ISSN 0021-9673

VOL. 214 NO. 3 OCTOBER 2, 1981

THIS ISSUE COMPLETES VOL. 214

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RNAL OF

CHROMATOGRAPHY

ERNATIONAL JOURNAL ON CHROMATOGRAPHY, ELECTROPHORESIS AND RELATED METHODS

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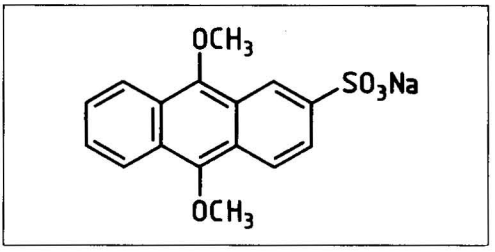

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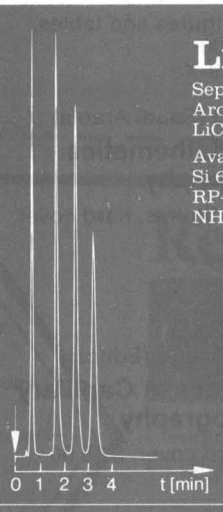

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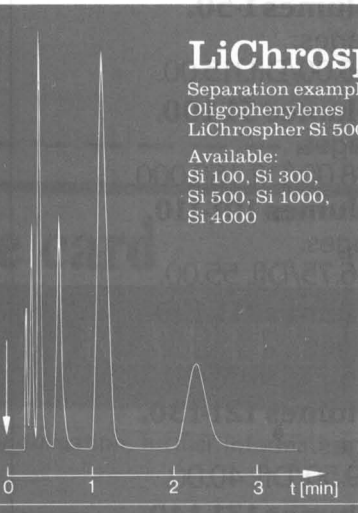

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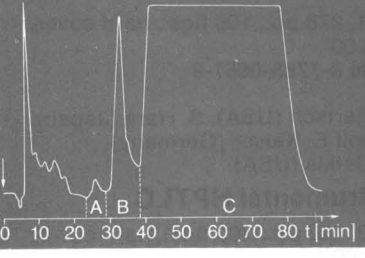

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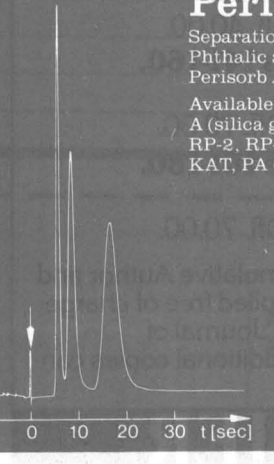

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GAS–SOLID CHROMATOGRAPHY OF ETHANE ON ACTIVATED CARBON AT 25°C

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(First received March 10th, 1981; revised manuscript received May 7th, 1981)

SUMMARY

The adsorption and diffusion of ethane in a chromatographic column packed with activated carbon were investigated for different concentrations and flow-rates. The adsorption isotherm of ethane can be represented by a three-parameter equation. The experimental data and theoretical asymptotic concentration profiles agree at high inlet concentrations and deviate at low inlet concentrations. The longitudinal diffusion coefficients were extracted from fitting the experimental data. Analytic criteria presented here for achieving an asymptotic concentration profile predict that the best result occurs at the flow-rate corresponding to the minimum of the height equivalent to a theoretical plate.

INTRODUCTION

Developments in gas–solid chromatography (GSC) progress less rapidly than in gas–liquid chromatography (GLC) because of the mathematical difficulty associated with a non-linear isotherm for gas–solid equilibrium, which is usually observed even at very low concentrations[1]. The analysis of GSC usually assumes that there is no variation of the gas-flow velocity in the radial direction and that longitudinal diffusion is negligible. These assumptions avoid the difficulty associated with a non-linear isotherm by permitting a first-order partial-differential equation which can be solved formally by a suitable transform of variables[2,3]. When longitudinal diffusion is taken into consideration, the differential equation is generally too complicated to solve analytically except for certain simple isotherms (*e.g.*, linear[4] or Langmuir[5]); however, some of the important physical properties such as adsorption capacities and diffusion coefficients can still be determined from transmission data for a step-function input provided the concentration has reached an asymptotic concentration profile.

After an adsorbate traverses a sufficiently long column, the adsorbate concentration reaches an asymptotic form called the asymptotic concentration profile. An asymptotic concentration profile occurs for a step-increase in the concentration of an adsorbate that exhibits a convex isotherm (*e.g.*, Langmuir or type I BET) and also for a step-decrease in the concentration of an adsorbate with a concave isotherm such as

0021-9673/81/0000–0000/$02.50

type III BET. An asymptotic concentration profile results when the effect of diffusion is offset by the effect of the curvature of the isotherm. For an observer moving with the average migration speed of the adsorbate, the concentration profile is independent of time. An asymptotic concentration profile can be measured as a function of the longitudinal coordinate by an observer moving along with the adsorbate or as a function of time by a laboratory observer. The possibility of approaching an asymptotic concentration profile was first noted by Sillen and Ekedahl[6]. Later Lapidus and Rosen[7] studied the asymptotic concentration profile of an ion-exchange column; Lightfoot[8] gave more quantitative mathematical discussions for an equilibrium system with longitudinal diffusion; and Acrivos[9] discussed a system with external mass-transfer resistance. Reports of experimental work on asymptotic concentration profiles are rare in the literature in comparison with theoretical discussions. In this paper, we present experimental results for the transmission of ethane on activated carbon at 25°C; calculate the adsorption capacity for ethane at different inlet concentrations; and compare transmission data with theoretical curves. Transmission is the ratio of the outlet concentration to the inlet concentration.

THEORY

For isothermal adsorption of a trace component in a constant carrier-gas flow, the equation for the gas-phase adsorbate concentration C can be written as[4,8,9]:

$$\frac{\partial}{\partial t}\left[\varepsilon C + (1 - \varepsilon)\, q\right] = D\frac{\partial^2 C}{\partial Z^2} - u\frac{\partial C}{\partial Z} \tag{1}$$

The initial and boundary conditions for an initially desorbed column are:

$$C\,(Z, 0) = 0 \tag{2}$$

$$q\,(Z, 0) = 0 \tag{3}$$

$$C\,(0, t) = C_0 \tag{4}$$

$$C\,(\infty, t) = 0 \tag{5a}$$

$$q\,(\infty, t) = 0 \tag{5b}$$

The symbol q is the solid-phase concentration of the adsorbate, ε is the void fraction in the packed column, u is the superficial flow velocity of the gas mixture, and D is the longitudinal diffusion coefficient. We used an infinite column length for the boundary condition in eqns. 5a and 5b because the asymptotic concentration profile can be attained only after a sufficiently long distance.

Let us introduce a new moving coordinate variable X defined as[8]:

$$X \equiv \frac{u}{D}\left[Z - \frac{C_0\, ut}{\varepsilon C_0 + (1 - \varepsilon)\, q_0}\right] \tag{6}$$

[where $q_0 = q(0, t)$]. For an asymptotic concentration profile, eqn. 1 becomes

$$\frac{d^2 C}{dX^2} - \frac{d}{dX}\left[\frac{Cq_0 - qC_0}{\frac{\varepsilon C_0}{1-\varepsilon} + q_0}\right] = 0 \tag{7}$$

Note that the concentration C is a function of X only. Eqn. 7 can then be integrated to yield

$$\frac{dC}{dX} - \frac{Cq_0 - qC_0}{\frac{\varepsilon C_0}{1-\varepsilon} + q_0} = 0 \tag{8}$$

where we used eqns. 5a and 5b and the fact that the slope $\left(\frac{dC}{dX}\right)_{C=0}$ of the asymptotic concentration profile at the leading-edge ($C \equiv 0$) is zero. The integration of eqn. 8 gives the formal solution for the asymptotic concentration profile[8]:

$$\frac{t_2 - t_1}{\tau} = \frac{X_1 - X_2}{\frac{\varepsilon C_0}{(1-\varepsilon)\, q_0} + 1} = \int_{C_1/C_0}^{C_2/C_0} \frac{d\, C/C_0}{q/q_0 - C/C_0} \tag{9}$$

where the definition of the time-constant τ is

$$\tau \equiv \frac{D\,[C_0\,\varepsilon + q_0\,(1-\varepsilon)]^2}{q_0\, C_0\, u^2\,(1-\varepsilon)} \tag{10}$$

The derivation of eqn. 9 does not depend on any specific relation between C and q. The only prerequisite is that the integral converges. Note that for the case of a convex isotherm, the integral has a negative value and the asymptotic concentration occurs for the desorption profile where C decreases with increasing values of the time coordinate t/τ. For a linear isotherm (*viz.*, q/C = constant), the integral diverges. Thus, eqn. 9 indicates a well-known fact that the profile of a linear-isotherm system diffuses continuously without forming an asymptotic concentration profile[10,11]; moreover, it does not demand an equilibrium relation between the gas-phase and the solid-phase. When the non-equilibrium relation prevails, both C and q still have asymptotic concentration profiles; but another equation is needed to obtain an expression for C and q as a function of the coordinate X. Garg and Ruthven[12] studied the non-equilibrium case for a Langmuir isotherm, which requires extensive numerical calculations. Since the flow-rates of the gas mixtures in this study were low, we treat here only the equilibrium relation between the gas and solid-phases[11]; furthermore, the approximations leading to eqn. 1 do not warrant introducing the intricate mass-transfer resistances in this study.

EXPERIMENTAL

The flow system and experimental procedures were described earlier[13]. The chromatographic column was a stainless-steel cylinder, 10 cm × 0.46 cm I.D., which

contained 0.588 gram of Columbia 4LXC 12/28 activated carbon (Union Carbide, New York, NY, U.S.A.). The carbon has intrinsic density 1.56 g/cm^3 and pore volume 0.51 cm^3/g. The composition of inlet ethane was controlled by two valves which separately adjusted the flow-rates of the pure helium and the calibrated ethane–helium mixture. Two calibrated ethane–helium mixtures were prepared by Matheson Gas Products (East Rutherford, NJ, U.S.A.) with nominal ethane concentrations of 500 and 10,000 ppm. The flow of each gas and the total flow-rate can be controlled to within an accuracy of $\pm 0.5\%$. The system pressure in the chromatographic column varied between 740 and 760 mmHg, but the pressure differences between the inlet and the outlet were always less than 10 mmHg. The activated carbon column was immersed in a two-layer water-bath which controlled the temperature at 25°C to within ± 0.02° C. The concentration of ethane at the outlet of the adsorber bed was measured at regular time intervals by a Varian 3700 gas chromatograph and a Spectra-Physics Minigrator. Transmissions at the outlet were corrected for detector non-ideality.

RESULTS AND DISCUSSION

Determination of the isotherm

The time-dependent transmission of ethane was measured for eight concentrations at various flow velocities; in addition, the transmission of the nominal 500 ppm mixture was measured at three flow velocities. Since the data show a regular trend, we list only four concentrations in Table I. The solid-phase concentration q_0 was calculated from the mass-balance equation

$$q_0 (1 - \varepsilon) L + C_0 \varepsilon L = u C_0 \int_0^{\infty} \left(1 - \frac{C}{C_0}\right) \mathrm{d}t \equiv u C_0 t_p \tag{11}$$

The propagation time t_p, which represents the average retention time of the adsorbate, was calculated from the transmission data by numerical integration. In eqn. 11, the integration term represents the adsorbate retained in the packed bed, while the first term and the second term of the left-hand side represent the adsorbate in the solid-phase and the gas-phase, respectively. From eqn. 11, the adsorption capacity K_0 is calculated as

$$K_0 \equiv \frac{q_0}{C_0} = \frac{1}{1 - \varepsilon}\left(\frac{u t_p}{L} - \varepsilon\right) \tag{12}$$

Gas-phase concentrations, solid-phase concentrations, and dimensionless adsorption capacities are listed in Table II for each run.

Fig. 1 is a plot of the relation between the solid-phase concentration and the gas-phase concentration. It is seen clearly that the solid-phase concentration increases with a decreasing slope when the gas-phase concentration increases. The gas–solid equilibrium relation does not follow either the Freundlich isotherm, which predicts a straight line on a logarithmic plot, or the Langmuir isotherm, which is shown as a dashed line fitted to the data for the two highest concentrations. In general, the Langmuir equation predicts a saturation of the solid-phase concentration at high gas-phase concentrations,and a proportional relation between gas- and solid-phase con-

TABLE I

TRANSMISSION *VERSUS* TIME OF FOUR DIFFERENT CONCENTRATIONS OF ETHANE IN A HELIUM CARRIER GAS FLOWING THROUGH AN ACTIVATED-CARBON ABSORBER BED AT 25°C

C_0 (10^{-8} moles/cm^3)							
39.2		10.1		1.98		0.47	
Run No.							
1		3		5B		7	
Time (sec)	Transmission	Time (sec)	Transmission	Time (sec)	Transmission	Time (sec)	Transmission
1920	0.00443	2120	0.00788	1670	0.00547	1650	0.00647
2020	0.0128	2220	0.0112	1770	0.0127	1770	0.0212
2120	0.0843	2320	0.0273	1870	0.0319	1890	0.0564
2220	0.349	2420	0.0721	1970	0.0732	2010	0.121
2320	0.668	2520	0.172	2070	0.150	2130	0.228
2420	0.856	2620	0.334	2170	0.267	2250	0.366
2520	0.940	2720	0.522	2270	0.415	2370	0.519
2620	0.975	2820	0.689	2370	0.569	2490	0.663
2720	0.989	2920	0.755	2470	0.705	2610	0.779
2820	0.9949	3020	0.888	2570	0.810	2730	0.864
2920	0.9978	3120	0.936	2670	0.883	2850	0.920
3020	0.9988	3220	0.964	2770	0.930	2970	0.956
		3320	0.980	2870	0.960	3090	0.975
		3420	0.988	2970	0.977	3210	0.987
		3520	0.9934	3070	0.990	3330	0.9920
		3620	0.99618			3550	0.9954

TABLE II

ETHANE CONCENTRATION IN THE GAS AND SOLID PHASES, THE DIMENSIONLESS ADSORPTION CAPACITY AND THE FLOW-RATE FOR EACH RUN

Run No.	Mole fraction of ethane (ppm)	Superficial flow-rate, u (cm/sec)	Gas-phase concentration, C_0 (10^{-9} mole/cm^3)	Solid-phase concentration, q_0 (10^{-5} mole/cm^3)	Adsorption capacity, K
1	9830	1.50	392	58.6	1500
2	5186	1.52	210	36.8	1760
3	2478	1.76	101	21.3	2100
4	1160	2.22	47.5	11.3	2380
5A	495	1.47	20.2	5.14	2550
5B	495	2.51	19.8	5.10	2580
5C	495	4.63	19.8	5.18	2620
6	229	2.52	9.29	2.58	2780
7	115	2.78	4.67	1.35	2900
8	44.7	3.05	1.82	0.54	2960

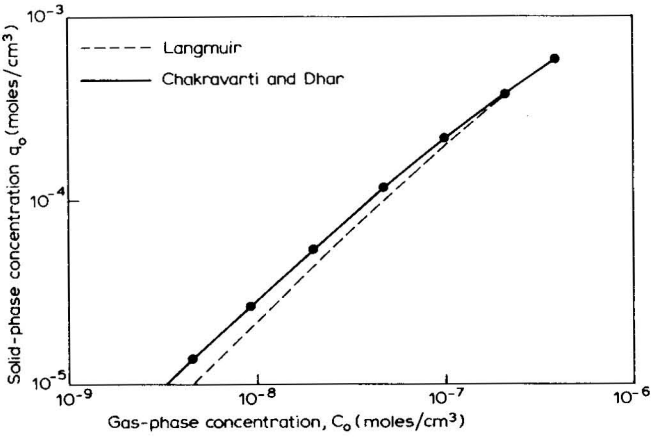

Fig. 1. The solid-phase concentration *versus* the gas-phase concentration. Circles are experimental results. The solid line is the isotherm of Chakravarti and Dhar[16]. The dashed line is the Langmuir equation fitted through the two points with the highest concentration.

centrations at low gas-phase concentrations. Our results on a logarithmic plot show a straight-line relation with a slope less than unity at low concentrations and a slightly convex curve at high concentrations. Similar experimental results were reported[14] in studies of the surface adsorption on non-polar solids at low concentrations of various gases; most of the isotherms[15], which were suggested to fit these data, contain the logarithm of the partial pressure of the adsorbate. Since the adsorbate concentration is zero initially, the inclusion of a logarithmic function causes a mathematical difficulty; therefore, to simplify the calculation we adopted the three-parameter isotherm of Chakravarti and Dhar[16]:

$$\frac{q_0}{q_0^s} = \frac{(K_m C_0)^v}{1 + (K_m C_0)^v} \tag{13}$$

Here q_0^s is the solid-phase concentration for monolayer coverage, and K_m and v are constants. Eqn. 13 will simplify to the Freundlich equation when $K_m C_0$ is very small. Also it simplifies to the Langmuir equation when $v = 1$.

The three parameters of eqn. 13 can be determined from experimental data. In order to calculate the parameters, we rewrite eqn. 13 as:

$$\log \frac{q_0}{q_0^s - q_0} - v \log K_m - v \log C_0 = 0 \tag{14}$$

The simultaneous determination of the three parameters (*viz.*, q_0^s, v, and K_m) requires a complicated calculation; however, the unique expression of eqn. 14 allows us to use a simple linear least-squares method to determine v and K_m provided q_0^s is known. In this method, we assume a value for q_0^s and calculate the best values for v and K_m for the assumed q_0^s. Also for each assumed q_0^s, we calculate the sum of the squares of the errors in the linear least-squares calculation. In Fig. 2, we plot the sum of squares of the errors for the assumed values of q_0^s ranging from $1.5 \cdot 10^{-3}$ to $2.5 \cdot 10^{-3}$ mole/cm^3.

The best value for q_0^s corresponding to the smallest error is $1.9 \cdot 10^{-3}$ mole/cm^3; according to this value, v and K_m are 0.927 and $1.06 \cdot 10^6$ cm^3/mole, respectively. The solid line in Fig. 1 is the calculated isotherm based on the above parameters.

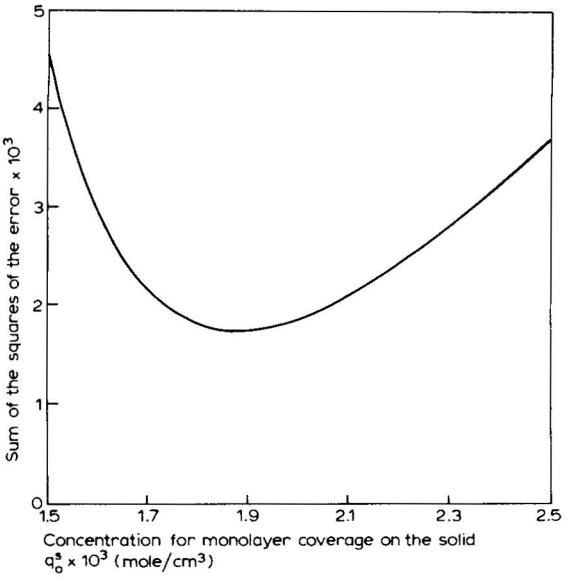

Fig. 2. The sum of the squares of the errors in the linear least-squares calculation *versus* the assumed concentration q_0^s for a monolayer coverage on the solid. For each q_0^s, the parameters v and K_m are calculated based on eqn. 14 and applied to calculate the error for the datum of an individual run.

Although eqn. 14 represents experimental data very well, we do not claim the superiority of this isotherm over others because data are needed over a wider range of concentrations and temperatures to support an isotherm. Also, the Chakravarti–Dhar isotherm is more applicable to chemisorption rather than to physical sorption[17]. These two adsorptions can be distinguished from their isosteric heats of adsorption which can be calculated from isotherm data at different temperatures. We are still conducting measurements at different temperatures; but the use of eqn. 13 is sufficient for this work.

Asymptotic concentration profile

Eqn. 9 is the general formula of the asymptotic concentration profile. The integral in eqn. 9 can be rewritten as

$$\frac{t_2 - t_1}{\tau} = \int_{C_1/C_0}^{C_2/C_0} \frac{\mathrm{d}C/C_0}{\left(\frac{C}{C_0}\right)^v \left[\frac{1 + B^v}{1 + B\left(\frac{C}{C_0}\right)^v}\right] - \frac{C}{C_0}} \tag{15}$$

where

$$B \equiv K_m C_0. \tag{16}$$

Even though a numerical calculation is required, the application of eqn. 15 is not difficult because it includes only one parameter B, which can be determined from an isotherm. Actually, if one assumes that the asymptotic concentration profile has developed, then one can obtain an isotherm (up to the input concentration) from data of a single run without having any *a priori* knowledge of the isotherm[18]. A similar conclusion was reached in the study of GLC where both a very long column and a very long propagation time are usually achieved[19,20]. In the next section, we discuss criteria for an asymptotic concentration profile.

With the knowledge of K_m and B values, the transmission of the adsorbate can be determined (within an integration constant) from eqn. 15. The only unknown parameter is τ. Specifically, for each run with known flow-rate and other physical data, the parameter τ depends on the diffusion coefficient. Determination of the diffusion coefficient separately from the isotherm avoids fitting data with excessive parameters. In Table III, we list average values of τ for each run. These numbers were determined by using two successive transmissions between 0.1 and 0.9 for evaluating the integral in eqn. 15. Note that large errors in τ would result from using transmissions near zero or unity. The time constants are then determined from eqn. 15.

TABLE III

TIME CONSTANT, LONGITUDINAL DIFFUSION COEFFICIENT, HETP AND DIMENSIONLESS PARAMETERS α AND β FOR EACH RUN

Run No.	*Time constant, τ (sec)*	*Longitudinal diffusion coefficient, D (cm^2/sec)*	*HETP, H (cm)*	*Dimensionless parameters*	
				α	β
1	36	0.24	0.32	0.028	0.14
2	36	0.21	0.28	0.034	0.17
3	30	0.20	0.22	0.042	0.20
4	23	0.21	0.19	0.049	0.23
5A	43	0.16	0.22	0.063	0.34
5C	22	0.24	0.19	0.058	0.29
5C	11	0.38	0.17	0.054	0.25
6	22	0.23	0.18	0.061	0.30
7	22	0.26	0.19	0.064	0.34
8	20	0.28	0.18	0.064	0.35

Fig. 3 is a plot of the transmission *versus* $(t - t_{1/2})/\tau$ for runs 1, 3, and 5B. Since the half-transmission point always corresponds to zero on the abscissa, the time constant τ is the only adjustable parameter for fitting the data. A comparison in Fig. 3 reveals that the fitting is very good for high concentrations even at both ends. For lower concentrations with a more diffuse theoretical curve, the fit becomes poorer; furthermore, the measured concentration is higher than the theoretical results. This fact indicates that the gas-phase concentration is higher than the equilibrium values. The higher concentration is possible because of a mass-transfer resistance, or because the profile has insufficient time to diffuse completely.

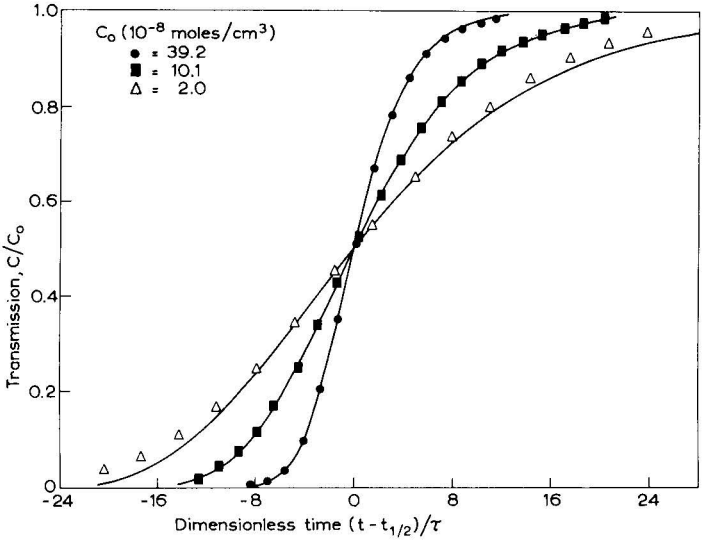

Fig. 3. The transmission *versus* the dimensionless time for runs 1, 3 and 5B.

Effect of flow-rate on transmission

To explore the possibility of non-equilibrium between gas and solid-phases, we varied the flow-rate for a fixed input concentration. For a given value of the input concentration C_0, the transmission data for different flow-rates can be superimposed on the same asymptotic concentration profile, as can be seen from eqn. 15. We measured the transmission of a 495 ppm mixture at three different flow-rates and plotted the results in Fig. 4. It can be seen that deviations exist for all runs, and that

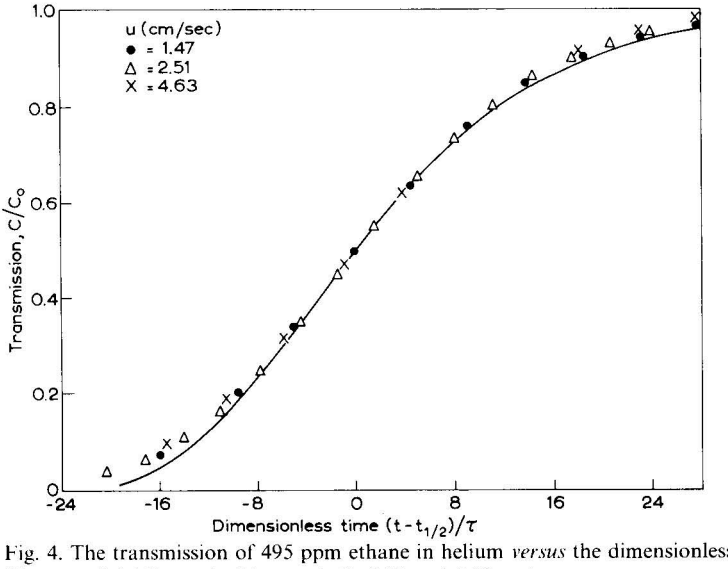

Fig. 4. The transmission of 495 ppm ethane in helium *versus* the dimensionless time for three flow-rates. The superficial flow velocities are 1.47, 2.51 and 4.63 cm/sec.

the deviations increase with the increasing flow-rate; however, the variations from one run to another are much smaller than the deviation from the theoretical results. This latter result indicates that the deviations in Figs. 3 and 4 are related to the applicability of eqn. 15 to low-concentration mixtures rather than to the effect of flow-rate.

The constant τ and the longitudinal diffusion coefficient D can be applied also to examine the effect of flow-rate on the transmission. Usually a high flow-rate will produce a steep transmission curve. In order to be able to superimpose data of the same input concentration (*e.g.*, runs 5A, 5B, and 5C) on a dimensionless time scale (in units of the time constant τ), the time constant τ must decrease with increasing flow-rate. The effect of diffusion in a chromatographic column is usually characterized by H, the height equivalent to a theoretical plate (HETP), which is defined as

$$H = \frac{2D}{u} \tag{17}$$

The relation between H and flow-rate is well-known in the literature[20]. The value of H first decreases sharply when the flow-rate increases and then increases gradually. The flow-rate dependences of H and D are shown in Fig. 5; the diffusion coefficient increases rapidly while H decreases. The decrease in H results from the combined effect of both flow-rate and longitudinal diffusion, but the effect of diffusion seems overwhelming. Although there is scatter in the values of the diffusion coefficients, they are roughly constant at low flow-rates and increase at high flow-rates. The constancy of the longitudinal diffusion coefficient at low flow-rates occurs because molecular diffusion dominates. Molecular diffusion is independent of flow-rate.

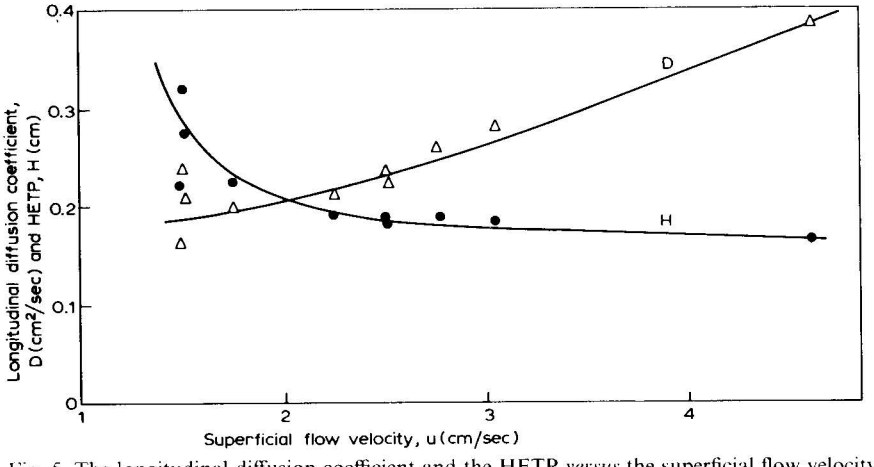

Fig. 5. The longitudinal diffusion coefficient and the HETP *versus* the superficial flow velocity. The solid lines are drawn to guide the eye.

At high flow-rates, the mass-transfer resistance between the gas-phase and the solid-phase causes a slow response for the solid-phase concentration which results in a wider concentration profile; consequently, the longitudinal diffusion coefficient in-

creases with flow-rate. The non-uniformity of the carrier gas flow in the radial direction of the chromatographic column also causes the longitudinal diffusion coefficient to increase with flow-rate. Since we saw that the comparison between the data and the theoretical curve is good for high-concentration mixtures and that the value of H is decreasing, it is concluded that the mass-transfer resistance is not the factor that causes the deviations in Fig. 3. Another factor, the speed of attaining an asymptotic profile, must be taken into consideration.

Analytic criteria for asymptotic concentration profiles

In order to achieve an asymptotic concentration profile, the adsorber must retain the adsorbate for a time long enough to develop the shape of the asymptotic profile. One expects a small flow-rate to result in a longer retention time and ease the development; however, a low flow-rate leads to a low diffusion coefficient which slows down the speed for development. Also, eqn. 9 points out that the asymptotic profile cannot be achieved for a linear isotherm in a finite time. The development of a concentration profile depends also on the curvature of the isotherm. The criterion for an asymptotic profile must involve a combination of these physical properties. From eqn. 8, one has

$$\frac{\partial C}{\partial Z} = \frac{u}{D} \frac{(Cq_0 - qC_0)}{\dfrac{\varepsilon C_0}{1 - \varepsilon} + q_0} \tag{18}$$

which is the slope of the theoretical transmission curve with respect to the column coordinate Z. The characteristic width W for the adsorption profile to be eluted completely can be measured from the reciprocal slope evaluated at $C = C_0/2$.

$$\text{Characteristic width } W = -\Delta C \left(\frac{\partial Z}{\partial C}\right)_{C = C_0/2} \tag{19}$$

Letting $\Delta C = C_0$ for a step change in the adsorbate concentration, we have

$$W = \frac{DC_0}{u} \left. \frac{\dfrac{\varepsilon}{1 - \varepsilon} C_0 + q_0}{(qC_0 - Cq_0)} \right|_{C = C_0/2} \tag{20}$$

The characteristic width W must satisfy two conditions for achieving an asymptotic profile: (1) the characteristic diffusion length δ must be larger than W so that the profile has enough time to develop; and (2) the column length L must be larger than W so that the end effect can be neglected.

During the elution time, Brownian motion diffuses the front of the profile; the characteristic diffusion length δ for the Brownian motion during a time t_p is

$$\delta \approx (Dt_p)^{1/2} \tag{21}$$

Based on the first condition, an analytical criterion for an asymptotic profile is

$$1 \gg \frac{W}{\delta} = \left(\frac{D}{uL}\right)^{1/2} \left.\frac{\left(\frac{\varepsilon}{1-\varepsilon} + K_0\right)}{\left(\frac{q}{C_0} - \frac{C}{C_0} K_0\right)\left(1 + \frac{K_0 (1-\varepsilon)}{\varepsilon}\right)^{1/2}}\right|_{C = C_0/2} \tag{22}$$

or, approximately

$$1 \gg \frac{W}{\delta} \approx \left(\frac{D}{uL}\right)^{1/2} \frac{2K_0^{1/2}}{K_{1/2} - K_0} \equiv \alpha \tag{23}$$

where $K_{1/2}$ is the dimensionless adsorption capacity at $C = C_0/2$. Eqn. 23 is valid in the approximation that $K_0 \gg \frac{\varepsilon}{1-\varepsilon} \approx 1$. Both K_0 and $K_{1/2}$ are calculated from eqn. 13.

The second condition for W is expressed as

$$1 \gg \frac{W}{L} = \frac{D}{uL} \left.\frac{\left(\frac{\varepsilon}{1-\varepsilon} + K_0\right)}{\left(\frac{q}{C_0} - \frac{C}{C_0} K_0\right)}\right|_{C = C_0/2} \tag{24}$$

or, approximately

$$1 \gg \frac{W}{L} \approx \left(\frac{D}{uL}\right) \frac{2K_0}{K_{1/2} - K_0} \equiv \beta \tag{25}$$

Eqn. 25 is valid in the same approximation leading to eqn. 23.

Both eqns. 23 and 25 indicate that the asymptotic profile is very sensitive to the curvature of the isotherm. For a linear isotherm, the adsorption capacity is constant ($K_{1/2} = K_0$); thus, the inequality is never satisfied. The ratio of the adsorption capacities in eqns. 23 and 25 dominates the values of α and β. In Table III we list values of α and β. Because of the square-root dependences in eqn. 23, the variation of α is smaller than the variation of β within our experimental conditions. Since the value of α is smaller than β even after considering the factor $\varepsilon/(1-\varepsilon)$, β is the stronger criterion. The inequality for β is best satisfied for the highest concentrations; however, for low concentrations, the increase in the value of β causes the inequality to be less well satisfied and accounts for the deviation between experiment and theory.

Eqns. 23 and 25 also point out that for the same concentration, the criteria decrease with D/u. Since D/u happens to have the same functional relation as H, we expect that the minimum of H gives the best fit to the theoretical curve. This result agrees with common practice in gas-chromatograph analysis where the minimum of H gives the narrowest peak and the best separation[20].

The combination of the effects of flow-rate and curvature also explains why the low-concentration mixture cannot give a good asymptotic profile. The β values for

495-ppm mixtures are higher than that for the 9830-ppm or the 2478-ppm mixtures. Increasing the flow-rate from 1.47 to 2.51 cm/sec decreased β from 0.34 to 0.29 for the 495-ppm mixture. A further increase of flow-rate to 4.63 cm/sec decreased β to 0.25. Since there is a minimum in the flow-velocity dependence of H, a further increase in the flow-rate would increase β again. The only possibility for achieving the asymptotic profile is to increase the column length L, which has no limitation. The comparison of β values does not explain why the result of run 5C, which has the lowest β value, is the worst among the three runs for the 495-ppm mixture; the reason may be that the mass-transfer resistance inside the carbon granules becomes relatively important at high flow-rates.

CONCLUSION

We measured the transmission of ethane in a helium carrier-gas flow for ethane compositions between 50 and 10,000 ppm on activated carbon; derived the adsorption isotherm of ethane at 25°C; and incorporated the adsorption isotherm into an asymptotic concentration profile formula. It is found that the transmission of high concentrations agrees with theory and that for low concentrations is higher than theory. Analytical expressions are suggested as the criteria for an asymptotic profile. We concluded that an asymptotic profile is easy to achieve with a high flow-rate, a long column length, a low diffusion coefficient, and a large curvature for the isotherm; however, the criteria cannot explain the small differences between results of different flow-rates for the same concentration. Further investigation may be warranted on the effect of the mass-transfer resistance on the asymptotic concentration profile.

ACKNOWLEDGEMENT

This work was supported in part by the U.S. Department of Energy.

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Journal of Chromatography, 214 (1981) 283–290
Elsevier Scientific Publishing Company, Amsterdam Printed in The Netherlands

CHROM. 14,005

OPEN-TUBULAR MICROCAPILLARY LIQUID CHROMATOGRAPHY WITH 20-μm I.D. COLUMNS

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(Received May 18th, 1981)

SUMMARY

Capillary columns of ethylene cyanohydrin, octadecylsilane and silica of I.D. 20 μm were prepared. For pre-treatment of a soda-lime glass capillary of I.D. 20 μm with sodium hydroxide solution a concentration of 0.5–0.7 *N* was optimal. A detection system with a small inner volume, such as 0.019 μl, was made. As extra-column effects increase with the fourth power of the ratio of the capillary column radius to the connections and cell radius, the latter values should be kept as small as possible.

INTRODUCTION

Although open-tubular microcapillary liquid chromatography is in the development stage[1–11], there is now increasing interest for its future ability. The practical inner diameters of capillary columns have decreased from 60–50 μm in 1978[2,3] to 40–30 μm in 1980[9,11]. A theoretical calculation of capillary column efficiency predicts that a smaller inner diameter column gives a higher theoretical plate number per unit time with reduced injection and detection volumes[8].

If the optimal conditions in open-tubular capillary gas chromatography are calculated for a linear velocity of 10 cm/sec with a 0.25-mm I.D. column for a solute with an interdiffusion coefficient assumed to be 10^{-1} cm^2/sec, these parameters should be reduced in open-tubular microcapillary liquid chromatography owing to the small interdiffusion coefficient, *e.g.*, 10^{-5} cm^2/sec. For example, if the flow velocity is set at 1 cm/sec, the I.D. of the capillary column in liquid chromatography should be about 8 μm.

We consider this size of column currently to be the final limit in capillary liquid chromatography. Before treating this very narrow capillary column, a 20-μm I.D. capillary column is discussed in this paper with respect to the development of a technique that would reduce extra-column effects and could be used for preparing the column. The cross-sectional area of a 20-μm I.D. column is about half that of a 30-μm I.D. column, and four times larger of that of a 10-μm I.D. column.

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EXPERIMENTAL

Preparation of capillary columns

Treatment of a soda-lime glass capillary with an alkaline solution gave a modified inner surface that worked as an adsorbent column either without further treatment[6] or followed by a stationary phase coating process[3,11]. The treatment process with an alkaline solution was as follows: sodium hydroxide solution was placed in the capillary tubing and kept at 50 °C for 18 h, then the capillary was washed with methanol, acetonitrile, dichloromethane and finally with *n*-hexane.

For filling, the alkaline solution was forced into capillary tubing by nitrogen gas at 15–70 atm. Under a pressure of 20 atm, connection of PTFE tubing (0.1 mm I.D.) with glass capillary or stainless-steel tubing (0.1 and 0.3 mm O.D.) could be used. However, above 20 atm one end of the capillary was kept in a 1/4-in. stainless-steel tube and the capillary was set in a polyimide ferrule (1/16 in.) using a 1/4–1/16 in. reducing union. PTFE tubing was attached to the capillary head and another PTFE tube, which was filled with the alkaline solution, was connected to the first PTFE tube by stainless-steel tubing (0.6 mm O.D.). The capillary head and alkaline solution were kept in the pressurized atmosphere, so there are no leakage problems.

To determine the amount of silica gel that was formed on the inner capillary tubing, the capacity factor (k') of β-naphthylamine was examined using *n*-hexane as the mobile phase. The results are shown in Fig. 1. The maximal effect was given by using *ca.* 0.7 *N* sodium hydroxide solution for 20-μm I.D. tubing, but 1 *N* solution for 50-μm I.D. tubing[6]. The k' value of β-naphthylamine for 20-μm I.D. tubing is about 10 times greater than that of same sample for 50-μm I.D. tubing under same treatment conditions. It is suggested that the phase ratio (mobile phase to adsorbent layer)

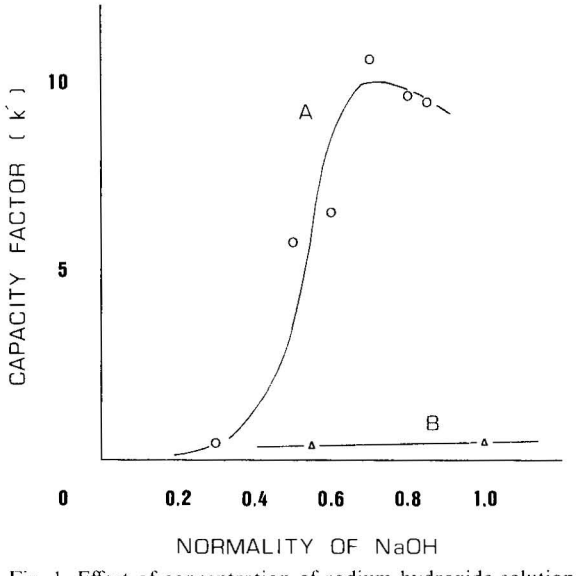

Fig. 1. Effect of concentration of sodium hydroxide solution on k' value of β-naphthylamine. The glass capillary was treated with sodium hydroxide solution of different concentrations for 18 h at 50 °C. A, 20-μm I.D. capillary column; B, 50-μm I.D. capillary column.

might be changed considerably. A concentration of sodium hydroxide solution above 1 *N* was not suitable for 20-μm I.D. capillary tubing, because the capillary often became clogged.

Ethylene cyanohydrin stationary phase

Ethylene cyanohydrin was coated by the modified dynamic coating method as follows[5]: four coils of glass capillary that had been pre-treated with alkaline solution were filled with dichloromethane, and a plug of 20% ethylene cyanohydrin–dichloromethane solution (about 10 μl) was forced into the capillary at a rate of about 3 cm/sec by using nitrogen at 70 atm. After coating, the capillary was kept under a flow of nitrogen, and then was placed in at oven with the temperature programmed at 0.25°C/min to 60°C and kept at 60°C overnight under a flow of nitrogen.

Octadecylsilane (ODS) stationary phase

After the capillary had been filled with xylene, about 50 μl of octadecyltrieth-oxysilane solution was passed through under a pressure of 18 atm (semi-static method)[7]. The concentration of ODS in xylene was examined in the range from 0.2 to 1.0% (v/v) and the reaction temperature from 65 to 110°C. The optimal conditions were found to be 0.5–1.0% and 95°C, respectively. Then nitrogen gas was passed through the capillary for 2–3 h with the temperature programmed at 0.5°C/min from 60 to 110°C, followed by moist nitrogen gas overnight at 130°C. Finally, the capillary was dried with nitrogen at 130°C for 2–3 h. The column was conditioned with dichloromethane, acetonitrile and methanol before analytical use. The use of octadecyltrieth-oxysilane instead of octadecyltrichlorosilane decreased the tendency for clogging to occur.

"In-column" injection

Split injection[1,11] and a new "in-column" injection technique were used. The latter method was as follows. The capillary column head was set in a vertical position using a Swagelock union and polyimide ferrule. The mobile phase in the capillary column head part (length about 5 cm) was removed with a hair-dryer. Then, using a microburner, the section up to 10 cm from the capillary column head was heated gently for several seconds in order to expand or partially vaporize the eluent in the capillary, until the level of the eluent fell nearly to the capillary head, then the microburner was removed. Immediately afterwards, the eluent started to rise owing to condensation of the vapour, and the capillary head was dipped in the sample solution so that the sample solution was sucked into the capillary column in a few seconds. After measuring the length of sample sucked in (an air bubble was used as the marker), the capillary column head was washed and connected to the pump, and a chromatographic run was started.

This new injection method is very simple and is completely free from an extra-column volume. If the capillary is kept in an oven, the microburner and dryer might be replaced by simply changing the oven temperature.

UV detection systems

Two different UV detection systems, type A and B, were made. The UV quartz cell in system A was coupled directly with the capillary column outlet using PTFE

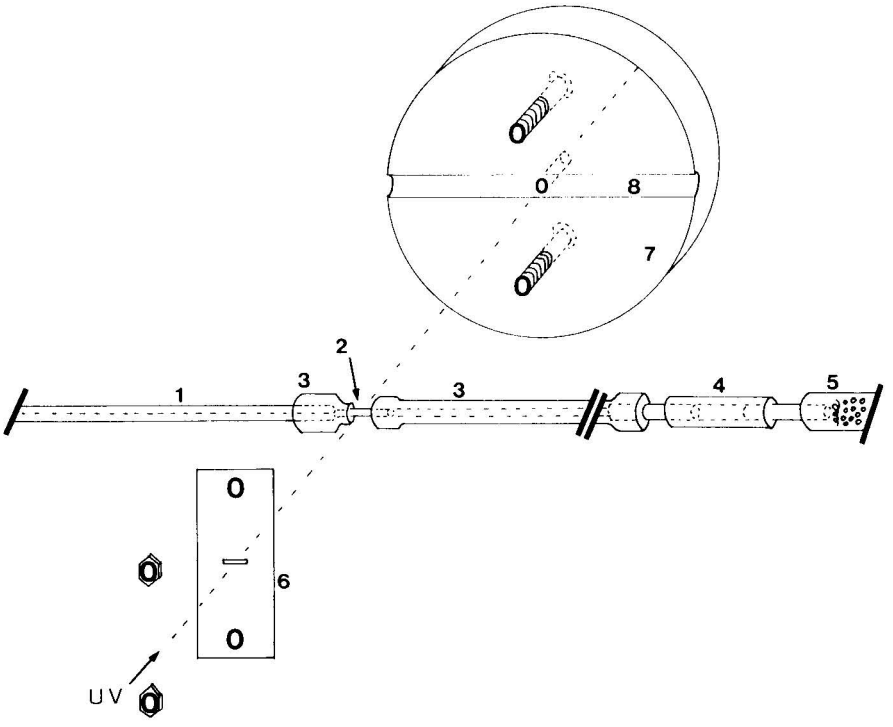

Fig. 2. Schematic diagram of UV detection system A. 1 = Capillary column; 2 = quartz cell, I.D. 0.1 mm, O.D. 0.3 mm; 3 = PTFE tubing, I.D. 0.1 mm, O.D. 1.6 mm; 4 = PTFE tubing, I.D. 0.5 mm, O.D. 1.0 mm; 5 = back-resistance, I.D. 0.5 mm × 1.5 mm O.D. × 20 mm length, PTFE tubing packed with 10-μm silica gel; 6 = slit; 7 = holder made of rubber; 8 = groove for quartz cell and its connections.

tubing, as shown in Fig. 2. A column outlet section (length 15 mm) and a quartz cell (I.D. 0.1 mm) were fixed by using adhesive on the cell holder, which was made of rubber. The length between the column outlet and the centre of the cell was 2 mm, so the volume of this part was 0.019 μl. System B was similar to system A except that tubing of different radius and extra connections were used for easy handling[3]. The column outlet was interfaced with stainless-steel tubing (I.D. 0.11 mm and length 7 mm) then PTFE tubing (I.D. 0.07 mm and length 20 mm). The last component was a quartz cell (I.D. 0.12 mm and length 2 mm) from the inlet to the centre of the UV light spot. The total volume of system B was 0.17 μl.

The other chromatographic operations were virtually the same as those used previously[11].

RESULTS AND DISCUSSION

Detection system B could be operated with a low noise level similar to that with the usual detectors, but system A showed much higher noise than system B (as shown in Figs. 4 and 5). One of the reasons might be vibration of the quartz cell, although this cell and the outlet part of the column were fixed tightly by adhesive.

Fig. 3 shows the differences in the chromatograms due to the different post-column systems. Chromatogram A was obtained by using a 20-μm I.D. capillary column and detection system A, and chromatogram B was obtained by using the same capillary column and detection system B. The k' values of the peaks are nearly zero and 0.05, respectively. The heights equivalent to a theoretical plate for the peaks in chromatogram A are 3–4 times larger than those in chromatogram B. The difference in the retention time of the first peak between chromatograms A and B is due to the extra time required for travelling between the column outlet and the UV cell. The extra-column effect, as shown in Fig. 3, is considerable for the peak with a small k' value, but it gradually decreases with increasing of k', as predicted by eqn. 1 below.

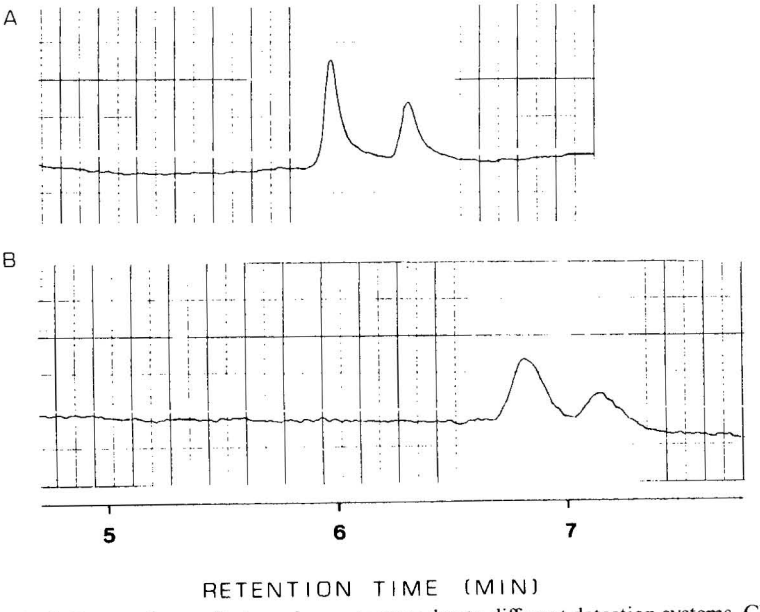

Fig. 3. Extra-column effect on chromatogram due to different detection systems. Chromatograms A and B were obtained by using detection system A and B, respectively, under same experimental conditions. Capillary column, I.D. 20 μm and length 5.5 m; mobile phase, *n*-hexane. First peak, N,N-diethylaniline; second peak, N-phenyl-α-naphthylamine.

Peak broadenings due to column and extra-column effects, which includes the connection and detection parts, are estimated by the factors f_c and f_{ext}, respectively, which were defined previously[11]. The ratio f_{ext}/f_c is

$$f_{ext}/f_c = [\Sigma L_{con,i}\,(r_{con,i}/r_c)^4 + L_d\,(r_d/r_c)^4]\,R_c^2 L_c^{-1}\,(6R_c^2 - 16R_c + 11)^{-1} \quad (1)$$

where L, r and R are the length, radius and the ratio of zone velocity to the velocity of the mobile phase, respectively. The suffixes con, d and c represent connection, detector and column part, respectively; i means a local part. As the radius term in eqn. 1 is raised to the fourth power, r_{con} and r_d should be kept as small as possible relative to r_c. The calculated values of f_{ext}/f_c for detection systems A and B are given in Table I.

TABLE I

CALCULATION OF EFFECT OF EXTERNAL COLUMN VOLUME* ON COLUMN EFFICIENCY

Column		*k'*	f_{ext}/f_c	
I.D. (μm)	*Length (m)*		*Detection system A*	*Detection system B*
20	30	0	0.042	0.40
		1	0.0023	0.022
		2	$2.3 \cdot 10^{-4}$	0.0070
	5	0	0.25	2.39
		0.5	0.37	0.36
		1	0.014	0.13
		2	0.0044	0.042
		3	0.0021	0.020
10	5	0	3.9	38
		1	0.22	2.1
		2	0.070	0.67
		3	0.034	0.33

* For the design of this part, see text.

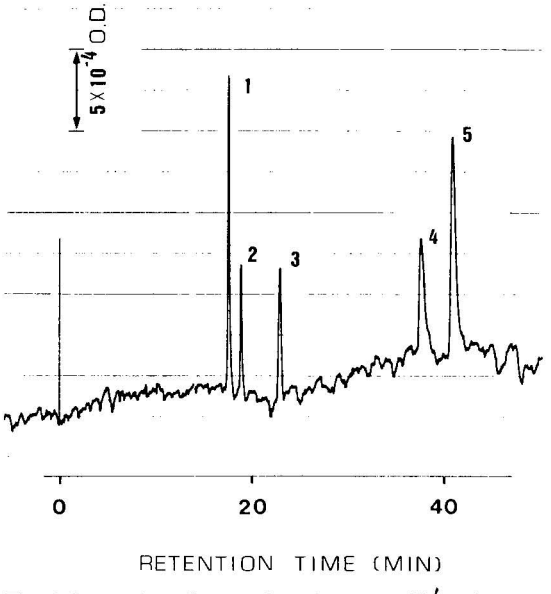

Fig. 4. Separation of aromatic amines on a silica column modified with hexylamine using detection system A. Column, I.D. 22 μm and length 560 cm; mobile phase, 0.05% hexylamine–*n*-hexane; linear velocity, 7 mm/sec. Samples: 1 = N,N-diethylaniline; 2 = N-phenyl-α-naphthylamine; 3 = N-phenyl-β-naphthylamine; 4 = α-naphthylamine; 5 = β-naphthylamine.

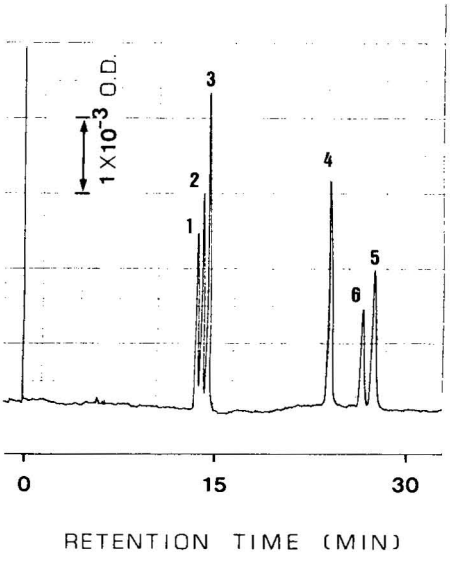

Fig. 5. Separation of aromatic amines on an ethylene cyanohydrin column using detection system B. Column, I.D. 23 μm and length 10.5 m; mobile phase, *n*-hexane saturated with ethylene cyanohydrin; linear velocity, 13 mm/sec. Samples: 1–5 as in Fig. 4; 6 = aniline.

Detection system A could be used even for a column of I.D. 10 μm and length 5 m for a sample with a k' value greater than 1. Detection system B for the column of I.D. 20 μm and length 5 m can only be used when k' is greater than 1 in the respect of extra-column effect.

Chromatograms obtained with the 20-μm I.D. capillary column are shown in Figs. 4 and 5 for detection systems A and B, respectively. The height equivalent to a theoretical plate (H) at a linear velocity of 1 mm/sec was calculated from the assumption for comparison of column efficiency that there is a first-order linear relationship with zero intercept between H and linear velocity in capillary liquid chromatography[3,5,6,11]. Calculated H values at a linear velocity of 1 mm/sec for N-phenyl-α-naphthylamine (k' = 0.03), N-phenyl-β-naphthylamine (k' = 0.07), α-naphthylamine (k' = 0.77), aniline (k' = 0.96) and β-naphthylamine (k' = 1.14) with an ethylene cyanohydrin column, shown in Fig. 5, were 0.029, 0.026, 0.016, 0.018 and 0.023 mm, respectively, at room temperature using *n*-hexane saturated with ethylene cyanohydrin as the mobile phase. H values at a linear velocity of 1 mm/sec for naphthalene (k' = 0.22), biphenyl (k' = 0.38), anthracene (k' = 0.51), phenanthrene (k' = 0.88) and pyrene (k' = 1.39) with an ODS column (I.D. 23 μm and length 4.4 m) were 0.25, 0.28, 0.23, 0.21 and 0.19 mm, respectively, at room temperature using water–acetonitrile (1:1) as the mobile phase. An ethylene cyanohydrin column gave almost one-tenth the H values for aromatic amines compared with those obtained with an ODS column for fused aromatics. Although the viscosity of water–acetonitrile solution is higher than that of *n*-hexane, the preparation procedure for the ODS column should still be improved. In Fig. 4 the mobile phase contained 0.05% of

hexylamine; without this addition the column worked as an adsorbent column, but with addition of hexylamine it worked as if it had a physically adsorbed layer of hexylamine, *i.e.*, as a liquid–liquid chromatographic column.

The chromatograms in Figs. 4 and 5 do not show any substantial broading of the later peaks. This suggests that narrow capillaries would have a high resolving ability, as predicted by theoretical considerations[1,8]. Capillary liquid chromatography using a column of I.D. 10 μm is now under investigation.

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Journal of Chromatography, 214 (1981) 291–306
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,015

STUDY OF RETENTION BEHAVIOUR OF PRIMARY, SECONDARY AND TERTIARY ANILINES IN NORMAL- AND REVERSED-PHASE LIQUID CHROMATOGRAPHY

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SUMMARY

A series of alkyl-substituted primary, secondary and tertiary anilines were chromatographed on three liquid chromatographic systems: one normal-phase system on a nitrile stationary phase and two reversed-phase systems on octadecyl- and octylsilane. On the nitrile stationary phase the elution order is tertiary, secondary and primary anilines and within each group the retention is mainly determined by the base strength, the number and size of *ortho*-alkyl groups and the size of the alkyl groups substituted at the nitrogen atom. On the reversed phases the anilines within each group are mainly eluted in order of increasing alkyl carbon number.

The relationship between the pK_b values of the anilines and $\log k'$ on the nitrile stationary phase is discussed and a comparison is made between k' for primary anilines and the corresponding phenols on the same stationary phase. Identification of different kinds of anilines by means of a two-phase plot is discussed and a method is described for the systematic separation of alkylanilines into structural types and individual compounds.

INTRODUCTION

In a previous paper[1], liquid chromatography (LC) was evaluated for the analysis of a series of alkyl-substituted phenols and some useful relationships were established between structure and elution order on various stationary phases. As an extension of this work, a similar study on alkylanilines is reported in this paper. It involved about 40 primary, secondary and tertiary alkylanilines, using both normal- and reversed-phase systems.

Previously, little attention has been paid to bonded-phase LC assay of these kinds of amines, in spite of the fact that they are widely used in the chemical industry and that their toxicity makes it essential to be able to control their occurrence in the environment. In the first successful application of a bonded stationary phase to the LC analysis of anilines, some phenylenediamines were separated on an "ether"-bonded phase[2].

0021-9673/81/0000–0000/$02.50

It was concluded that this stationary phase in combination with cyclopentane–methanol as carrier was useful for the separation of compounds containing NH groups. Later, Sleight[3] made a systematic survey of the elution of some phenols and a few alkylanilines from Durapak OPN and Durapak Carbowax 400 bonded stationary phases. However, these stationary phases are now obsolete for bonded-phase LC. Recently, Frohliger *et al.*[4], reported the separation and quantitation of some aromatic amines that occur in the working environment by means of ion-exchange chromatography, using a surface-sulphonated cation exchanger and perchloric acid as the eluent.

EXPERIMENTAL

Apparatus

The LC pump used was a Varian Model 4100 (Varian, Palo Alto, CA, U.S.A.) and the detector was a Laboratory Data Control Model 1285 UV monitor (Laboratory Data Control, Riviera Beach, FL, U.S.A.) used at 280 nm. Sample application was accomplished by a valve injector (Rheodyne, Berkeley, CA, U.S.A.) with a 20-μl loop.

Columns

The bonded-phase packing materials were the commercially available LiChrosorb RP-8 (10 μm) (E. Merck, Darmstadt, G.F.R.), Nucleosil C_{18} (5 μm) and Nucleosil CN (5 μm) (Machery, Nagel & Co., Düren, G.F.R.). LiChrosorb RP-8 was packed by the balanced density technique using tetrabromoethane. Nucleosil C_{18} and Nucleosil CN were packed in accordance with the upward-slurry packing technique[5,6]. All columns consisted of precision-bore stainless-steel tubing (200 × 4.4 mm I.D.). The columns were used at room temperature.

For accurate work it is necessary to reactivate the columns regularly, especially the nitrile column. This was done according to recommended procedures. The stability of the columns was tested daily using a mixture of phenol, 2,6-dimethylphenol and 4-*tert.*-butylphenol for the reversed phases and a mixture of diphenylamine, triphenylamine and N-methyl-2-methylaniline for the nitrile phase.

Chemicals

Isooctane (certified ACS grade; Fischer Scientific, Fairlawn, NJ, U.S.A.), methanol (analytical-reagent grade; May & Baker, Dagenham, Great Britain), 2-propanol (pro analysi grade; E. Merck, Darmstadt, G.F.R.), sodium dihydrogen orthophosphate, $NaH_2PO_4 \cdot 2H_2O$ (99%) (BDH, Poole, Great Britain), disodium hydrogen orthophosphate, $Na_2HPO_4 \cdot 2H_2O$ (according to Sörensen; E. Merck) and orthophosphoric acid (pro analysi grade; E. Merck) were used for preparing the LC eluents.

The anilines were of the best grade commercially available. Some of them were further purified by distillation or recrystallization. N,N-Dimethyl-2,6-dimethylaniline, N,N-diethyl-2-ethylaniline and N-ethyl-2-ethylaniline were prepared in this labotarory.

Mobile phases

For reversed-phase LC on C_8 and C_{18} stationary phases methanol–aqueous buffer was used and for normal-phase LC on the nitrile stationary phase isooctane containing 0.2% (v/v) of 2-propanol was applied.

Methanol–aqueous buffer (60:40) (pH 7.0). A 15-ml volume of 0.025 *M* Na_2HPO_4 + 250 ml of 0.025 *M* NaH_2PO_4 + 397.5 ml of methanol; the pH was adjusted to 7.0 with small amounts of orthophosphoric acid and 1 *M* sodium hydroxide solution.

Methanol aqueous buffer (70:30) (pH 7.0). A 15-ml volume of 0.025 *M* Na_2HPO_4 + 250 ml of 0.025 *M* NaH_2PO_4 + 618.33 ml of methanol; the pH was adjusted as above.

Methanol–aqueous buffer (80:20) (pH 7.0). A 15-ml volume of 0.05 *M* Na_2HPO_4 + 250 ml of 0.05 *M* NaH_2PO_4 + 1060 ml of methanol; the pH was adjusted as above.

Procedure

Anilines were dissolved in methanol or isooctane to give concentrations of approximately 0.02–0.04 $mg \cdot ml^{-1}$ and 20 µl of the solutions were injected on to the LC columns. The capacity factors given are mean values from at least three injections with a relative standard deviation of about 3%. Retention times of unretained solutes were determined by injecting *n*-hexane on the nitrile phase and sodium nitrate solution (0.05%, w/w) on the reversed phases. The capacity factor, k', was calculated from the equation

$$k' = \frac{t_R - t_0}{t_0} \quad (1)$$

where t_R is the retention time of the sample and t_0 that of the unretained solute.

The systematic separation procedure described later involves separation into primary, secondary and tertiary anilines on the nitrile phase, followed by a separation according to alkyl carbon number on the C_{18} phase. The fractions of anilines in isooctane solution collected from the nitrile phase cannot be introduced directly on to the C_{18} phase, but have to be transferred to methanol before injection. About 200 µl of methanol were added and the mixture was shaken in order to extract the anilines. The methanolic solution was then injected on to the C_{18} phase.

Acetylation procedure for the separation of secondary and tertiary anilines

The mixture of secondary and tertiary anilines eluted from the nitrile phase with isooctane was collected in a test-tube. The volume was reduced by about 75% by blowing a gentle stream of nitrogen over the surface at room temperature. A 1-ml volume of acetic anhydride was added and the mixture heated over a small flame nearly to boiling for 1 min. The excess of acetic anhydride was then removed with a stream of nitrogen as above nearly to dryness.

In order to separate the tertiary anilines from the acetylated secondary anilines, *ca.* 100 µl of isooctane were added and the solution was injected on top of a short glass column (50 × 4 mm), filled with nitrile stationary phase (slurry packed without pressure). Tertiary anilines were eluted with 2–3 ml of isooctane, then acetylated secondary anilines with the same volume of ethanol, with separate collection.

The tertiary aniline fraction can be injected on to the nitrile phase after concentration, but the acetylated secondary anilines have to be hydrolysed first. A 2-ml volume of 50% sulphuric acid was added and the mixture heated nearly to boiling for 10 min. After cooling, the acid was neutralized with excess of dilute sodium hydroxide solution and the free secondary anilines were extracted with a few millilitres of iso-octane. After concentration with a stream of nitrogen as before, the solution is ready for injection on to the nitrile phase.

RESULTS AND DISCUSSION

The liquid chromatographic investigation involved three chemically bonded stationary phases: one normal-phase system with a nitrile phase and two reversed-phase systems with octyl- and octadecylsilane. With the reversed-phase systems, two eluents with different proportions of methanol and aqueous buffer were investigated.

Normal-phase liquid chromatography

The results of the runs on the nitrile phase are given in Table I. Within the group of primary anilines, the compounds are listed in order of increasing number of alkyl groups on the benzene ring, and within the secondary and tertiary groups of anilines in order of increasing size of substituents on the nitrogen atom.

It can be seen from Table I and from Fig. 7 that primary anilines travel more slowly on the nitrile phase than any of the secondary and tertiary anilines, and secondary more slowly than tertiary anilines, with a few exceptions. This result indicates that the migration is governed by the ease of access to the nitrogen atom. This conclusion is further confirmed by an examination of the order of elution within each group of anilines.

For primary anilines, *i.e.*, anilines without alkyl groups on the nitrogen atom, the order of elution is determined mainly by the number and size of *ortho*-alkyl groups. Thus, primary anilines with two *ortho*-alkyl groups have the lowest retentions, and those without such groups the highest. Among the latter, *para*-substituted anilines travel more slowly than the corresponding *meta*-substituted anilines and the size of the alkyl group seems to be of minor importance for the retention. This fact is demonstrated by the series of 4-alkyl-substituted primary anilines.

The difference in retention between *para*-substituted primary anilines without *ortho*-substituents and the corresponding *meta*-substituted anilines can be ascribed to the fact that anilines belonging to the former group are stronger bases and accordingly more ionized in the eluent than are the members in the latter group.

The influence of *ortho*-substitution on the interaction of primary anilines with the nitrile phase is demonstrated in Fig. 1, where log k' is plotted against alkyl carbon number. It can be seen that the anilines fall into three groups according to the number of *ortho*-substituents, *viz.*, non-*ortho*-, mono-*ortho*- and di-*ortho*-substituted compounds, separated by the broken lines in Fig. 1. In a previous study the same relationship was found to apply to phenols[1].

For secondary anilines the retention on the nitrile phase is mainly governed by two factors, *viz.*, the size of the alkyl group substituted on the nitrogen atom and the presence or absence of *ortho*-alkyl groups and by their size. Thus, log k' decreases linearly with N-alkyl carbon number for N-alkylanilines without nuclear substituents

TABLE I

CAPACITY FACTORS (k') FOR ALKYLANILINES IN FOUR LC SYSTEMS

Normal-phase system: Nucleosil CN. Mobile phase: 0.2% (v/v) 2-propanol in isooctane. Reversed-phase systems: (a) Nucleosil C_{18}, mobile phase methanol–aqueous buffer (80:20 and 70:30) (pH 7.0); (b) LiChrosorb RP-8, mobile phase methanol–aqueous buffer (60:40) (pH 7.0).

No.	*Aniline (substituent)*	k'			
		Nitrile phase	C_{18} *phase*		C_8 *phase, (60:40) eluent*
			(80:20) eluent	*(70:30) eluent*	
	Primary anilines				
1	None	7.53	0.41	0.55	0.60
2	2-Methyl	4.84	0.55	0.85	0.92
3	2-Ethyl	3.65	0.71	1.20	1.40
4	2-Isopropyl	3.14	0.86	1.63	2.07
5	3-Methyl	8.07	0.51	0.83	0.94
6	3-Ethyl	7.02	0.67	1.20	1.49
7	4-Methyl	9.62	0.54	0.85	0.97
8	4-Ethyl	8.72	0.71	1.26	1.59
9	4-Isopropyl	8.87	0.90	1.75	2.46
10	4-*n*-Butyl	8.72	1.38	3.11	4.84
11	2,3-Dimethyl	5.48	0.74	1.18	1.31
12	2,4-Dimethyl	6.04	0.78	1.25	1.49
13	2-Methyl-4-*n*-butyl	5.07	1.91	4.71	7.40
14	2,5-Dimethyl	4.45	0.75	1.26	1.43
15	2,6-Dimethyl	2.83	0.78	1.31	1.43
16	3,4-Dimethyl	9.51	0.69	1.16	1.40
17	2,4,6-Trimethyl	2.96	1.08	1.99	2.34
	Secondary anilines				
18	N-Methyl	2.32	0.68	1.12	1.22
19	N-Methyl-2-methyl	1.43	0.97	1.68	1.86
20	N-Methyl-3-methyl	2.16	0.89	1.62	1.86
21	N-Methyl-4-methyl	2.73	0.90	1.63	1.93
22	N-Ethyl	1.49	0.87	1.54	1.77
23	N-Ethyl-2-methyl	0.82	1.34	2.57	2.91
24	N-Ethyl-3-methyl	1.40	1.14	2.26	2.71
25	N-Ethyl-4-methyl	1.72	1.15	2.26	2.73
26	N-Ethyl-2-ethyl	0.67	1.86	3.80	4.61
27	N-Acetyl		0.41	0.62	0.71
28	N-Acetyl-4-methyl		0.54	0.87	1.09
29	N-Propyl	1.06	1.23	2.35	3.00
30	N-*n*-Butyl	0.90	1.69	3.70	5.04
31	N-Phenyl	2.50	1.52	3.45	4.86
32	N-Benzyl	1.65	1.50	3.42	4.79
	Tertiary anilines				
33	N,N-Dimethyl	0.65	1.35	2.42	2.68
34	N,N-Dimethyl-2-methyl	0.45	1.76	3.48	3.99
35	N,N-Dimethyl-3-methyl	0.68	1.82	3.61	4.17
36	N,N-Dimethyl-4-methyl	0.79	1.80	3.64	4.17
37	N,N-Dimethyl-2,6-dimethyl	0.14	4.20	10.1	
38	N,N-Diethyl	0.50	2.32	5.21	6.44
39	N,N-Diethyl-2-methyl	0.29	3.45	7.71	8.89
40	N,N-Diethyl-3-methyl	0.54	3.08	7.31	9.18
41	N,N-Diethyl-4-methyl	0.90	2.95	6.81	8.06
42	N,N-Diethyl-2-ethyl	0.21	5.21	12.8	16.8
43	N,N-Diphenyl	0.39	7.40	25.9	

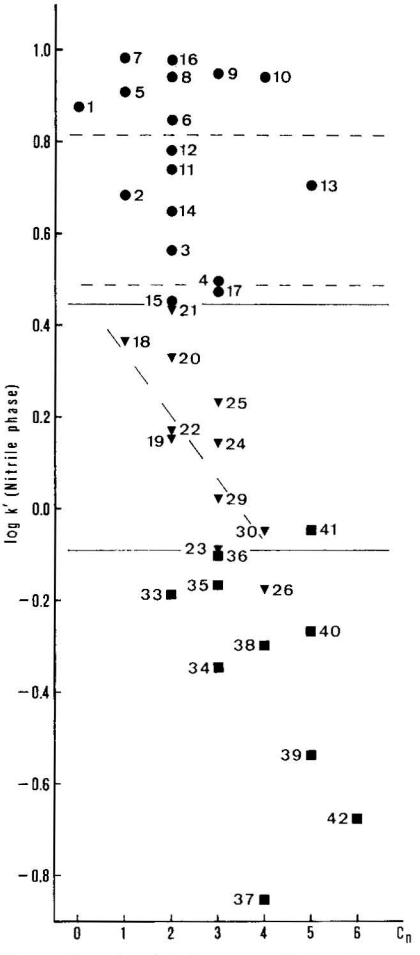

Fig. 1. Relationship between alkyl carbon number (C_n) and log k' of alkylanilines on the nitrile stationary phase (Nucleosil CN). Mobile phase, 0.2% (v/v) 2-propanol in isooctane; 45 ml h^{-1}. ● = Primary anilines; ▼ = secondary anilines; ■ = tertiary anilines. The numbers refer to Table I.

(N-alkyl = methyl to butyl, Nos. 18, 22, 29 and 30 in Fig. 1), and a further decrease then results from *ortho*-substitution of hydrogen for alkyl groups. As for primary anilines, the effect of *meta*-substitution on retention is slight while *para*-substitution increases retention.

For the retention of tertiary anilines on the nitrile phase the same rules apply as for secondary anilines. Accordingly, retention is a function of the size of the alkyl groups on the nitrogen atom, decreasing with increasing size. In the same way, *ortho*-substitution of alkyl groups decreases retention. Among tertiary anilines, those with one or two *ortho*-alkyl groups can be clearly distinguished from secondary anilines by their lower retention, whereas there is some mixing between other kinds of tertiary and secondary anilines (Fig. 1).

Relationship between pK_b of anilines and log k' on the nitrile phase

It is interesting that if log k' on the nitrile phase is plotted against pK_b of the primary anilines[7–9], a semi-linear correlation is obtained (Fig. 2). A similar relationship between pK_b and log k' on silica gel was reported for toluidines and phenylenediamines[10]. Among secondary anilines, N-methylanilines form a group of their own, whereas N-alkylanilines with ethyl and larger alkyl groups align themselves around a lower line.

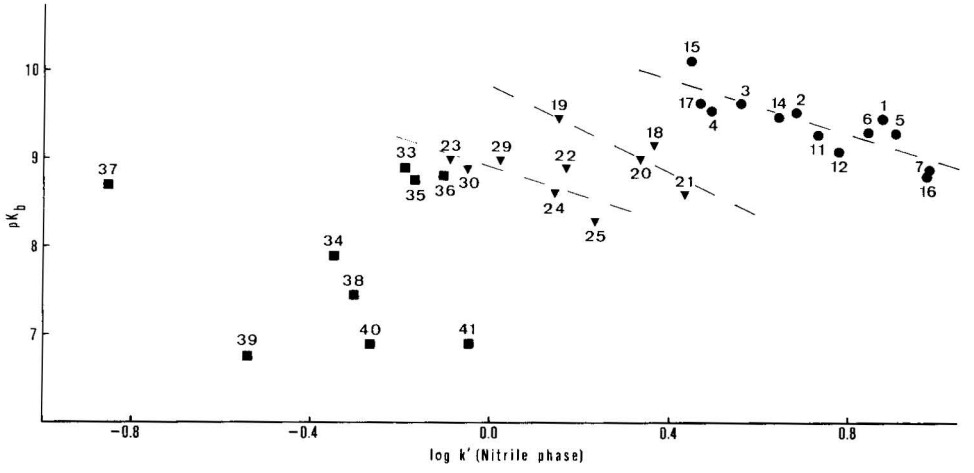

Fig. 2. Relationship between pK_b and log k' of alkylanilines on the nitrile stationary phase (Nucleosil CN). Mobile phase, 0.2% (v/v) 2-propanol in isooctane; 45 ml h^{-1}. ● = Primary anilines; ▼ = secondary anilines; ■ = tertiary anilines. The numbers refer to Table I.

To this group also belong some tertiary anilines, *viz.*, N,N-dimethylanilines without *ortho*-alkyl groups. For tertiary anilines with greater steric hindrance around the nitrogen atom, which appear at the bottom of the plot, the spread of points is considerable. This group consists of N,N-dimethylanilines with *ortho*-methyl groups and N,N-diethylanilines.

Within each of the above-mentioned groups of anilines there is a general trend of increasing k' values with decreasing pK_b values, *i.e.*, with increasing base strength of the compounds. This would be expected as the pK_b value is a measure of the ability of the amino group to accept a proton and the normal-phase k' value is mainly a measure of its ability to interact with a nitrile group in the bonded phase.

Because of the different steric requirements of the proton and the nitrile group, the semi-linear relationship between pK_b and log k' is only valid for sterically equivalent anilines, which causes a subdivision into structural groups as illustrated in Fig. 2. Accordingly, the k' value of an alkylaniline on the nitrile phase system is primarily a function of its base strength and of the substitution pattern around the nitrogen atom, the latter mainly determining the subdivision into structural groups, whereas the position of an aniline within a group is governed by both the base strength and steric factors.

Reversed-phase liquid chromatography

Three systems were studied, two on a C_{18} stationary phase with methanol–

aqueous buffer (80:20 and 70:30) as eluents and one on a C_8 stationary phase with methanol–aqueous buffer (60:40) as eluent. In all instances the pH was adjusted to 7.0. The results are given in Table I. It is evident that for primary anilines the alkyl carbon number determines the retention whereas the position of the alkyl groups in the benzene ring is of minor importance.

On moving alkyl groups from the nucleus to the nitrogen atom a distinct increase in retention occurs. Thus, secondary anilines invariably have higher retentions than primary anilines with the same total alkyl carbon number, and the same applies to tertiary *vs.* secondary anilines. Accordingly, if retention on the C_{18} phase is plotted against total alkyl carbon number, primary, secondary and tertiary anilines align themselves along three parallel lines (Fig. 3).

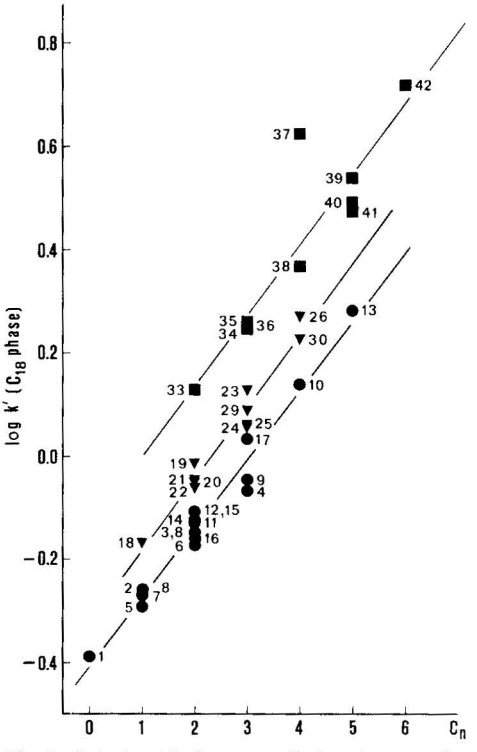

Fig. 3. Relationship between alkyl carbon number (C_n) and log k' of alkylanilines on the C_{18} stationary phase (Nucleosil C_{18}). Mobile phase, methanol–aqueous buffer (80:20) (pH 7.0); 45 ml h^{-1}. ● = Primary anilines; ▼ = secondary anilines; ■ = tertiary anilines. The numbers refer to Table I.

Of the two solvent systems used for the C_{18} phase, methanol–aqueous buffer (80:20) elutes lower primary anilines too rapidly for a good resolution to be obtained, and methanol–aqueous buffer (70:30) elutes higher tertiary anilines too slowly to be of practical value. A compromise between the two solvent systems should therefore best meet the case of a more universal isocratic solvent for alkylanilines on the C_{18} phase.

Using the C_8 stationary phase with methanol–aqueous buffer (60:40) (pH 7.0)

as eluent, alkylanilines behave much as on the C_{18} phase with methanol–aqueous buffer (70:30), the retentions being slightly higher (Table I).

Comparison between primary anilines and phenols

Primary anilines are structurally analogous to phenols and it is therefore not surprising to find their behaviour in LC to be similar. For phenols it was concluded that differences in the strength of hydrogen bonding to the proton-accepting cyano group was responsible for the division into non-*ortho*-, mono-*ortho*- and di-*ortho*-substituted compounds, when run on the nitrile phase[1]. The same explanation should be valid for the corresponding primary anilines, although the division into structural classes is less sharp.

In Fig. 4, k' values for alkylphenols on a nitrile stationary phase, taken from ref. 1, are plotted against k' values for the corresponding primary anilines, taken from Table I. It can be seen that there is a considerable spread of points, indicating different behaviours of primary anilines and phenols on the nitrile phase. Although the two nitrile phases used are different, it is felt that certain conclusions can be drawn from Fig. 4.

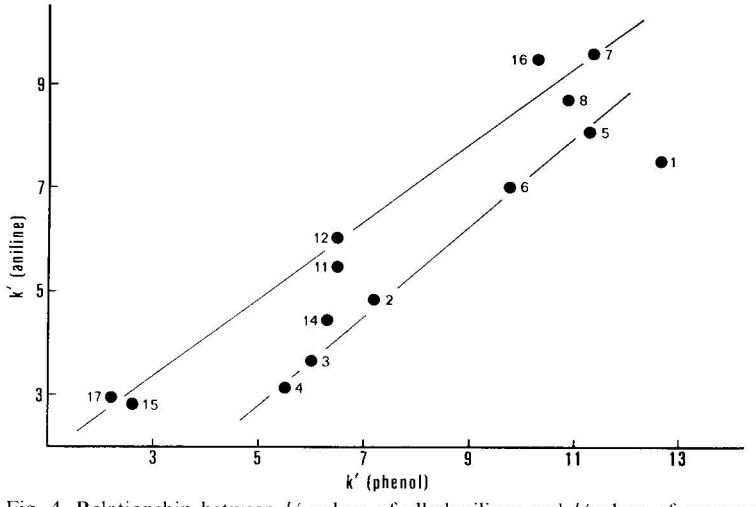

Fig. 4. Relationship between k' values of alkylanilines and k'values of corresponding alkylphenols on nitrile phases. Anilines: stationary phase, Nucleosil CN; mobile phase, 0.2% (v/v) 2-propanol in isooctane; 45 ml h^{-1}. Phenols: stationary phase, Cyano Sil-X-1; mobile phase, 0.5% (v/v) 2-propanol in isooctane; 40 ml h^{-1}. The numbers refer to Table I.

The points are more or less aligned along two straight lines. Along the upper line are mainly found di-*ortho*-alkyl-substituted compounds and compounds having a *para*-alkyl group. For these compounds there is no great difference between the k' values of corresponding anilines and phenols. In the former instance this can be ascribed to the fact that the *ortho*-alkyl groups partly inhibit hydrogen bonding, making the chromatographic properties of the aniline and the phenol more similar. A levelling effect on the hydrogen bonding properties would also be exerted by a *para*-alkyl group, which tends to weaken the acidic properties of phenols and strengthen the basic properties of anilines[7].

Along the lower line are mainly compounds with a single *ortho*- or *meta*-alkyl group. For these compounds there is a considerable difference between the k' values for anilines and the corresponding phenols. In this instance the levelling effect of the alkyl groups is smaller, and the stronger hydrogen bonding of the phenolic hydroxyl group causes phenols to move more slowly than the corresponding anilines on the nitrile phase. This difference is most pronounced for phenol and aniline themselves with their points lying far outside the two regions discussed above, as can be seen from Fig. 4.

In reversed-phase chromatography of primary anilines and phenols the influence of the two functional groups is small, retention being mainly determined by the number of alkyl groups. This fact is demonstrated in Fig. 5, which shows k' values on the C_{18} phase for corresponding compounds. The slightly higher k' values of anilines on this phase can be ascribed to the presence of residual acidic silanol groups in the C_{18} phase, which interact with the basic amino groups, and to the fact that phenols are stronger electrolytes.

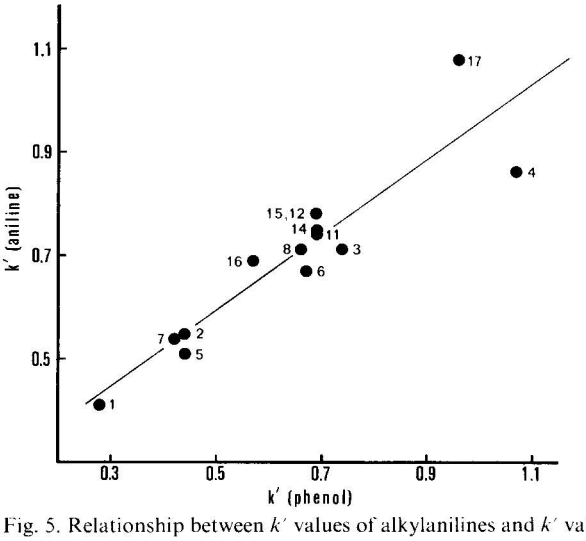

Fig. 5. Relationship between k' values of alkylanilines and k' values of corresponding alkylphenols on C_{18} phases. Anilines: stationary phase, Nucleosil C_{18}; mobile phase, methanol–aqueous buffer (80:20) (pH 7.0); 45 ml h^{-1}. Phenols: stationary phase, μBondapak C_{18}; mobile phase, ethanol–water (60:40); 20 ml h^{-1}. The numbers refer to Table I.

Use of two-phase plot for the identification of alkylanilines

In Fig. 6, log k' for the C_{18} phase system is plotted against log k' for the nitrile stationary phase system. It appears that the plot can be divided into zones for different kinds of anilines. Thus, all primary anilines fall to the right of vertical line 1 (zone P), and the horizontal line divides the secondary and tertiary anilines into two zones below and above the line (zones S and T, respectively). The P zone may in turn be divided by two vertical lines into zones for non-*ortho*-substituted compounds (to the right of line 3), mono-*ortho*-substituted compounds (between lines 2 and 3) and di-*ortho*-substituted compounds (between lines 1 and 2).

Although the boundaries between some of the zones are rather narrow and

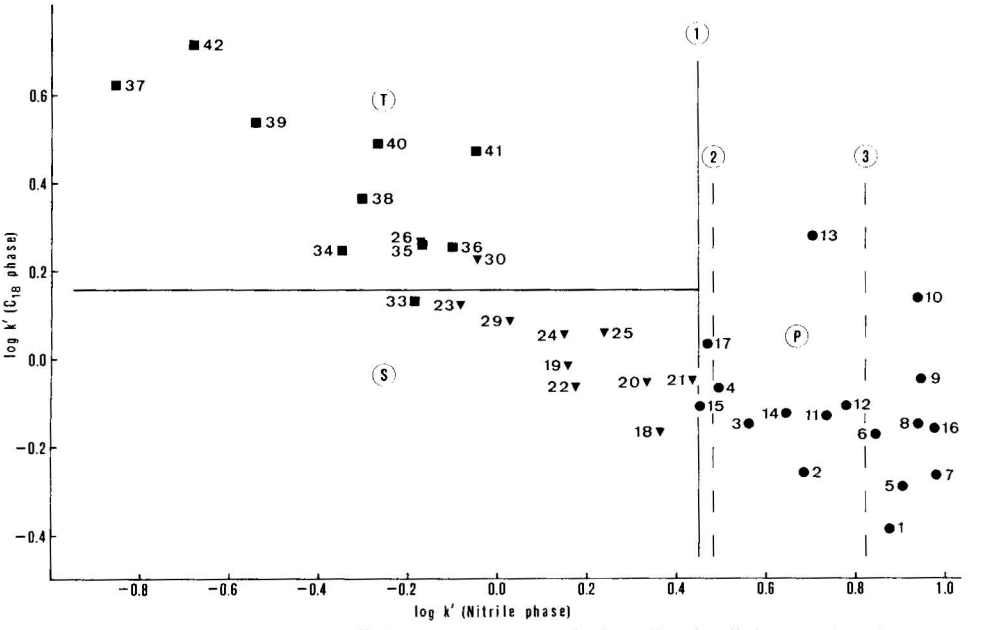

Fig. 6. Two-phase plot of log k' of alkylanilines on reversed phase [Nucleosil C_{18}, methanol–aqueous buffer (80:20) (pH 7.0); 45 ml h^{-1}] *versus* log k' on normal phase [Nucleosil CN, 0.2% (v/v) 2-propanol in isooctane; 45 ml h^{-1}]. ● and P = primary anilines; ▼ and S = secondary anilines; ■ and T = tertiary anilines. The numbers refer to Table I.

some mixing of compounds occurs, it is felt that Fig. 6 would be useful for identification purposes. The fact that different kinds of alkylanilines fall into different zones will make it possible to distinguish to a great extent between primary, secondary and tertiary alkylanilines and between the three structural classes of primary anilines. In addition, the zone diagram gives information about alkyl carbon number, anilines with the lowest carbon number appearing at the bottom of each zone and those with the highest carbon number at the top.

There is just one tertiary aniline falling into the S zone; as it is the lowest member in the series of tertiary anilines, *viz.*, N,N-dimethylaniline, it is safe to conclude that no other tertiary aniline will appear in this zone. The T zone contains some secondary anilines. This is unavoidable, as the horizontal separation between secondary and tertiary anilines, *i.e.*, the separation on the nitrile phase, is insufficient and the vertical position of a secondary aniline in the diagram is determined solely by the alkyl carbon number.

It can be concluded that it is impossible to decide whether an aniline falling in the T zone is in fact a secondary aniline. For this purpose other methods must be resorted to. A simple test to distinguish between the two classes of amines is to heat the sample for about 2 min with an excess of acetic anhydride and then chromatograph the product on the nitrile phase system. If no change in retention is observed, the aniline is tertiary and, if no peak is obtained, or the retention time is much increased, the amine is secondary. The reason for this behaviour is that only secondary anilines react with acetic anhydride and that the acetylated amine travels very slowly on the nitrile column (see below).

Systematic separation of alkylanilines into primary, secondary and tertiary anilines and individual compounds

As pointed out previously, the investigated primary alkylanilines may be separated as a class from secondary and tertiary anilines on the nitrile phase, and the latter two classes are also largely resolved on the same phase. Accordingly, the problem of separating an unknown mixture of alkylanilines by LC should be solved by first running the mixture on the nitrile phase, isolating the compounds according to class, and then running the three classes separately on the C_{18} phase, where a separation according to alkyl carbon number is achieved. When this procedure was applied to the 41 anilines (Nos. 1–26 and 29–43) listed in Table I, the chromatograms shown in Figs. 7 and 8 were obtained.

If the contents in the peaks representing the different carbon numbers are collected from the C_{18} phase and re-run on the nitrile phase, the peaks are largely resolved into individual compounds, as demonstrated in Figs. 9 and 10. In order to distinguish between primary, secondary and tertiary anilines, 2,6-dimethylaniline (No. 15) and N-propylaniline (No. 29) are admixed for marking the boundaries between the three structural classes.

In Fig. 8 the tertiary aniline fraction was run as obtained from the nitrile phase, which means that it contains some secondary anilines. However, for mixtures of unknown composition it is recommended first to separate the two groups of anilines by the acetylation method described below, and then to run them separately on the C_{18} phase.

The acetylation procedure, as detailed under Experimental, involves treatment

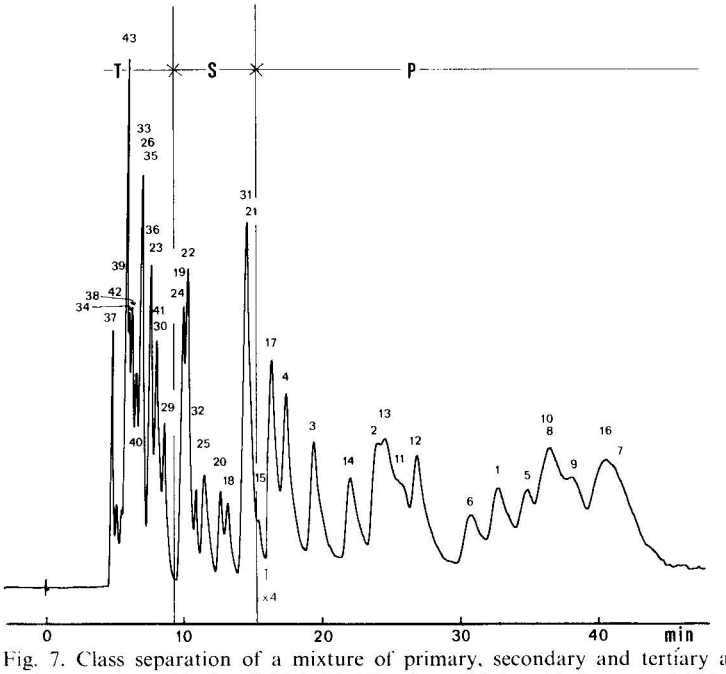

Fig. 7. Class separation of a mixture of primary, secondary and tertiary alkylanilines on the nitrile stationary phase (Nucleosil CN). Mobile phase, 0.2% (v/v) 2-propanol in isooctane; 45 ml h^{-1}. P = primary anilines; S = secondary anilines; T = tertiary anilines. The numbers refer to Table I.

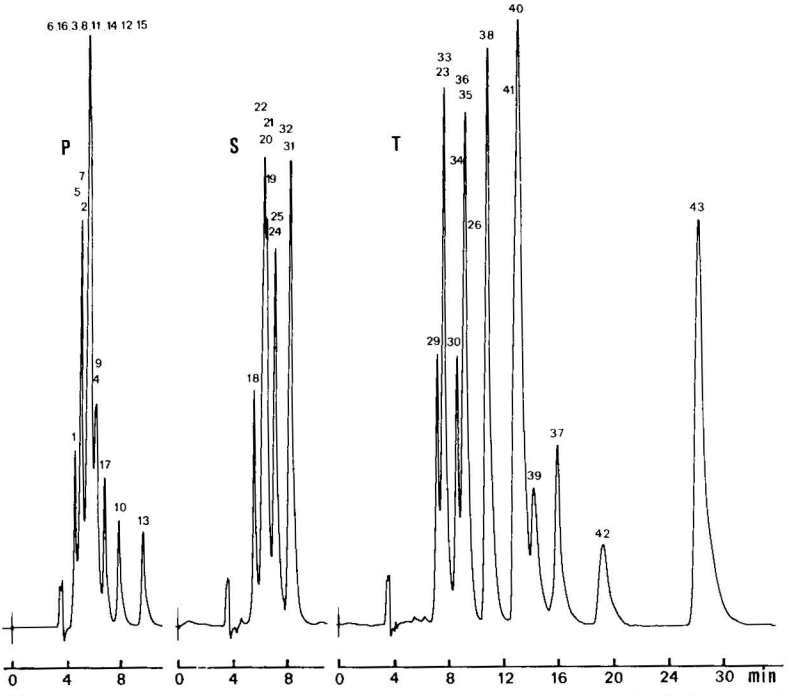

Fig. 8. Carbon number separation on the C_{18} stationary phase (Nucleosil C_{18}) of primary, secondary and tertiary anilines isolated from the run on the total mixture on the nitrile phase (Fig. 7). Mobile phase, methanol–aqueous buffer (80:20) (pH 7.0); 45 ml h^{-1}. P = primary anilines; S = secondary anilines; T = tertiary anilines. The numbers refer to Table I.

of the mixture of secondary and tertiary anilines with acetic anhydride in order to convert the secondary anilines into acetyl derivatives. The mixture is then passed through a short column packed with nitrile phase. Unchanged tertiary anilines are rapidly eluted with isooctane, whereas acetylated secondary anilines travel more slowly and are eluted in a second step with a stronger solvent, ethanol. On hydrolysis of the latter mixture, free secondary anilines result.

The application of the acetylation procedure to a mixture of eight secondary

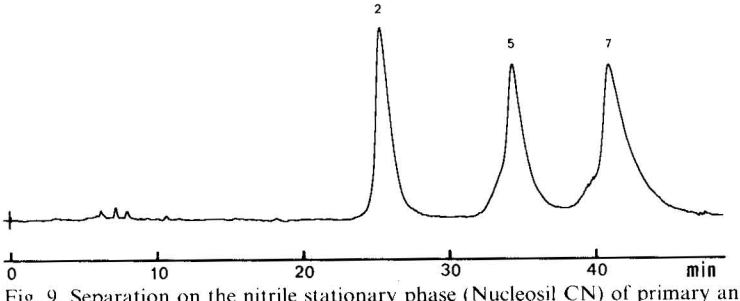

Fig. 9. Separation on the nitrile stationary phase (Nucleosil CN) of primary anilines present in the peak with carbon number 1, isolated from the carbon number run on primary anilines (Fig. 8). Mobile phase, 0.2% (v/v) 2-propanol in isooctane; 45 ml h^{-1}. The numbers refer to Table I.

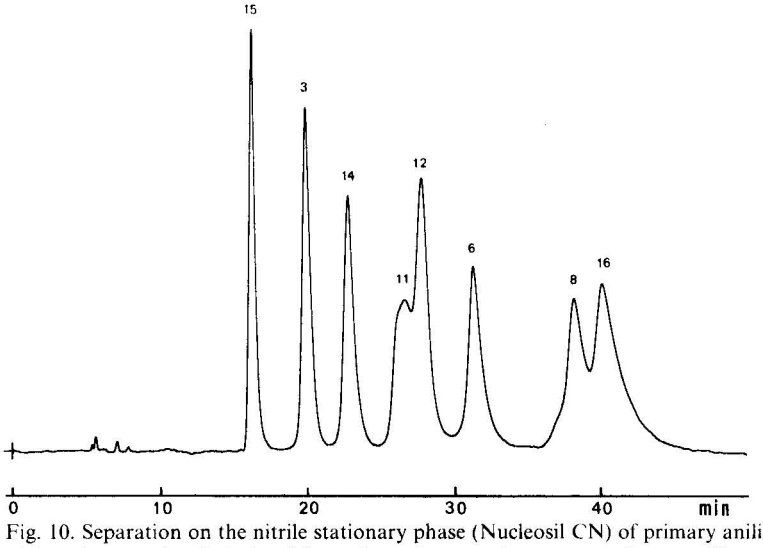

Fig. 10. Separation on the nitrile stationary phase (Nucleosil CN) of primary anilines present in the peak with carbon number 2, isolated from the carbon number run on primary anilines (Fig. 8). Mobile phase, 0.2% (v/v) 2-propanol in isooctane; 45 ml h^{-1}. The numbers refer to Table I.

and tertiary anilines is demonstrated in Fig. 11. This mixture contains all four secondary anilines (Nos. 23, 26, 29 and 30) that fall into the tertiary group in the original class separation on the nitrile phase (Fig. 8). The chromatogram of the total mixture on the nitrile phase is given in Fig. 11 (S + T). After acetylation, separation and hydrolysis, the two chromatograms in T and S for tertiary and secondary anilines, respectively, result. It can be seen that the class separation achieved is complete. The separation procedure outlined above will permit the class separation of complex mixtures of primary, secondary and tertiary anilines and will also largely resolve the mixtures into individual compounds. In addition, information is obtained about alkyl carbon number and, to a certain extent, about the number and position of nuclear alkyl groups.

As shown by the chromatograms in Fig. 8, the investigated primary and secondary anilines are eluted from the C_{18} phase in order of increasing alkyl carbon number. For tertiary anilines there is some deviation from this rule, as N,N-dimethyl-2,6- dimethylaniline (No. 37) with an alkyl carbon number of 4 appears after the three tertiary anilines Nos. 39–41 with an alkyl carbon number of 5. It seems that a high degree of steric hindrance around the nitrogen atom will increase the retention on the C_{18} phase, resulting in a disturbance of the regular elution according to alkyl carbon number.

N-Phenyl- and N-benzylanilines

The investigated material contains some anilines with aromatic groups substituted on the amino group. These anilines appear together with other secondary and tertiary anilines in the class separation on the nitrile phase (Fig. 7). On the following carbon number separation on the C_{18} phase they are eluted last of the investigated compounds in their respective groups (Fig. 8). In the two-phase plot in Fig. 6 these

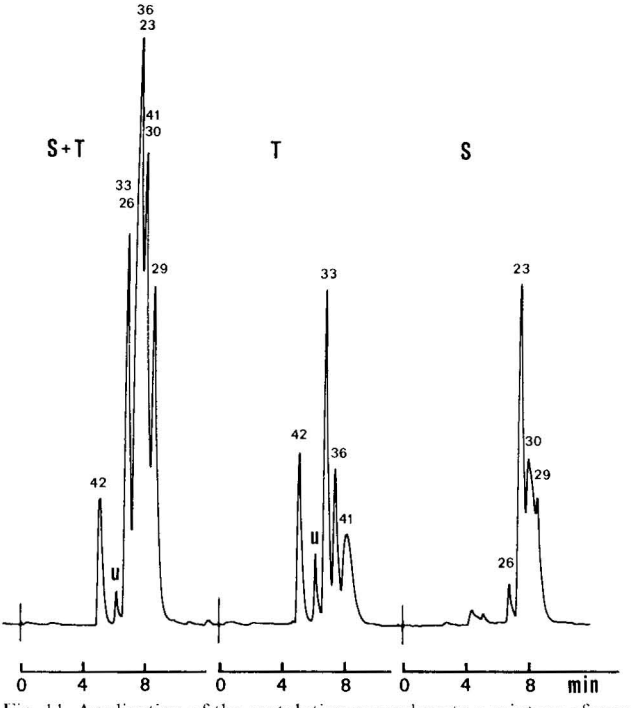

Fig. 11. Application of the acetylation procedure to a mixture of secondary (S) and tertiary (T) anilines. (S + T) = chromatogram of mixture on the nitrile stationary phase (Nucleosil CN); S and T = chromatograms of the isolated secondary and tertiary anilines, respectively, on the nitrile phase. Mobile phase, 0.2% (v/v) 2-propanol in isooctane; 45 ml h^{-1}. The numbers refer to Table I. u = Unknown.

compounds would appear in the T zone and cannot be distinguished from other kinds of secondary and tertiary anilines.

CONCLUSIONS

Bonded-phase normal- and reversed-phase LC, using nitrile and C_{18} stationary phases, can be applied to the separation of complex mixtures of alkylanilines and the identification of individual compounds. The separation on the nitrile phase proceeds in the order tertiary, secondary and primary anilines, and these classes can then be resolved on the C_{18} phase in order of increasing alkyl carbon number.

For confirming the structure of an alkylaniline, several useful relationships are available, *viz.*, two-phase plots and correlations between log k' and carbon number and between log k' and pK_b values of the anilines. By these means it is possible to ascertain the identity of an alkylaniline in terms of class and general structure with a fair degree of certainty.

ACKNOWLEDGEMENT

The experimental assistance of Mrs. Kerstin Svensson is gratefully acknowledged.

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Journal of Chromatography, 214 (1981) 307–315
Elsevier Scientific Publishing Company, Amsterdam Printed in The Netherlands

CHROM. 13,992

ASSAY OF THE COMBINED FORMULATION OF ERGOMETRINE AND OXYTOCIN BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(Received May 18th, 1981)

SUMMARY

The present British Pharmacopoeial monograph for the combined formulation of ergometrine and oxytocin requires a spectrophotometric assay for the ergometrine and a biological assay for the oxytocin content. Simultaneous spectrophotometric assay of the two ingredients has not previously been practicable, owing to the widely different amounts of each. Two high-performance liquid-chromatographic separations have been developed by which both ingredients can be assayed at different dilutions in both systems. One separation is on the strong cation-exchange bonded phase Partisil 5 SCX, where reproducibility of injection gave a peak-height coefficient of variation of 0.8% for ergometrine and 2.5% for oxytocin. An alternative system involves use of the ion-pair reagent, sodium tetradecyl sulphate, with a reversed-phase packing, giving a coefficient of variation for repeat injections of 1.0% (peak height) for ergometrine and 2.5% (peak area) for oxytocin.

INTRODUCTION

Combined formulations often require different assay procedures for each active component. However, if each could be quantitated by the same method, assay might be simpler, quicker and more cost-effective. The British Pharmacopoeial monograph for the combined formulation, ergometrine and oxytocin injection, requires a spectrophotometric assay with dimethylaminobenzaldehyde for the alkaloid[1] and a biological assay by milk ejection pressure in the lactating rat for oxytocin[1,2]. Dimethylaminobenzaldehyde is a colorimetric reagent specific for the indole nucleus and so also reacts to give coloured derivatives with breakdown products of ergometrine, which are thus measured by the assay. A high-performance liquid-chromatographic (HPLC) system that resolved ergometrine from these products would provide a more specific and accurate assay of ergometrine activity. The rats needed for the bioassay of oxytocin in the presence of ergometrine must be at the correct stage of lactation (*i.e.*, between 3 and 21 days post partum) and are not always readily available. An HPLC assay for the peptide would be subject neither to such biological constraints, nor to the accompanying ethical considerations. Good correlation has been obtained

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between HPLC assay results and bioassay (rat uterus contraction and cockerel blood pressure) for oxytocin injection[3,4] and may be expected to be applicable to analysis of other preparations. Separate HPLC procedures for both oxytocin and ergometrine have been published[4–6], and in principle the two assays specified in the monograph might be replaced by a single chromatographic analysis. However, two main obstacles to this are, firstly, that ergometrine is present at a 50-fold higher concentration than oxytocin and, secondly, that ergot alkaloids typically form breakdown products on storage and exposure to light[8] in quantities that might interfere with measurement of the oxytocin. This paper reports two HLPC assays that circumvent these problems.

EXPERIMENTAL

Apparatus

An Altex 110 or LDC mini-pump was used with a Cecil 272 or Hewlett-Packard 1030B variable-wavelength UV monitor. Fluorescence was monitored with a Perkin-Elmer 2000 fluorescence monitor. Unless stated otherwise, a Rheodyne 7125 or Waters U6K loop injector was used.

Materials

Partisil 10 ODS2 (Whatman, Maidstone, Great Britain) and μBondapak C_{18} (Waters Assoc., Milford, MA, U.S.A.) were purchased pre-packed. Stainless-steel columns (100 or 250 mm × 4.6 or 5 mm) were slurry-packed at 300 bar with the following reversed-phase packings: LiChrosorb RP-18 (10 μm; Merck, Darmstadt, G.F.R.), Nucleosil C_8 and C_{18} (both 5 μm; Macherey, Nagel & Co., Düren, G.F.R.), Hypersil SAS, Hypersil ODS (Shandon Southern Instruments, Runcorn, Great Britain), Spherisorb S5 ODS (Phase Separations, Queensferry, Great Britain), and HSCP C_{18} (5 μm, not capped; HS Chromatography Packings Ltd., Bourne End, Great Britain) and the following cation-exchange packings: Partisil 5 SCX, Partisil 10 SCX (Whatman), LiChrosorb KAT (10 μm, Merck), Nucleosil SA (5 μm; Macherey, Nagel & Co.) and Vydac cation-exchange (The Separations Group, Hesperia, CA, U.S.A.). A LiChrosorb Si 60 (10 μm, Merck) column was also packed. A pre-column (50 mm × 4.6 mm) containing silica (LiChroprep Si 60, 5–25 μm was used before the injector with all columns.

AnalaR ammonia and orthophosphoric acid were supplied by BDH (Poole, Great Britain). Triethylamine (Et_3N) was obtained from Koch-Light Labs. (Colnbrook, Great Britain) and acetonitrile (far-UV HPLC grade) from Fisons (Loughborough, Great Britain). Methanesulphonic acid was obtained from Aldrich (Milwaukee, WI, U.S.A.), the sodium salts of pentane- and heptane-sulphonic acids and sodium tetradecyl sulphate (STS) from Eastman-Kodak (Rochester, NY, U.S.A.), and sodium dodecyl sulphate (SDS) from BDH. Ephedrine was supplied by Sigma (St. Louis, MO, U.S.A.) and thermolysin by Merck (for use in solution) and by Sigma (for immobilisation).

Oxytocin concentrate, ergometrine and the [D-Tyr^2]-, [D-Gln^4]- and [D-Tyr^2, D-Gln^4]-oxytocin analogues were gifts from Drs. K. Krummen and H. G. Leeman (Sandoz, Basle, Switzerland). Ergometrine and oxytocin injection BP (Sandoz) was purchased locally.

Mobile phases

Reversed-phase system. A calculated volume of ion-pairing agent (10%, w/v, in water) was added to the buffer (0.83 m*M* H_3PO_4 adjusted to pH 5.0 with Et_3N) to give a final concentration, after the addition of acetonitrile, of 0.05% (w/v); normally, the acetonitrile concentration was 40% (v/v). The temperature of the mobile-phase reservoir was maintained at 18°C, or above, to keep the ion-pairing agent in solution. Before use, reversed-phase columns were washed with approximately 100 ml of [8.3 m*M* H_3PO_4–Et_3N (pH 5.0)]–acetonitrile (60:40) 0.1% in ion-pair reagent. After use, the reversed-phase columns were washed with 5–10 column volumes of 83 m*M* H_3PO_4–acetonitrile (60:40, v/v) to remove Et_3N.

Cation-exchange system. The mobile phase was 8.3 m*M* H_3PO_4 adjusted to pH 5.0 with NH_3–acetonitrile (88:12).

Dilution of formulations

Reversed-phase system. The formulation was diluted 1:10, and to 1 1-ml portion were added 1.5 ml of water and 2.5 ml of ephedrine solution (0.02 mg ml^{-1}). The injection volume for the quantitation of ergometrine was 80 μl. No internal standard was used for oxytocin, and 50 μl of the undiluted formulation were injected direct.

Cation-exchange system. A 1-ml portion of the formulation was diluted with 2.5 ml of ephedrine solution (0.2 mg ml^{-1}) and 1.5 ml of water, and 50-μl aliquots were injected into the HPLC system to quantitate ergometrine. To another portion of the formulation (950 μl) were added 50 μl of ephedrine solution (0.1 mg ml^{-1}), and 50-μl aliquots were injected to assay oxytocin.

Digestion of oxytocin

Soluble enzyme. A freshly prepared solution (1 mg ml^{-1}) of thermolysin (Merck) in water was added to the formulation, or to a solution of oxytocin and ergometrine in water at the same concentrations, to give a final thermolysin concentration of 125 μg ml^{-1}. Digestion was allowed to proceed at room temperature in the dark for the times noted below.

Immobilised enzyme. Thermolysin (Sigma) was immobilised by linkage to cyanogen bromide activated Sepharose 4B[9,10]. A 0.2-ml volume of a 50% (v/v) suspension of immobilised thermolysin in 0.03 *M* sodium borate–5 m*M* $CaCl_2$ (pH 7.0) was added to an equal volume of formulation (or a control solution of the same concentrations of oxytocin and ergometrine in water), and digestion was allowed to proceed at room temperature in the dark for the stated periods.

RESULTS AND DISCUSSION

Since the concentration of oxytocin in the combined formulation was low (10 μg/ml), it was desirable to monitor the UV absorption at the shortest practicable wavelength. For this reason, the separations developed by Larsen *et al.*[5] and Sondack[6], with acetate in the mobile phase, were not suitable. In addition, to maximise sensitivity for oxytocin, it was preferable for the peptide to be eluted before ergometrine. Investigation of other published systems[4,7] showed that ergometrine emerged close to the solvent front and oxytocin was eluted afterwards, too near the alkaloid for quantitation and sometimes together with an impurity peak.

Ion-pairing on reversed-phase

Knox and Jurand[11] and Sood *et al.*[12] obtained good separations of ionizable compounds by incorporating hydrophobic pairing ions containing alkyl groups into the mobile phase, and this approach has since found many applications[13,14]. The addition of a counter-anion containing such a group to the mobile phase reversed the order of elution of ergometrine and oxytocin from reversed-phase packings[15]. The retentions of the nonapeptide and ergometrine increased with the length of the alkyl group attached to the ion-pair (Fig. 1) as has been observed by other investigators[13,14,16,17]. The deviation in retention of oxytocin in the presence of STS may have been due to impurities present in the surfactant[18,19]. Good resolution of ergometrine and oxytocin in the formulation was obtained with a mobile phase containing 0.01% (w/v) SDS and 0.25 m*M* phosphate–Et_3N (pH 5). However, with repeated injections, the retention of the oxytocin peak drifted towards that of trace amounts of chlorbutol (a preservative in the oxytocin concentrate used to prepare the formulation), which progressively interfered with quantitation. This drift was not eliminated by thermostatic control of the system. A similar separation could be obtained on HSCP C_{18} and Partisil ODS at a higher ionic strength (0.1% SDS, 8.3 m*M* phosphate–Et_3N at pH 5) without such drifting, but with other packings the separation was inadequate. Substitution of STS (0.05%, w/v) for SDS in the mobile phase gave good separation of the two drugs at higher ionic strength (0.83 m*M* phosphate–Et_3N). However extra peaks, attributed to breakdown products of ergometrine and to chlorbutol were eluted close to oxytocin.

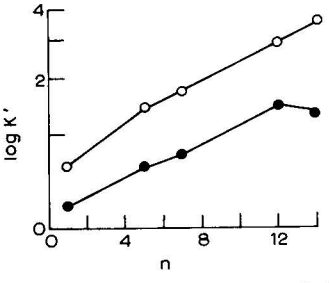

Fig. 1. Relationship between the chain length of counter-ion and capacity ratio (k'). C_1, C_5 and C_7 alkyl chain lengths as sulphonates, the remainder as sulphates. Chromatographic conditions: support ODS Hypersil (50 × 4.6 mm; a freshly packed column for each homologue); mobile phase: 0.05% (w/v) STS, 0.83 m*M* H_3PO_4–Et_3N pH 5.0, 40% (w/v) acetonitrile. Flow-rate: 0.5 ml min^{-1}. ○ = Ergometrine; ● = oxytocin.

When the relative retentions of the two drugs were compared on different commercial packings (Fig. 2), the variations between packings were less pronounced for STS than for SDS at the lower ionic strength[15]. Such differences in behaviour between reversed-phase packings from different manufacture have been widely observed and commented upon[20–22]. Simultaneous monitoring by UV absorption and fluorescence of the separation on Nucleosil C_{18} using STS demonstrated that no fluorescent peak typical of ergometrine breakdown products was co-eluted with oxytocin. A formulation was digested with a preparation of immobilised thermolysin in order to check that oxytocin was not co-eluted with other peaks on three different commercial packings. No peak of significant height was co-eluted with the peptide

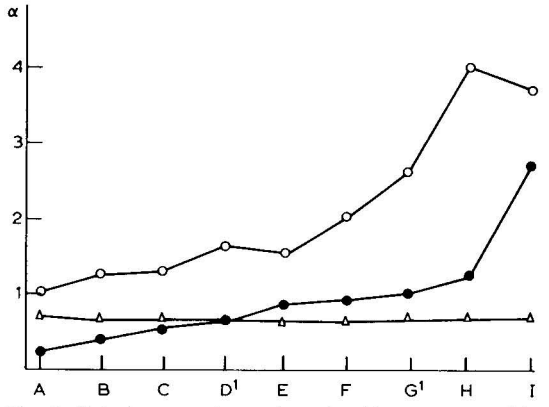

Fig. 2. Relative capacity ratios (α) with respect to chlorocresol of different commercial packings. A, Partisil 10 ODS; B, LiChrosorb 10 RP-18; C, μBondapak 10, C_{18}; D, Nucleosil 5 C_8; E, Nucleosil 5 C_{18}; F, ODS Hypersil; G, SAS Hypersil; H, Spherisorb S5 ODS; I, HSCP C_{18}. Mobile phase as in Fig. 1; flow-rate 1 ml min^{-1}. ○ and ● as in Fig. 1; △ = chlorbutol. Superscript 1, 25% (v/v) of acetonitrile.

from μBondapak C_{18}; however, with Nucleosil C_{18} and Spherisorb ODS, it was necessary to reduce the acetonitrile concentration (to 37% and 35%, respectively) to prevent significant interference.

Three diastereoisomers, [D-Tyr^2]-, [D-Gln^4]- and [D-Tyr^2,D-Gln^4]-oxytocin, were either partially or wholly resolved from oxytocin on Nucleosil C_{18} (Table I). The order of elution was the reverse of that obtained on LiChrosorb RP-8 and μBondapak C_{18} with no hydrophobic ion-pair reagent[3,23]. Resolution of oxytocin from these diastereoisomers was not affected by inclusion in a formulation.

TABLE I

SEPARATION OF OXYTOCIN DIASTEREOISOMERS

	k'	
	Nucleosil C_{18}	*Partisil 5 SCX*
[D-Tyr^2]-Oxytocin	1.78	12.20
[D-Tyr^{2}DGln^4]-Oxytocin	2.30	10.44
[D-Gln^4]-Oxytocin	2.75	13.40
Oxytocin	3.43	15.60

It was possible to obtain separations similar to the ion-pair reversed-phase systems on LiChrosorb Si 60 (mobile phase 8.3 m*M* H_3PO_4 adjusted to pH 7.0 with NH_3; 5% v/v acetonitrile), but with reduced resolution of the peaks. Since problems with column stability and variation between different silica packings were experienced, this separation was not investigated further.

Cation exchange

In view of the above results and published work[24], it seemed worthwhile to examine bonded ion-exchange packings in the hope of obtaining more favourable

selectivity. On Partisil 5 SCX, oxytocin was eluted before ergometrine, and many of the contaminating peaks appeared close to the solvent front. No fluorescent peak was co-eluted with oxytocin. Treatment of the formulation with thermolysin showed that no other peak was co-eluted with oxytocin (Fig. 3). A similar separation was obtained on Partisil 10 SCX, but not on other cation exchangers.

The three diastereoisomers were separated from oxytocin on Partisil 5 SCX (Table I), both separately and when incorporated in a formulation.

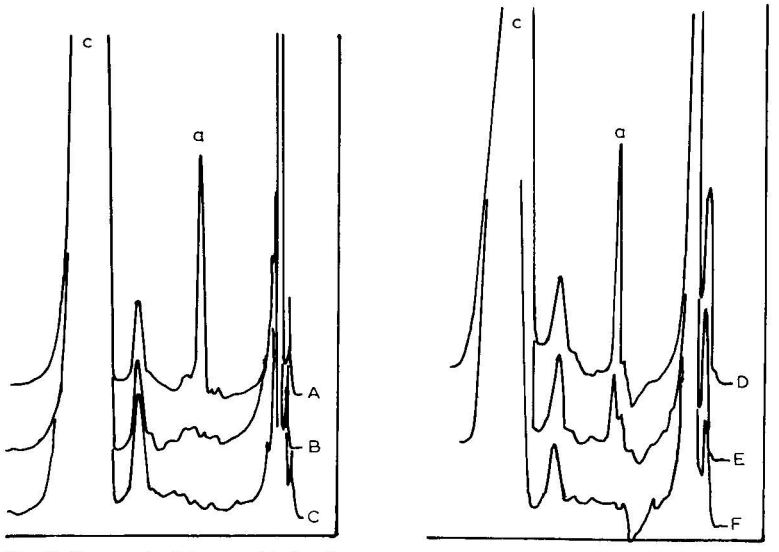

Fig. 3. Removal of the peptide by digestion with thermolysin. Stationary phase: Partisil 5 SCX (50 × 4.6 mm; mobile phase: 8.3 m*M* H_3PO_4–NH_3 (pH 5.0); 12% (v/v) acetonitrile; flow-rate 1 ml min^{-1}. A, 800 ng of oxytocin + 4 μg of ergometrine; B, 800 ng of oxytocin + 4 μg of ergometrine treated with thermolysin for 1.25 h; C, 4 μg of ergometrine treated with thermolysin overnight; D, 80 μl of formulation; E, 80 μl of formulation treated with thermolysin for 1.25 h; F, 80 μl of formulation treated with thermolysin overnight.

Reproducibility and linearity of response

Examination of the chromatograms in Figs. 4 and 5 shows that any suitable internal standard for the ion-pair reversed-phase system must emerge after ergometrine, but might be eluted between oxytocin and ergometrine in the cation-exchange system. Fortunately, ephedrine exhibited just this behaviour, and was therefore a suitable internal standard for either system. The coefficient of variation of five or more replicate injections of oxytocin on Partisil 5 SCX (50-mm column, septum injection) was 2.5% (peak-height measurements, with ephedrine as internal standard). For the ion-pair system (Nucleosil C_{18} column, 37% acetonitrile), reproducibility was better for oxytocin without an internal standard, and the coefficient of variation was 2.9% for peak-height and 2.5% for peak-area measurements. The coefficient of variation for ergometrine with ephedrine as internal standard using peak-height measurement was 1.0% for the ion-pair system and 0.8% for the cation-exchange (100-mm) column.

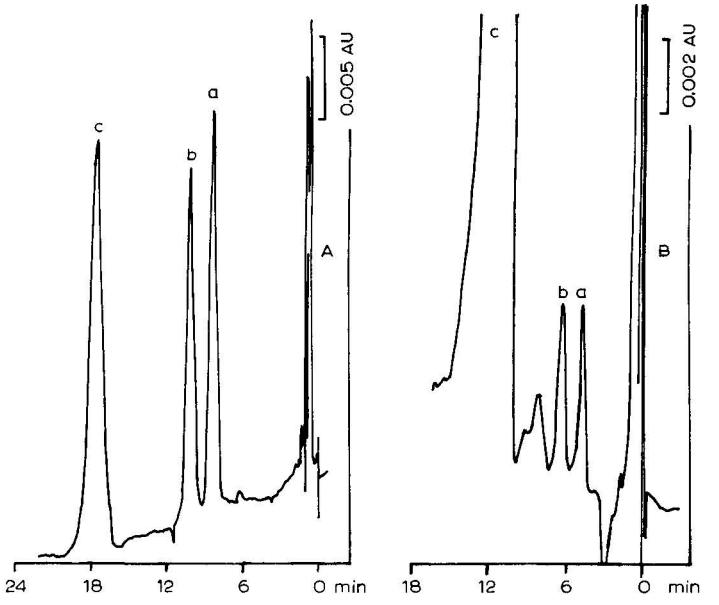

Fig. 4. Chromatograms obtained by ion-pair reversed-phase HPLC. Support: Nucleosil C_{18}. Mobile phase: as in Fig. 1, except with 35% acetonitrile for Fig. 4A and 37% (v/v) of acetonitrile for Fig. 4B. Flow-rates 2.5 ml min^{-1} for Fig. 4A and 1 ml min^{-1} for Fig. 4B. a = Oxytocin; b = ephedrine; c = ergometrine.

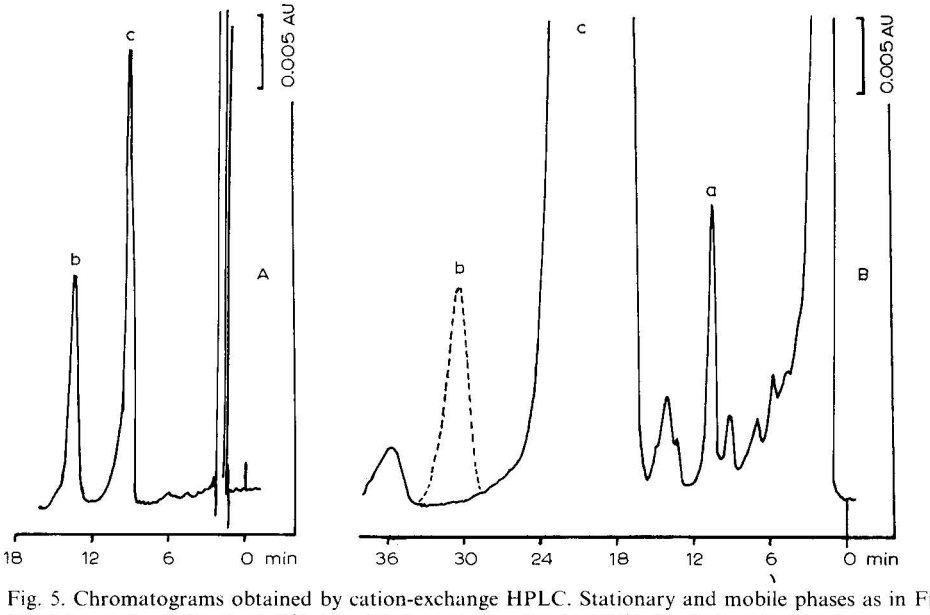

Fig. 5. Chromatograms obtained by cation-exchange HPLC. Stationary and mobile phases as in Fig. 3. Fig. 5A, standards (1 ml min^{-1}); Fig. 5B, formulation (2 ml min^{-1}). Symbols as in Fig. 4.

Injections of oxytocin (50–1000 ng) containing ergometrine at the concentration in the formulation were made by septum on to Partisil 5 SCX (50-mm column; ephedrine as internal standard) and by valve on to Nucleosil C_{18} (37% acetonitrile; no internal standard). For both systems, variation in peak height with oxytocin concentration displayed a linear relationship and the best fit passed through the origin. Likewise, the variation in peak height with ergometrine concentration was linear for both procedures (1.0–7.5 μg, Partisil 5 SCX, 100-mm column; Nucleosil C_{18}, 37% acetonitrile). Extrapolation of the line did not pass through the origin for the ion-pair system, and, with increase in concentration of ergometrine, a corresponding decrease in the peak height of the standard and increase in height of an unidentified peak was observed. Use of an alternative standard (chlorocresol) gave a non-linear response. Injection of ergometrine at lower quantities (160–1200 mg) gave a variation in peak height that was both linear and passed through the origin.

The coefficients of variation for five repeated dilutions for the cation-exchange system were 1.1% for ergometrine (100-mm column) and 3.7% for oxytocin (50-mm column, septum injection). The coefficient of variation on Partisil 5 SCX (100-mm column) between vials from the same batch was 1.6% for ergometrine (mean: 109.7% of the labelled dose) and 3.0% for oxytocin (mean: 105.1% of the labelled dose).

Both separation procedures are sufficiently reproducible and sensitive to determine the active ingredients quantitatively. The separation on Partisil 5 SCX was good, and use of a valve injector might improve the reproducibility for measurements of the peptide. This packing is not, as yet, generally available, but, in principle, Partisil 10 SCX should give similar results. The ion-pair separation is a preferable alternative to the cation-exchange system, since adequate separations were obtained on several commercial packings. A disadvantage experienced with the reversed-phase system was that the relative elution position of oxytocin changed with alteration of pH. The presence of Et_3N will make the pH of the mobile phase sensitive to fluctuation in temperature. In this study, the ambient temperature varied by at least 4°C; consequently, improvement in the reproducibility of the oxytocin peak might be obtained by thermostatic control of the column.

We believe that, subject to appropriate assessments, one or other of the procedures described should be a suitable replacement for the assay methods at present official in the British Pharmacopoeia.

ACKNOWLEDGEMENTS

R.A.P.-H. was supported by a grant from the Lord Dowding Fund. The generous gifts of oxytocin concentrate and the oxytocin analogues by Drs. K. Krummen and H. G. Leeman of Sandoz Ltd. are gratefully acknowledged.

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Journal of Chromatography, 214 (1981) 317–325
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,007

ISOLATION OF HUMAN HAEMOPEXIN BY BIOAFFINITY CHROMATOGRAPHY ON HAEME-SEPHAROSE

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(Received May 18th, 1981)

SUMMARY

A preparative procedure was developed for the isolation of human apohaemopexin from Cohn fraction IV or blood serum, based on bioaffinity chromatography on haeme-Sepharose. The isolation is carried out in the pH range 4–8; hence the possibility of degradation of the carbohydrate moiety of the glycoprotein in the acidic media used in other isolation procedures is decreased. Owing to the conditions of the separation and the good stability of the affinity support, the column can be used repeatedly for long periods without a significant loss of binding capacity. The reversibility of the conformational changes that haemopexin undergoes in acidic media was examined by hydrophobic chromatography. The original hydrophobic characteristics were restored only approximately 48 h after haemopexin had been brought into a neutral medium.

INTRODUCTION

Haemopexin is a single-chain protein with a relative molecular weight of *ca.* 57,000[1], of which roughly 20% represents the carbohydrate moiety[2]. Haemopexin belongs to specific blood transport proteins whose concentration in the blood serum is 0.6–1.0 g/l[3], and binds very strongly the haeme released in the bloodstream during the degradation processes. Its physiological function and physico-chemical characteristics have been reported earlier[4,5].

Haemopexin has been isolated from blood serum by several procedures, making use of selective precipitation and ion-exchange chromatography[6–13], in yields below 20%. Bioaffinity chromatography, based on the binding of the carbohydrate moiety of the molecule to wheat germ lectin-Sepharose[14] or on the interaction of apohaemopexin with the haeme bonded to Sephadex G-100[15], Bio-Gel P200[16] or Sepharose 4B[17], has also been used for the isolation of a haemopexin. The aim of this study was to improve the isolation by bioaffinity chromatography on a support with immobilized haeme[16,17], in order to avoid the action of strongly acidic media on haemopexin, and by increasing the lifetime of the column by employing a novel mode of bonding the haeme to the affinity support.

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EXPERIMENTAL

Sepharose 4B, Sepharose CL-4B and Sephadex G-10, G-100, and G-25 Fine were obtained from Pharmacia (Uppsala, Sweden), and Spheron P300 (63–100 μm) from Lachema (Brno, Czechoslovakia). Octyl-Sepharose was prepared from Sepharose CL-4B according to Hjertén *et al.*[18]. Polyamide layer sheets were purchased from BDH (Poole, Great Britain). Acrylamide, N,N′-methylenebisacrylamide and sodium dodecyl sulphate were purchased from Koch-Light (Colnbrook, Great Britain), Tris [N-tris(hydroxymethyl)aminomethane] and 1-dimethylaminonaphthalene-5-sulphonyl chloride from Serva (Heidelberg, G.F.R.) and Amido black 10B from Merck (Darmstadt, G.F.R.). Transferrin was a B-grade product of Calbiochem (San Diego, CA, U.S.A.). Horse antihuman sera (anti-haemopexin, anti-albumin, anti-transferrin and anti-human serum) were obtained from Sevac (Prague, Czechoslovakia). A standard preparation of Cohn fraction IV in lyophilized form and human serum albumin (salt-free, 98%) were obtained from Imuna (Šarišské Michalany). Rivanol (2-ethoxy-6,9-diaminoacridine lactate) was purchased from Zdravotnické Zásobování (Prague, Czechoslovakia), epichlorohydrine (pure) from Jenapharm (Jena, G.D.R.), sodium borohydride from Metallgesellschaft (Frankfurt/Main, G.F.R.), 2-mercaptoethanol (pure) from Loba Chemie (Vienna, Austria), barium hydroxide (analytical-reagent grade) from Merck and 1,4-dibromobutane (pure) from Fluka. Dicyclohexylcarbodiimide was prepared as described elsewhere[18]. The remaining chemicals were analytical-reagent grade materials from Lachema.

Preparation of haeme-Sepharose

Haemin (protoporphyrin IX chloride) was prepared by the method described elsewhere[19] but using a volume of acetone three times greater than that reported by the authors. The product was recrystallized twice from pyridine–acetic acid–chloroform[19]. Haeme-Sepharose was prepared according to the scheme shown in Fig. 1. SH-Sepharose was prepared by a procedure described elsewhere[20]. The content of SO_3H groups in our preparation, determined by acidimetric titration prior to the reduction, was 0.4 mmol per 100 ml. This material can be replaced with a commercial preparation of thiopropyl-Sepharose 4B (Pharmacia).

Preparation of 8-amino-2-hydroxy-4-thiooctyl-Sepharose (V)

Thiopropyl-Sepharose (III) was washed with water, filtered off and transferred into a mixture of 200 ml of ethanol, 70 ml of water, 30 ml of 0.1 *M* ammonium hydrogen carbonate containing 1 m*M* EDTA, and 25 ml of 1,4-dibromobutane. The mixture was stirred thoroughly and allowed to stand for 45 min at 50°C. The slurry was washed with ethanol–water (4:1) and transferred into 250 ml of 10% ammonia solution. The reaction mixture was set aside for 10 h at room temperature and then washed thoroughly with water.

Binding of haeme

The gel (V, 150 ml) was washed with 250 ml of dimethylformamide (DMF), filtered off, then treated with 300 ml of DMF together with a solution of 50 mg of haemin in 30 ml of DMF and a filtered solution of 3 g of dicyclohexylcarbodiimide in 30 ml of DMF. The reaction mixture was set aside for 15 h at room temperature. The

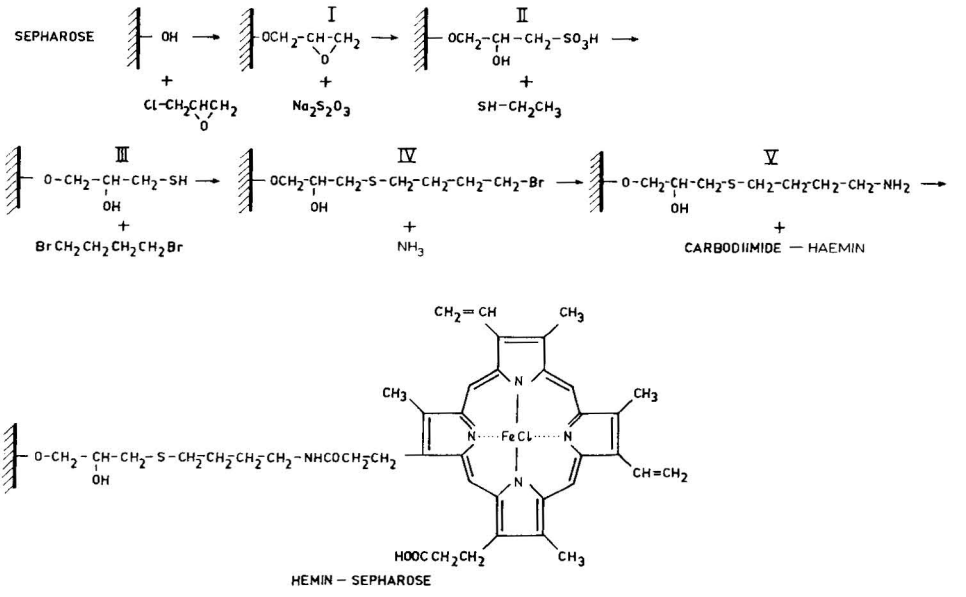

Fig. 1. Scheme of preparation of haeme-Sepharose.

gel was filtered off and the reaction was repeated with 50 mg of haemin dissolved in a mixture of 100 ml of DMF, 50 ml of water and 300 ml of ethanol. The gel was washed with two 200-ml portions of DMF, two 200-ml portions of methanol, water and a saturated solution of sodium tetraborate until the filtrate became only slightly discoloured. The remaining haeme not bound covalently was removed from the column by repeatedly washing it stepwise with several 50-ml portions of 1 % serum albumin, 200-ml portions of 0.05 *M* Tris–hydrochloric acid buffer (pH 7.0) and 100-ml portions of 0.1 *M* citrate buffer (pH 4.0) until the red colour of any of the washing solutions had disappeared.

Isolation of haemopexin from Cohn fraction IV

Lyophilized Cohn fraction IV (5 g) was suspended in 50 ml of water and the pH of the turbid solution was adjusted to 8.0 by the addition of 0.5 *M* sodium hydroxide solution. The solution was cooled to 4 °C and 75 ml of pre-cooled 1.68 % rivanol solution (pH 8.0) was added in portions. The albumin precipitate was removed by filtration through a cotton-wool pad. The filtrate was treated with sodium chloride added to a final concentration of 5 %. The precipitate of the insoluble rivanol salt was separated by filtration (Schleicher and Schüll filter-paper No. 589) and the traces of rivanol remaining in the solution were removed by the addition of 4 g of Spheron P300. After stirring for 20 min the Spheron was separated by filtration and the pure filtrate was adjusted to pH 7.0 and applied to a column of haeme-Sepharose (20 × 2.5 cm), equilibrated with 0.05 *M* Tris–hydrochloric acid buffer (pH 7.0) containing 0.2 *M* sodium chloride. Unretained proteins were washed out with 350 ml of the same buffer. The protein attached was displaced with 0.1 *M* citrate buffer (pH 4.0); the effluent fractions were assayed by absorbance measurement at 280 nm. The course of the separation is shown in Fig. 2. The fractions pooled were desalted on Sephadex G-

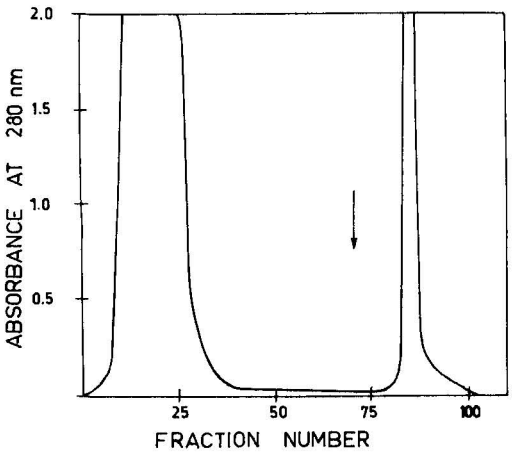

Fig. 2. Affinity chromatography of rivanol supernatant of Cohn fraction IV on haeme-Sepharose.

25 in dilute ammonia solution (pH 8); the yield was 55 mg of the lyophilized preparation.

Isolation of haemopexin from blood serum

The pooled blood serum obtained from donors (200 ml) was adjusted to pH 8.0 and albumin was precipitated by the addition of 1.68% rivanol solution. The conditions of the experiment were identical with those described above for the treatment of Cohn fraction IV as starting material. The volume of serum used (200 ml) afforded 124 mg of the lyophilized preparation (yield about 85%).

The preparations obtained were of high purity and contained a negligible amount of a mixture of immunoglobulins. As described by other workers[16,17], this mixture can be removed by ion-exchange chromatography. Wherever necessary this removal was effected on a small DEAE-cellulose column (6 × 0.8 cm), equilibrated with 0.01 *M* Tris–hydrochloric acid buffer (pH 7.0). When a sample solution in the same buffer was applied, the mixture of immunoglobulins was not retained and was washed from the column. Haemopexin was displaced as a sharp peak by the same buffer containing 0.4 *M* sodium chloride. The total yield of this purification procedure was 70% based on the original serum.

Analytical procedures and evaluation of the individual preparations

The composition of the pooled fractions was checked by discontinuous electrophoresis in 7.5% polyacrylamide gel in Tris–glycine buffer (pH 8.3)[21] and by discontinuous electrophoresis in 10% polyacrylamide gel of reduced samples in the presence of 1% sodium dodecyl sulphate in 0.1 *M* phosphate buffer (pH 7.2)[22]. Unlike the former electrophoresis, under these conditions haemopexin can be separated from transferrin. The protein bands were stained with Amido Black 10B or with Coomassie Brilliant Blue G-250 (the latter procedure). The purity of the preparations obtained was checked by immunoelectrophoresis[23] and by capillary isotachophoresis using the apparatus developed in this Institute[24], equipped with a PTFE capillary of I.D. 0.45 mm and length 45 cm.

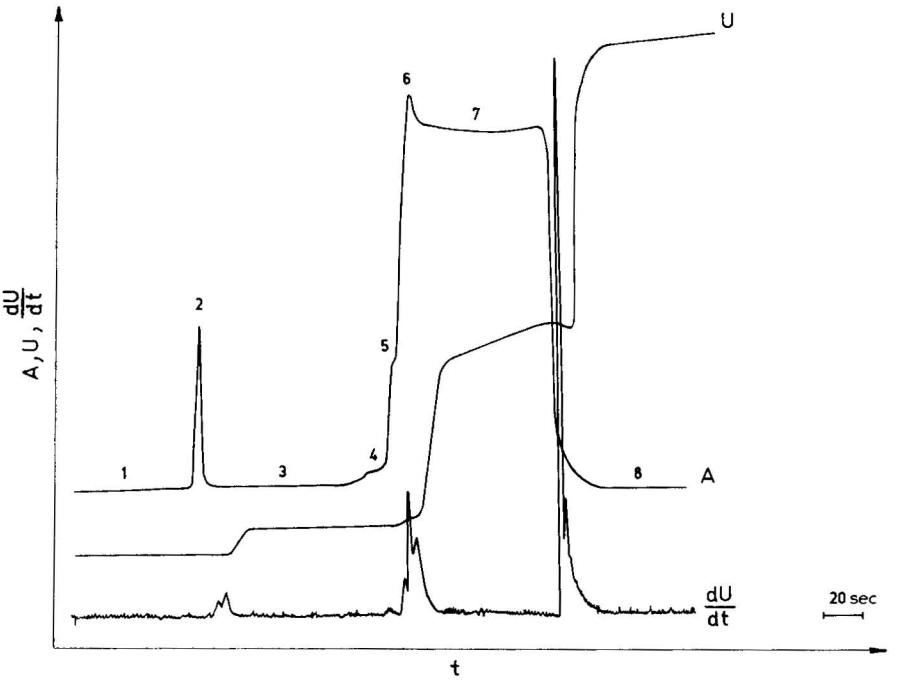

Fig. 3. Analysis of haemopexin (HX) by capillary isotachophoresis. 1 = Chlorides; 2, 4, 5, 6 = unidentified contaminants (6 = probably a dimer of HX); 3 = carbonates; 7 = HX; 8 = Gly^-. A = absorbance at 254 nm; U = potential gradient; dU/dT = derivation of potential gradient; t = time. Conditions of separation: leading electrolyte, 0.01 M hydrochloric acid–0.02 M Tris; leading anion, Cl^-; counter ion, $Tris^+$; pH, 8.3; additive, poly(vinyl alcohol) (Mowiol; Merck), 0.02% (w/v); terminating electrolyte, 0.01 M glycine, pH 10.2, adjusted with saturated barium hydroxide solution; the amount of sample applied was 62.5 μg in 5 μl of water. The analysis was carried out at a constant temperature of 15°C under a constant current whose level was changed stepwise in the course of the separation as follows: 5 min, 50 μA; 20 min, 80 μA; 5 min, 50 μA; 20 μA during the detection.

The zones were detected by a contact universal potential gradient detector and by an absorption detector at 254 nm. Additional details are given in Fig. 3. The N-terminal amino acid residues were determined as 1-dimethylaminonaphthalene-5-sulphonyl (DANS) derivatives[25] by thin-layer chromatography on polyamide layer sheets[26]. The spectrophotometric measurements were carried out with a Zeiss PMQ II spectrophotometer.

Chromatographic studies of haemopexin renaturation

Haeme-haemopexin (0.7 mol of haeme per mole of protein) was dissolved in water (0.77 mg/ml) and the pH was adjusted to 2.2 with 0.5 M hydrochloric acid. After haemopexin had been exposed to the acidic conditions for 150 min at 20°C, the pH of the solution was increased to 8.0 with 0.5 M sodium hydroxide solution. At pre-determined time intervals 75-μl samples of the solution were applied to a 130 × 8 mm I.D. column of octyl-Sepharose CL-4B. The elution was effected with 0.05 M phosphate buffer (pH 8.0) containing 0.15 M sodium chloride. The chromatographic equipment has been described elsewhere[27].

RESULTS AND DISCUSSION

The predominant part of the proteins in the starting material for haemopexin isolation, in either Cohn fraction IV or blood serum, consists of albumin. Like other workers[17], we employed precipitation with rivanol as the first isolation step. The removal of the last traces of rivanol from the supernatant was facilitated by the addition of Spheron P300, replacing filtration through a Sephadex G-25 column. The composition of the starting material after the rivanol fractionation is shown in Fig. 4. During affinity chromatography (Fig. 2) the haemopexin adsorbed is liberated under relatively mild conditions (pH 4.0) and the danger of deterioration of the sugar moiety of haemopexin in acidic media is thus diminished. No additional protein-containing material was released by elution of the column by a buffer of pH 2.4[17].

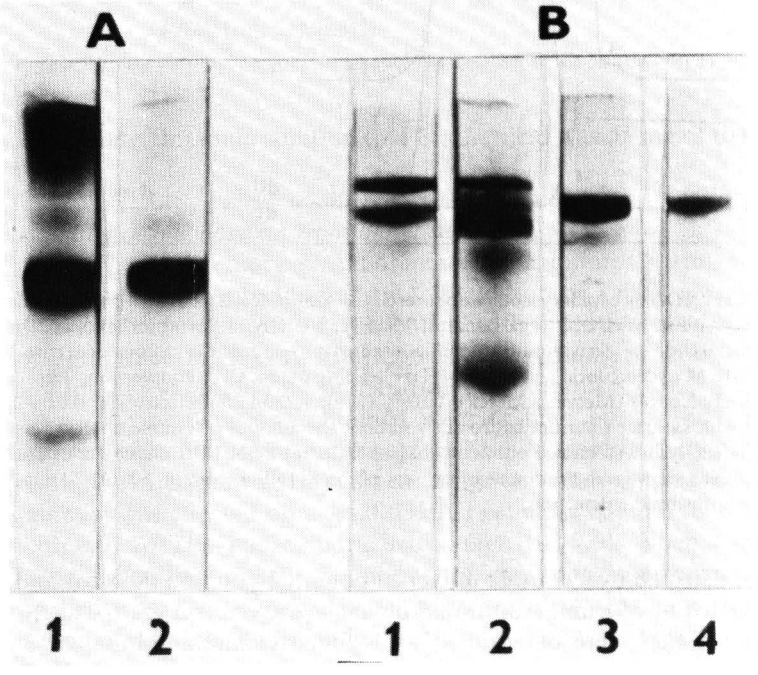

Fig. 4. Discontinuous electrophoresis of the preparations obtained. (A) Electrophoresis at pH 8.3; 1 = rivanol supernatant of Cohn fraction IV; 2 = mixture of hemopexin and transferrin (fraction "E" obtained in earlier work[28]). Haemopexin and transferrin remain unseparated under the conditions of this electrophoretic experiment. (B) Electrophoresis of reduced samples in the presence of 1 % sodium dodecyl sulphate (pH 7.2); 1 = mixture of transferrin (top band) and haemopexin (bottom band; *cf.*, A-2); 2 = rivanol supernatant of blood serum; 3 = preparation of haemopexin after affinity chromatography; 4 = haemopexin purified on DEAE-cellulose.

The modification of the haeme bonding to Sepharose results in good efficiency of the column. As the spacer used did not introduce any electric charges, the protracted tailing of the protein was minimized. Haemopexin was eluted as a sharp peak in a small volume and the exposure of the protein to acidic conditions was short. As the attachment of the haeme to the matrix involved only one bond sensitive to hydrol-

ysis and as mild elution conditions were chosen, the affinity column showed excellent stability: during 1 year of use no significant decrease in its capacity was observed. The advantageous properties of the affinity support can be ascribed to the modified binding of the haeme to the matrix. The binding to Sepharose via the propionyl residue in position 6 or 7 (Fig. 1) obviously does not affect the binding ability of the immobilized haeme. The spacer is sufficiently long and flexible to provide for good exposure of the porphyrin ring necessary for the interaction with haemopexin.

The yield of haemopexin after the precipitation and affinity chromatography was very good, about 85% when haemopexin was isolated from blood serum. The preparation obtained was very pure (Figs. 3–5) and a negligible amount of a mixture of immunoglobulins was detected. This mixture has also been detected by Suttnar *et al.*[17] in their preparation obtained by affinity chromatography using an affinity support with a different haeme bonding. The total yield of our preparation after purification by chromatography on DEAE-cellulose was about 70%; the use of DEAE-Sepharose CL-6B[17] seems more advantageous. The final preparation did not contain any protein contaminants, as confirmed by the determination of only one N-terminal end group (threonine).

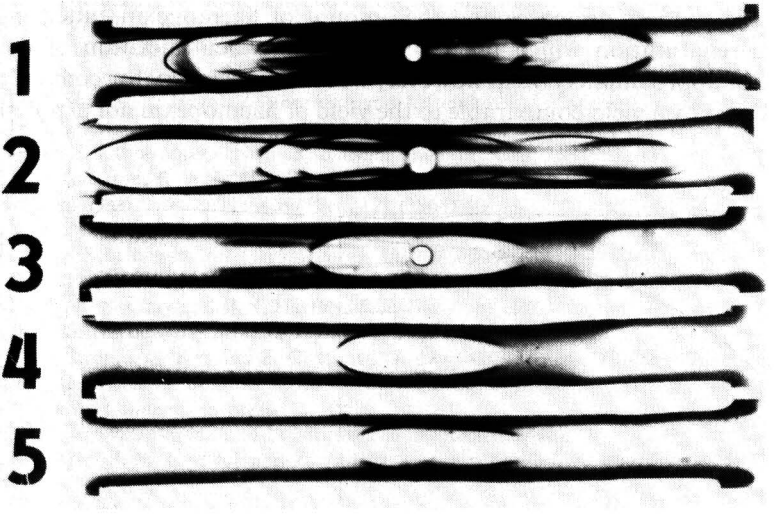

Fig. 5. Immunoelectrophoresis of the individual preparations. 1 = Blood serum; 2 = rivanol supernatant of blood serum; 3 = preparation of haemopexin obtained by affinity chromatography; 4 = haemopexin purified on DEAE-cellulose (all against human serum antiserum); 5 = purified haemopexin (sample 4) against human haemopexin antiserum.

In an earlier study[28] we investigated the isolation of haemopexin from a material containing this protein predominantly as haeme-haemopexin, which cannot be purified by bioaffinity chromatography on supports with immobilized haeme. The remaining content of transferrin could be removed by the procedures used only with difficulty. The liberation of apo-haemopexin from the strong complex with the haeme and the isolation of the free protein is difficult. Evidence has been obtained[29] that this can be approached to a certain extent in the presence of material competing for the

haeme liberated. The most successful has been the procedure which involves dissolution of the haeme-haemopexin preparation in 0.1 *M* glycine–hydrochloric acid buffer (pH 2.4) and the separation of the haeme liberated by filtration through a column of Spheron H1000 (Laboratory Instruments Works, Prague, Czechoslovakia). By this procedure the free apo-haemopexin content of the preparations was considerably enriched and the latter could be isolated in pure form by affinity chromatography as described above; the contamination with immunoglobulins was removed by the preceding procedure.

The effect of acidic media on haemopexin has been studied by Hrkal *et al.*[30]. They concluded that the unfolding of the haemopexin molecule and the dissociation of the haeme from the binding site are reversible processes and the restoration of the native conformation proceeds relatively quickly. Our experiments with hydrophobic chromatography on octyl-Sepharose (Fig. 6) have shown that the reconstitution of certain amino acid residues in the spatial positions is slower than the transitions followed in terms of changes of the absorption spectrum in the Soret region. These changes which take place in acidic media made certain hydrophobic residues accessible to interaction with the chromatographic support and result in complete hold-up of haemopexin in the column. The interaction of the hydrophobic residues with the chromatographic support decreases and the amount of haemopexin eluted increases during the renaturation which takes place after the neutralization of the solution. A period of approximately 48 h, however, would be necessary for complete renaturation, indicated by a yield comparable to the yield of haemopexin not exposed to acidic media.

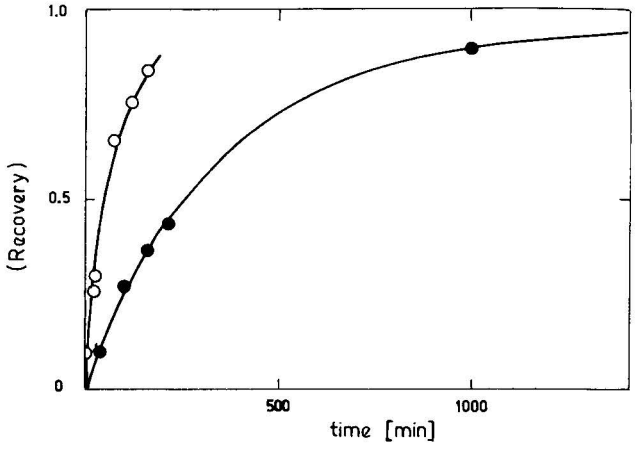

Fig. 6. Renaturation of haemopexin after conversion from acidic to neutral pH. Open symbols: renaturation of haemopexin as a function of time examined spectrophotometrically at 414 nm as the ability to bind the heme. Recovery $= [A_{414(t)} - A_{414(t=0)}]/[A_{414(t=\infty)} - A_{414(t=0)}]$, where $A_{414(t)}$ is the absorbance at time t, $A_{414(t=0)}$ the absorbance of the haemopexin sample immediately after conversion to neutral pH and $A_{414(t=\infty)}$ the absorbance of the unacidified haeme-haemopexin solution; all solutions in 0.05 *M* Tris-hydrochloric acid buffer (pH 8.0). Closed symbols: renaturation of haemopexin as a function of time examined by hydrophobic chromatography. The recovery is represented by the ratio of the eluted acid-treated to untreated haemopexin.

ACKNOWLEDGEMENTS

We are indebted to Dr. Z. Hrkal and Mrs. J. Rejnková (Institute of Haematology and Blood Transfusion, Prague) for the comparative immunoelectrophoretic tests.

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Journal of Chromatography, 214 (1981) 327–334
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,010

C_{18} REVERSED-PHASE TRACE ENRICHMENT OF CHLORINATED PHENOLS, GUAIACOLS AND CATECHOLS IN WATER

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(First received March 2nd, 1981; revised manuscript received May 18th, 1981)

SUMMARY

Reversed-phase adsorption with octadecyl-modified silica gel was found to give quantitative recoveries of di-, tri-, tetra- and pentachlorophenol, tri- and tetrachloroguaiacol and tri- and tetrachlorocatechol from water. After desorption with acetone the chlorophenolic compounds were determined as their acetyl derivatives, using quartz capillary column gas chromatography. Different aqueous acetylation methods were compared, the highest yields being obtained with potassium carbonate solution. The procedure described has been applied to natural and industrial waste waters.

INTRODUCTION

Chlorinated phenolic compounds enter the environment from a variety of sources, including both accidental and non-accidental discharges from industrial activities. Pentachlorophenol, together with 2,3,4,6-tetra- and 2,4,6-trichlorophenol, are widely used as pesticides, mainly as fungicides for wood protection. Tri- and tetrachloroguaiacols and catechols have been identified in effluents from pulp and paper industries, where they are formed during bleaching[1–3].

The interest in chlorophenolic as environmental contaminants is due mainly to the high toxicity of chlorophenols[4–6], guaiacols and catechols[7–10]. Also, bioaccumulation properties have been established for chlorophenols[11–13] and chloroguaiacols[9,14].

The release of chlorophenolics through waste waters into water recipients has created a need for monitoring these substances in industrial effluents and natural waters. Sensitive and selective methods based on gas chromatography have therefore been developed.

Many methods have been used successfully for the isolation and concentration of chlorophenolic compounds from water samples. Usually, liquid–liquid extractions

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have been applied for the determination of pentachlorophenol[15–20] and for the determination of chlorinated guaiacols and catechols[3,9]. The main problem with liquid–liquid extraction methods, when applied to waste and natural waters, is the formation of emulsions which tend to complicate the phase separation. Also, the partition coefficients for relatively water-soluble substances, *e.g.*, chlorinated catechols, are sometimes unfavourable and may result in inadequate extraction efficiencies.

Other methods that have been employed for the isolation of chlorophenols from water samples are ion-exchange techniques[21–24] and reversed-phase adsorption on cross-linked polystyrene resins[25], although the latter technique in certain instances has been less successful[26].

Reversed-phase chemically bonded stationary phases offer an attractive alternative for the isolation and concentration of organic substances. The development of such stationary phases for high-performance liquid chromatography (HPLC) has also resulted in commercially available cartridges packed with octadecyl-modified silica gel. Such cartridges have been applied successfully to the isolation of chlorinated hydrocarbons[27] and benzidine and related compounds[28].

In this work, the possibility of applying C_{18} cartridges to the trace enrichment of chlorophenolics has been investigated. After the isolation, chlorinated phenols, guaiacols and catechols are converted into their acetyl derivatives and analysed by gas chromatography. The method has been applied to industrial waste waters and natural water.

EXPERIMENTAL

Reagents and materials

Methanol, acetone, sulphuric acid, 0.1 *M* potassium carbonate solution, *n*-hexane, acetic anhydride, sodium sulphate (anhydrous), ascorbic acid, Sep-Pak C_{18} cartridges (9 × 9 mm I.D.) (Waters Assoc., Milford, MA, U.S.A.) and a 10-ml Hamilton gas-tight 1010 W syringe with a T-tube connection (Waters Assoc.) were used. All reagents and materials were of analytical-reagent grade and tested in blank procedures. Reference compounds were synthesized as described earlier[9,29,30]. 2,6-Dibromophenol (Fluka, Buchs, Switzerland) was used as an internal standard.

Trace enrichment step

Before use, a C_{18} cartridge is activated by passing methanol (2 ml) through it, followed by distilled water (5 ml). The water sample (100 ml or less) is transferred into a glass beaker and, if chlorocatechols are to be included in the analysis, ascorbic acid (100–200 mg) is added to the water sample to prevent auto-oxidation. After acidification to pH < 5 by dropwise addition of sulphuric acid, the water sample is passed through the cartridge at a flow-rate of 30–40 ml/min with the aid of a glass syringe. The cartridge is disconnected and eluted in the reverse direction with acetone, and 4 ml of eluate are collected. If desired, the acetone extract is gently concentrated at room temperature to approximately 1 ml using a gentle stream of nitrogen.

Derivatization step

Before the gas chromatographic analyses, the chlorophenolics are converted

into their acetate derivatives, mainly according to the procedure described by Chau and Coburn[18] and Voss *et al.*[31]. The acetone extract (1 ml) is transferred into a test-tube containing 0.1 *M* potassium carbonate (3 ml). After the addition of *n*-hexane (1–2 ml) containing internal standard and acetic anhydride (50 μl), the test-tube is immediately shaken for 1 min. After centrifugation the *n*-hexane extract is transferred into another test-tube and a few crystals of sodium sulphate are added.

Gas chromatographic determination

The *n*-hexane extracts were injected into a Varian 3700 gas chromatograph with a ^{63}Ni electron-capture detector. The capillary quartz column (25 m × 0.2 mm I.D., SE-30 methylsilicone) was maintained at 200°C and the injector and detector temperatures were 250°C. Helium was used as the carrier gas (0.6 ml/min) and argon–methane (95:5) as the make-up gas (30 ml/min). The injector splitting ratio was usually 1:20.

Recovery experiments

Natural water (100 ml, pH 8.3) taken from the Baltic Sea was poured into a glass beaker and an ethanolic solution (10 μl) containing the substances listed in Table I was added. Concentration levels of 0.1–1 μg per litre of water were thus obtained. The sample was then stirred and analysed as described. The beaker was rinsed with acetone (10 ml) to dissolve compounds that might be partially adsorbed on the glass walls. The acetone extract was then gently concentrated to 1 ml. After acetylation, the extract was injected into the gas chromatograph.

TABLE I

COMPARATIVE STUDY OF SOME ACETYLATION PROCEDURES

The yields are reported relative to those obtained by the procedure described here. The results are expressed as mean values of triplicate determinations.

Compound	*Relative yield*		
	0.1 M potassium carbonate buffer[18]	*0.1 M borax buffer*[16]	*1.25 M sodium hydroxide buffer*[33]
2,4-Dichlorophenol	0.98	1.0	0.94
2,4,6-Trichlorophenol	0.98	0.97	0.98
2,3,4,6-Tetrachlorophenol	0.97	0.76	1.1
Pentachlorophenol	1.0	0.58	1.1
4,5,6-Trichloroguaiacol	1.0	0.89	0.89
Tetrachloroguaiacol	0.97	0.67	0.84
3,4,5-Trichlorocatechol	0.95	0.13	0.12
Tetrachlorocatechol	0.80	0.10	0.20

RESULTS AND DISCUSSION

Trace enrichment and recoveries

In trace enrichment techniques, based on column chromatography, a maxi-

mum sample volume is mainly determined by the retention volume for each individual component. No attempt was made, however, to determine the elution volumes of the substances studied. For practical reasons a 100-fold concentration of the water sample was assumed to be sufficient for most purposes, and recovery experiments were therefore carried out using 100-ml water samples. In a series of five such experiments, the recoveries for all substances indicated in Table I exceed 90%. No adsorption on the glass wall occurred and no breakthrough on the C_{18} cartridge was noticed. These observations indicated that the chlorophenols were quantitatively trapped on the cartridge. Fig. 1 illustrates the device used for the enrichment technique. The enrichment can be carried out rapidly at the sampling location and the cartridge sent to the laboratory for further processing.

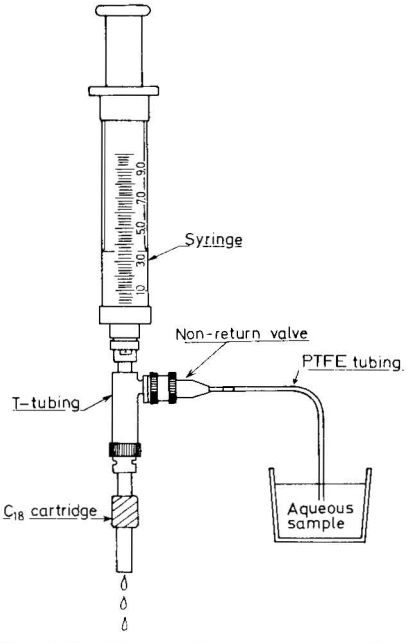

Fig. 1. Enrichment of an aqueous sample on a C_{18} cartridge by means of a syringe.

In the desorption step, acetone was chosen as elution solvent as no interference from acetone was noticed in the subsequent acetylation step. To minimize the volume of the elution solvent required, the cartridge was eluted in the reverse direction. All substances were desorbed quantitatively with 4 ml of acetone.

Additional recovery experiments with tri- and tetrachloroguaiacol and tetra- and pentachlorophenol, using 1000 ml of water, resulted in recoveries of 85% and higher. The strong retention of these compounds probably corresponds to their relatively strong hydrophobicities in comparison with chlorocatechols.

In order to prevent auto-oxidation of the chlorocatechols into the corresponding chloro-*o*-benzoquinones, which may be caused by dissolved oxygen, a small amount of ascorbic acid (which serves as a weak reducing agent) was added to the water samples. Without this treatment, the recoveries of the chlorocatechols varied irregularly. Similar observations with tetrachlorinated hydroquinone and catechols, present

as metabolites in urine samples, were reported by Edgerton *et al.*[32], who employed sodium disulphite as the reducing agent.

If other factors, *e.g.*, sample matrix, are suspected to influence the recovery, it is recommended that two cartridges be coupled in series during the adsorption step, and then eluted separately. If no breakthrough occurs into the second column the adsorption can be assumed to be quantitative.

Acetylation

The most popular derivatization procedures employed for the gas chromatographic determination of chlorophenols are alkylation with diazoalkanes[3,11–15,22] and acetylation with acetic anhydride[9,16,18,26,31,33]. The latter procedure was preferred for this study as acetylation was found to be rapid and non-contaminating with no hazardous reagents involved. Although not always recognized, acetylation of phenols in alkaline aqueous solutions was first described by Chattaway[34]. This procedure has been applied to residue analyses of chlorinated phenols[16,26,31,33], chloroguaiacols[9,31] and chlorocatechols[31]. When evaluating the different acetylation procedures described in the literature, we found that the yield varied considerably. The results are summarized in Table I. The procedure used by Voss *et al.*[31] seemed to be the most satisfactory.

The choice of aqueous phase, together with the anhydride to buffer molar ratio, was found to be most critical. This is probably due to differences in reaction rates between the acylation of the phenolate ion and the hydrolysis of the anhydride, *e.g.*, if the pH is too high in the aqueous phase the acetic anhydride will be destroyed before the acetylation process is completed.

On the other hand, a lower pH (at pH $>$ pK_a) corresponds to lower concentrations of the reactive phenolate ions and thus the reaction rate is considerably reduced, leading to much lower yields. The decrease in pH, as a function of time, of an aqueous potassium carbonate solution mixed with acetic anhydride is shown in Fig. 2. As the pH changes from about 10 to 7 within a few seconds, the acetylation reaction must be completed within that time. It is therefore important to have the

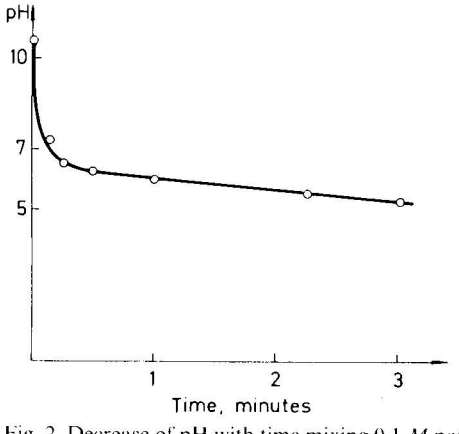

Fig. 2. Decrease of pH with time mixing 0.1 *M* potassium carbonate solution (15 ml) and acetic anhydride (125 µl).

TABLE II

THE RELATIVE MEAN VALUES OF THE ELECTRON-CAPTURE DETECTOR RESPONSES, RETENTION TIMES AND STANDARD DEVIATIONS OF SIX REPLICATE DETERMINATIONS OF CHLOROPHENOLIC COMPOUNDS IN SPIKED AQUEOUS SOLUTIONS

Acetylated compound	*Relative response factor*	*Relative retention time*	*Relative standard deviation (%)*
2,4-Dichlorophenol	10.1	0.79	8
2,4,6-Trichlorophenol	2.67	0.91	5
2,6-Dibromophenol (internal standard)	1.00	1.00	–
4,5-Dichloroguaiacol	13.5	1.16	7
2,3,4,6-Tetrachlorophenol	1.47	1.29	5
3,4,5-Trichloroguaiacol	1.27	1.52	3
4,5,6-Trichloroguaiacol	1.35	1.60	4
3,4,5-Trichlorocatechol	1.52	1.90	7
Pentachlorophenol	0.74	1.97	7
Tetrachloroguaiacol	0.83	2.08	2
Tetrachlorocatechol	1.33	2.60	10

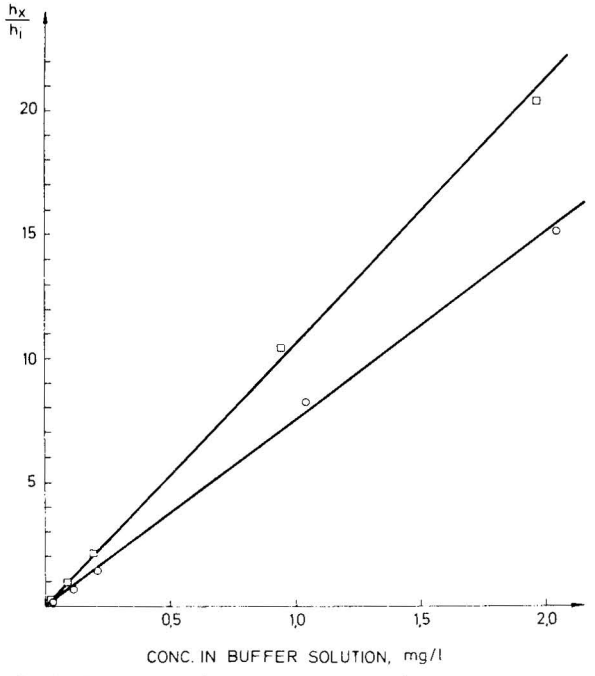

Fig. 3. Formation of acetyl derivatives of 3,4,5-trichlorocatechol (○) and tetrachlorocatechol (□) at various concentrations in waters. h_x and h_i are the peak heights of the chlorocatechols and the internal standard, respectively.

optimal anhydride to buffer molar ratio, particularly when derivatization of polyhydroxy groups in the benzene ring is carried out.

Of all the substances studied, the acetylation of chlorocatechols seems to be the most critical. Commonly used catalysts for acylation reactions, *e.g.*, pyridine, were found to reduce the yield considerably. In Table I, results for the evaluation of different acetylation methods are summarized.

Gas chromatography

To decrease the analytical time required and increase the resolution, ordinary glass capillary columns were chosen for the gas chromatographic separation. The precision of the gas chromatographic analysis was very low, however, probably owing to partial and irregular degradation of the relatively alkali-labile ester derivatives on the surface of the glass column. On replacing the glass capillary columns with quartz capillary columns the precision increased considerably. The relative mean detector response, retention time and standard deviation from six replicate determinations of chlorophenolic compounds in aqueous solutions are given in Table II.

The linearity of the response for each compound was also investigated. As an example, the relationship between the concentration and the peak-height ratio for the chlorocatechols and internal standard (0.1 mg/l) are shown in Fig. 3. Similar results were obtained for the other chlorophenolic compounds.

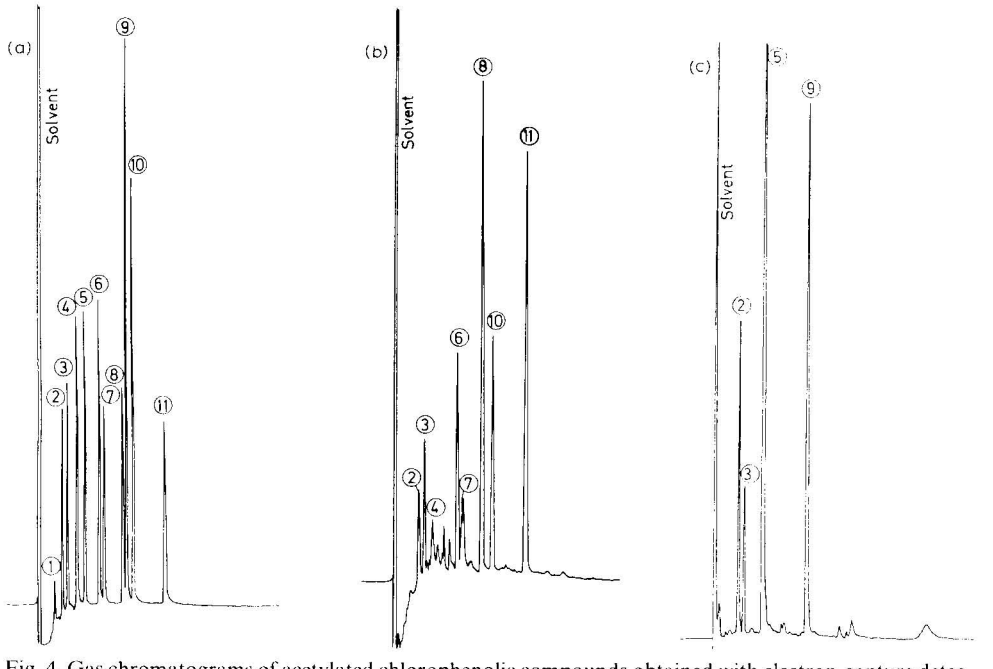

Fig. 4. Gas chromatograms of acetylated chlorophenolic compounds obtained with electron-capture detection for different samples. (a) Standard mixture: 1 = 2,4-dichlorophenol (0.10 μg/ml); 2 = 2,4,6-trichlorophenol (0.12 μg/ml); 3 = 2,6-dibromophenol (internal standard) (0.054 μg/ml); 4 = 4,5-dichloroguaiacol (1.1 μg/ml); 5 = 2,4,5,6-tetrachlorophenol (0.11 μg/ml); 6 = 3,4,5-trichloroguaiacol (0.12 μg/ml); 7 = 4,5,6-trichloroguaiacol (0.084 μg/ml); 8 = 3,4,5-trichlorocatechol (0.11 μg/ml); 9 = pentachlorophenol (0.14 μg/ml); 10 = tetrachloroguaiacol (0.13 μg/ml); 11 = tetrachlorocatechol (0.10 μg/ml). (b) Effluent at the outflow of an aerated lagoon at a kraft pulp bleach plant. The levels are 8.0, 31, 6.1, 1.1, 18, 4.0 and 14 μg per litre of water, corresponding to peaks 2, 4, 6, 7, 8, 10 and 11 in (a). (c) Lake water, downstream of a saw mill, taken 1 week after an accidental discharge of a chlorophenolic wood-protecting formulation. The levels are 0.5, 1.8 and 0.3 μg per litre of water, corresponding to peaks 2, 5 and 9 in (a).

Applications

The method described has been applied to several types of water samples, including industrial waste waters and natural waters. The chromatogram in Fig. 4b shows the presence of chlorinated guaiacols and catechols in the effluent of an aerated lagoon at a Swedish kraft pulp bleachery. The chromatogram in Fig. 4c originates from a lake-water sample taken downstream of a saw mill 1 week after an accidental discharge of a wood protection formulation containing tri-, tetra- and pentachlorophenol.

ACKNOWLEDGEMENTS

The authors are indebted to Carin Wahlberg and Lillemor Johansson for their skillful assistance.

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Journal of Chromatography, 214 (1981) 335–341
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,026

DETERMINATION OF CHLOROPHACINONE IN FORMULATIONS BY REVERSED-PHASE ION-PAIR CHROMATOGRAPHY

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(First received January 27th, 1981; revised manuscript received May 25th, 1981)

SUMMARY

The retention behaviour of chlorophacinone [2-(*p*-chlorophenyl)phenylacetyl-1,3-indandione] has been studied using an octadecylsilica column and different eluents, *e.g.*, methanol–water, methanol–aqueous phosphoric acid, methanol–aqueous McIlvaine buffer–tetramethylammonium bromide and tetrahydrofuran–aqueous McIlvaine buffer–tetrabutylammonium bromide. The dependence of log k' on the organic modifier content and the apparent pH of the eluents was determined. The retention behaviour could be interpreted by assuming that chlorophacinone existed mostly as a weakly acidic enol that could dissociate depending on the pH of the eluent. Chlorophacinone could be separated and determined quantitatively in 0.25–25% (w/w) oily formulations and in 10–100 ppm crushed grain bait using diphacinone [2-(diphenylacetyl)-1,3-indandione] as an internal standard.

INTRODUCTION

Chlorophacinone [2-(*p*-chlorophenyl)-phenylacetyl-1,3-indandione] is a powerful anticoagulant rodenticide and most formulations contain up to 5% (w/w) of active ingredient. A Hungarian patent[1] described the preparation of concentrates containing up to 25% (w/w) of active ingredient by adding long-chain trialkylamines in vaseline oil to chlorophacinone. On the other hand, certain formulations, especially crushed corn baits, contain chlorophacinone in concentrations as low as 75 ppm. Therefore, a flexible analytical method is required for the determination of chlorophacinone in a number of sample matrices at widely varying concentration levels.

Existing photometric[2] and electroanalytical[3,4] methods are not sufficiently selective and sensitive. Known thin-layer chromatographic methods[5–7] were not designed for low-level formulations. Two gas chromatographic (GC) methods have been published[8–10], based on the oxidation by chromic acid in acidic medium of chlorophacinone to chlorobenzophenone, separation and detection with an

0021-9673/81/0000–0000/$02.50

electron-capture detector[8,9], and the GC separation of brominated chlorophacinone[10]. Both are tedious and require chemical modification of chlorophacinone. Grant and Pike[11] published a reversed-phase (RP) high-performance liquid chromatographic (HPLC) method using a pellicular C_{18} packing and methanol–water containing 0.75% of ammonia as the eluent. The separation between chlorophacinone and matrix peaks arising from the corn-based rodenticide bait was not complete, but they excluded the possibility of using microparticulate octadecylsilica on account of its instability in the alkaline eluent used.

It was felt that with a more judicious choice of the composition of the mobile phase microparticulate reversed-phase packings could perform better in the analysis of various rodenticide formulations with widely varying concentration levels.

EXPERIMENTAL

Chemicals

Chlorophacinone, diphacinone, Redentin samples, methanol, tetrahydrofuran and acetone (all analytical-reagent grade) were obtained from Reanal (Budapest, Hungary). Tetramethylammonium chloride and tetrabutylammonium bromide were purchased from BDH (Poole, Great Britain) and Fluka (Buchs, Switzerland), respectively.

Apparatus and procedure

A Varian LC 5020 liquid chromatograph (Varian Aerograph, Walnut Creek, CA, U.S.A.) equipped with a 10-μl loop injector was used, connected to a Type LC-55 variable-wavelength detector (Perkin-Elmer, Norwalk, CT, U.S.A.) set at 285 nm and a Model 9176 dual-channel recorder (Varian). Separations were carried out on a 250 × 4.0 mm I.D. stainless-steel column (Labor MIM, Budapest, Hungary) packed[12] with 10-μm RP-18 material (Merck, Darmstadt, G.F.R.). The column was jacketed[13] and thermostated at 30.0°C by a Type U10 circulating water bath (MLW, Medingen, G.D.R.).

Redentin samples were dissolved in a mixture of tetrahydrofuran (THF) and eluent. Crushed corn bait samples were extracted for 3 h with 100 ml of acetone in a Soxhlet apparatus, the acetone was evaporated and the residue was dissolved in THF and made up to volume with eluent.

A combined glass electrode, calibrated with aqueous buffers (pH 4 and 7), and a Type OP-204/1 pH meter (Radelkisz, Budapest, Hungary) was used to measure the pH of the buffer solutions and the eluents. The latter uncorrected (apparent) values as measured in the hydro-organic medium are reported here.

RESULTS AND DISCUSSION

Aqueous methanol eluents

Chlorophacinone was strongly retained in pure methanol ($k' = 10$) and produced a poor peak shape. It is known that chlorophacinone in its enolic form behaves like a weak acid, so this large retention and unfavourable peak shape can be attributed to strong interactions between chlorophacinone and the residual hydroxyl groups of the packing. Addition of 1% concentrated (85%) phosphoric acid to estab-

lish an apparent pH of 2.2 decreased the retention dramatically and improved the peak shape. By decreasing the methanol concentration of the eluent, the capacity factor (k') of chlorophacinone increased, as shown in Fig. 1.

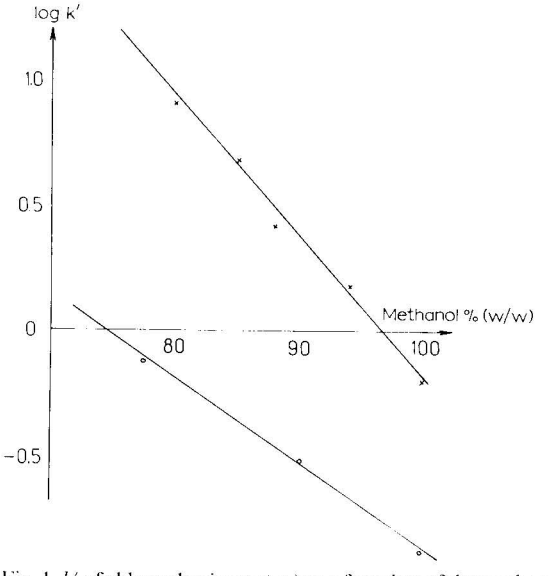


Fig. 1. k' of chlorophacinone (×) as a function of the methanol concentration in the eluent. Column: RP-18 (250 × 4.0 mm I.D.), thermostated at 30.0°C. Eluent: pH 2.2 (apparent) aqueous methanol. ○, Nitrohexane.

For comparison purposes the behaviour of a simple compound, nitrohexane, is also shown. As the width of the chlorophacinone peak was still wider than that of a simple compound of comparable retention, the measured pH of the eluent was adjusted to about 6.3 by mixing varying amounts of methanol and aqueous McIlvaine buffer (0.2 *M* disodium hydrogen orthophosphate and 0.1 *M* citric acid). The k' values obtained are given in Table I. Compared with the values obtained in eluents of low pH, the k' values are significantly lower and the peak shape was also improved.

TABLE I

k' VALUES OF CHLOROPHACINONE AS A FUNCTION OF THE METHANOL CONCENTRATION IN THE ELUENT

Column: RP-18 (250 × 4.0 mm I.D.), thermostated at 30.0°C. Eluent: pH 6.3 (apparent) methanol–McIlvaine buffer–water.

Methanol concentration (%, w/v)	*Retention volume (ml)*	k'
67	2.6	0.24
62.2	3.22	0.53
53.8	5.08	1.42
53.8*	5.80	1.76

* Plus tetramethylammonium chloride (0.05 *M*).

This behaviour can be rationalized by assuming that chlorophacinone exists mostly in the enolic form. This weakly acidic enolic form can dissociate at high pH, resulting in an ionic species that is only slightly retained by the column, while at low pH the enol form is believed to stabilize by intermolecular hydrogen bonding, forming a chelate ring that is better retained by the RP-18 column. To substantiate this assumption, tetramethylammonium chloride (TMA) was added to the previous 53.8% (w/v) methanol–water eluent. As shown in Table I, the addition of TMA increased the k' of chlorophacinone, *i.e.*, ion-pair formation could be postulated.

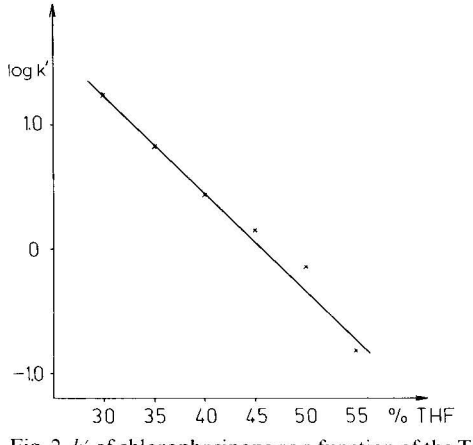
Although the retention of chlorophacinone could be easily controlled in this phase system, the eluent failed to dissolve the Redentin samples composed from vaseline oil, tridecylamine and chlorophacinone. Redentin samples could not be dissolved in aqueous acetonitrile either, only in THF. Therefore, the retention of chlorophacinone in aqueous THF eluents was investigated.

Aqueous tetrahydrofuran eluents

At first the k' of chlorophacinone in THF–McIlvaine buffer (1:1) (pH 6.3) as eluent was investigated without and with tetrabutylammonium bromide (TBA) as ion-pair reagent. Chlorophacinone eluted very close to the dead volume in the absence of TBA, whereas k' increased to 0.72 at a TBA concentration of 0.05 *M*, a behaviour similar to that observed with aqueous methanol eluents.

The k' values of chlorophacinone as a function of the THF concentration using 0.05 *M* TBA eluent of pH 6.3 are shown in Fig. 2. The retention can be easily controlled by changing the organic modifier content of the eluent.

The pH dependence of the k' of chlorophacinone in a 35.09% (w/v) THF eluent containing 0.05 *M* TBA is shown in Fig. 3. The shape of the curve is similar to



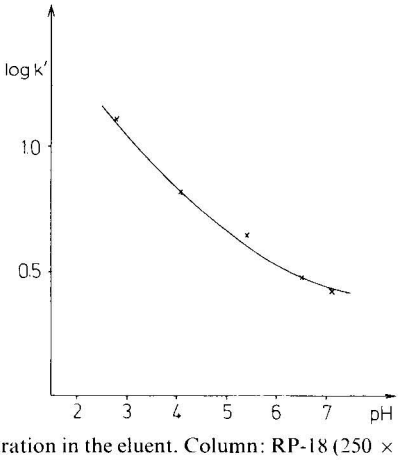


Fig. 2. k' of chlorophacinone as a function of the THF concentration in the eluent. Column: RP-18 (250 × 4.0 mm I.D.), thermostated at 30.0°C. Eluent: pH 6.3 (apparent) THF–McIlvaine buffer–water containing 0.05 *M* tetrabutylammonium bromide.

Fig. 3. k' of chlorophacinone as a function of the pH of the eluent. Column: RP-18 (250 × 4.0 mm I.D.), thermostated at 30.0°C. Eluent: 35.09% (w/v) THF–McIlvaine buffer–water containing 0.05 *M* tetrabutylammonium bromide.

that predicted by Terweij-Groen and Kraak[4] for weak acids. In the intermediate pH range (3.5–5) the peak of chlorophacinone became very wide and at some point a separate shoulder appeared.

As the peak shape was best at pH > 6, pH 6.5 was selected for the analysis of the Redentin samples.

It is believed that chlorophacinone forms an adduct with long-chain trialkylamines[1]. However, when the amine-chlorophacinone adduct and pure chlorophacinone were injected successively in the eluent of pH 6.5 they had identical k' values, indicating that the adduct decomposed in this eluent. This is an additional practical advantage of the eluent system developed, as the same eluent can be used for the analysis of both the free chlorophacinone and adduct-containing formulations.

Quantitative analysis

Oily Redentin formulations in the range 0.25–25% (w/w) could be readily analysed using a peak-height calibration graph measured at 285 nm. The equation for the calibration graph was

$$h = 1.103 + 6.794\ c\ (r^2 = 0.99926)$$

where h is the peak height (mm) and c is the concentration of chlorophacinone (μg/ml).

The oily samples were dissolved in 25 ml of THF, and the sample weight was varied in the range 0.05–2 g, depending on the concentration level to be determined. Portions of 200 μl of these solutions were added to 1800 μl of a 1:1 mixture of THF and eluent. Erroneous results were obtained when the injected samples were dissolved in pure THF only. The presence of oil caused no apparent problems in the quantitation procedure. The proposed sample amounts and the analytical results (the averages for three independent solutions, each injected twice) are given in Table II.

TABLE II

RESULTS FOR ANALYSIS OF REDENTIN FORMULATIONS

Nominal sample concentration (%, w/w)	*Amount added to 25 ml of THF (g)*	*Chlorophacinon concentration found (%, w/w)*
0.25	1.5–2.0	0.25 ± 0.03
1.0	0.4–0.5	1.01 ± 0.01
25	0.08–0.1	23.5 ± 0.2

Crushed corn bait Redentin contains 50–100 ppm of chlorophacinone. A 10-g amount of crushed untreated corn was extracted with 100 ml of acetone as described under Experimental. This solution was used as a blank to determine the presence or absence of interfering peaks and components originating from the sample matrix. No peak of significant intensity eluted at the retention volume of chlorophacinone and diphacinone. The latter was added to the crushed corn sample as an internal standard prior to extraction.

The equation of the calibration graph was

$$\frac{h_{CF}}{h_{DF}} = 0.002 + 0.6641 \cdot \frac{c_{CF}}{c_{DF}} \qquad (r^2 = 0.999783)$$

where h_{CF} and h_{DF} are the peak heights of chlorophacinone and diphacinone, respectively, and c_{CF} and c_{DF} are their concentrations.

The recovery for 0.5 μg of chlorophacinone per gram of crushed corn was 105 ± 10% (average of three separate extractions). The chromatograms of untreated crushed corn and a Redentin corn bait are shown in Figs. 4 and 5.

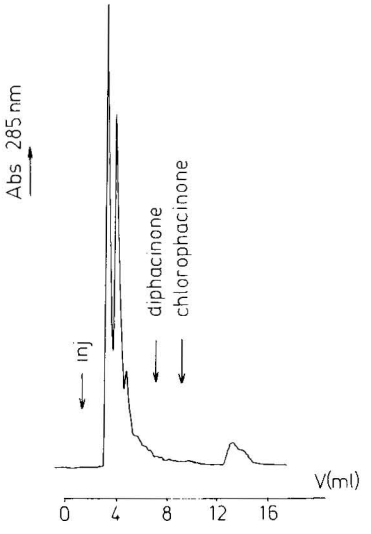


Fig. 4. Chromatogram of untreated crushed corn extract.

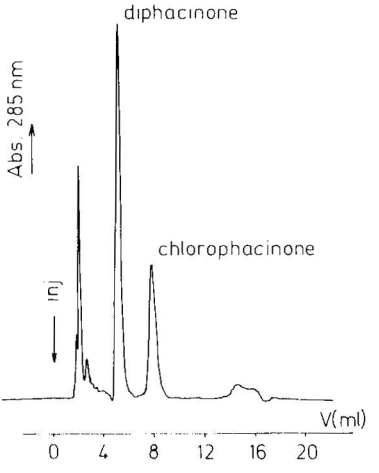


Fig. 5. Chromatogram of crushed Redentin 75 bait extract.

The same column could be used for over 6 months provided that the buffer-containing eluent was replaced with an aqueous THF eluent of identical THF concentration when the column was not being used. As another preventive measure, the column was washed free of contaminants with pure THF each week.

CONCLUSIONS

The retention of chlorophacinone as a function of the methanol and tetrahydrofuran content and pH of the eluent and the absence or presence of tetraalkylammonium cation ion-pair reagent was investigated. The observed behaviour could be readily interpreted by assuming that chlorophacinone existed mostly in a weakly acidic enolic form which, depending on the pH of the eluent, could dissociate and form ion pairs.

The tetrahydrofuran-aqueous McIlvaine buffer (pH 6.5) eluent system developed allows the quantitative determination of the concentration of the active ingredient in products prepared with free chlorophacinone, chlorophacinone–trialkylamine adducts and crushed corn bait.

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Journal of Chromatography, 214 (1981) 343–348
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,009

SEPARATION OF URUSHIOL BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ON AN 8% OCTADECYLSILANE CHEMICALLY BONDED SILICA GEL COLUMN WITH ELECTROCHEMICAL DETECTION

ANALYSIS OF URUSHIOL IN THE SAP OF LAC TREES (*RHUS VERNICIFERA*) AND THAT IN THE JAPANESE LAC-MAKING PROCESS

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(First received April 22nd, 1981; revised manuscript received May 18th, 1981)

SUMMARY

Urushiol, a major component of a sap of lac trees (*Rhus vernicifera*), is resolved into four components due to differences in the degree of unsaturation by high-performance liquid chromatography on Hitachi 3053 gel (an 8% octadecylsilane chemically bonded silica gel), using 1% lithium perchlorate in acetonitrile–water (7:3) as eluent and electrochemical detection. This method was applied to the analysis of the urushiol components of two samples of sap and in the lac-making process.

INTRODUCTION

It was established by Majima[1] and Sunthanker and Dawson[2] that Japanese lac urushiol is made up of four components with a 3-pentadecanylcatechol skelton with different numbers of double bonds (0–3) in the side-chain.

Urushiol is converted into either the dimethyl ether or the diacetate, which have been resolved into components with differences in the olefinic structures of the side-chains by liquid chromatography[2] and high-performance liquid chromatography (HPLC)[3]. Recent progress in developing gels for HPLC is likely to make it possible to resolve urushiol by HPLC without chemical modification. The first resolution of urushiol into its components by HPLC was performed on a μBondapak C_{18} column with UV detection[4].

While studying the resolution of urushiol by HPLC in our laboratory, the resolution was found to be dependent on the level of octadecylsilane (ODS) chemically bonded to silica gels. Urushiol can be resolved into its components on Hitachi 3053 gel (an 8% ODS chemically bonded silica gel) column using a acetonitrile–water (7:3) as the mobile phase, which can be monitered quantitatively using electrochemical detection based on voltammetry in the presence of 1% of lithium perchlorate as the supporting electrolyte in the mobile phase.

In this work, HPLC for urushiol with electrochemical detection was establish-

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ed and applied to the analysis of components of urushiol in the sap of lac trees (*Rhus vernicifera*) and in the lac-making process.

EXPERIMENTAL

Reagents

Tetrahydrofuran, chloroform and acetonitrile were commercial products of extra-pure grade.

Materials

Two samples of sap from lac trees (Chinese and Japanese) were supplied by Saito (Tokyo, Japan).

Crude urushiol, obtained as the acetone-soluble part of the sap, was dissolved in excess of *n*-hexane. The solution was filtered with a Fluoropore filter (pore size 0.1 μm, type FP-100; Sumitomo Electric Ind., Tokyo, Japan) and *n*-hexane was removed from the filtrate to give a dark-brown oily material as the residue. A solution (3 ml) of the oil (1 g) in chloroform (4 ml) was submitted to gel permeation chromatography (GPC) in 20-μl portions and urushiol monomer was separated from the oligomer and obtained by evaporating the fractions to dryness under vacuum.

In the process for making lac from the sap, the sap is stirred in a open vessel at room temperature for 30 min and then at temperatures below 45°C for 6–8 h. Samples were taken from the sap during this process after 0, 30, 70, 90, 120 and 150 min, the oily material was obtained from them as in the separation of crude urushiol monomer, and was subsequently resolved into components by HPLC.

Hydrourushiol as a standard was obtained by hydrogenation of urushiol followed by recrystallization from ethanol according to the method in the literature[5] (m.p. 57.5–58.5°C; lit.[5], 58–59°C).

Mass spectrometry

Mass spectra were obtained with an RMS-4 mass spectrometer (Hitachi, Tokyo, Japan).

GPC

The preparative system consisted of a Type KSD-P-45 pump (Kyowa Seimitsu, Tokyo, Japan), two 60 × 2.2 cm I.D. stainless-steel columns packed with TSK G2000HG (Toyo Soda, Tokyo, Japan), and a Type RI-2 refractive index (RI) detector (JAI, Tokyo, Japan). The mobile phase was chloroform.

The analytical system consisted of a Type 2396-57 pump (Milton Roy, Philadelphia, PA, U.S.A.), three 60 × 0.76 cm I.D. stainless-steel columns (packed with TSK G4000H6, G2000H8 and G10000H8 gel) and an RI detector as used in the preparative system. The mobile phase was tetrahydrofuran.

HPLC

The system consisted of a Type 2396-57 pump (Milton Roy), a 350 kg/cm^2 pressure gauge (Umetani Seiki, Osaka, Japan), a Type DAM bellows-type damper (Umetani Seiki), a Rheodyne Model 7120 20-μl syringe-loading sample injector, a Type VMD-101 electrochemical detector (Yanagimoto, Tokyo, Japan) and a 15 ×

0.4 cm I.D. stainless-steel column packed with Hitachi 3053 gel (Hitachi, Tokyo, Japan). Peak areas were calculated with a Shimazu Chromatopack E1A integrator (Shimazu Seisakusho, Kyoto, Japan). Acetonitrile–water (7:3) containing 1 % of lithium perchlorate (extra-pure grade) mixture was used as the mobile phase.

RESULTS AND DISCUSSION

A current–potential curve is shown in Fig. 1 for hydrourushiol as a standard ($15 \cdot 10^{-4}$ *M*) using 1 % lithium perchlorate in acetonitrile–water (7:3) as eluent and a rotating disk electrode (Nikko Keisoku, Yokohama, Japan).

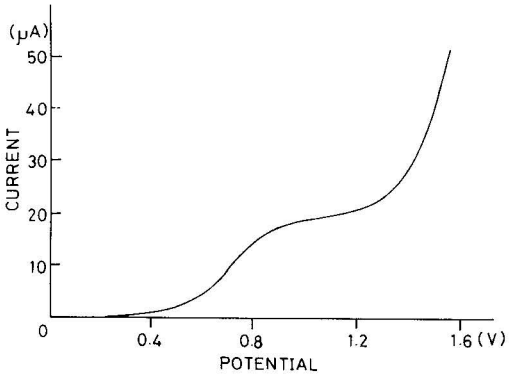

Fig. 1. Current–potential curve for hydrourushiol. Concentration, $1.52 \cdot 10^{-4}$ *M*; solvent, 1 % lithium perchlorate in acetonitrile–water (7:3); apparatus, rotating disk electrode, platinum electrode (500 rpm) and scanning speed 60 sec/V.

Based on this curve, electrochemical detection was performed at 400 or 800 mV for the resolved urushiol components, because the oxidation potential of these components is correlated with the oxidation of their catechol nucleus, and is the same regardless of the double bonds located in the side-chain far from the catechol nucleus.

As the positions and intensities of the UV absorption bands of urushiol components differ owing to the different structures of the side-chains, it is clear that a UV detector at a certain wavelength cannot detect the peaks of urushiol components quantitatively.

In Fig. 2, chromatograms obtained by HPLC with the Hitachi 3053 column are compared with those with electrochemical (at 800 mV) and UV (at 218 nm) detection for a sample of urushiol (0.72 μg, loaded in 2 μl of acetonitrile solution) using 1 % lithium perchlorate in acetonitrile–water (7:3) as eluent, indicating that electrochemical detection is much more sensitive than UV detection (the UV wavelength was selected from λ_{max} = 218 nm in *n*-hexane for hydrourushiol diacetate[3]).

Prior to assignment of the peaks, complete agreement of the peaks detected with the UV and electrochemical detectors was confirmed; peak a, both of the two separated peaks of b, peak c and peak d, detected with the UV detector (at 254 nm), were fractionated and 8 μl of each fraction were re-chromatographed on the same column using the electrochemical detector at 800 mV (see Fig. 4), using 1 % lithium perchlorate in acetonitrile–water (7:3) as eluent. It is clear that peaks a, b, c and d

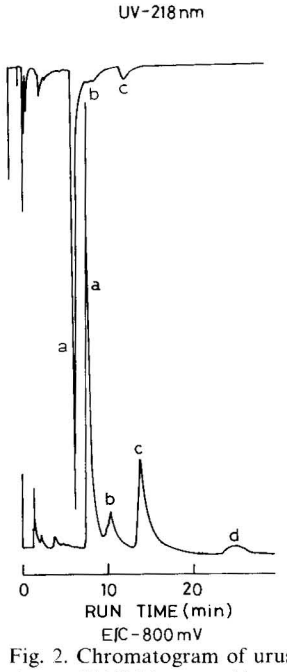

Fig. 2. Chromatogram of urushiol. HPLC conditions: column, Hitachi 3053 (5 μm), 15 × 0.4 cm I.D.; eluent, 1% lithium perchlorate in acetonitrile–water (7:3); flow-rate, 1.0 ml/min; electrochemical (EIC) detector, applied at 800 mV *vs.* Ag/AgCl, sensitivity 10 nA, UV 218 nm, 0.16 a.u.f.s.; loading, 0.72 μg.

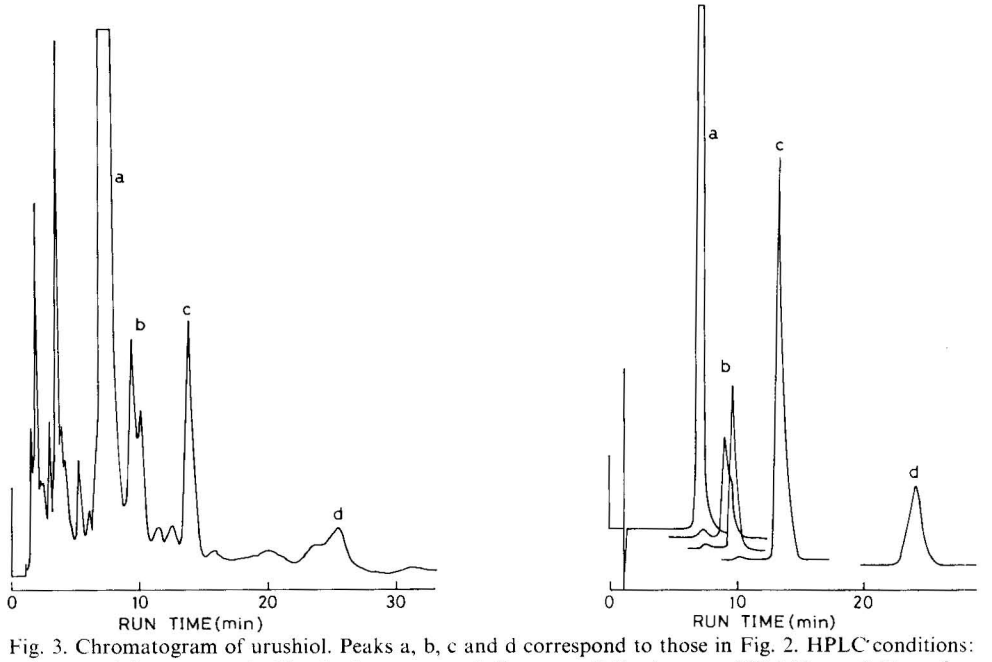

Fig. 3. Chromatogram of urushiol. Peaks a, b, c and d correspond to those in Fig. 2. HPLC conditions: column and flow-rate as in Fig. 2; eluent, acetonitrile–water (7:3); detector, UV 254 nm, 2.56 a.u.f.s.; loading, 3.0 mg.

Fig. 4. Results of re-chromatography with an electrochemical detector of fractionated peaks in Fig. 3. HPLC conditions: column, eluent and flow-rate as in Fig. 2; electrochemical detector, applied at 800 mV *vs.* Ag/AgCl, sensitivity 1 nA; loading, 8 μl of each fraction.

correspond well in the chromatograms obtained with the two types of detector (see Figs. 3 and 4).

In order to identify the peaks, peaks a, b, c and d were fractionated using acetonitrile–water (7:3) as eluent and using the UV detector (at 254 nm) (see Fig. 3), as urushiol as a substrate is oxidized in electrochemical detection. A 20-μl volume of an acetonitrile solution containing 3 mg of urushiol monomer was loaded on the column. By removal of the used eluent from the resolved peaks, fractionated materials were obtained. Mass spectrometry gave the following m/e values for the peaks: a, 314; b, 316; c, 318; and d, 320. These correspond to triolefinic, diolefinic, monoolefinic and saturated side-chain urushiol, respectively.

Calibration graphs for determining each of the resolved urushiol components using an electrochemical detector with measurement at 400 and 800 mV, are shown in Figs. 5 and 6, respectively. The data were obtained by injecting 20 μl of an acetonitrile solution containing a given amount of hydrourushiol ($0.125 \cdot 10^{-3}$–$3.00 \cdot 10^{-3}$ mg) on to the analytical HPLC column. The coefficient of variation of the peak area in HPLC was 1.29% at 400 mV and 0.79% at 800 mV; in each instance 20 μl of a solution of hydrourushiol (0.125 g) in 10 ml of acetonitrile was loaded using the present HPLC system.

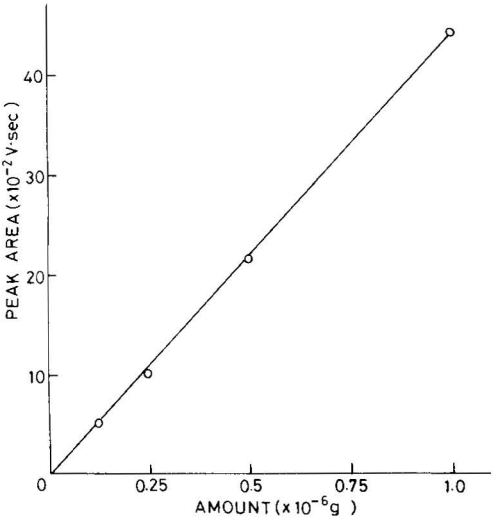

Fig. 5. Calibration graph for hydrourushiol in HPLC; correlation between peak area and amount of hydrourushiol as solute. HPLC conditions: column, eluent and flow-rate as in Fig. 2; electrochemical detector, applied at 400 mV *vs.* Ag/AgCl, sensitivity 10 nA.

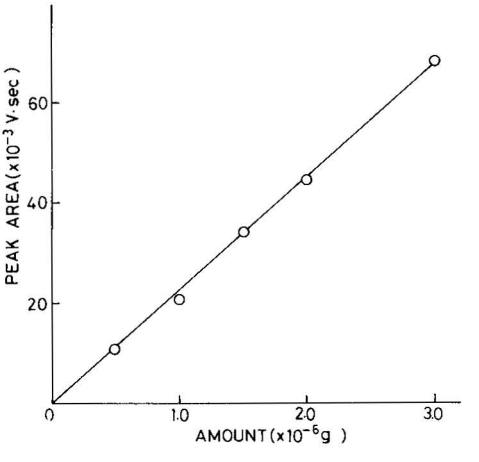

Fig. 6. Calibration graph for hydrourushiol in HPLC; correlation between peak area and amount of hydrourushiol as solute. HPLC conditions: column, eluent and flow-rate as in Fig. 2; electrochemical detector, applied at 800 mV *vs.* Ag/AgCl, sensitivity 10 nA.

Table I shows the results of analyses with electrochemical detection of urushiol obtained from two samples of the sap (Chinese and Japanese) (the constituents of urushiol do not correspond to those in the original sap of lac trees, because 20% of oligomeric urushiol was already involved in the sap used.)

From Fig. 7, it is clear that components of urushiol are oxidized in proportion

TABLE I

QUANTITATIVE ANALYSIS OF URUSHIOL COMPONENTS BY HPLC

HPLC conditions: column, eluent and flow-rate, as in Fig. 2; electrochemical detector, applied at 800 mV, sensitivity, 10 nA; loading, 1.0 μg.

Origin of sap	*Proportion (%)*			
	Triolefinic	*Diolefinic*	*Monoolefinic*	*Saturated*
Japanese	52.3	10.3	30.0	7.7
Chinese	66.0	5.1	21.5	6.7

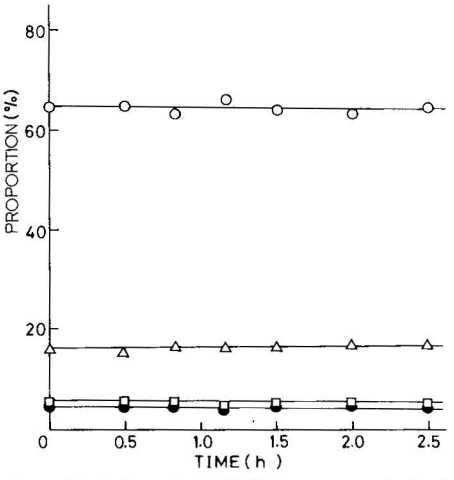

Fig. 7. Variation of urushiol components in the lac-making process, determined by HPLC: (○) triolefinic, (□) diolefinic, (△) monoolefinic and (●) saturated urushiol components, HPLC conditions as in Fig. 5; loading, 0.5 μg.

to the urushiol components in the sap used in the lac-making process. This result confirmed the previous report[6] that in the early stage of the lac-making process polymerization starts from the enzymic oxidation of urushiol into the corresponding quinone, followed by a coupling reaction with urushiol.

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Journal of Chromatography, 214 (1981) 349–359
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,003

THIN-LAYER CHROMATOGRAPHIC ASSAY OF PHOTOACTIVE COMPOUNDS (FUROCOUMARINS) USING THE FUNGUS *PENICILLIUM EXPANSUM* AS A TEST ORGANISM

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(Received May 19th, 1981)

SUMMARY

A simple method using silica gel thin layers for the detection and determination of phototoxic compounds has been developed, using the fungus *Penicillium expansum* as a test organism. The method is suitable for the determination of phototoxic components in mixtures or in impure compounds, as the chromatographic separation and the growth-inhibition test based on photoactivity are carried out on the same thin-layer plate. The highly photoactive linear furocoumarins psoralen, bergapten, xanthotoxin and trioxalen can be detected even more sensitively on the thin-layer plate by means of this photoactivity bioassay than by examination under UV light at 366 nm. The occurrence of xanthotoxin and bergapten as minor constituents in the extract of *Ammi visnaga* could be detected using this method. Sphondin was weakly photoactive. We could not find any photoactivity for the linear furocoumarins imperatorin and isopimpinellin, which have been described by other authors to be slightly photoactive. Samples of these compounds, however, were found to be contaminated with minor amounts of the highly photoactive furocoumarins xanthotoxin and bergapten, respectively.

INTRODUCTION

Some fungi[1,2], green algae[3] and bacteria[4,5] have been shown to be excellent test organisms for assaying the phototoxic properties of furocoumarins and related compounds. The method applied for the detection of photoactivity in all of these studies is similar to that used for testing the antimicrobial properties of compounds. In the photoactivity test, however, the sample to be tested is irradiated with UV light at 366 nm in the presence of the test organism on agar dishes. A limitation of the method on

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agar dishes is that with mixtures (or impure compounds) it is not possible to distinguish between active and inactive components.

In this respect, improvement is achieved by combining a chromatographic procedure with detection of the antimicrobial properties of the separated components of the sample under investigation. Several studies using the same thin-layer plates for a chromatographic separation and the subsequent antimicrobial test have been described[6–9]. In particular, sporulating fungi have been shown to be excellent test organisms for this purpose[6].

In a previous study we used the innoxious fungus *Penicillium expansum* for the visualization of the fungitoxicity of polyene antibiotics and saponins[10] on silica gel thin-layer chromatographic (TLC) plates. To study samples containing photoactive compounds we have developed a similar bioassay directly on TLC plates using *P. expansum* as a test organism. This paper describes this method and the results obtained particularly with furocoumarins and furochromones.

EXPERIMENTAL

UV apparatus

A Desaga UVIS UV–visible spectrophotometer, supplied with two Sylvania F T4/BLB UV lamps (366 nm), was used.

Samples tested

Furocoumarins. The following were used: xanthotoxin (Carl Roth, Karlsruhe, G.F.R.; batch No. 4580277), imperatorin (Carl Roth; batch No. 3006462), trioxsalen (TMP) (Paul B. Elder Co., Bryan, OH, U.S.A.; rec. 23148), psoralen (synthesized by N. J. de Mol, University of Leiden, Leiden, The Netherlands), bergapten and isopimpinellin (isolated by N. J. de Mol from *Heracleum mantegazzianum* Somm. et Lév.), pimpinellin and isobergapten (F. C. Fischer; isolated from fruits of *H. mantegazzianum* Somm. et Lév.) and angelicin (synthesized by N. J. de Mol).

Furochromones. Khellin was obtained from O. P. G. (Utrecht, The Netherlands) and visnagin from Carl Roth (batch No. 1930347; donated by Dr. O. Schimmer).

Plant extracts. The extract of *Heracleum sphondylium* L. ssp. *orsinii* Guss. [1 g of fresh roots per 0.9 ml of 70% (v/v) aqueous ethanol] contained per ml about 0.5 mg of isobergapten, 0.5 mg of sphondin, 0.5 mg of angelicin, 0.2 mg of bergapten, 0.2 mg of xanthotoxin, 0.9 mg of isopimpinellin and 1.6 mg of pimpinellin per millilitre, determined by means of gas–liquid chromatography and high-performance liquid chromatography[11].

Ammi visnaga $D_1 = \varnothing$ [1 g of dried fruits per 10 ml of 70% (v/v) aqueous ethanol] was a commercial plant extract (VSM, Zaandam, The Netherlands).

Sorbent layers. The following pre-coated TLC plates were obtained from Merck (Darmstadt, G.F.R.): silica gel 60 F-254 (5 × 20 and 20 × 20 cm), silica gel silanized F-254 (5 × 20 cm) and aluminium oxide (type T) (5 × 20 cm).

Test organism. Penicillium expansum was used.

Conidial spray suspension[6]

Conidio-spores of the test organism culture, suspended in a solution of inorganic salts and glucose (30%) in water, were used. The salt solution contained 7 g of

potassium dihydrogen orthophosphate, 3 g of disodium hydrogen orthophosphate, 4 g of potassium nitrate, 1 g of magnesium sulphate and 1 g of sodium chloride per litre of water. A conidial suspension of 35 ml (30 ml of salt solution and 5 ml of glucose solution) is sufficient for spraying four TLC plates (20 × 20 cm).

Optimization

Sample solutions. A 1-mg amount of xanthotoxin (8-MOP) was dissolved in 1 ml of methanol, and from this solution 1/10, 1/100, 1/250, 1/500, 1/1000 and 1/2500 dilutions were prepared. Volumes of 2 μl of the sample solutions were applied as spots on 5 × 20 cm silica gel, silanized silica gel and aluminium oxide TLC plates.

Irradiation. Identically prepared TLC plates with the xanthotoxin samples were irradiated with UV light at 366 nm (distance to the UV lamp, 10 cm) either immediately or 24 h after spraying the plates with the conidal spray suspension and for 5, 10, 20, 40, 60, 120 or 240 min.

The influence of the usual laboratory illumination (fluorescent light) on the phototoxic activity was tested only on silica gel TLC plates. The different durations of exposure to laboratory illumination were 0, 10, 20, 40, 60, 120 and 240 min. The distance to the TL tubes (Philips TL 40W/34 De Luxe) was about 4 m.

Incubation. The TLC plates were kept for 2–3 days in a moist atmosphere at about 25°C in the dark.

Evaluation. Photoactivity was observed as white zones of inhibition of growth on the TLC plates. Optimal conditions were judged by comparing the presence and areas of inhibition zones.

Photoactivity bioassays

Sample solutions. Amounts of 1 mg each of TMP, psoralen, bergapten and xanthotoxin were dissolved in 1 ml of methanol and from each of the solutions dilutions of 1/10, 1/100, 1/250, 1/500, 1/1000 and 1/2500 were prepared. Amounts of 1 mg each of angelicin, imperatorin, pimpinellin, isopimpinellin, khellin and visnagin were dissolved in 1 ml of methanol and from each of the solutions dilutions of 1/10, 1/25, 1/50, 1/100 and 1/250 were prepared. From the extract of *Heracleum sphondylium* dilutions of 1/2, 1/5, 1/10, 1/25, 1/50 and 1/100 were prepared.

From each sample solution 2 μl were spotted on the 20 × 20 cm silica gel TLC plates.

Solvent systems. The following solvent systems were used: (a) toluene–ethyl acetate (9:1)[2]; (b) hexane–ethyl acetate (7:3 and 7.5:2.5)[12]; (c) dichloromethane–ethanol (99:1); (d) dichloromethane–ethanol (98.5:1.5); (e) dichloromethane[3]; (f) chloroform[3]; (g) chloroform–ethanol (98.5:1.5)[13]; and (h) ethyl acetate[14].

Development. An unsaturated chamber was used, with a solvent migration distance of 15 cm.

Detection. Detection was effected by using UV light of wavelength 366 and 254 nm, the spots being marked with a pencil, and by photoactivity bioassay.

Photoactivity bioassay. The well dried thin-layer chromatograms were sprayed with the conidial spray suspension and irradiated for 1 h with UV light at 366 nm from a distance of 10 cm to the UV lamp. Incubation was performed by keeping the thin-layer chromatograms for 2–3 days in a moist atmosphere at about 25°C in the dark.

The results were evaluated by observing the presence or absence of inhibition zones (white spots on a green background). Photoactivity of a compound was detected by comparing its effects on two identical chromatograms, one of which had been exposed to UV irradiation (366 nm) and the other kept in the dark.

RESULTS AND DISCUSSION

Optimization

Xanthotoxin (8-MOP, methoxsalen), a compound known to be highly phototoxic to many organisms[2–5], was used as a test compound throughout the pilot experiments and the optimization of the assay. Amounts ranging between 1 and 2000 ng, dissolved in 2 μl of methanol, were applied as spots on the TLC plates. Variables tested included the nature of the sorbent on the TLC plate, the timing of irradiation and the duration of UV treatment. The plates were always incubated in the dark.

On the non-irradiated TLC plates xanthotoxin was found to be not fungitoxic in any of the amounts tested. On the irradiated TLC plates the photo-fungitoxic activity could be optimally demonstrated by using silica gel layers, irradiated with UV light at 366 nm for about 60 min immediately after spraying with the conidial suspension. Amounts down to 2 ng of xanthotoxin were found to be photoactive under these test conditions; amounts as small as this remain undetected as far as their fluorescence under UV light at 366 nm is concerned before spraying with the conidial

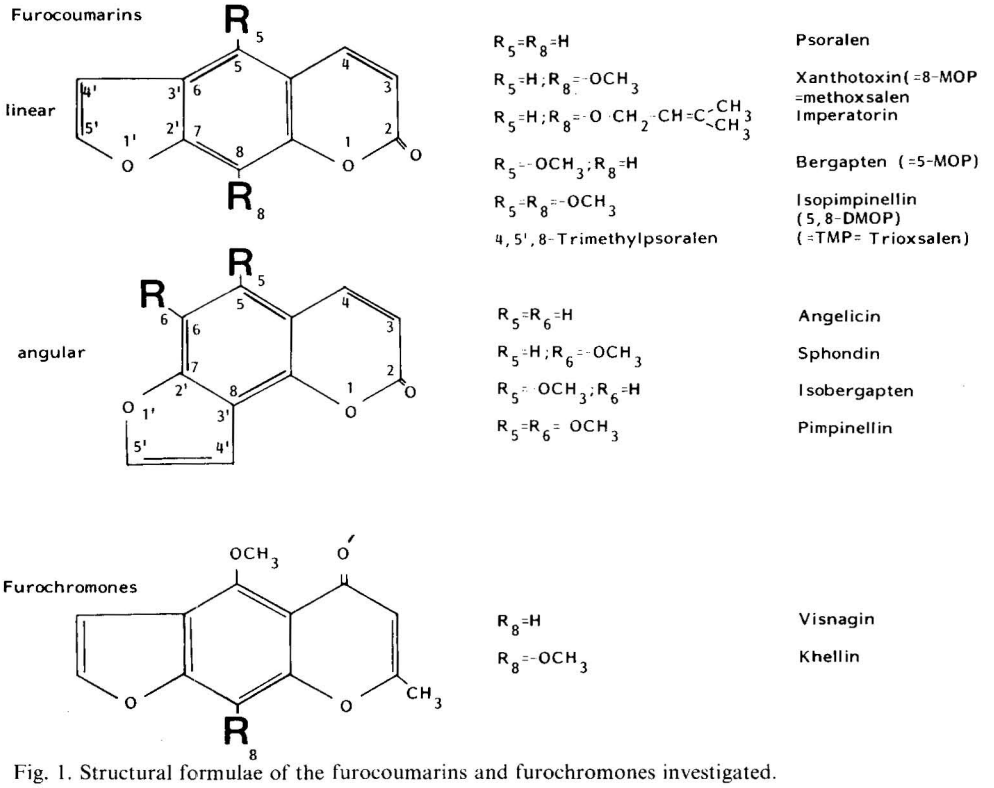

Fig. 1. Structural formulae of the furocoumarins and furochromones investigated.

suspension (the fluorescence detection limit is about 20 ng). A shorter period of irradiation and also a much longer period (*e.g.*, 4 h) reduce the sensitivity of the detection of photoactivity. Irradiation 24 h after spraying with the conidial suspension also results in a decrease in sensitivity of detection. The inhibition zones are larger, however, probably owing to increased diffusion of xanthotoxin.

On aluminium oxide plates the bioassay is also much less sensitive and the inhibition zones are much larger. Moreover, the mycelium covers aluminium oxide plates less smoothly than it covers silica gel plates.

On silanized silica gel plates photo-fungitoxic activity cannot be detected for any of the amounts of xanthotoxin tested. The phenomenon that fungitoxic compounds like polyene antibiotics and saponins fail to show their activity in a bioassay on silanized silica gel layers has been described earlier[10].

Although less effective than long-wavelength UV light (366 nm), irradiation with daylight and fluorescent lighting (TL tubes) also induce xanthotoxin to exhibit photofungitoxic activity on silica gel TLC plates. Xanthotoxin applied to a silica gel plate and exposed for 1 h to ordinary room illumination with fluorescent light (TL tubes) on the laboratory bench exhibits comparable photoactivity to that when it is

(a)

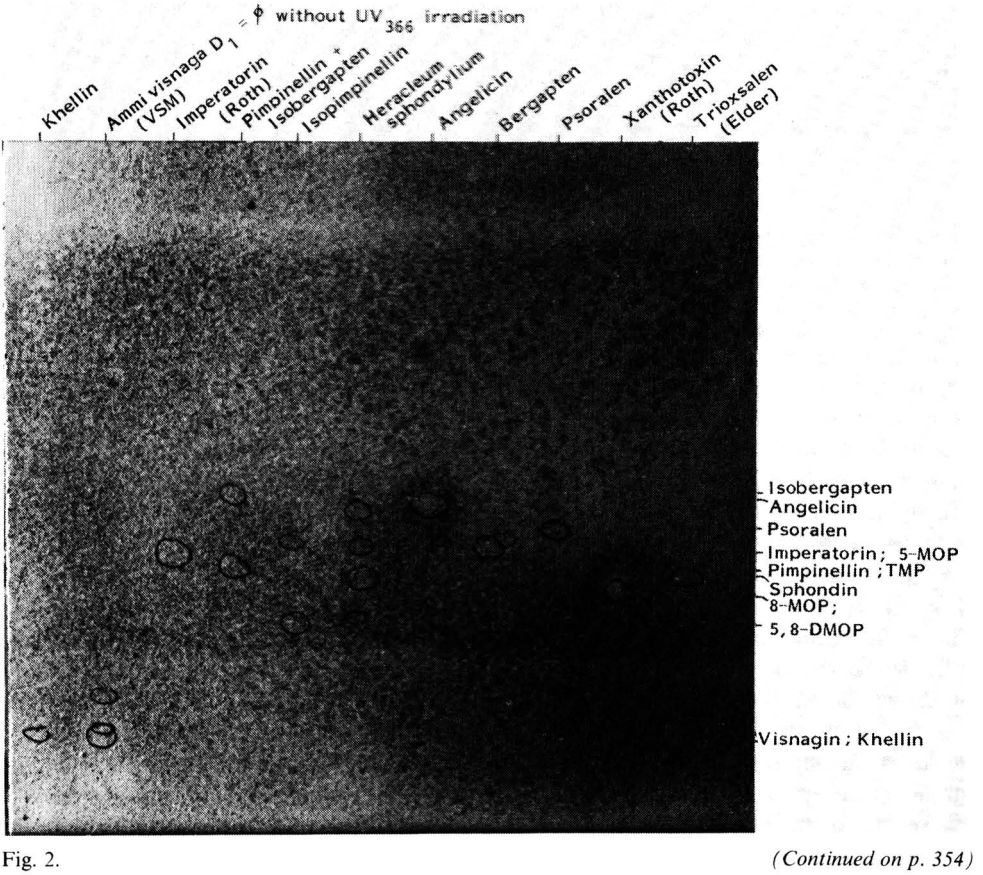

Fig. 2.

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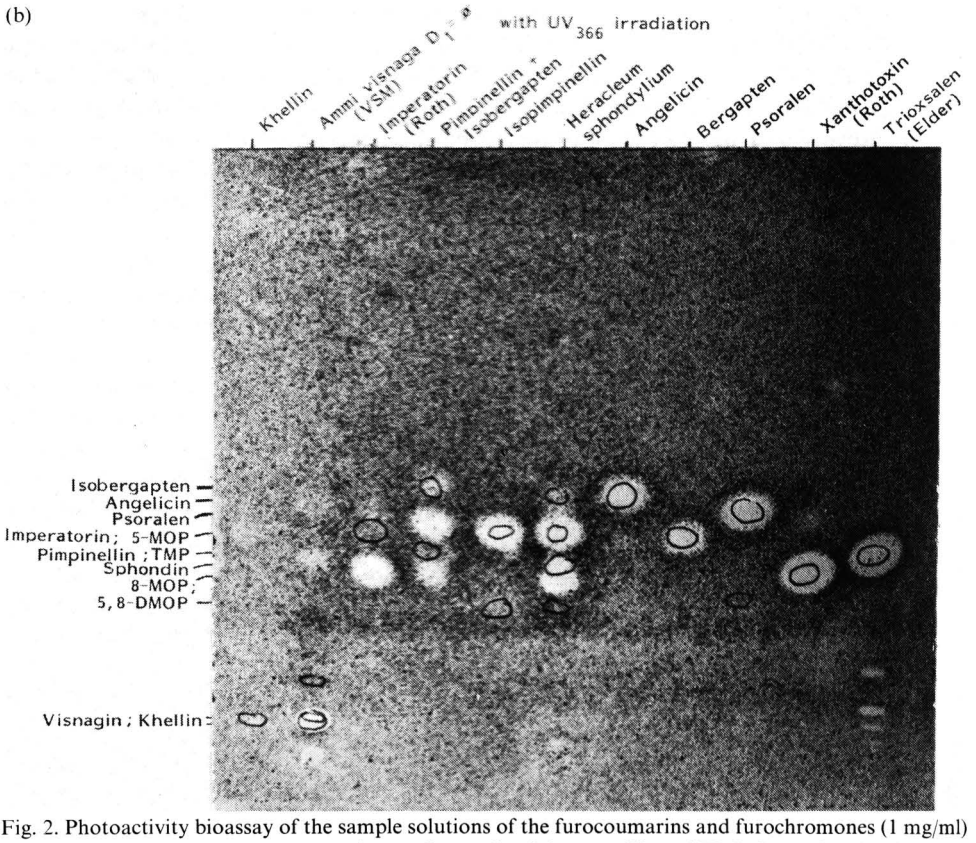

Fig. 2. Photoactivity bioassay of the sample solutions of the furocoumarins and furochromones (1 mg/ml) and of the extracts of *Ammi visnaga* and *Heracleum sphondylium* on silica gel TLC plates after development with solvent system c. Ringed areas show areas of compound spots detected under UV light (366 nm), before spraying with the conidial suspension. (a) Without UV irradiation; (b) with UV irradiation (366 nm).

irradiated for 10 min with UV light at 366 nm; under these test conditions 20 ng of xanthotoxin is about the smallest amount that shows photoactivity.

Johnson *et al.*[9] found xanthotoxin to be strongly fungitoxic against *Helminthosporium carbonum*. Our experiments indicate that incomplete exclusion of light during the biological tests caused the fairly high fungitoxicity of xanthotoxin for this test organism.

Photoactivity bioassays of the samples after thin-layer chromatographic separation

Several furocoumarins and furochromones (Fig. 1) were tested. As no sample of sphondin was available, an extract of *Heracleum sphondylium* with a known content of sphondin was used for the bioassay of this compound.

A limitation of the bioassay is set by the solvents which can be used in the chromatographic separation procedure on thin layers prior to irradiation and the growth inhibition test. In general, only solvents that can be evaporated quantitatively and easily from the thin layer and solvents that are non-fungitoxic themselves can be used[6].

Different solvent systems were tested for this purpose and the results obtained with three different solvent systems are shown in Figs. 2–4. None of the solvent systems tested separated all compounds. Hence, different solvent systems have to be used in order to characterize mixtures of furocoumarins and furochromones.

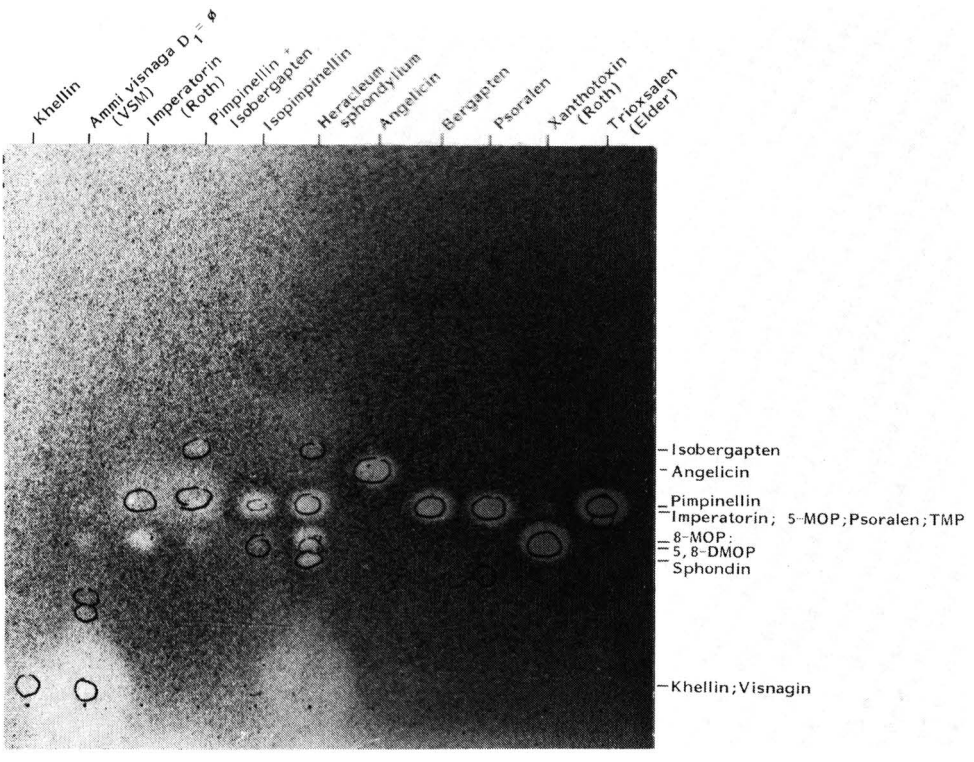

Fig. 3. Photoactivity bioassay of the sample solutions of the furocoumarins and furochromones (1 mg/ml) and of the extracts of *Ammi visnaga* and *Heracleum sphondylium* on silica gel TLC plates after development with solvent system a. Ringed areas as in Fig. 2.

With the solvent systems tested only psoralen and bergapten could be separated and even then hardly with solvent systems containing chloroform or dichloromethane. Dichloromethane–1 % ethanol gave the best results (Fig. 2).

With the solvent systems tested only ethyl acetate[14] is able to separate visnagin and khellin from each other; however, under these conditions most other compounds are not separated from each other.

In our experiments khellin, pimpinellin, isopimpinellin and imperatorin were found not to be photo-fungitoxic in the amounts tested. The samples of the other compounds were tested over a wide range of concentrations after chromatographic development with solvent systems a and c.

The minimal amounts for which photo-activity is detectable were determined. The detection limits of a compound based on its photo-fungitoxicity were compared with those based on its fluorescence under UV light at 366 nm on the chromatogram before spraying with the conidial suspension. The results are summarized in Table I

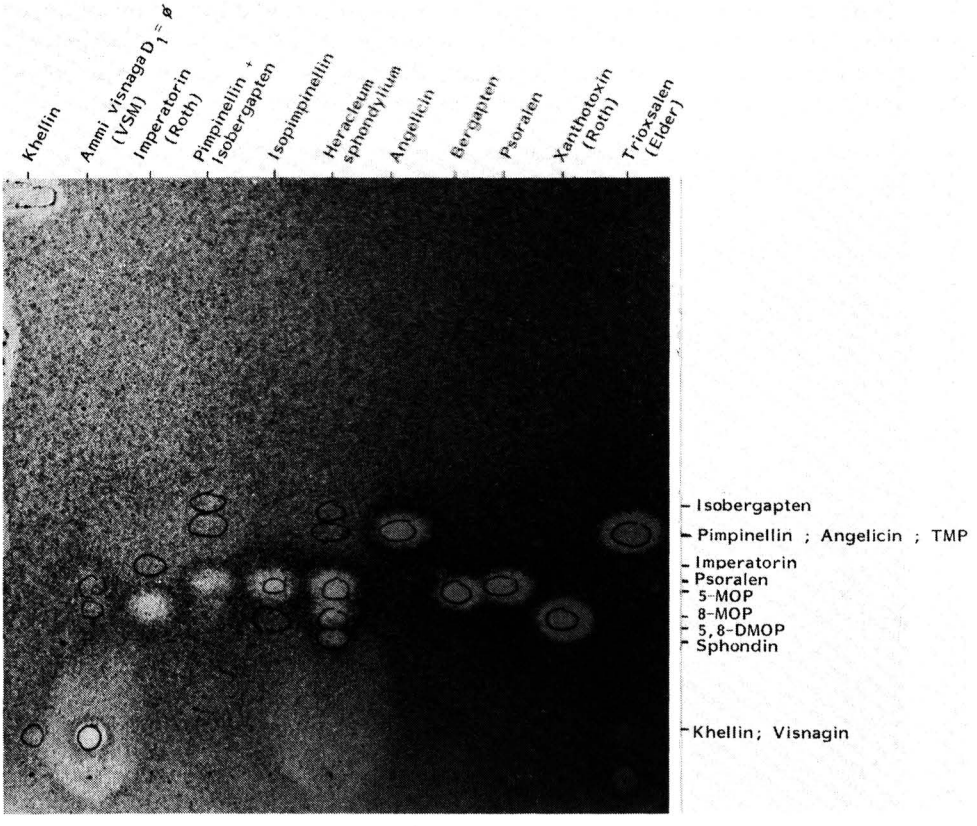

Fig. 4. Photoactivity bioassay of the sample solutions of the furocoumarins and furochromones (1 mg/ml) and the extracts of *Ammi visnaga* and *Heracleum sphondylium* on silica gel TLC plates after development with solvent system b. Ringed areas as in Fig. 2.

and an example of these experiments with TMP is shown in Fig. 5. It can be seen that the sample of TMP contains at least three other photoactive compounds. Although the detection limits of the compounds are in principle dependent on the extent of diffusion on the TLC plate, as a consequence of chromatographic development there is no great difference in the results obtained with the two solvent systems tested.

As shown in Table I, xanthotoxin, bergapten, psoralen and TMP are strongly phototoxic towards the test organism. These compounds can be detected even more sensitively by means of the photoactivity bioassay than by examination under UV light at 366 nm. This demonstrates that the photoactivity bioassay is suitable as a very specific and for some compounds also very sensitive detection method for photoactive compounds on thin-layer chromatograms.

With this method the occurrence of xanthotoxin and bergapten as minor constituents in the extract of *Ammi visnaga* (Figs. 2–4) could be detected. The results of an investigation of several commercial plant extracts and also extracts of plants belonging to the Umbelliferae and Rutaceae will be published elsewhere[11].

Most of the data given in Table I are in good agreement with literature data

TABLE I

DETECTION LIMITS OF FUROCOUMARINS AND FUROCHROMONES ON SILICA GEL TLC-PLATES AFTER DEVELOPMENT WITH SOLVENT SYSTEM a OR c, DETERMINED UNDER UV LIGHT AT 366 nm AND WITH THE BIOASSAY

Compounds	*UV (366 nm)*		*Bioassay detection limit (ng)**	
	Colour	*Detection limit (ng)**	*With UV irradiation (366 nm)***	*Without irradiation***
Furocoumarins				
(a) Linear				
Psoralen	Blue	40–80	4–8	–***
Bergapten (5-MOP)	Yellow	20–40	4–8	–***
Xanthotoxin (8-MOP)	Yellow	20–40	2–4	–***
Isopimpinellin (5,8-DMOP)	Brown	80–200	–***	–***
Imperatorin	Yellow	40–80	–***	–***
TMP	Blue	4–8	1–2	–***
(b) Angular				
Angelicin	Blue	80–200	80–200	–***
Isobergapten	Yellow	20–40	±(2000)	–***
Pimpinellin	Brown	80–200 §	–***	–***
Sphondin	Blue	10–20	40–100	–***
Furochromones				
Khellin	Brown	10–20	–***	–***
Visnagin	Yellow	10–20	10–20	–***

* The higher figure represents an easy detectable amount; the lower figure represents a non-detectable amount.

** –, Inactive; ±, very weakly active.

*** Maximum amount tested = 2000 ng.

§ More sensitively detectable in UV light at 254 nm: detection limit = 8–20 ng.

concerning photoactivity against microorganisms[1–5,15] and skin photo-sensitizing activity[16,17]. However, our results did not agree with data on photoactivity described for imperatorin[2,3] and for isopimpinellin[4]. Imperatorin itself showed no photoactivity in our bioassay, but the sample we used was found to be contaminated with xanthotoxin (Figs. 2–4). This casts some doubts on reports of a low photoactivity for imperatorin[2,3]; in at least one instance the sample used[3] was from the same source as ours. Our sample of isopimpinellin was slightly contaminated with bergapten (Figs. 2–4); the same sample was used by De Mol[4], who found very weak photoactivity for this sample.

Sphondin was weakly photo-active. To our knowledge, no data have been published previously concerning the photoactivity of this compound.

Although furocoumarins have been described as stable in long-wavelength UV light, in contrast to the also highly photoactive phenylheptatriyne[18], a negative result in the photoactivity bioassay could be due to instability of the compound in UV light at 366 nm. To test this, UV spectra of the compound adsorbed on silica gel plates were measured before and after 2 h of UV irradiation at 366 nm. The spectra of all compounds except imperatorin coincided before and after irradiation. Although

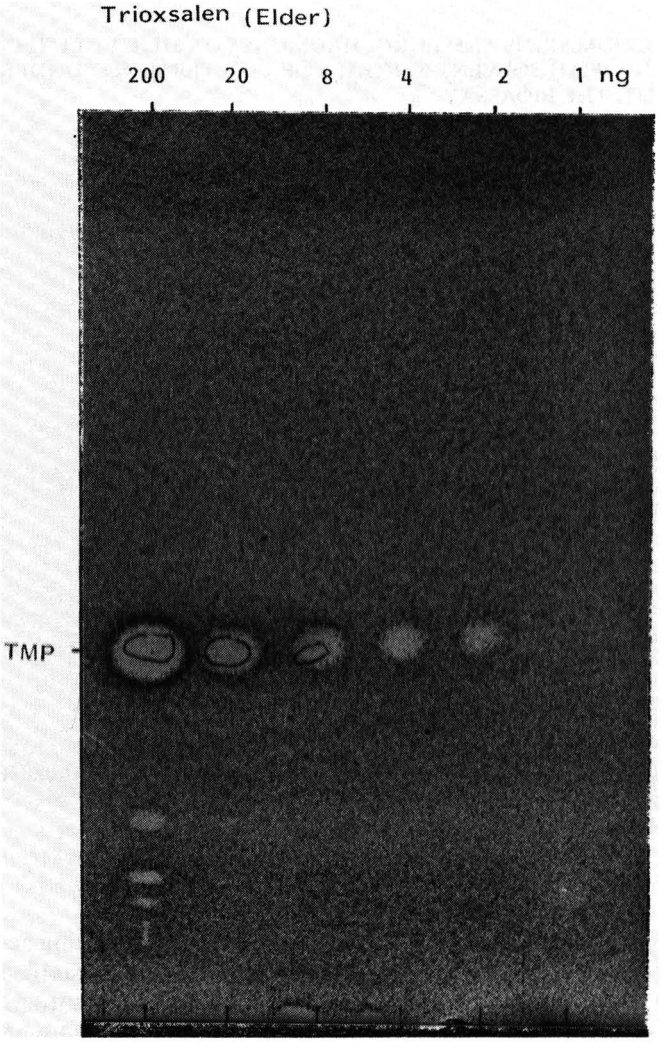

Fig. 5. Photoactivity bioassay of different amounts of trioxsalen (Elder) on silica gel TLC plates after development with solvent system c. Ringed areas as in Fig. 2.

the colour of the spot of imperatorin turned yellow, only a small part of the imperatorin decomposed; on the thin-layer chromatogram, developed two-dimensionally with the same solvent system before and after irradiation, the amount of imperatorin had not diminished appreciably and the decomposition product was observed as a non-migrating yellow spot.

ACKNOWLEDGEMENTS

The authors thank Dr. N. J. de Mol (Subfaculteit Farmacie, Rijksuniversiteit Leiden), Dr. O. Schimmer (Institut für Botanik und Pharmazie, Erlangen, G.F.R.)

and the Paul B. Elder Co. (Bryan, OH, U.S.A.) for their kind gifts of authentic samples, and VSM (Zaandam, The Netherlands) for the commercial plant extract. They are also indebted to Mr. P. van Dorp van Vliet for the drawings and photographs.

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Journal of Chromatography, 214 (1981) 360
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,973

Book Review

Analytical techniques in environmental chemistry (Proc. Int. Congr., Barcelona, November 1978), edited by J. Albaiges, Pergamon, Oxford, New York, Toronto, Sydney, Paris, Frankfurt, 1980, XII + 646 pp., price £ 37.50, US$ 85.00, ISBN 0-08-023809-2

This book contains most of the papers presented at the *International Congress on Analytical Techniques in Environmental Chemistry* held in Barcelona in November, 1978. The scope of environmental chemistry is by definition so incredibly vast and disparate that no single congress or conference could hope to do justice to it. As a consequence, the 60 papers included in this volume is a comparative smörgåsbord, so to speak, of topics that cover an enormous area of endeavors, some rather superficially, others in more detail. The problem can be gleaned from a consideration of topical areas and analytical techniques that cover the expanse of anthropogenic and synthetic organic chemicals (*e.g.*, isoprenoid alkanes, hydrocarbons, polycyclic aromatic hydrocarbons, alkane halides, polychlorinated biphenyls and related derivatives) from industrial and pesticidal classes of utility as waste contaminants in environmental sinks including all aspects of aquatic environments, coastal waters, seas, microflora and microfauna, plant waste streams, incinerator effluents, etc. Coupled to this are the whole panoply of analytical methodologies including: UV spectral analysis, luminescence, gas chromatography, gas chromatography–mass spectrometry, high-performance liquid chromatography, microwave plasma either in monitoring or automated analytical modes. The result makes for a lack of a cohesive theme or sectioning of the book with concomittant confusion to the reader and hence it would appear to be of limited or restricted interest.

Jefferson, AR (U.S.A.) L. FISHBEIN

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Errata

J. Chromatogr., 209 (1981) 7–13

Page 9, line 14 from bottom, "regression coefficients" should read "correlation coefficients".

Page 10, 1st and 3rd lines below eqn. 11, "regression" should read "correlation".

Page 10, Table I, R_4 = H for all barbiturates; the substituents R_1 and R_2 for allobarbital should read "$CH_2CH=CH_2$".

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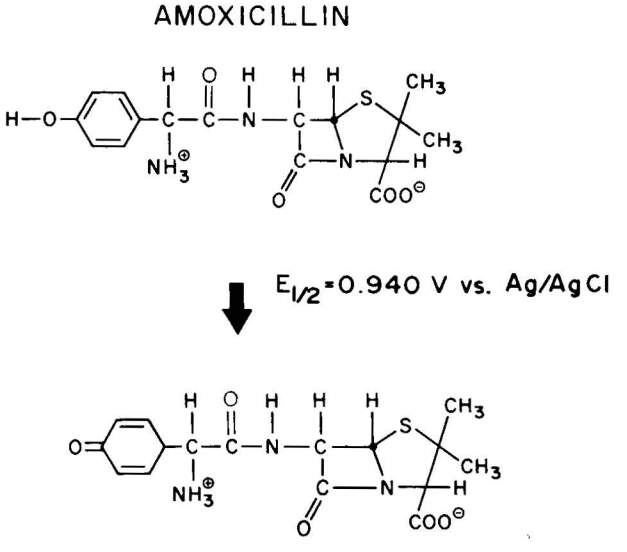
Page 531, Fig. 1, should be:



PUBLICATION SCHEDULE FOR 1981

Journal of Chromatography (incorporating *Chromatographic Reviews*) and *Journal of Chromatography, Biomedical Applications*

MONTH	N 1980	D 1980	J	F	M	A	M	J	J	A	S	O	N	D
Journal of Chromatography			203 204 205/1 205/2	206/1 206/2 206/3	207/1 207/2 207/3	208/1 208/2 209/1	209/2 209/3 210/1	210/2 210/3 211/1	211/2 211/3 212/1 212/2	212/3 213/1 213/2	213/3 214/1 214/2	214/3 215 216	217 218 219/1	219/2 219/3
Chromatographic Reviews							220/1					220/2		220/3
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(Detailed *Instructions to Authors* were published in Vol. 209, No. 3, pp. 501–504. A free reprint can be obtained by application to the publisher.)

Types of Contributions. The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications:* Regular research papers (Full-length papers), Short communications and Notes. Short communications are preliminary announcements of important new developments and will, whenever possible, be published with maximum speed. Notes are usually descriptions of short investigations and reflect the same quality of research as Full-length papers, but should preferably not exceed four printed pages. For reviews, see page 2 of cover under Submission of Papers.

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