (Cl_6), 394 (Cl_7), 428 (Cl_8), 462 (Cl_9) and 496 (Cl_{10} not detected) for the remaining homologous series. In this instance all of the chlorobiphenyl isomers were detected in the Aroclor mixture.

3.7. Variation of detector responses

A common problem with electron-capture, NICI-MS and EI-MS (SIM, LMS) detectors is the large variation that has been observed between the individual chlorobiphenyl isomers, both within and between homologous series[1,15–18]. The relative response factors (RRFs) for a few individual chlorobiphenyl isomers obtained with high-resolution GC–ECD (HRGC–ECD) have been reported[18]. Most isomers have

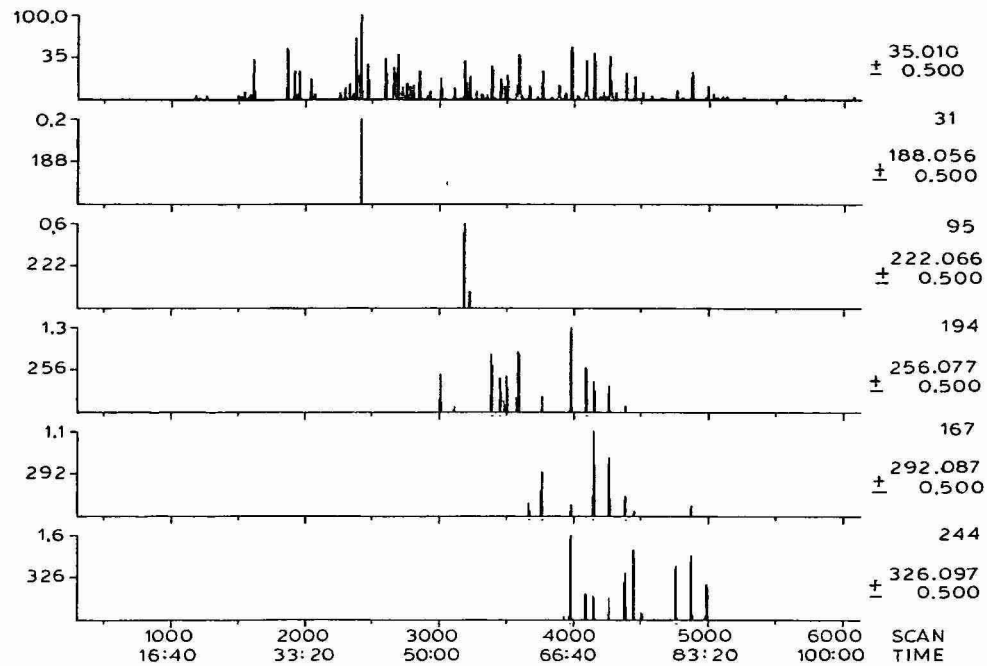

Fig. 12. Mass chromatogram for parent ions of each PCB homologue (Cl_1–Cl_5) in Aroclor 1016–1254–1260 mixture.

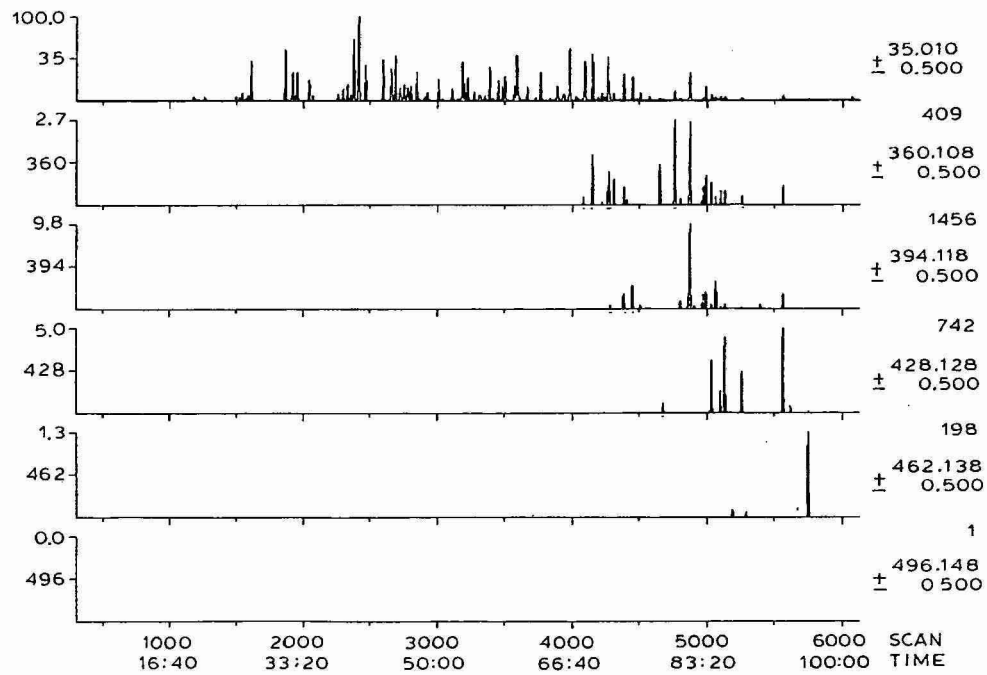

Fig. 13. Mass chromatogram for parent ions of each PCB homologue (Cl_6–Cl_{10}) in Aroclor 1016–1254–1260 mixture.

TABLE 2

RELATIVE RESPONSE FACTORS FOR INDIVIDUAL PCB ISOMERS USING HRGC–ECD

Homologue (No.)	*PCB isomer No.*	*RRF* (relative to OCN)*		*SE-54 column*				*Apiezon M column*
				RRF (relative to MBB)		*RRF (relative to TBB)*		*RRF (relative to DCN)*
		Mean	*RSD (%)*	*Mean*	*RSD (%)*	*Mean*	*RSD (%)*	*Mean*
1-Cl (3)	001	27.319	4.4	13.344	1.5	24.956	2.4	5.965
	002	47.158	5.2	20.152	3.7	38.534	3.9	9.435
	003	55.970	2.3	22.950	5.7	43.296	7.3	10.735
2-Cl (12)	004	10.696	15.2	4.798	10.2	9.153	11.5	2.703
	005	12.659	2.5	5.189	5.0	9.788	6.6	1.628
	006	2.622	1.5	2.851	1.3	5.556	2.5	1.049
	007	2.266	1.8	1.047	1.1	2.013	2.0	0.465
	008	3.958	2.6	2.040	0.3	3.836	1.5	0.798
	009	3.711	3.8	1.726	0.6	3.293	1.1	0.719
	010	5.202	0.0	2.054	0.8	3.920	1.9	0.717
	011	8.519	1.8	3.653	1.4	6.399	1.2	2.054
	012	4.322	4.8	2.211	1.1	4.162	1.4	0.929
	013	7.495	1.5	2.851	1.3	5.556	2.5	1.049
	014	4.956	3.4	2.395	1.3	4.578	1.9	0.987
	015	5.620	3.0	2.678	2.2	5.151	3.1	1.104
3-Cl (24)	016	2.678	3.0	1.087	1.1	2.088	1.9	0.378
	017	2.085	3.1	1.079	1.6	2.029	2.8	0.418
	018	2.543	6.0	1.129	3.1	2.152	3.2	0.651
	019	4.283	3.2	2.193	1.0	4.125	2.2	0.871
	020	1.344	3.6	0.670	2.5	1.260	3.5	0.283
	021	1.157	4.5	0.516	1.0	0.986	0.4	0.221
	022	0.757	5.5	0.341	3.1	0.627	1.5	0.139
	023	1.056	7.0	0.435	0.5	0.843	0.9	0.273
	024	2.198	3.6	0.480	0.5	1.627	1.4	0.273
	025	4.734	5.5	2.043	1.6	3.736	0.7	0.806
	026	2.001	2.5	1.046	0.7	1.969	1.9	0.388
	027	3.067	4.0	1.324	1.9	2.423	4.1	0.490
	028	1.449	8.3	0.747	0.6	1.433	0.9	0.315
	029	2.137	2.6	0.844	1.6	1.611	1.4	0.273
	030	0.742	4.8	0.392	0.3	0.743	0.4	0.155
	031	2.130	5.5	1.100	1.6	2.096	2.8	0.439
	032	2.633	4.9	1.136	0.7	2.079	1.7	0.421
	033	2.600	2.1	1.113	0.8	2.098	2.4	0.370
	034	1.144	3.2	0.590	0.5	1.109	1.5	0.231
	035	2.319	3.2	1.188	2.9	2.234	4.0	0.472
	036	1.351	21.7	0.780	1.7	1.428	3.5	0.319
	037	3.880	4.2	1.615	1.3	2.984	0.9	
	038	1.216	4.1	0.647	1.4	1.227	1.7	0.252
	039	3.813	3.1	2.000	1.1	3.768	0.3	0.778

			Statistics of RRFs (relative to OCN) for homologue					
	RRF (relative to TCN)		*Range*		*Mean*	*Standard deviation*	*RSD (%)*	*N*
RSD (%)	*Mean*	*RSD (%)*	*Low*	*High*				
0.8	27.695	1.4	27.319	55.970	43.482	14.675	33.7	3
2.9	46.291	1.6						
3.2	47.269	1.4						
1.7	11.929	2.3	2.266	12.659	6.002	3.225	53.7	12
7.1	7.171	7.3						
1.2	4.315	0.9						
4.7	1.986	0.5						
2.6	3.766	0.7						
1.3	3.452	0.7						
0.8	2.961	2.8						
3.3	9.941	1.1						
0.6	4.393	1.3						
1.2	4.315	0.9						
2.9	4.626	2.0						
4.1	4.178	0.6						
1.0	1.555	0.6	0.657	4.734	2.217	1.117	50.4	24
2.0	1.975	0.5						
1.9	2.872	2.7						
1.9	4.111	0.7						
2.3	1.336	0.3						
2.5	1.085	1.3						
1.4	0.649	0.1						
0.7	1.192	1.4						
0.7	1.192	1.4						
4.2	3.356	4.1						
0.9	1.862	5.7						
2.4	2.040	6.3						
1.3	1.801	1.2						
0.7	1.192	1.4						
0.5	0.686	2.2						
3.3	2.489	1.4						
2.8	1.753	5.5						
1.7	1.755	1.0						
2.0	1.088	0.7						
2.6	2.226	0.8						
6.7	1.327	1.4						
	—	—						
4.0	1.114	4.4						
0.9	3.690	0.4						

(Continued on p. 296)

TABLE 2 *(continued)*

Homologue (No.)	*PCB isomer No.*	*RRF* (relative to OCN)*		*SE-54 column*				*Apiezon M column*
		Mean	*RSD (%)*	*RRF (relative to MBB)*		*RRF (relative to TBB)*		*RRF (relative to DCN)*
				Mean	*RSD (%)*	*Mean*	*RSD (%)*	*Mean*
4-Cl (42)	040	1.525	3.8	0.707	0.9	1.353	0.8	0.278
	041	1.688	3.0	0.879	2.2	1.652	1.2	0.336
	042	1.465	4.1	0.747	1.1	1.375	2.0	0.317
	043	0.331	4.4	0.138	2.2	0.273	2.0	0.045
	044	2.184	3.3	0.758	1.4	1.485	1.7	0.353
	045	2.319	5.1	0.996	1.2	1.909	2.4	0.339
	046	0.866	0.0					0.209
	047	1.219	4.4	0.584	5.4	1.102	6.1	0.247
	048	1.831	4.1	0.888	2.7	1.749	2.5	0.371
	049	1.713	9.2	0.691	8.3	1.330	8.9	0.270
	050	0.876	5.5					0.375
	051	1.200	1.9	1.428	0.8	2.784	2.0	0.480
	052	1.400	6.7	0.679	2.9	1.294	3.3	0.328
	053	2.156	5.3	1.054	1.4	1.876	2.7	0.424
	054	2.409	3.7	1.224	1.2	2.356	1.7	0.503
	055	1.481	4.2	0.663	2.5	1.237	2.8	0.282
	056	1.239	2.2	0.622	0.8	1.170	0.8	0.259
	057	1.405	3.4	0.742	1.0	1.399	0.3	0.284
	058	2.338	2.8	0.835	0.7	–	–	0.640
	059	1.405	3.4	0.742	1.0	1.399	0.3	0.284
	060	1.464	0.4	0.626	2.4	1.196	2.6	0.221
	061	0.990	4.0	0.450	1.0	0.860	0.1	0.185
	062	1.031	5.4	0.535	1.4	1.026	2.6	0.197
	063	1.209	2.3	0.656	2.3	1.236	2.0	0.246
	064	1.209	2.3	0.656	2.3	1.236	2.0	0.246
	065	1.090	3.5	0.502	1.6	0.957	0.6	0.213
	066	1.765	4.1	0.724	0.73	1.366	9.0	0.148
	067	1.405	3.4	0.742	1.0	1.399	0.3	0.284
	068	1.635	4.3	0.760	0.4	1.405	1.7	
	069	1.683	3.1	0.585	0.9	1.146	1.5	0.271
	070	1.377	3.3	0.616	1.6	1.151	2.1	0.159
	071	2.605	4.6	1.089	1.9	2.147	1.8	0.444
	072	1.114	6.9	0.491	8.3	0.944	7.4	0.279
	073	1.547	3.2	0.796	2.5	1.498	3.6	0.313
	074	1.209	2.3	0.656	2.3	1.236	2.0	0.246
	075	1.286	4.1	0.650	0.9	1.198	1.9	0.273
	076	1.707	6.6	0.861	0.9	1.670	1.5	0.313
	077	2.036	34.7	0.758	35.0	1.327	34.8	0.544
	078	1.481	4.2	0.663	2.5	1.237	2.8	0.282
	080	2.020	2.6	0.835	0.7	–	–	
	080	0.587	14.5					0.252
	081	1.008	12.7	0.490	12.5	0.922	13.5	0.219

			Statistics of RRFs (relative to OCN) for homologue					
	RRF (relative to TCN)		*Range*		*Mean*	*Standard deviation*	*RSD (%)*	*N*
RSD (%)	*Mean*	*RSD (%)*	*Low*	*High*				
1.5	1.366	0.3	0.331	2.605	1.488	0.490	32.9	42
2.5	1.585	0.4						
1.1	1.477	0.7						
2.6	0.207	3.7						
1.8	1.859	0.9						
0.8	1.605	1.2						
3.5	0.969	5.1						
2.5	1.087	0.3						
4.9	1.722	3.6						
19.8	1.151	17.2						
1.9	1.871	1.1						
1.0	1.975	0.6						
1.8	1.448	1.9						
2.6	1.867	3.6						
1.1	2.179	1.3						
5.9	1.231	0.8						
2.9	1.220	0.5						
1.4	1.347	1.0						
3.6	2.972	0.7						
1.4	1.347	1.0						
10.3	1.037	12.5						
1.6	0.910	0.3						
2.1	0.933	0.3						
2.4	1.124	0.3						
2.4	1.124	0.3						
1.4	1.026	1.0						
4.0	0.652	5.0						
1.4	1.347	1.0						
1.7	1.430	0.8						
6.8	0.703	5.6						
2.7	2.063	0.6						
2.4	1.208	0.5						
2.1	1.475	0.7						
2.4	1.124	0.3						
3.3	1.547	0.5						
1.8	1.366	0.6						
1.0	2.635	2.1						
5.9	1.231	0.8						
15.6	1.259	16.5						
3.1	1.035	1.5						

(Continued on p. 298)

TABLE 2 *(continued)*

Homologue (No.)	*PCB isomer No.*	*RRF* (relative to OCN)*		*SE-54 column*				*Apiezon M column*
		Mean	*RSD (%)*	*RRF (relative to MBB)*		*RRF (relative to TBB)*		*RRF (relative to DCN)*
				Mean	*RSD (%)*	*Mean*	*RSD (%)*	*Mean*
5-Cl (46)	082	1.242	3.2	0.588	3.9	1.106	3.1	0.124
	083	1.802	5.9	0.686	7.8	1.338	8.9	0.264
	084	1.802	5.9	0.686	7.8	1.338	8.9	0.314
	085	1.325	5.5	0.580	2.4	1.048	1.6	0.265
	086	1.073	6.0	0.553	3.3	1.053	2.7	0.222
	087	0.934	5.4	0.494	1.4	0.951	1.0	0.183
	088	1.249	6.1	0.605	0.7	1.156	0.5	0.248
	089	0.830	2.7	0.359	3.4	0.656	5.1	0.099
	090	2.305	7.7	0.480	3.8	1.630	4.0	0.321
	091	1.824	5.6	0.798	2.3	1.442	2.9	0.335
	092	1.790	5.9	0.834	2.8	1.644	2.1	0.382
	093	1.727	3.7	0.598	1.6	1.172	1.5	0.280
	094	3.071	9.8	0.936	7.3	1.782	6.7	0.370
	095	1.654	4.6	0.939	1.4	1.792	0.3	0.352
	096	2.093	3.6	1.015	0.9	1.941	1.0	0.415
	097	1.614	3.1	0.655	0.5	1.258	1.9	0.264
	098	1.329	2.1	0.684	0.8	1.288	1.0	0.263
	099	2.305	7.7	0.480	3.8	1.630	4.0	0.289
	100	1.437	3.9	0.598	0.6	1.105	0.3	0.235
	101	1.847	2.1	0.835	0.5	1.574	1.4	0.270
	102	3.071	9.8	0.936	7.3	1.782	6.7	0.370
	103	1.329	3.9	0.688	1.9	1.268	3.4	0.282
	104	3.704	3.4	1.658	0.6	3.095	1.8	0.378
	105	1.285	2.1	0.651	1.5	1.225	1.8	0.260
	106	1.058	2.7	0.554	2.0	1.045	1.0	0.216
	107	1.026	2.5					0.247
	108	1.142	1.6	0.625	2.3	1.177	0.9	0.230
	109	0.931	5.0	0.442	2.9	0.843	2.4	0.172
	110	1.087	2.3					0.262
	111	1.295	2.0	0.705	2.2	1.328	0.9	0.263
	112	1.111	4.8	0.582	0.7	1.088	0.5	0.223
	113	1.017	7.6	0.547	1.2	1.020	1.5	0.186
	114	0.947	1.4	0.419	1.6	0.735	1.2	0.222
	115	1.251	3.5	0.434	1.5	0.851	1.2	0.202
	116	0.510	6.6	0.271	1.4	0.513	1.0	0.106
	117	1.078	5.7	0.459	2.0	1.027	1.4	0.224
	118	1.238	2.5	0.657	3.5	1.236	2.3	0.236
	119	1.276	5.6	0.559	3.2	1.009	2.0	0.245
	120	1.295	2.0	0.705	2.2	1.328	0.9	0.263
	121	1.270	3.8	0.589	0.8	1.127	0.4	0.232
	122	1.644	2.9	0.853	2.5	1.625	2.3	0.338
	123	1.189	5.4	0.598	6.8	1.119	7.8	0.251
	124	1.078	3.1	0.512	3.2	0.977	1.8	0.204
	125	0.753	28.5	0.359	3.4	0.656	5.1	0.238
	126	2.491	5.0	1.201	7.4	2.297	7.3	0.498
	127	1.142	1.6	0.625	2.3	1.177	0.9	0.230

			Statistics of RRFs (relative to OCN) for homologue					
	RRF (relative to TCN)		*Range*		*Mean*	*Standard deviation*	*RSD (%)*	*N*
RSD (%)	*Mean*	*RSD (%)*	*Low*	*High*				
2.3	0.545	3.1	0.510	3.704	1.489	0.640	43.046	46
1.5	1.166	1.9						
0.7	1.292	0.6						
3.6	1.083	1.5						
3.4	1.258	0.4						
4.3	0.793	2.3						
4.0	1.162	2.0						
6.6	0.410	1.6						
0.9	1.398	0.8						
2.1	1.369	0.6						
6.7	1.773	4.6						
1.5	1.473	0.8						
3.6	2.070	0.6						
1.5	1.453	2.1						
3.8	1.944	3.2						
1.5	1.166	1.9						
1.3	1.262	6.0						
4.9	1.261	6.4						
2.0	1.343	0.5						
2.2	1.238	0.8						
3.6	2.070	0.6						
1.4	1.313	1.1						
1.5	1.811	1.5						
2.3	1.246	6.1						
0.8	1.026	0.5						
3.3	1.147	0.6						
2.9	1.049	0.8						
4.5	0.847	4.2						
3.0	1.216	0.3						
2.7	1.199	0.3						
3.2	1.034	3.2						
7.2	0.889	8.0						
2.2	1.075	0.6						
1.3	1.064	0.3						
2.6	0.469	3.4						
1.9	1.038	0.5						
2.5	1.131	7.3						
3.0	1.001	1.2						
2.7	1.199	0.3						
1.5	1.138	0.4						
3.6	1.913	0.5						
0.5	1.167	1.2						
3.0	0.981	2.6						
5.3	0.994	10.1						
4.8	2.336	3.3						
2.9	1.049	0.8						

(Continued on p. 300)

TABLE 2 *(continued)*

Homologue (No.)	*PCB isomer No.*	*RRF* (relative to OCN)*		*SE-54 column*				*Apiezon M column*
		Mean	*RSD (%)*	*RRF (relative to MBB)*		*RRF (relative to TBB)*		*RRF (relative to DCN)*
				Mean	*RSD (%)*	*Mean*	*RSD (%)*	*Mean*
6-Cl (42)	128	1.309	29.7	0.808	29.2	1.517	27.4	0.215
	129	0.987	3.4	0.520	1.6	0.992	0.9	0.249
	130	1.423	3.9	0.762	1.3	1.407	2.4	0.261
	231	0.919	1.9	1.153	1.0	2.019	0.6	0.218
	132	1.423	2.4	0.743	1.2	1.387	2.0	0.271
	133	0.827	8.4					0.259
	134	1.213	2.2	0.631	0.5	1.186	1.1	0.242
	135	1.564	1.8	0.595	1.9	1.149	2.9	0.259
	136	1.538	3.3	0.803	1.7	1.480	2.8	0.323
	137	1.055	3.2	0.489	1.8	0.934	1.3	0.193
	138	1.161	2.9	0.601	2.5	1.107	1.8	0.249
	139	1.253	3.4	0.582	1.3	1.112	0.5	0.229
	140	1.179	7.2	0.604	4.8	1.160	6.2	0.229
	141	0.475	6.7	0.259	2.1	0.490	1.8	0.096
	142	0.993	3.8	0.509	1.0	0.960	1.8	0.207
	143	1.196	3.4	0.583	3.1	1.114	3.2	0.236
	144	1.277	3.9	0.664	1.0	1.265	2.0	0.261
	145	1.539	2.7	0.801	1.8	1.581	0.9	0.318
	146	2.521	1.1	0.996	1.4	1.900	0.9	0.436
	147	1.260	5.8	0.674	0.8	1.242	1.8	0.263
	148	2.258	8.2	1.049	4.8	1.939	5.5	0.362
	149	1.564	1.8	0.595	1.9	1.159	2.9	0.557
	150	2.045	2.6	1.105	0.6	2.063	2.5	0.370
	151	1.011	3.8	0.473	1.3	0.901	0.1	0.246
	152	1.441	3.1	0.754	3.5	1.421	2.5	0.295
	153	1.606	4.7	0.499	3.6	0.950	3.3	0.148
	154	1.502	4.0	0.524	1.8	1.027	1.1	0.241
	155	2.158	5.7	0.926	1.5	1.776	3.0	0.315
	156	0.993	2.0	0.511	2.4	0.953	2.4	0.193
	157	1.118	5.7	0.556	1.3	1.065	2.9	0.227
	158	0.954	9.1	0.463	3.2	0.883	1.6	0.176
	159	1.227	7.3	0.616	5.1	1.181	6.6	0.246
	160	0.846	5.7	0.403	3.0	0.770	2.9	0.172
	161	1.010	2.2	0.279	2.7	0.914	1.1	0.192
	162	0.838	5.3	0.502	10.5	0.988	9.7	0.116
	163	1.271	2.3	0.659	1.5	1.231	2.0	0.244
	164	1.294	3.1	0.661	2.9	1.166	1.3	0.247
	165	1.677	3.5	0.720	1.8	1.381	2.2	0.197
	166	0.971	9.7	0.485	2.7	0.926	1.3	0.173
	167	1.195	5.3	0.502	10.5	0.988	9.7	0.116
	168	1.046	3.1	0.525	1.6	1.003	1.8	0.198
	169	1.428	3.2	0.678	5.4	1.297	5.5	0.291

			Statistics of RRFs (relative to OCN) for homologue					
	RRF (relative to TCN)		*Range*		*Mean*	*Standard deviation*	*RSD (%)*	*N*
RSD (%)	*Mean*	*RSD (%)*	*Low*	*High*				
5.5	0.932	4.5	0.475	2.521	1.286	0.402	31.342	42
4.1	1.028	0.9						
4.5	1.235	5.6						
1.2	1.056	2.1						
1.7	1.296	0.6						
8.0	1.280	6.9						
3.0	1.142	0.6						
8.0	1.280	6.9						
1.6	1.530	1.4						
1.9	0.949	0.8						
2.7	1.161	2.0						
1.7	1.122	0.6						
1.1	1.084	1.1						
11.6	0.422	10.1						
4.3	1.174	1.0						
2.6	1.104	2.5						
3.7	1.480	0.3						
0.4	1.506	0.2						
3.2	1.801	0.7						
1.2	1.246	1.0						
1.4	1.712	2.0						
19.8	2.675	19.6						
1.3	1.771	0.9						
2.0	1.087	2.6						
0.9	1.398	0.5						
14.5	0.641	16.2						
1.7	1.270	0.7						
1.1	1.493	0.9						
1.8	0.924	0.8						
2.7	1.077	1.1						
9.5	0.846	9.3						
2.6	1.164	0.9						
4.2	0.804	1.8						
2.1	0.290	1.7						
3.1	0.537	0.7						
1.7	1.168	0.7						
2.9	1.184	2.4						
1.8	0.934	0.4						
10.1	0.831	9.8						
3.1	0.537	0.7						
3.4	0.929	3.5						
4.9	1.365	2.3						

(Continued on p. 302)

TABLE 2 *(continued)*

Homologue (No.)	*PCB isomer No.*	*RRF* (relative to OCN)*		*SE-54 column*				*Apiezon M column*
		Mean	*RSD (%)*	*RRF (relative to MBB)*		*RRF (relative to TBB)*		*RRF (relative to DCN)*
				Mean	*RSD (%)*	*Mean*	*RSD (%)*	*Mean*
7-Cl (24)	170	1.698	3.6	0.466	2.5	0.858	3.1	0.414
	171	1.033	3.6	0.542	1.3	1.032	2.2	0.209
	172	1.722	2.3	0.802	2.1	1.482	3.5	0.253
	173	0.852	6.4	0.452	1.5	0.833	0.9	0.180
	174	1.344	6.4	0.724	1.3	1.333	0.8	0.279
	175	9.084	2.0	2.868	26.1	5.406	26.8	1.809
	176	0.652	4.9					0.279
	177	0.292	1.9	0.314	1.8	0.595	1.3	0.057
	178	2.418	3.1	1.007	0.6	1.861	0.9	0.403
	179	1.203	9.9	0.624	0.3	1.153	0.5	0.269
	180	1.113	4.1	0.516	1.1	0.984	0.8	0.274
	181	1.142	2.6	0.437	2.0	0.846	0.7	0.161
	182	1.123	6.7	0.596	1.6	1.097	0.6	0.237
	183	1.305	6.3	0.648	1.4	1.194	0.9	0.221
	184	0.978	2.8	0.499	1.5	0.959	1.7	0.203
	185	0.813	1.9	0.441	3.3	0.841	2.2	0.167
	186	0.964	0.7	3.121	13.7	5.466	13.5	0.226
	187	0.904	10.8	0.376	10.7	0.659	10.4	0.223
	188	1.444	6.1	0.770	0.7	1.418	1.8	0.303
	189	1.993	3.2	0.830	7.0	1.534	9.0	0.036
	190	0.862	1.4	0.429	1.6	0.808	0.7	0.178
	191	0.490	2.5	0.253	2.6	0.480	2.1	0.106
	192	0.736	5.3	0.379	0.9	0.698	2.1	0.161
	193	0.868	1.5	0.295	2.2	0.517	2.1	0.165
8-Cl (12)	194	0.967	1.5	0.533	0.7	1.005	1.7	0.196
	195	2.376	1.4	1.124	3.7	2.141	2.5	0.574
	196	1.263	3.1	0.582	1.9	1.113	1.1	0.233
	197	1.116	4.1	0.537	1.2	1.034	0.4	0.251
	198	0.831	32.9	0.415	36.4	0.807	37.0	0.232
	199	0.969	2.4	0.443	0.5	0.852	1.8	0.201
	200	2.261	3.2	1.124	1.8	2.071	2.5	0.505
	201	1.655	3.2	0.760	1.9	1.454	1.1	0.306
	202	1.244	2.7	0.692	1.3	1.303	1.5	0.223
	203	0.910	3.3	0.399	4.8	0.721	5.5	0.201
	204	2.595	5.0	1.433	4.0	2.699	2.5	0.471
	205	0.883	1.8	0.415	4.1	0.783	5.7	0.184
9-Cl (3)	206	0.953	1.8	0.329	1.2	0.645	1.5	0.155
	207	0.764	3.5	0.378	1.1	0.725	1.9	0.260
	208	1.032	1.5	0.473	2.0	0.890	0.8	0.092
10-Cl (1)	209	0.659	2.2	0.335	2.9	0.638	1.7	0.264

* These values were obtained by averaging the RRFs obtained from the two columns in most instances.

			Statistics of RRFs (relative to OCN) for homologue					
	RRF (relative to TCN)		*Range*		*Mean*	*Standard deviation*	*RSD (%)*	*N*
RSD (%)	*Mean*	*RSD (%)*	*Low*	*High*				
5.0	1.823	2.6	0.292	9.084	1.452	1.695	116.7	24
4.2	1.185	0.9						
1.8	1.196	2.9						
1.2	0.850	0.5						
1.2	1.320	0.2						
2.1	8.690	6.9						
3.1	1.393	1.2						
3.0	0.250	1.5						
2.5	2.306	0.4						
3.2	1.539	1.0						
2.3	1.207	3.2						
2.5	0.707	1.2						
2.8	1.122	2.4						
1.8	1.164	0.8						
2.5	0.881	0.9						
2.7	0.736	3.3						
2.4	1.095	0.7						
3.9	1.079	1.7						
1.0	1.434	0.4						
3.3	0.206	1.1						
2.3	0.855	5.6						
2.5	0.468	0.9						
1.3	0.760	0.4						
2.6	0.799	0.9						
4.0	0.850	3.1	0.831	2.595	1.423	0.640	45.0	12
2.7	2.532	4.0						
2.1	1.142	0.9						
3.3	1.088	1.7						
2.4	0.928	1.6						
5.8	0.861	1.7						
3.1	2.353	2.5						
2.0	1.501	0.8						
3.0	1.061	0.9						
3.7	0.470	5.1						
3.6	2.235	1.4						
3.5	0.810	1.4						
1.8	0.815	1.0	0.764	1.032	0.916	0.138	15.1	3
3.7	1.039	2.3						
1.7	0.405	0.7						
4.5	1.055	2.9						

been analyzed on SE-54 and Apiezon M fused-silica capillaries and the RRF compared (Table 2). The RRF was calculated as follows:

$$RRF = \frac{Amt_i}{A_i} \cdot \frac{A_{i.s.}}{Amt_{i.s.}}$$

where RRF = relative response factor for PCB isomer i; Amt_i = amount of PCB isomer i injected; A_i = peak area; $A_{i.s.}$ = peak area for internal standard; $Amt_{i.s.}$ = amount of internal standard.

The relative standard deviation for the RRFs within a homologous series ranged from 32 to 117%. Thus, for accurate quantification of individual chlorobiphenyl isomers by HRGC–ECD, the appropriate RRF must be employed. The largest variation is observed with the Cl_7 homologous series. As additional chlorine substituents are introduced on to the biphenyl nucleus, the limits of detection do not significantly decrease and the response variation decreases as ring substitution pattern no longer plays a large role in determining the magnitude of response. This is not the case, of course, with the lower homologous series where the ring substitution pattern and number of chlorine substituents are important determinants of electron-capture detector response.

In a parallel study, the relative molar response (RMR, for m/z 35) has been determined for individual chlorobiphenyl isomers using high-resolution GC–NICI-MS. The operating conditions were as previously given in Table 1. The RMR was calculated as follows:

$$RMR_i = \frac{A_i}{A_{i.s.}} \cdot \frac{MW_i}{MW_{i.s.}} \cdot \frac{Amt_{i.s.}}{Amt_i}$$

where A_i = peak area of PCB isomer i; $A_{i.s.}$ = peak area of internal standard; MW_i = molecular weight of PCB isomer i; $MW_{i.s.}$ = molecular weight of internal standard; $Amt_{i.s.}$ = amount of internal standard; Amt_i = amount of PCB isomer i.

The relative standard deviation for the RMR_i values within a homologous series ranged from 33 to 125% (Table 3). It is apparent that appropriate RMR factors must be employed for accurate quantification.

A summary comparison of response factors is given in Table 4 for electron-capture, NCI-MS and EI-MS (SIM) detection. Literature values for EI-MS were normalized to the lowest response within a homologous series. Thus, a comparison between homologs was not possible[4,5]. On the other hand, it can readily be seen that EI-MS exhibited the lowest relative standard deviation within a homologous series when compared with ECD and NICI-MS. Some caution is needed in this comparison as the number of chlorobiphenyl isomers used in the study was small. This observation is consistent with the expectation that EI-MS produces the smallest variation of response between chlorobiphenyl isomers. No comparable data for positive ion chemical ionization have been reported; however, the variations in responses should be similar to EI-MS.

The extreme RRF_i values determined with electron-capture detection were approximately 190:1. The range of RRF values was not as large as reported by other investigators[1,15–18]. This difference may reflect differences amongst instrumental systems.

The extreme RMR_i values (the calculation includes MW) determined with NICI-MS were approximately 320:1.

The importance of using appropriate response factors for quantifying individual chlorobiphenyl isomers is evident from the above observations.

4. CHLOROBIPHENYL ISOMERS AND INSTRUMENT CALIBRATION

4.1. Primary standards

The issue of instrument calibration for chlorobiphenyl isomer quantification in environmental, biological and process stream samples has been recognized, and for this reason the synthesis of the individual isomers was performed[57].

Primary standards have been also needed for establishing reference data (spectral) banks for NICI-MS, EI-MS, FTIR, etc., for use in qualitative analysis or for verification purposes. They have been needed to establish relative retention indices to standardized fused-silica capillary GC (FSCGC) operating conditions.

The large-scale synthesis of chlorobiphenyl isomers would obviously be an expensive proposition, particularly if the supply must accommodate the needs of many analytical laboratories performing isomer quantification in environmental, biological and process stream samples. Hence research on devising alternative techniques for instrument calibration has been needed.

4.2. Secondary standards

One approach has been to determine whether a secondary standard for calibrating instruments for quantification could be used so that this secondary standard could be widely distributed among laboratories involved in chlorobiphenyl isomer analysis. The initial availability of the primary standards (209) has been the key to developing a secondary standard.

Two approaches have been investigated. One is the characterization of an Aroclor "cocktail" using primary standards[58–63]; the other employs a "clustering" of similar response factors to derive a small subset of chlorobiphenyls from the 209 primary standards, with a chlorobiphenyl serving as a surrogate for several isomers[64] Each of these concepts is discussed here.

4.3. Characterization of Aroclor mixtures

As ample amounts of various commercial Aroclors have been available for distribution, the use of an Aroclor "cocktail" that was thoroughly characterized with respect to isomer speciation and amount has been studied.

An Aroclor 1016–1254–1260 mixture (2.5:2.0:1.0, w/w) was used. For characterization the use of relative retention time data for primary standards and matching with those in the Aroclor cocktail mixture for three different HRGC–ECD systems and one HRGC–NICI-MS system were employed. In addition, the molecular ions and mass spectra obtained from HRGC–NICI-MS and HRGC–EI-MS analysis of the Aroclor cocktail mixture were used to establish the identities of the components in each of the chromatographic peaks.

A more complete Aroclor cocktail characterization (qualitatively and quantitatively) would be desirable and allow its use as a secondary standard for instrument

TABLE 3

RELATIVE MOLAR RESPONSES (*m/z* 35) FOR INDIVIDUAL PCB ISOMERS USING GLASS CAPILLARY GC–NICI-MS

*Homologue (No.)**	*PCB isomer No.***	*RRF_i*	*(RSD, %)*	*Range*	*Mean ± S.D. (RSD, %)*	*N****
1Cl (3)	1	0.356	(6.8)§	0.091–0.356	0.184 ± 0.15 (81)	3
	2	0.105	(6.2)			
	3	0.091	(5.0)			
2Cl (12)	5	0.574	(10)§	0.574–4.223	1.662 ± 1.23 (74)	8
	7	4.223	(11)			
	9	2.064	(0.6)			
	10	2.430	(4.4)			
	11	0.574	(4.6)			
	12	1.452	(5.0)			
	14	1.209	(1.3)			
	15	0.773	(1.0)			
3Cl (24)	18	0.340	(12)§§	0.340–5.143	1.378 ± 1.72 (125)	7
	21	0.842	(3.1)			
	22	5.143	(2.6)			
	26	0.623	(3.1)			
	29	0.462	(4.3)			
	30	1.686	(4.4)			
	31	0.555	(1.9)			
4Cl (42)	40	1.240	(6.3)§§	0.048–2.013	0.971 ± 0.48 (50)	6
	42	1.208	(3.0)			
	44	0.920	(4.7)			
	47	1.447	(2.5)			
	53	0.048	(16)			
	54	0.358	(0.5)			
	55	0.779	(5.3)			
	60	2.013	(1.9)			
	61	1.562	(2.3)			
	65	0.789	(0.6)			
	69	1.055	(4.6)			
	70	0.989	(8.4)			
	72	0.840	(18)			
	75	1.038	(1.7)			
	77	0.364	(3.9)			
	81	0.893	(1.7)			
5Cl (46)	85	1.077	(2.0)§§§	0.465–1.216	0.805 ± 0.27 (33)	12
	87	0.564	(8.0)			
	93	0.528	(4.6)			
	101	0.818	(8.0)			
	106	1.123	(4.8)			
	112	0.667	(3.1)			
	114	1.137	(4.0)			
	116	1.216	(1.8)			
	117	0.839	(2.5)			
	118	0.465	(8.4)			
	121	0.634	(5.5)			
	122	0.591	(2.3)			

TABLE 3 *(continued)*

*Homologue (No.)**	*PCB isomer No.***	*RRF_i*	*(RSD, %)*	*Range*	*Mean ± S.D. (RSD, %)*	*N****
6Cl (42)	128	1.011	(4.8)§§§	0.369–1.440	0.817 ± 0.29 (36)	16
	129	0.901	(8.4)			
	131	1.256	(4.8)			
	133	0.559	(2.7)			
	136	0.396	(4.9)			
	137	0.911	(4.6)			
	139	0.747	(4.1)			
	141	1.440	(8.6)			
	147	1.042	(6.8)			
	151	1.030	(7.3)			
	155	0.369	(2.9)			
	159	0.525	(0.7)			
	160	0.735	(2.5)			
	161	0.719	(2.3)			
	163	0.688	(4.4)			
	165	0.738	(3.9)			
7Cl (24)	170	0.451	(10)	0.236–1.192	0.703 ± 0.30 (43)	13
	173	0.659	(0.4)			
	175	0.305	(2.5)			
	180	0.679	(11)			
	184	0.708	(7.0)			
	185	0.755	(16)			
	186	0.236	(4.9)			
	187	1.102	(11)			
	188	0.503	(5.1)			
	191	1.192	(3.0)			
	192	0.542	(5.0)			
	193	1.096	(8.0)			
8Cl (12)	194	0.637	(1.3)§§§	0.241–1.116	9.573 ± 0.26 (46)	8
	196	0.608	(3.9)			
	197	1.116	(9.0)			
	199	0.393	(6.3)			
	200	0.486	(6.8)			
	201	0.413	(5.3)			
	202	0.692	(5.9)			
	204	0.241	(9.1)			
9Cl (3)	206	0.431	(8.0)§§§	0.066–0.565	0.354 ± 0.26 (73)	3
	207	0.066	(26)			
	208	0.565	(20)			
10Cl (1)	209	0.418	(7.5)§§§	–	–	–

* (No.) = number of theoretical isomers possible.
** See ref. 58 for assignments.
*** N = number of isomers studied; each isomer was measured in triplicate.
§ Internal standard = 1,2-dichloronaphthalene.
§§ Internal standard = 1,2,3,4.tetrachloronaphthalene.
§§§ Internal standard = octachloronaphthalene.

TABLE 4

COMPARISON OF RELATIVE RESPONSE FACTORS BETWEEN GLASS CAPILLARY GC–ECD, GC–EI-MS (MOLECULAR ION) AND GLASS CAPILLARY GC–NICI-MS (*m/z* 35) FOR HOMOLOGOUS SERIES OF PCBs

Homologous series	*Glass capillary GC–ECD**			*Glass capillary GC–NICI-MS**			*GC–EI-MS***		
	*Range****	*Mean ± S.D. (RSD, %)*	*N*§	*Range****	*Mean ± S.D. (RSD, %)*	*N*§	*Range*§§	*Mean ± S.D. (RSD, %)*	*N*§
1Cl(3)§§§	27.319–55.970	43.48 ± 14.67 (34)	3	0.456–1.787	0.924 ± 0.75 (81)	3	1.000–1.090	1.050 ± 0.04 (3.8)	3
2Cl(12)	2.666–12.659	6.00 ± 3.22 (54)	9	2.881–21.199	8.343 ± 6.17 (74)	8	1.000–2.062	1.736 ± 0.30 (17)	10
3Cl(24)	0.657–4.734	2.22 ± 1.12 (50)	9	0.721–10.901	2.921 ± 3.64 (125)	7	1.000–1.627	1.400 ± 0.24 (17)	9
4Cl(42)	0.331–2.605	1.49 ± 0.49 (33)	31	0.102–4.267	2.058 ± 1.02 (50)	16	1.000–2.146	1.549 ± 0.33 (21)	11
5Cl(46)	0.510–3.704	1.49 ± 0.64 (43)	35	0.465–1.216	0.805 ± 0.27 (33)	12	1.000–1.013	1.004 ± 0.01 (0.7)	3
6Cl(42)	0.475–2.521	1.29 ± 0.40 (31)	37	0.369–1.440	0.817 ± 0.29 (36)	16	1.000–1.321	1.153 ± 0.11 (9.6)	7
7Cl(24)	0.292–9.084	1.45 ± 1.69 (117)	21	0.236–1.192	0.703 ± 0.30 (43)	13	–	–	0
8Cl(12)	0.831–2.595	1.42 ± 0.64 (45)	10	0.241–1.116	0.573 ± 0.26 (46)	8	1.000–1.359	1.179 ± 0.25 (22)	2
9Cl(3)	0.764–1.032	0.92 ± 0.14 (15)	3	0.066–0.565	0.354 ± 0.26 (73)	3	–	–	0
10Cl(1)	–	1.168	1	–	0.418	1	–	–	0
	Overall: 0.292–55.97 (≈190:1)			Overall: 0.066–21.199 (≈320:1)					

* From Tables 2 and 3, respectively.
** From ref. 45.
*** All values are relative to octachloronaphthalene.
§ *N* = number of PCB isomers included in measurement.
§§ Responses were relative to lowest response for each group.
§§§ Values in parentheses are numbers of theoretical isomers possible.

calibration, provided that identical high-resolution chromatographic conditions are employed in sample analysis as used for the Aroclor characterization.

4.4. Clustered secondary standard

Another reported approach is the development of a secondary standard that contains a small subset of individual chlorobiphenyl isomers that can be used to develop instrument responses for all PCBs of interest[64].

5. APPLICATION TO THE ANALYSIS OF PCBs AND PPBs IN ENVIRONMENTAL AND BIOLOGICAL SAMPLES

With these recent parallel developments, laboratories have begun to characterize and quantify chlorobiphenyl isomers in environmental[65–72], biological[73–77] and process stream samples. A few examples of the combined state-of-the-art technology described above are discussed here.

The benefit of high-resolution GC over conventional packed column GC for analysis of sera and adipose tissue is exemplified by Figs. 14 and 15, respectively[77]. Fig. 14 depicts the profile for a human serum sample extract chromatographed on a packed column (top) and a capillary (bottom). Improved resolution and hence a better signal-to-noise ratio are achieved with high-resolution GC. Fig. 15 shows that the low-resolution profile has many hidden isomers in its chromatographic peaks, which are revealed by high-resolution GC.

Identification of specific chlorobiphenyl isomers in human tissues may be important for two reasons: for the assessment of long-term persistence and for the evaluation of potential health effects as suggested by toxicological studies on individual isomers. Recently, the disposition of PCB isomers in occupationally exposed persons has been reported[78]. The concentrations of PCBs in adipose tissue and plasma were related to the duration and intensity of exposure in the workplace. It was reported that PCB levels in adipose tissue were proportional to those in plasma (total PCB ratio 190:1). The distribution of specific chlorobiphenyl isomers between plasma and adipose tissue was reported, however, to be related to specific ring position substitution, differing among isomers.

These state-of-the-art techniques are currently being applied to the analysis of samples of rainwater, surface water, sediment, fish, human milk, maternal cord blood, etc.[35].

Finally, an area of considerable activity involves the development of computerized data analysis systems. With the use of automated gas chromatographs, a considerable amount of data are generated when using FSCGC.

Computerization is not only being introduced to facilitate calculations of PCB isomer and total PCB levels in samples, but is also being developed to assist in answering comparative questions about samples[79]. Questions relating to biotransformation, distribution and fate through an ecosystem and sources require sophisticated pattern recognition techniques for comparing and relating PCB information between environmental and biological samples[79].

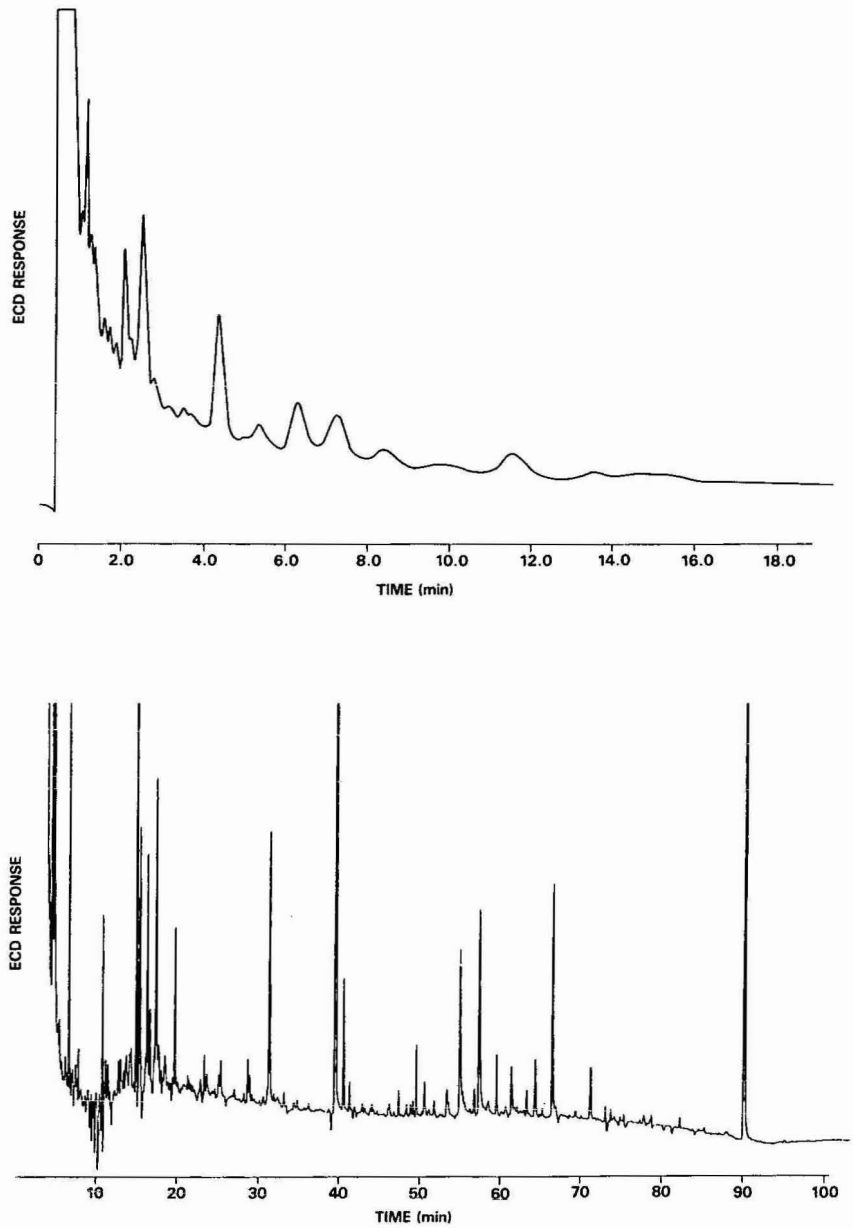

Fig. 14. Chromatograms of human serum sample. Top, packed column; bottom, capillary.

6. CONCLUSION

Significant studies have been performed during the past 10 years in developing: (a) high-resolution GC capillaries tailor-made for PCB analysis; (b) ultra-sensitive electron-capture detectors compatible with capillary column flow-rates; (c) an ex-

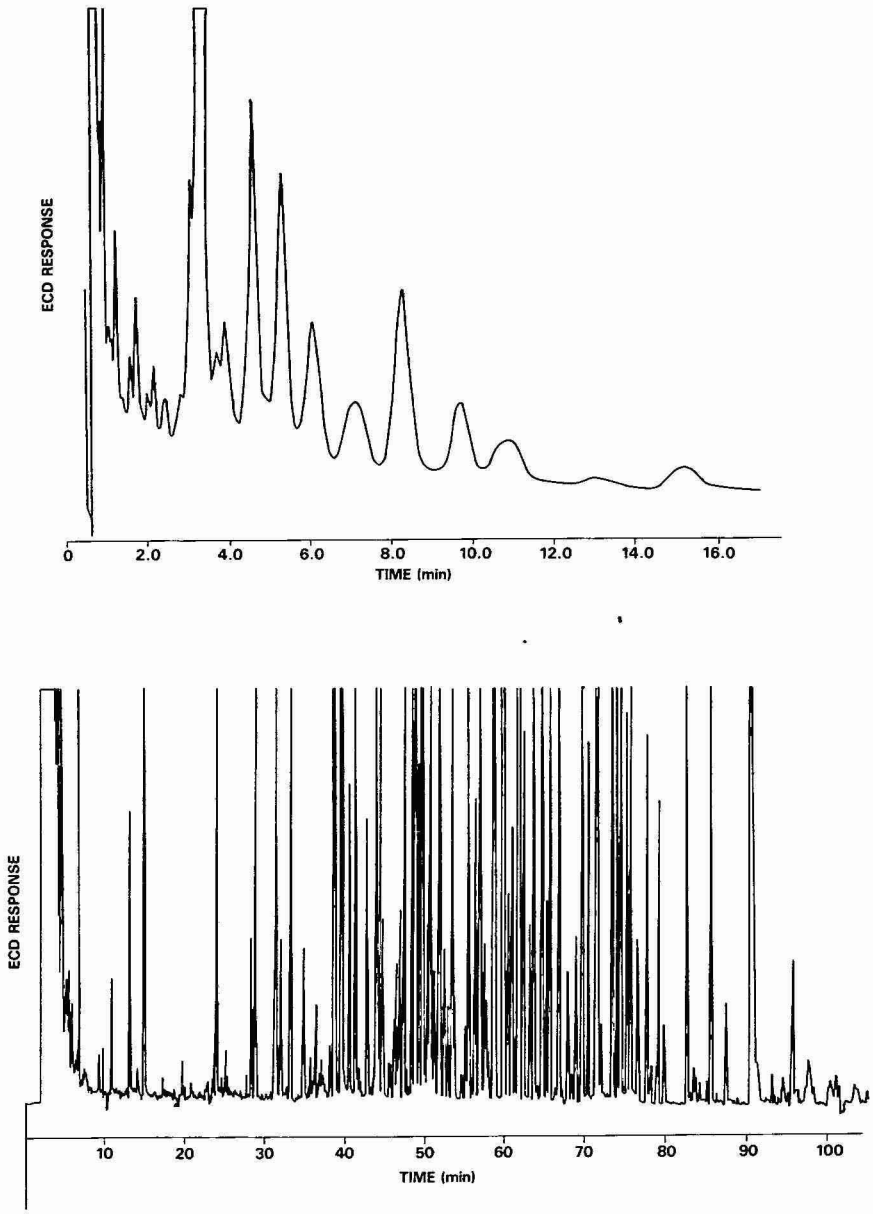

Fig. 15. Chromatograms of human adipose tissue. Top, packed column; bottom, capillary.

panded linear dynamic range of operation for the electron-capture detector; (d) the operating conditions for NICI-MS necessary to measure and characterize PCBs in environmental and biological samples; and (e) the development of primary and secondary PCB standards for calibration of instrumentation. Research in the last area continues as more work needs to be done. Investigators are just beginning to reap

the fruits of their labor by applying this advanced analytical methodology to real-world problems.

7. ACKNOWLEDGEMENTS

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8. SUMMARY

Analytical advances in the detection, identification and quantification of polychlorinated biphenyl isomers (PCBs) are reviewed. High-resolution gas chromatography, with specific reference to capillary column development and support "phases", methodologies, detector systems and the comparative advantages and limitations of each combination, is covered. Problems associated with instrument calibration, general non-availability of primary PCB standards and the use of secondary standards are discussed. Typical applications of these newer methods to environmental, biological and process stream samples are presented.

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