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# CHROMATOGRAPHIC REVIEWS (Vol. 24, No. 1)

edited by

Michael Lederer

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## PUBLICATION SCHEDULE FOR 1980

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

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| Chromatographic Reviews | | | 184/1 | 184/2 | | | | | | | | | |
| Biomedical Applications | | 181/1 | 181/2 | 181/ 3-4 | 182/1 | 182/2 | | | | | | | |

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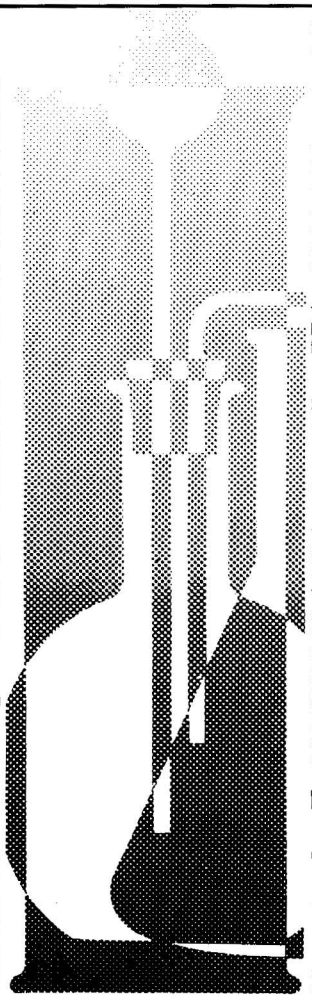

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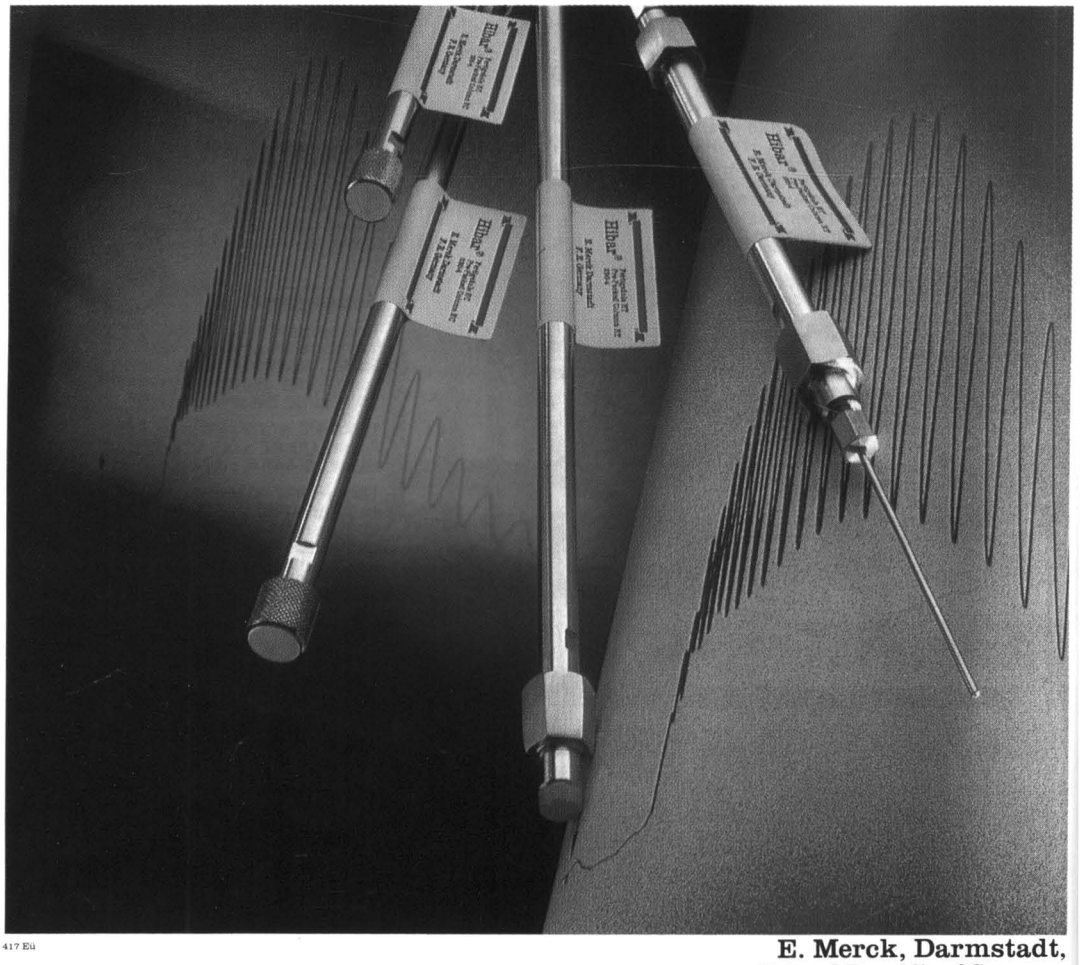

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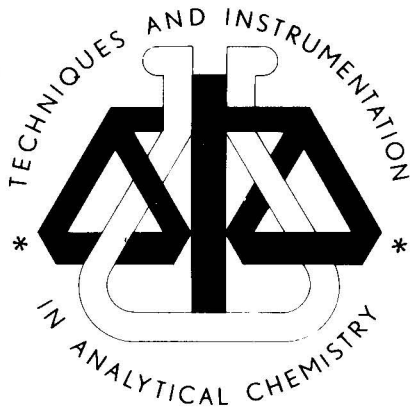


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ELECTROPHORESIS AND RELATED METHODS


## CHROMATOGRAPHIC REVIEWS

edited by

Michael Lederer


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CHREV. 127

# EVALUATION OF PROCEDURES FOR THE ESTIMATION OF DEAD TIME

M. S. WAINWRIGHT and J. K. HAKEN

*Departments of Industrial Chemistry and Polymer Science, University of New South Wales, P.O. Box 1, Kensington, N.S.W. 2033 (Australia)*

(Received August 21st, 1979)

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## 1. INTRODUCTION

The presentation of gas chromatographic data in a precise manner is important for comparative inter-laboratory studies and therefore several methods of presentation have been introduced. Irrespective of the retention scheme used, a knowledge of the column dead time, $t_m$, is necessary because retention times are dependent on this dead time, which is a function of the flow-rate used and the void volume of the experimental apparatus. Therefore the retention times of components must be corrected by subtracting the dead time from them, giving the adjusted retention time, $t'_R$.

Adjusted retention times (which are still dependent on several variables, such as flow-rate, pressure drop, liquid phase and column temperature) are then used in a variety of ways to obtain a method of presentation that is dependent only on the column temperature and the stationary phase used.

## 2. METHODS OF PRESENTATION OF RETENTION DATA

Several methods of presentation have been introduced, but the most widely used are relative retention data[1] and the retention index system introduced by Kováts[2].

Relative retention is presented as a volume or time relative to that of some standard compound, while Kováts retention indices are calculated by numerical

interpolation, by means of the following general equation proposed by Kováts[2]:

$$I = 100\left(n + \frac{\log t'_r - \log t'_n}{\log t'_{n+1} - \log t'_n}\right) \quad (1)$$

where $t'_R$ is the corrected retention time of the substance, $t'_n$ and $t'_{n+1}$ are the corrected retention times of two *n*-alkanes ($t'_n < t'_r < t'_{n+1}$), $I$ is the retention index and $n$ is the carbon number of the first alkane used.

Kováts retention indices can also be calculated by graphical interpolation within the linear relationship between $\log t'_R$ and the number of carbon atoms[3], $n_c$, according to

$$\log t'_R = a\,I + b \quad (2)$$

where $a$ and $b$ are constants and Kováts retention indices for *n*-alkanes are defined as $100Z$ for every temperature and every liquid phase ($Z$ is the carbon number of the *n*-alkane).

However, as can be seen from eqns. 1 and 2, the corrected retention time and thus the column dead times, must be known.

## 3. MEASUREMENT OF COLUMN DEAD TIME

Dead times are determined experimentally by the injection of air, methane or some other substance that is not significantly retarded by the column[4]. A flame-ionization detector does not ordinarily produce a signal with air and the use of methane introduces a slight error as it is retarded to some extent. Hilmi[4] found that when the carrier gas is presaturated with a low-volatility organic solvent, negative air peaks can be readily detected with a hydrogen flame-ionization detector. By measuring the retention time of the air peak and making allowance for the vapour pressure of the solvent, the column dead time can be determined. The method, which suffers from considerable experimental difficulties, requires large injections of air (of the order of 1 $cm^3$) and has not found wide acceptance.

## 4. CALCULATION OF COLUMN DEAD TIME

The inability of the flame-ionization detector to produce an air peak and the questionable accuracy of methane retention as a measure of the dead time have lead to the development of several methods for calculating the dead time.

### *A. Classical methods*

Of the many classical methods reported, the most widely used include the following.

(1) Linearization of a logarithmic plot for homologous *n*-alkanes by graphical trial and error, as reported by Evans and Smith[5]. This method was time consuming and was soon superseded by mathematical treatment of the alkane lines.

(2) The use of a linear relationship derived by Peterson and Hirsch[6], which

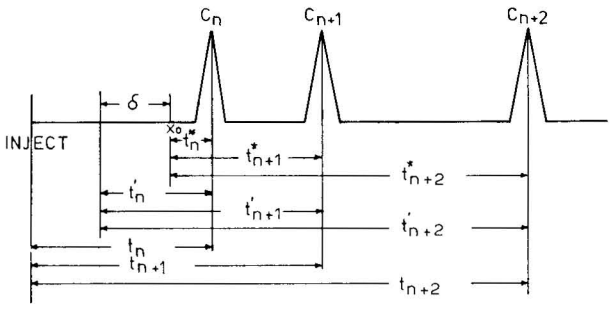


Fig. 1. Schematic chromatogram. $t$ = uncorrected retention time; $t^*$ = retention time relative to origin $x_0$; $t'$ = corrected retention time.

requires three evenly spaced homologues. This method is illustrated in Fig. 1, where three consecutive alkane peaks are used. An arbitrary reference origin ($x_0$) is used and distances are determined from this origin. If the origin corresponds to the true column dead time, then $\delta = 0$. However, in the general case the distance to a given peak is not measured from the true carrier gas but from the origin. In this case the following equation is established:

$$t'_n = t^*_n + \delta \tag{3}$$

The linear relationship between retention time and carbon number is now shown by eqns. 4–6:

$$\log(t^*_n + \delta) \propto n \tag{4}$$

when the three adjacent homologues are used,

$$\frac{t^*_{n+1} + \delta}{t^*_n + \delta} = \frac{t^*_{n+2} + \delta}{t^*_{n+1} + \delta} \tag{5}$$

or

$$\delta = \frac{t^{*2}_{n+1} - t^*_n t^*_{n+2}}{t^*_{n+2} + t^*_n - 2t^*_{n+1}} \tag{6}$$

Thus, one can measure from any arbitrary point ($x_0$) to the peaks of three homologues and record the distances as $t^*_n$, $t^*_{n+1}$ and $t^*_{n+2}$, and solve for $\delta$ and measure from this arbitrary origin to the point which should correspond to the column dead time, $t_m$. If $\delta$ is positive, $t_m$ precedes $x_0$; if $\delta$ is negative, $t_m$ follows $x_0$.

Peterson and Hirsch[6] further modified their method by suggesting the use of the second peak as the origin of reference. In this case, $t^*_{n+1} = 0$. Then, the distance measured between the peaks of homologues 2 and 1 multiplied by that between homologues 2 and 3, divided by their sum ($t^*_n$ is a negative number in this formulation), is the distance from homologue 2 to $t_m$.

(3) The method of Peterson and Hirsch[6] was modified by Gold[7] who described, a method that did not require the restriction of using equally spaced homologues. The

method is based on the fact that as $\log(t - t_m)$ is proportional to the carbon number, $n$, then

$$n = m \log(t - t_m) + k \tag{7}$$

If the difference between the carbon numbers of the first homologues is given by $\Delta n_{1,2} = n_2 - n_1$, then

$$\Delta n_{1,2} = m[\log(t_2 - t_m) - \log(t_1 - t_m)] \tag{8}$$

where $t_1$ and $t_2$ are uncorrected retention times of the first and seconds peaks. Then,

$$m = \frac{\Delta n_{1,2}}{\log (t_2 - t_m)/(t_1 - t_m)} \tag{9}$$

Equn. 8 can be solved for $t_m$ by taking exponentials, giving

$$t_m = \frac{t_2 - e^{(\Delta n_{1,2}/m)} t_1}{1 - e^{(\Delta n_{1,2}/m)}} \tag{10}$$

Eqns. 9 and 10 are simultaneous and can be solved by the method of successive approximations, but such a method is extremely tedious.

Gold[7] also suggested a graphical procedure by using the relationship shown in eqn. 9 for $\Delta n_{1,3}$, which leads to two equations for $m$. Various values of $t_m$ are assumed and plotted against the resulting value of $m$ for each of the two equations. The common solution is the point at which the two lines intersect. The method has little to recommend it and offers little improvement over the trial and error procedure of Evans and Smith[5]. The method of Gold has been adapted for use on a Hewlett-Packard HP65 programmable pocket calculator by Ebel and Kaiser[8].

(4) Hafferkamp[9] and Hansen and Andresen[10] have proposed a method that uses equally spaced homologues as proposed by Petersen and Hirsch[6]. However, they eliminated the need for choosing an arbitrary origin. The method simply uses the uncorrected retention times to give a direct calculation of $t_m$, as shown in the equation

$$t_m = \frac{t_{n+2} \cdot t_n - t_{n+1}^2}{t_{n+2} + t_n - 2t_{n+1}} \tag{11}$$

Reference to eqn. 6 shows the comparison between this method and that proposed earlier by Peterson and Hirsch[6].

(5) Recently, a further method similar to that of Gold has been described[11], in which the retention times of three successive homologues are used to estimate $t_m$. The relationship shown in eqn. 12 can be expressed in the form of eqn. 13.

$$\log(t_n - t_m) = an + b \tag{12}$$

$$t_n - t_m = e^{(an+b)} \tag{13}$$

Hence, for two successive homologues $n = n$ and $n = n + 1$, the expressions shown in eqns. 14–19 are derived.

$$t_n = t_m + e^{(an+b)} \tag{14}$$

and

$$t_{n+1} = t_m + e^{[a(n+1)+b]} \tag{15}$$

Therefore,

$$\Delta t_{n,n+1} = t_{n+1} - t_n = e^{(an+b)}(e^a - 1) \tag{16}$$

Similarly, for the pair $n = n + 1$ and $n = n + 2$

$$t_{n+2} = t_m + e^{a(n+2)+b} \tag{17}$$

Hence,

$$\Delta t_{n+1,n+2} = t_{n+2} - t_{n+1} = e^{a(n+1)+b}(e^a - 1) \tag{18}$$

and

$$\frac{\Delta t_{n+1,n+2}}{\Delta t_{n,n+1}} = e^a \tag{19}$$

Therefore, by using the differences in retention times for three consecutive homologues, $a$ can be evaluated from the equation

$$a = \log\left(\frac{\Delta t_{n+1,n+2}}{\Delta t_{n,n+1}}\right) \tag{20}$$

Having evaluated $a$, $b$ is evaluated using eqn. 16 (or 17), which is rewritten as

$$b = \log\left(\frac{\Delta t_{n,n+1}}{e^a - 1}\right) - an \tag{21}$$

Then $t_m$ is calculated from

$$t_m = t_n - e^{(an+b)} \tag{22}$$

For all pairs of homologues employed a consistent result confirms the validity.

The method does not provide any advantage in accuracy over that of Hansen and Andresen[10] and involves more effort in calculation.

*B. Statistical and iterative methods*

The previous classical methods were limited in accuracy as only three alkanes could be used or a graphical trial and error was needed. Therefore, several more sophisticated methods have been developed.

(1) The method of Grobler and Balizs[12], which uses two successive linear regressions. Although the derivation of the expressions used is not given in the paper, the following derivation gives the required equations (eqns. 23–35).

Restating eqn. 2, we have

$$\log t'_R = bZ_i + c \tag{23}$$

Thus

$$\log (t_R - t_m) = bZ_i + c \tag{24}$$

$$t_R - t_m = e^{bZ_i + c} \tag{25}$$

$$t_{R+1} - t_m = e^{bZ_{i+1} + c} \tag{26}$$

But $Z_{i+1} = Z_i + 1$. Therefore, the previous equation becomes

$$t_{R+1} - t_m = e^{b(Z_i+1) + c} \tag{27}$$

Subtraction of eqn. 25 from eqn. 27 gives

$$(t_{R+1} - t_m) - (t_R - t_m) \tag{28}$$

which

$$= e^{b(Z_i+1)) + c} - e^{bZ_i + c} \tag{29}$$

$$= e^{bZ_i} e^b e^c - e^{bZ_i} e^c \tag{30}$$

$$= e^c e^{bZ_i} e^{(b-1)} \tag{31}$$

Therefore,

$$t_{R+1} - t_R = A e^{bZ_i} \tag{32}$$

where

$$A = e^c e^{(b-1)} \tag{33}$$

Taking logarithms, we obtain

$$\log(t_{R+1} - t_R) = \log A + bZ_i \tag{34}$$

Therefore, a linear regression on eqn. 34 of $\log(t_{R+1} - t_R)$ against $Z_i$ will give $b$ as the the slope. We then obtain

$$b = \frac{(n-1) \sum_{i=Z_1}^{Z_n} Z_i \log [t_{R(i+1)} - t_{R(i)}] - \sum_{i=Z_1}^{Z_{n-1}} Z_i \sum_{i=Z_1}^{Z_{n-1}} \log [t_{R(i+1)} - t_{R(i)}]}{(n-1) \sum_{i=Z_1}^{Z_{n-1}} Z_i^2 - \left( \sum_{i=Z_1}^{Z_{n-1}} Z_i \right)^2} \tag{35}$$

This is eqn. 1 in the paper by Grobler and Balizs[12] (it should be noted that there is a mistake in that paper; the $n$ in the numeration should be $n-1$ as in eqn. 35 above).

Now, from eqn. 2,

$$t_R = t_m + e^{bZ} e^c \tag{36}$$

$$t_R = t_m + Aq^Z \tag{37}$$

where

$$A = e^c \text{ and } q = e^b \tag{38}$$

We can perform another linear regression on $t_R$ against $q^Z$ and $t_m$ will be the intercept. Therefore, we obtain

$$t_m = \frac{\sum_{i=Z_1}^{Z_n} q^{Z_i} \sum_{i=Z_1}^{Z_n} t_{R(i)} q^{Z_i} - \sum_{i=Z_1}^{Z_n} q^{2Z_i} \sum_{i=Z_1}^{Z_n} t_{R(i)}}{\left(\sum_{i=Z_1}^{Z_{n-1}} q^{Z_i}\right)^2 - n \sum_{i=Z_1}^{Z_{n-1}} q^{2Z_i}} \tag{39}$$

This is eqn. 4 in the paper by Grobler and Balizs[12].

Finally, $c$ can be found from a linear regression on eqn. 39 of $\log t_R$ against $Z_i$. This gives

$$c = \frac{\sum_{i=Z_1}^{Z_n} \log t'_{R(i)} - b \sum_{i=Z_1}^{Z_n} Z_i}{n} \tag{40}$$

This is eqn. 5 in the paper by Grobler and Balizs[12].

Thus, eqns. 35, 39 and 40 give the dead time, $t_m$, and the slope, $b$, and intercept, $c$, of the plot of the logarithm of the adjusted retention times against carbon number, where:

$t_{R(i)}$ = uncorrected retention time of the $i$th $n$-alkane;
$t'_{R(i)}$ = corrected retention time of the $i$th $n$-alkane;
$Z_i$ = carbon number of the $i$th $n$-alkane;
$q$ = antilog $b$;
$n$ = number of $n$-alkanes used.

Thus, the retention index for any compound can be calculated from the equation

$$I = 100 (\log t'_R - c)/b \tag{41}$$

(2) The method of Guardino *et al.*[13] requires an iteration to be carried out on $T_m$, while a least-squares fit is applied to $b$ and $c$. The best values of $T_m$, $b$ and $c$ are determined by minimizing the sum of squares of the difference between the known and the calculated $I$ values. Fig. 2 shows a flow diagram, where UPLIM and LOWLIM are the upper and lower limits, respectively, of the sum of squares of the deviation,

$T_m$ is the dead time, INC is the increment in the dead time, IC is the calculated Kováts retention index, SUM is the sum of squares of the deviations, TR is the unadjusted retention times of the compounds, $I$ is the known Kováts retention index ($100Z$, where $Z$ is the carbon number) and PREC is the precision to which the answer is required.

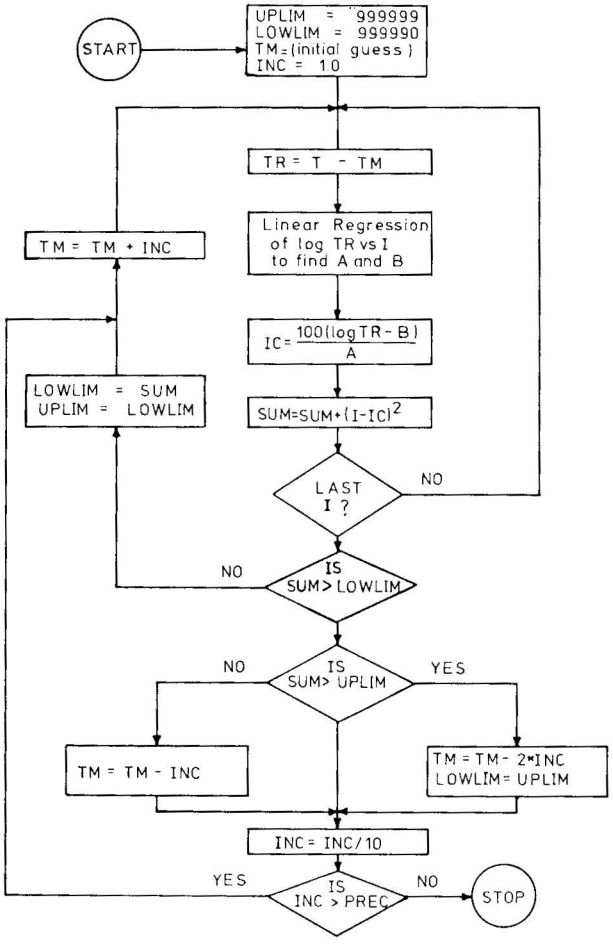


Fig. 2. Flow chart for calculation of mathematical dead time by Guardino *et al.*'s method[13].

(3) The non-linear least-squares estimation of $t_m$, $b$ and $c$ simultaneously[14,15] by the use of numerical minimization[16]. The objective function that is minimized is the sum of squares of the differences between the known and calculated $I$ values for the series of *n*-alkanes.

## 5. EVALUATION OF PROCEDURES FOR THE CALCULATION OF DEAD TIME

### *A. Comparison of calculated dead time with methane retention*

The use of methane retention as an estimate has been criticized[6,17,18]. If one

accepts the linearity of the plot of the logarithm of the corrected retention times of homologous series *versus* carbon number, then methane as the first *n*-alkane homologues must have a finite corrected retention time. That is, methane provides a poor estimate of dead time, being the sum of dead time plus the retention of methane. Further, if one accepts the evidence that the use of any homologous series can provide the same estimate of dead time[19], then the logical conclusion is that the retention of methanol and similar oxygenated $C_1$ compounds should be a good estimate of dead time.

The controversy surrounding the comparative merits of methane injection and mathematical dead time estimates has recently been raised by Sharples and Vernon[20]. They pointed out inaccuracies in mathematical dead time estimates when the method of Peterson and Hirsch[6] is applied to retention times for *n*-alkanes measured by a stop-watch. They have shown that when stop-watch measurement of retention is employed, the retentions of air and methane are identical for polar and non-polar phases over a wide temperature range.

The errors attributed to the method of Peterson and Hirsch[6] are caused in two ways. Firstly, the method is one using the analysis of three equally spaced points and therefore the centre point is weighted excessively and so small errors in retention times measurement (particularly with the second alkane) leads to gross errors in $t_m$. This error in the estimation procedure can be overcome by the more statistically sound approach of Grobler and Balizs[12], which weights all points equally. The second source of error is that of measuring retention times. Sharples and Vernon[20] suggest that the reason that other workers[10,18] measured methane retentions in excess of the calculated dead time was because of errors in measurement of retention times. However, Smith *et al.*[15] found similar excess retentions of methane in a study in which a computer was used to measure the retention time of the *n*-alkanes and in which a proved method of statistical estimation of $t_m$ was employed[12].

Smith *et al.*[15] calculated the dead time for six alkanes ($C_5$–$C_{10}$) and over a series of 18 injections obtained a mean estimate of $t_m$ of 54.0 sec with a standard deviation of 0.5 sec. The same number of air and methane injections gave retentions of 55.1 and 55.5 sec, respectively, both with a standard deviation of 0.7 sec. In all instances the calculated dead time was less than both the air and methane injections.

Recently, Wainwright *et al.*[21] used a mixture of methane and $C_5$–$C_9$ *n*-alkanes in nitrogen to compare the mathematical dead time with methane retention. This method, in which methane and the *n*-alkanes are injected simultaneously onto the column, gives an accurate comparison under identical operating conditions. Calculated dead times and methane retention were measured on polar (OV-25) and non-polar (SE-30) columns at several temperatures and carrier gas flow-rates. In all instances the methane retention was in excess of the calculated value of $t_m$. The results of this study are consistent with those of Garcia Dominguez *et al.*[17], who found that methane was retained on various chromatographic columns of different polarity for the temperature range 100–180°. The results are also consistent with those of an earlier study[3] in which it was observed that the use of methane retention time as an estimate of column dead time produced curvature in the logarithmic plot of the *n*-alkanes. The solubility of methane in liquid phases has been shown elsewhere[22].

*B. Comparison of methods of computing mathematical dead time*

The classical methods described earlier in general use the uncorrected retention times of three equally spaced homologues to calculate the retention times. A recent paper[11] compared methane retention with a calculation method that uses only two alkanes. Data were presented for the uncorrected retention times of $C_1$–$C_4$ *n*-alkanes together with the retention times calculated by eqns. 14–19. These values are presented in Table 1 for two different column packings. The retention data for $C_2$–$C_4$ *n*-alkanes have been used here to calculate $t_m$ by the method of Peterson and Hirsch[6]. It is evident from Table 1 that both methods of calculation give essentially the same dead times, all providing estimates of $t_m$ that are less than methane retention. The error in using methane retention is highlighted with the non-polar (squalane) stationary phase, where the differente is approximately 7% compated with a value of 0.5% for dinonyl phthalate.

TABLE 1

COMPARISON OF DIFFERENT METHODS OF ESTIMATING DEAD TIMES

Data from ref. 11.

| *n-Alkane* | *Squalane* | | | | | *Dinonyl phthalate* | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $t_R$ | $t'_R$ | $t_m$* | $t_m$** | $t_m$*** | $V_R$ | $V'_R$ | $V_m$* | $V_m$** | $V_m$*** |
| $C_1$ | 21.5 | 1.5 | 20.0 | 20.2 | 19.8 | 19.05 | 0.25 | 18.95 | 18.95 | 18.64 |
| $C_2$ | 26.5 | 6.4 | 20.0 | | | 19.68 | 0.75 | 18.95 | | |
| $C_3$ | 46.5 | 26.0 | 20.1 | | | 21.10 | 2.15 | 18.95 | | |
| $C_4$ | 129.5 | 109.1 | 20.4 | | | 25.29 | 6.34 | 18.95 | | |

* Estimated by the method of ref. 11.
** Estimated by the method of ref. 6 (for $C_2$–$C_4$).
*** Estimated by the method of ref. 12 (for $C_1$–$C_4$).

Haken *et al.*[14] made a comparison of the different methods for calculating dead times. These results are summarised in Table 2. It is evident that the three iterative methods described above give essentially the same dead times (to the fourth significant figure). The values of $t_m$ calculated by the classical methods of Peterson and Hirsch[6] and Gold[7] differ from the iterative methods by less than 0.2 sec in 60 sec.

TABLE 2

COMPARISON OF DIFFERENT METHODS FOR CALCULATING DEAD TIMES[14]

Methods: A = non-linear regression using Simplex; B = method of Grobler and Balizs[12]; C = method of Guardino *et al.*[13]; D = method of Peterson and Hirsch[6]; E = method of Gold[7].

| *Run* | *Dead time (sec)* | | | | |
|---|---|---|---|---|---|
| | *A* | *B* | *C* | *D* | *E* |
| 1 | 59.79 | 59.77 | 59.74 | 59.95 | 59.95 |
| 2 | 60.58 | 60.50 | 60.53 | | |
| 3 | 62.91 | 63.04 | 62.86 | | |
| 4 | 69.74 | 69.75 | 69.73 | 69.62 | 69.62 |

In a subsequent paper[15] a more detailed study of the iterative procedures was performed following the comments of Gassiot *et al.*[23]. The three methods were written in Fortran IV and run on a Cyber 72-26 digital computer to ensure maximum accuracy.

Seven series of calculation were conducted using the retention times of the following alkanes for each of the 46 sets of data: (a) $C_5$–$C_7$, (b) $C_5$–$C_8$, (c) $C_6$–$C_9$, (d) $C_7$–$C_{10}$, (e) $C_5$–$C_9$, (f) $C_6$–$C_{10}$ and (g) $C_5$–$C_{10}$.

Flexible Simplex and the method of Guardino *et al.*[13] both use an iterative technique and thus require an objective function to be defined which is then minimized. In a previous paper[14] we used the objective function suggested by Guardino *et al.*[13] which is the sum of squares of the differences between the known and calculated Kováts retention indices as shown in the equation

$$\text{Objective function} = \Sigma(I - I_c)^2 \tag{42}$$

where $I$ is the known and $I_c$ is the calculated Kováts retention index.

However, as $I$ is defined as 100 times the carbon number, $Z$, for $n$-alkanes, its value is known, and it is thus the independent variable. The objective function should, however, be based on the dependent variable, which in this instance is the retention time, $t_R$. This would suggest the use of the sum of squares of the differences between the experimental and calculated retention times as defined in the equation

$$\text{Objective function} = \Sigma(t_R - t_{R_c})^2 \tag{43}$$

where $t_R$ is the experimental and $T_{R_c}$ is the calculated retention time. However, this function weights those alkanes with longer retention times and which are the least accurate, as discussed in a previous paper[14]. Therefore, to overcome this difficulty and to take account of the logarithmic nature of our model (eqn. 23), an objective function based on the sum of squares of the difference between the logarithms of the experimental and calculated connected retention times was chosen, as shown in eqn. 23:

$$\text{Objective function} = \Sigma(\log t'_R - \log t'_{R_c})^2 = \sum \left( \log \frac{t_R - t_m}{t_{R_c} - t_m} \right)^2 \tag{44}$$

where $t_R$ = the experimental retention time, $t_{R_c}$ = the calculated retention time and $t_m$ = the calculated dead time.

The difference between the objective functions shown in eqns. 43 and 44 can be seen by reducing each to a simpler form, eqns. 45 and 46 respectively:

$$\Sigma(t_R - t_{R_c})^2 = \Sigma(t'_R - e^{bZ+c})^2 \tag{45}$$

$$\sum \left( \log \frac{t_R - t_m}{t_{R_c} - t_m} \right)^2 = \Sigma(\log t'_R - \log e^{bZ+c})^2 = \Sigma(\log t'_R - bZ - c)^2 \tag{46}$$

Table 3 compares the three objective functions studied, where it is apparent that the objective function used by Guardino *et al*[13]. gives identical dead times to the Simplex method to five significant figures. When the objective work function is sub-

TABLE 3

COMPARISON OF DEAD TIMES CALCULATED BY THE ITERATIVE METHODS USING DIFFERENT OBJECTIVE FUNCTIONS[15]

| *Run* | $(I - I_c)^2$ | | | $(t_{R_c} - t_R)^2$ | | $ln\left(\frac{t_R - t_m}{I_{R_c} - t_m}\right)$ | |
|---|---|---|---|---|---|---|---|
| | *Grobler and Balizs*[12] | *Flexible Simplex* | *Guardino et al.*[13] | *Flexible Simplex* | *Guardino et al.*[13] | *Flexible Simplex* | *Guardino et al.*[13] |
| 1 | 53.735 | 53.772 | 53.772 | 54.351 | 53.936 | 53.769 | 53.735 |
| 2 | 55.001 | 55.067 | 55.067 | 55.277 | 55.117 | 55.067 | 55.001 |
| 3 | 54.664 | 54.480 | 55.480 | 54.386 | 54.494 | 54.478 | 54.664 |
| 4 | 54.982 | 55.008 | 55.008 | 55.136 | 55.037 | 55.007 | 54.982 |
| 5 | 54.677 | 54.626 | 54.626 | 54.692 | 54.650 | 54.625 | 54.677 |
| 6 | 54.282 | 54.255 | 54.255 | 54.036 | 54.200 | 54.255 | 54.282 |
| 7 | 53.626 | 53.814 | 53.814 | 54.295 | 53.916 | 53.812 | 53.626 |
| 8 | 53.784 | 53.924 | 53.924 | 53.994 | 53.918 | 53.923 | 53.784 |
| 9 | 54.259 | 54.181 | 54.180 | 53.880 | 54.110 | 54.180 | 54.259 |
| 10 | 54.228 | 54.250 | 54.250 | 54.259 | 54.247 | 54.250 | 54.228 |
| 11 | 54.147 | 53.969 | 53.969 | 53.916 | 53.916 | 53.968 | 54.147 |
| 12 | 53.547 | 53.670 | 53.670 | 53.934 | 53.723 | 53.670 | 53.547 |
| 13 | 53.836 | 53.837 | 53.837 | 54.201 | 53.936 | 53.835 | 53.836 |
| 14 | 53.234 | 53.404 | 53.404 | 53.916 | 53.512 | 53.402 | 53.234 |
| 15 | 53.958 | 53.904 | 53.904 | 53.716 | 53.864 | 53.903 | 53.958 |
| 16 | 53.546 | 53.573 | 53.573 | 53.376 | 53.504 | 53.546 | 53.546 |
| 17 | 53.911 | 53.834 | 53.834 | 53.725 | 53.813 | 53.833 | 53.911 |
| 18 | 53.889 | 53.871 | 53.871 | 53.885 | 53.881 | 53.871 | 53.889 |

stituted in both methods, it is evident that the method of Guardino *et al.* gives identical dead time estimations to those of Grobler and Balizs[12].

## 6. PROBLEMS OF ACCURACY OF MATHEMATICAL DEAD TIME ESTIMATION

Haken *et al.*[14] reported inaccuracies in the estimation of $t_m$ using the method of Grobler and Balizs.[12] When determining $I$ values on a 12 ft. $\times$ $^1/_4$ in. column packed with 10% squalane and operating at 120°, it became apparent that small differences (of the order of 2 sec) in uncorrected retention times of $C_7$–$C_{10}$ alkanes led to large differences (up to 10 sec) in $t_m$ values. Typical results are given in Table 4.

The principle of the calculation of mathematical dead time involves the extrapolation of the least-squares line through the logarithms of the corrected retention

TABLE 4

VARIATION OF DEAD TIMES FOR RUNS USING $C_7$–$C_{10}$ *n*-ALKANES[14]

| *Run* | *Uncorrected retention time (sec)* | | | | *Calculated dead time (sec)* |
|---|---|---|---|---|---|
| | $C_7$ | $C_8$ | $C_9$ | $C_{10}$ | |
| 1 | 182 | 291 | 497 | 887 | 59.77 |
| 2 | 182 | 291 | 497 | 889 | 60.50 |
| 3 | 182.5 | 290 | 495.5 | 884 | 63.04 |
| 4 | 184 | 290 | 494.5 | 888.5 | 69.75 |

TABLE 5

EFFECT OF SMALL PERTURBATIONS IN RETENTION TIMES[14]

| *n-Alkane* | *Experimental data* | *Perturbed data* | | | |
|---|---|---|---|---|---|
| | *Retention time (sec)* | *Retention time (sec)* | *Difference (sec)* | *Retention time (sec)* | *Difference (sec)* |
| $C_5$ | 86 | 84 | −2 | 88 | +2 |
| $C_6$ | 110 | 109 | −1 | 111 | +1 |
| $C_7$ | 156 | 157 | +1 | 155 | −1 |
| $C_8$ | 245 | 247 | +2 | 243 | −2 |
| Dead time (sec) | 60.13 | 55.60 | −4.53 | 64.18 | +4.05 |
| $C_7$ | 182 | 180 | −2 | 184 | +2 |
| $C_8$ | 291 | 290 | −1 | 292 | +1 |
| $C_9$ | 497 | 498 | +1 | 496 | −1 |
| $C_{10}$ | 887 | 889 | +2 | 885 | −2 |
| Dead time (sec) | 59.79 | 55.60 | −4.19 | 63.98 | +4.17 |

times to zero carbon number. Therefore, small changes in the slope of this line will lead to large variations in calculated values of $t_m$ and hence to large variations in *I*-values[18]. In order to quantify the effect of small systematic changes in retention times of the *n*-alkanes, small perturbations (of the order of 1 or 2 sec) were made to uncorrected retention data for two sets of alkanes, $C_5$–$C_8$ and $C_7$–$C_{10}$. These results are presented in Table 5. It is obvious from these results that small changes in the alkane retention times lead to large changes in the dead time value.

Of the several stationary phases investigated, squalane was the only one to give significant differences in dead time values. Table 6 presents typical values of dead time for an OV-7 column that was coupled to the squalane column via a splitter. The results show that although the retention times of the alkanes show variations of similar magnitude on both columns, the differences in dead time for OV-7 are significantly lower. The mean dead time for squalane over a large number of determinations was approximately 60 sec and the value for OV-7 was approximately 67 sec. The greater inaccuracy in the dead time determination for squalane appeared to be associated with the relatively long retention times of the $C_7$–$C_{10}$ alkanes. Therefore, $C_5$–$C_8$ alkanes were used and Table 7 lists the dead time values calculated over a 6-h period of Kováts retention index calculations. It can be seen that the variation in dead time is not significant.

TABLE 6

RETENTION AND DEAD-TIME MEASUREMENTS MADE ON SQUALANE AND OV-7 COLUMNS FOR $C_7$–$C_{10}$ *n*-ALKANES[14]

| *Column* | *Uncorrected retention time (sec)* | | | | *Dead time (sec)* |
|---|---|---|---|---|---|
| | $C_7$ | $C_8$ | $C_9$ | $C_{10}$ | |
| Squalane | 182 | 291 | 497 | 887 | 59.77 |
| | 184 | 290 | 494.5 | 888.5 | 69.75 |
| OV-17 | 113.5 | 150.5 | 216.5 | 335.5 | 66.93 |
| | 113 | 150 | 215 | 332.5 | 65.52 |

TABLE 7

EFFECT ON MATHEMATICAL DEAD TIME WHEN $C_5$–$C_8$ *n*-ALKANES ARE USED[14]

| *Uncorrected retention time (sec)* | | | | *Dead time (sec)* |
|---|---|---|---|---|
| $C_5$ | $C_6$ | $C_7$ | $C_8$ | |
| 85 | 108 | 152 | 236.5 | 59.92 |
| 85.5 | 109 | 153 | 239 | 59.88 |
| 85.5 | 108.5 | 153 | 238.5 | 60.70 |
| 86.0 | 110 | 156.5 | 246 | 60.21 |
| 86 | 110 | 156 | 244.5 | 59.93 |
| 86 | 110 | 156 | 245 | 60.10 |

It is apparent from the results in Tables 6 and 7 that significant differences in dead time result from using alkanes that have long retention times. Longer retention times obviously mean a greater extrapolation and a larger error. Therefore, it is desirable to use low-molecular-weight alkanes, provided that the retention times can be measured with sufficient accuracy.

Recently, Sharples and Vernon[20] have shown similar inaccuracies in mathematical dead times calculated by the method of Peterson and Hirsch[6]. The hypothetical system having small variations in uncorrected retention times of three consecutive *n*-alkanes is illustrated in Table 8. It can be seen that a small error in the measurement of the retention time of the second alkane can produce an error of 3.5 sec in the estimation of a dead time of 30 sec. This calculation points to the weakness of the method of Peterson and Hirsch when crude methods of measuring retention times are used.

TABLE 8

HYPOTHETICAL SYSTEM HAVING A 30.0-sec DEAD TIME SHOWING THE EFFECTS OF SMALL ERRORS IN A PEAK TIMING ON THE DEAD TIME AS CALCULATED BY THE METHOD OF PETERSON AND HIRSCH[6] (REF. 20)

| *n-Alkane* | $t_R$ *(sec)* | *Error (sec)* | $t_R$ *(sec)* | *Error (sec)* | $t_R$ *(sec)* | *Error (sec)* | $t_R$ *(sec)* | *Error (sec)* |
|---|---|---|---|---|---|---|---|---|
| $C_8$ | 87.0 | 0 | 87.0 | 0 | 87.0 | 0 | 86.0 | −1 |
| $C_{8+1}$ | 127.5 | 0 | 128.0 | +0.5 | 126.2 | −1.3 | 128.0 | +0.5 |
| $C_{8+2}$ | 196.8 | 0 | 196.8 | 0 | 198.6 | +1.8 | 195.3 | −1.5 |
| $t_m$(calc.) | 30.0 | | 26.5 | | 40.7 | | 16.3 | |

Guberska[18,24] has made several studies of the determination of dead time. In his second study[18] he found that the retention time of methane is greater than the dead time calculated by the method of Hansen and Andresen[10]. However, he found that the precision of estimating $t_m$ by the same method[10] was less than the measurement of methane retention, particularly at high temperatures. He therefore devised a scheme to estimate $t_m$ using the retention time of methane.

Assuming that the retention time of methane in the stationary phase is dependent on the amount of the stationary phase, without regard to the type of phase, and

using experimentally obtained retention data, Guberska[18] developed the following empirical equation to determine dead time from the total retention time of methane:

$$t_m = t_{CH_4} - \left(\frac{t_{CH_4} M}{163}\right) \tag{47}$$

where $M$ (wt.-%) is the amount of stationary phase in the column packing.

Table 9 compares the results of dead time determination using the Hansen and Andresen method (column 4) with dead time results (column 5) calculated according to eqn. 47 from the total retention time of methane (column 3). The absolute errors of dead time determination using eqn. 47 in relation to the dead time obtained with the Hansen and Andresen method (column 7), and the corresponding percentage errors (column 9), are also given.

In order to verify the proposed eqn. 47, results found in the literature for the dead time and retention time of methane (rows 1 and 2; columns 3 and 4), which were obtained under different conditions than those used in this work, were also used in comparison.

It can be seen from the error comparison (columns 6–9) that the values of $\bar{t}'_m$ calculated by eqn. 47 are much closer to $\bar{t}_m$ than to $\bar{t}_{CH_4}$.

The method of Guberska[18], whilst having no fundamental basis does make some attempt to correct methane retention to take into account the fact the methane is retained on stationary phases. However, the assumption that the correction term is independent of the type of stationary phase is unlikely to be valid. Further work on this type of modification of methane retention as an estimate of $t_m$ should take into account both the polarity of the stationary phase and its amount.

## 7. CALCULATION OF ADJUSTED RETENTION TIME WITHOUT ESTIMATION OF DEAD TIME

In two recent papers, Sevčik[25] and Sevčik and Löwentap[26] have described the accurate calculation of adjusted retention time by using the ratio, $A$, of the time differences for neighbouring $n$-alkanes in a homologous series. It is claimed that the procedures used to indicate the time of injection have a marked effect on gross retention times and this causes errors in $t_m$, either measured or calculated, even if the gas chromatographic conditions remain unchanged.

The concept of the calculation of adjusted retention time is based on the fact that the adjusted retention time is related only to the path of the compound through the column and not to contributions caused by the gas chromatograph or recording system. The adjusted retention time is therefore related to a non-retarded substance for which $I = 0$.

The method is best illustrated by reference to Fig. 3. The adjusted retention time of any peak in a chromatogram can be expressed as the sum of the time differences between neighbouring peaks. Assuming that $t'_{n+1}$, $t'_{n-1}$ and $t_{n+1}$, $t_n$, $t_{n-1}$ are the adjusted retention times and gross retention times of $n$-alkanes with carbon numbers $n+1$, $n$ and $n-1$, respectively, then

$$\Delta_{n+1} = t'_{n+1} - t'_n = t_{n+1} - t_n \tag{48}$$

$$\Delta_n = t'_n - t'_{n-1} = t_n - t_{n-1} \tag{49}$$

TABLE 9

COMPARISON OF DEAD TIMES OBTAINED BY THE METHODS OF HANSEN AND ANDRESEN[10] AND GUBERSKA[18] USING THE RETENTION TIME OF METHANE[18]

| *Column* | *Column temperature (°C)* | $\bar{t}_{CH_4}(n)$ *(mm)* | $\bar{t}_m(n)$ *(mm)* | $\bar{t}'_m$ *(mm)* | $t_{CH_4} - t_m$ *(mm)* | $\bar{t}'_m - \bar{t}_m$ *(mm)* | $\frac{(\bar{t}_{CH_4} - \bar{t}_m) \cdot 100}{\bar{t}_m}$ | $\frac{(\bar{t}'_m - \bar{t}_m) \cdot 100}{\bar{t}_m}$ |
|---|---|---|---|---|---|---|---|---|
| 10% Squalane (length 2 m) | 50 | 5.70 (4) | 5.40 (4) | 5.35 | +0.30 | −0.05 | +5.55 | −0.9 |
| 10% Carbowax 20M (length 3 m) | 130 | 11.10 (2) | 10.60 (2) | 10.40 | +0.50 | −0.20 | +4.59 | −1.83 |
| 20% DC-200 silicone oil (length 2.7 m) | 140, 154, 160, 174 | 12.78 (4) | 11.23 (4) | 11.05 | +1.55 | −0.18 | +13.80 | −1.60 |
| | 140, 154, 160, 174, 140 → 234 | 12.69 (5) | 11.35 (5) | 11.13 | +1.33 | −0.23 | +11.60 | −2.02 |
| | 140, 154, 160 | 12.78 (3) | 11.22 (3) | 11.21 | +1.56 | −0.01 | +14.00 | −0.09 |
| | 140 | 12.69 (10) | 10.81 (10) | 11.13 | +1.88 | +0.32 | +17.40 | +2.96 |
| | 140 → 234 at 4°/min | 12.47 (10) | 10.85 (10) | 10.94 | +1.62 | +0.09 | +14.70 | +0.83 |

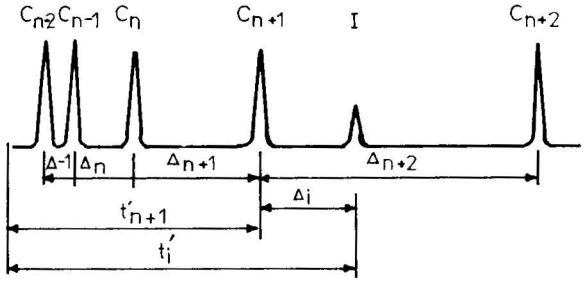


Fig. 3. Schematic chromatogram. $t'$ = adjusted retention time; $\Delta_n$ = time difference between the $n$-alkanes in row; $\Delta_i$ = time difference between the peaks not in row[25].

The adjusted retention time, $t'_{n+1}$, can then be expressed by

$$t'_{n+1} = \Delta_1 + \Delta_2 + \Delta_3 + \ldots + \Delta_n + \Delta_{n+1} \tag{50}$$

where $\Delta_1$ is the time difference between the elution of a substance with $I = 0$ and one with $I = 100$, etc.

From eqn. 50, the adjusted retention time can be calculated if all of the time differences are known. Then,

$$t_i = \sum_0^I \Delta_x \tag{51}$$

Experimentally, it was found that the ratio of neighbouring time differences, $\Delta_{n\pm x}$ in a homologous series is constant. Therefore,

$$\Delta_{n+x} = A\,\Delta_{n+x-1} \tag{52}$$

Hence a graph of the logarithm of the time difference, $\log \Delta_x$, *versus* $I_x$ is a straight line. Assuming eqn. 52 is valid for all members of the homologous series from $I = 1$, we can calculate the time differences, $\Delta_x$, between all peaks until we reach the start of separation where $I = 0$. It therefore follows that

$$\Delta_1 = \Delta_n A^{-(n-1)} \tag{53}$$

$$\Delta_2 = \Delta_n A^{-(n-2)} \tag{54}$$

$$\Delta_3 = \Delta_n A^{-(n-3)} \tag{55}$$

$$\Delta_n = \Delta_n A^{-(n-n)} = \Delta_n \tag{56}$$

$$\Delta_{n+1} = \Delta_n A^{-[n-(n+1)]} = \Delta_n A \tag{57}$$

$$\Delta_{n+2} = \Delta_n A^2 \tag{58}$$

$$\Delta_{n+x} = \Delta_n A_x \tag{59}$$

By summing these time differences, the equation for the adjusted retention time of the peak due to a molecule containing $n$ carbon atoms can be written as

$$t_n' = \Delta_n[A^{-(n-1)} + A^{-(n-2)} + \ldots + 1] \tag{60}$$

or

$$t_n' = \Delta_n \left[\frac{A^{n+1} - A}{A^{n+1} - A^n}\right] \tag{61}$$

which is expressed for ease of calculation as

$$t_n' = \Delta_n \left[\frac{1 - \left(\frac{1}{A}\right)^n}{1 - \left(\frac{1}{A}\right)}\right] \tag{62}$$

The validity of the method was determined by injection of a mixture of $C_7$–$C_{12}$ $n$-alkanes and the results are presented in Table 10. It is interesting that this method, which is not related to factors other than retention in the column, predicts a finite retention for methane.

TABLE 10

CALCULATION OF ADJUSTED RETENTION TIME USING A MIXTURE OF $n$-ALKANES[25]

| $C_n$ | $I$ | $\Delta$ | $A^*$ | $\log \Delta$ | $\log A$ | $t'$ |
|---|---|---|---|---|---|---|
| 12 | 1200 | | | | | 656.78 |
| | | 336.5 | | 2.5270 | | |
| 11 | 1100 | | 2.0644 | | 0.3148 | 320.10 |
| | | 163.0 | | 2.2122 | | |
| 10 | 1000 | | 2.0375 | | 0.3091 | 155.95 |
| | | 80.0 | | 1.9031 | | |
| 9 | 900 | | 2.0513 | | 0.3120 | 75.95 |
| | | 39.0 | | 1.5911 | | |
| 8 | 800 | | 2.0526 | | 0.3123 | 36.95 |
| | | 19.0 | | 1.2788 | | |
| 7 | 700 | | | | | 17.95 |
| | | 9.26 | | 0.96675 | | |
| 6 | 600 | | | | | 8.69 |
| | | 4.52 | | 0.65470 | | |
| 5 | 500 | | | | | 4.17 |
| | | 2.20 | | 0.34265 | | |
| 4 | 400 | | | | | 1.97 |
| | | 1.07 | | 0.03060 | | |
| 3 | 300 | | | | | 0.89 |
| | | 0.52 | | −0.28145 | | |
| 2 | 200 | | | | | −0.37 |
| | | 0.25 | | −0.59350 | | |
| 1 | 100 | | | | | 0.12 |
| | | 0.12 | | −0.90555 | | |
| 0 | 0 | | | | | 0 |

* $\bar{A} = 2.0514$.

The procedure described above allows the calculation of the adjusted retention times of a series of *n*-alkanes using the time differences between three successive *n*-alkanes. As the calculation is related to $I = 0$, the calculated adjusted retention times serve as fixed points on the time axis of the chromatogram. In relation to these points, the difference $\Delta_i$ can be measured and the adjusted retention time, and hence Kováts retention index, $I$, can be calculated for a substance as illustrated in Fig. 3.

In a later paper, Sevčik and Löwentap[26] produced further evidence for the validity of the method. They computed adjusted retention times based on four *n*-alkanes using four methods, as shown in Table 11. The methods of Sevčik[25] and Peterson and Hirsch[6] for $C_5$–$C_7$ *n*-alkanes give excellent agreement, However, the use of methane for an estimate of $t_m$ gives low values of $t'$, as expected. The use of $C_6$–$C_8$ *n*-alkanes when calculating $t_m$ gives higher values of adjusted retention times. This result is not unexpected, as Haken *et al.*[14] have shown that the choice of *n*-alkanes having high retention times can lead to errors in the estimation of $t_m$.

TABLE 11

RETENTION TIMES AND THEIR ESTIMATED STANDARD DEVIATIONS CALCULATED BY METHODS 1–4* FOR FOUR *n*-ALKANES[26]

The data are mean values of five repeated measurements.

| $C_8$ | $t$ (sec) | $t'$ (sec) | | | |
|---|---|---|---|---|---|
| | | *1* | *2* | *3* | *4* |
| 5 | 180.54 ± 0.195 | 46.04 ± 0.207 | 48.30 ± 0.283 | 50.44 ± 0.681 | 48.03 ± 0.205 |
| 6 | 242.04 ± 0.358 | 107.56 ± 0.358 | 109.82 ± 0.477 | 111.96 ± 0.618 | 109.66 ± 0.205 |
| 7 | 381.94 ± 0.699 | 247.44 ± 0.677 | 249.70 ± 0.778 | 251.84 ± 0.850 | 249.04 ± 0.390 |
| 8 | 696.58 ± 1.915 | 562.08 ± 1.914 | 564.34 ± 2.060 | 566.48 ± 1.660 | 564.25 ± 1.324 |

* Methods:
1 = methane retention as estimate of $t_m$;
2 = method of Peterson and Hirsch[6] using $C_5$–$C_7$ *n*-alkanes;
3 = method of Peterson and Hirsch[6] using $C_6$–$C_8$ *n*-alkanes;
4 = method of Sevčik[25] for adjusted retention times.

## 8. CONCLUSIONS AND RECOMMENDATIONS

The method used for estimation of mathematical dead time is dependent on the method of measurement of uncorrected retention times and the precision required in the calculated retention indices. Many laboratories now have minicomputers and microprocessors for data acquisition. These instruments can be used to determine $t_m$ most accurately by using at least four *n*-alkanes and the calculation method of Grobler and Balizs[12].

However, if stop-watch timing of peaks or measurement of chart distances is used, thereby involving off-line calculations, the methods using three consecutive *n*-alkanes can be employed. These involve the direct calculation method first described by Peterson and Hirsch[6] or the inferential method of Sevčik[25] and Sevčik and Löwentap[26].

The use of the retention of methane as an estimate of $t_m$ must be severely questioned, particularly for highly non-polar columns. Should this method be adopted,

some correction based on the amount[18] and nature of the stationary phase should be made.

## 9. SUMMARY

In order to determine the exact retention of compounds in gas chromatographic studies some method of determining the column dead time must be employed. This paper reviews direct measurement techniques using methane injection as well as mathematical determination of dead-time from retention data for *n*-alkanes. A critical evaluation of these procedures is made along with recommendations concerning the choice of evaluation method to be adopted by the chromatographer.

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# MASS, CHARGE, MOMENTUM AND ENERGY CONSERVATION IN ELECTROPHORETIC FRACTIONATION SYSTEMS*

G. COLANTUONI

*Department of Chemical Engineering, University of Virginia, Charlottesville, Va. 22901 (U.S.A.)*

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## 1. INTRODUCTION

As an analytical and small-scale preparative technique (*i.e.*, at the milligram to microgram level), electrophoresis has acquired a prominent status among modern physico-chemical separation methods on account of its versatility, ease of implementation and, most importantly, its extremely high resolving power. Since the adaptation by Tiselius[1] in 1937 of the moving-boundary method for the fractionation and analysis of complex protein mixtures, progress has accelerated rapidly. Numerous applications to chemical, biological, biochemical and medical systems have led to a significant number of important discoveries and to the synthesis of other electrophoretic apparatus with improved resolving capacity and operating characteristics[2,3].

As a large-scale preparative technique, on the other hand, electrophoresis has not achieved the same degree of success. Because of the high non-linear interactions among the governing mass, charge, momentum and energy transport processes, straightforward scale-up procedures are not effective or may be completely inapplicable. The main difficulty stems from the Joule heating mechanism, which is inherently present in any system through which an electric current is conducted. As the physical dimensions of the apparatus are increased, it becomes increasingly

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difficult to remove this internally dissipated energy, which generally varies proportionally to the square of the local electric field strength or, equivalently, to the square of the current. An attempt to reduce the Joule heat effect, then, would necessarily have to emanate from either an increase in the cooling capacity of the system (which has the disadvantages of spatially varying temperature distributions and enhanced instrumentation complexity) or from a reduction in the current or field strength. Buffer systems of the appropriate ionic strength may be selected in order to accomplish the latter. However, it is evident that this is in direct contrast to the requirements of rapid and high-resolution separations. The optimization criteria are consequently apparent: maximize the Joule heat dispersion and transfer with the system's surroundings, minimize the thermal convection currents and maximize the electric field strength in order to achieve rapid fractionations with the best possible resolution.

It must be further pointed out, however, that even with the attainment of a successful solution to this optimization problem, other significant practical difficulties remain. An example is the recovery of fractionated components of mixtures in polyacrylamide gel electrophoresis.

In this review, previously reported investigations are summarized, and the fundamental mass, momentum and energy transport relationships applicable to multi-component flowing mixtures are presented. These relationships are subsequently limited to macromolecular mixtures with non-interacting components and, on dimensionally analysing the resulting equations, two dimensionless parameters are found, one of which is associated with the mass transport mechanism ($E_f$) and the other with the thermal energy dissipation mechanism ($Je$).

## 2. ANALYSIS OF PREVIOUS INVESTIGATIONS

The combined effect of the various transport processes present in electrophoretic fractionation systems are considered from two standpoints: one at the single particle or microscopic level, where the primary objective is to determine the interactions among the uniformly applied electrostatic field, the composition of the electrolytic solution, the structure of the electrical double layer and the induced electrophoretic mobility, and the other at the macroscopic level where quantitative expressions are sought for the concentration distributions of the macromolecular mixture components and the associated velocity, temperature and electrical potential distribution.

The behavior of single isolated particles has been studied extensively. Smoluchowski[4,5] at the turn of the century, derived an expression for the electrophoretic mobility of a non-conducting particle with a small double layer thickness when compared with the particle's radius of curvature throughout its surface. A decade later, Hückel[4,6] obtained a similar expression applicable to small spherical particles for which the ratio of radius of curvature to double layer thickness is small, and the frictional resistance on the particle by the medium is given by Stokes' law. Subsequently, Henry[7,8], Booth[7,9] and Overbeek and co-workers[7,10] generalized the above theories by incorporating into the analysis the four principal forces believed to be acting on a particle in a stationary state of electrophoretic motion. These included the force exerted by the electrostatic field on the charge of the particle, the Stokes'

frictional resistance, a retardation force due to the influence of the electrostatic field on the ions in the diffuse double layer and a relaxation force which attempts to restore the original symmetrical configuration of the electrical double layer. The specific form of the electrophoretic mobility relationships obtained by these investigators are given in a later section. Other extensions to non-spherical particles and to systems with intra-particle interactions are also available[11–13] and can be consulted for further details on the diversity and complexity of the phenomena.

At the macroscopic level, many investigations have been conducted to elucidate the importance of the various factors that affect electrophoretic separations, including buffer composition, ionic strength, pH, temperature, electric field intensity, fractionation time and zone stability. Analytical studies addressed to quantitative descriptions of such factors, however, have been scarce and mostly confined to isothermal systems with constant physical parameters and mobilities. Cann and Goad[14] considered mass transport in non-reacting and reversibly reacting systems under isothermal conditions and constant electrostatic field strength. Typically, they stipulated that the components of the macromolecular mixture interact either through acid–base dissociative reactions of the type

$$PH_i \rightleftarrows PH_{i-1} + H^+$$

or through associative reactions of the form

$$P + iHA \rightleftarrows P(HA)_i$$

with each species possessing a characteristic net charge, electrophoretic mobility and diffusion coefficient. Integrating these chemical reactions with diffusion and electromigration processes, they derived mass conservation conditions as

$$\frac{\partial C_i}{\partial t} = \frac{\partial}{\partial x}\left(D_i \frac{\partial C_i}{\partial x}\right) - \frac{\partial(\mu_i E\, C_i)}{\partial x} + r_i \tag{1}$$

which they solved analytically or numerically to arrive at the electrophoretic patterns. Following a similar procedure for isotachophoresis, Coxon and Binder[15] also considered systems modeled by the above differential relationship. The chemical reaction terms were absent from their analysis, however, and the applied electric force followed Poisson's equation:

$$\nabla \cdot E = \frac{z}{\varepsilon} \Sigma_i C_i$$

More recently, Ries *et al.*[16] extended the work of Philpot[17] and described quantitatively the combined effects of diffusion, convection and electromigration under isothermal conditions and constant transport properties, including electrophoretic mobilities. The bulk velocity profile in the planar, forced-flow electrophoretic system was taken as parabolic with diffusion in all three coordinate directions, *i.e.*, the transient distribution of the mixture constituents was stated as

$$\frac{\partial C_i}{\partial t} + v_x \frac{\partial C_i}{\partial x} - \mu \frac{\partial C_i}{\partial z} = D_i\left(\frac{\partial^2 C_i}{\partial x^2} + \frac{\partial^2 C_i}{\partial y^2} + \frac{\partial^2 C_i}{\partial z^2}\right) \tag{2}$$

with the appropriate boundary and initial conditions. This model was recognized by the authors as the slit-flow analog of the classical Taylor dispersion problem[18]. Their analytical solution reflected quantitatively a significantly large Taylor dispersion dependence, and qualitatively a decrease in fractionation efficiency with increasing electromigration velocities and Peclet numbers. Other investigations addressed to isothermal systems described by convective–diffusion differential relationships of the above type include those by Weiss and Rodbard[19] (pore gradient electrophoresis), Lee and Lightfoot[20] and Krishnamurthy and Subramanian[21] (field-flow fractionation), and Ries and Lightfoot[22] and Lee *et al.*[23] (ultrafiltration and electrophoresis).

Quantitative studies of temperature distributions within electrophoretic cells have been confined to one-dimensional conductive systems with no convective energy transport and constant density, specific heat and thermal conductivity:

$$\frac{\partial T_E}{\partial t} = k_E \nabla_{e_1}^2 T_E + Q_E(T_E)/\varrho_E C_{pE} \text{ (electrolytic solution)}$$

$$\frac{\partial T_w}{\partial t} = k_w \nabla_{e_1}^2 T_w \text{ (cell wall)}$$

Martin and Everaerts[24], with negligible cell wall effects, analysed the parabolic distributions which arise under additional constraints of constant electrical conductivity and uniform wall temperature. Coxon and Binder[25], Hinckley[26] and Brown and Hinckley[27] treated cells of both circular and rectangular cross-section under transient and stationary conditions. The energy source was held constant and the electrical conductivity was taken to vary linearly with cell temperature, *i.e.*,

$$Q_E(T_E) = E^2 \sigma_E^\circ (1 + \alpha T_E)$$

## 3. FUNDAMENTAL TRANSPORT RELATIONSHIPS

The application of the fundamental principles of mass, momentum and energy conservation to a flowing mixture, either via the arbitrary control volume approach or the more classical theory[28,29], gives rise to the following continuity, motion and energy equations, respectively:

Overall continuity:

$$\frac{\mathrm{D}\varrho}{\mathrm{D}t} + \varrho(\nabla \cdot v) = 0 \tag{3}$$

Component continuity:

$$\frac{\mathrm{D}\varrho_i}{\mathrm{D}t} = -\nabla \cdot j_i - \varrho_i(\nabla \cdot v) + r_i \; (i = \text{fluid mixture components}) \tag{4}$$

Motion:

$$\varrho \frac{\mathrm{D}v}{\mathrm{D}t} = -\nabla p - \nabla \cdot \tau + \varrho g + \Sigma_i \varrho_i \hat{F}_i \tag{5}$$

Energy:

$$\varrho \frac{DU}{Dt} = -\nabla \cdot q - (pI + \tau)/\nabla v + \Sigma_i j_i \cdot \hat{F}_i$$

For purposes of thermal systems analysis, the last expression can be more conveniently rewritten as

$$\varrho C_v \frac{DT}{Dt} = -\nabla \cdot q - (pI + \tau)/\nabla v + \delta \nabla \cdot v + \\ + \Sigma_i[(\nabla \cdot j_i) + r_i]\,[\overline{U}_i/M_i + \delta \overline{V}_i/M_i] + \Sigma_i j_i \cdot \hat{F}_i \tag{6}$$

with

$$\delta = p - T(\partial p/\partial T)$$

Following Hirschfelder *et al.*[28], the mass flux of the individual species can be broken down into a contribution due to the concentration (ordinary diffusion), temperature (thermal diffusivity, the Soret effect) and pressure (pressure diffusion) gradients present locally within the fluid mixture, and a contribution due to the externally applied forces (forced diffusion):

$$j_i = j_i^{(g)} + j_i^{(F)} = j_i^{(c)} + j_i^{(T)} + j_i^{(p)} + j_i^{(F)} \tag{7}$$

Similarly, the thermal energy flux can be broken down into contributions emanating from ordinary thermal conduction and energy transport due to concentration gradients (the Dufour effect), molecular diffusion and radiation:

$$q = q^{(T)} + q^{(c)} + q^{(d)} + q^{(r)} \tag{8}$$

The last term in eqn. 5 represents the momentum imparted on the fluid mixture due to the influence of the externally applied forces, and the last term in eqn. 6 corresponds to the work done (energy dissipated) by the migrating molecules in overcoming the external forces.

## 4. ELECTROPHORETIC TRANSPORT RELATIONSHIPS FOR NON-INTERACTING MACROMOLECULAR MIXTURES

General expressions for the mass and energy flux components included in eqns. 7 and 8 have been obtained by Hirschfelder *et al.*[28] through the thermodynamics of irreversible processes and the Onsager reciprocal relations approach. Of such fluxes, specifically as related to electrophoretic systems, mass fluxes resulting from temperature and pressure diffusion mechanisms, and also energy fluxes resulting from concentration, molecular diffusion and radiation mechanisms, are neglected. Consequently, as a function of the corresponding transport coefficients, the following expressions hold:

Ordinary diffusion:

$$j_i^{(c)} = \frac{C^2}{\varrho RT} \sum_j M_i M_j D_{ij} \left[ x_j \sum_{\substack{k \\ k \neq j}} \left( \frac{\partial \bar{G}_j}{\partial x_k} \right)_{T,P,x_{s \neq j,k}} \right] \nabla x_k$$

Forced diffusion:

$$j_i^{(F)} = -\frac{C^2}{\varrho RT} \cdot \sum_j M_i M_j D_{ij} \left[ x_j M_j \left( \hat{F}_j - \sum_k \frac{\varrho_k}{\varrho} \cdot \hat{F}_k \right) \right]$$

Heat conduction:

$$q^{(T)} = -k \nabla T$$

In this form these expressions are rather unmanageable, as the partial molal Gibbs free energies are temperature, pressure and composition dependent, and the transport coefficients, $D_{ij}$, are multi-component diffusion parameters whose values are difficult to obtain either theoretically or experimentally. Note that in addition, generally $D_{ij} \neq D_{ji}$ for other than binary systems.

To arrive at a quantitatively suitable set of transport equations, it will be assumed that the interactions along the macromolecular mixture constituents are negligible and that, consequently, the mixture behaves ideally. For each constituent, then, the combined binary form of the concentration and forced diffusion flux is

$$J_i = -D_i \nabla C_i + \left( \frac{D_i C_i}{RT} \right) F_i \tag{9}$$

Also, if viscous dissipation mechanisms and pressure variations are insignificant, eqn. 6 may be transformed into

$$\varrho C_v \frac{\mathrm{D}T}{\mathrm{D}t} = \nabla \cdot (k \nabla T) + \Sigma_i \bar{U}_i r_i + \Sigma_i J_i \cdot F_i \tag{10}$$

The functionality between the externally applied electric forces and the net migration imparted on the individual components of the electrolytic solution must now be established. Pertaining to the ionic species, they may be viewed as point charges and, consequently,

$$F_i = z_i F (-\nabla \Phi)$$

Pertaining to the charged macromolecular components, on the other hand, the resultant force is directly dependent on the character of the particles' double layer and on the magnitude of the zeta potential. In its simplest form, assuming the presence of single, rigid and non-conducting particles with uniform dielectric constants and solution viscosity coefficients[4,10], the zeta potential can be expressed as

$$\zeta = \frac{Q}{\varepsilon a} - \frac{Q}{\varepsilon (a + 1/\lambda)}$$

For large ratios of radius of curvature to diffuse double layer thickness ($\lambda a$),

$$\zeta = (3/2)Q/\varepsilon a$$

and, on equating the electrical and viscous forces affecting the particle, Smoluchowski's equation is obtained for the particle electrophoretic mobility:

$$v = \zeta\varepsilon/4\pi\mu$$

For small ratios of the radius of curvature to the diffuse double layer thickness,

$$\zeta = Q/\varepsilon a$$

Similarly, if only forces of electrical and frictional origin are accounted for, Hückel's equation derives:

$$v = \zeta\varepsilon/6\pi\mu$$

Incorporating surface conductivity distributions, solvent retardation effects and diffuse double layer asymmetries into the analysis, Henry[8], Booth[9] and Overbeek and co-workers[7,10] derived generally flexible zeta potential relationships of the form

$$\zeta = \frac{Q}{\varepsilon a} \mathrm{f}(\lambda a)$$

and for the electrophoretic mobility

$$v = \frac{\zeta\varepsilon}{6\pi\mu} \mathrm{f}(\lambda a)$$

where both analytical and graphical representations of $\mathrm{f}(\lambda a)$ for Henry's equation (relaxation effects not included and $\lambda a$ small or large) and the more general case can be found in the references cited.

Generally then, the force exerted on the components of the electrolytic medium will be

$$F_i = \begin{cases} z_i \mathrm{F}(-\nabla\Phi) & \text{ionic species} \\ [\zeta\varepsilon a \mathrm{f}(\lambda \mathrm{a})]_i \tilde{\mathrm{N}}(-\nabla\Phi) & \text{charged particles or macromolecules} \end{cases}$$

$$= \gamma_i(-\nabla\Phi)$$

Consequently, the component continuity and energy equations result in

$$\frac{\mathrm{D}C_i}{\mathrm{D}t} = \nabla \cdot (D_i \nabla C_i) + \nabla \cdot \left(\frac{D_i C_i \gamma_i}{RT}\right)\nabla\Phi - C_i(\nabla \cdot v) + r_i \tag{11}$$

$$\varrho C_v \frac{\mathrm{D}T}{\mathrm{D}t} = \nabla \cdot (k\nabla T) + \Sigma_i(D_i\gamma_i\nabla C_i)\nabla\Phi + (\Sigma_i D_i C_i \gamma_i^2/RT) \tag{12}$$
$$(\nabla\Phi \cdot \nabla\Phi) + \Sigma_i \overline{U}_i r_i$$

In addition to mass, momentum and energy conservation, electrophoretic systems must also uphold the electrical charge conservation principle. To arrive at an expression for such a principle, eqn. 11 is formally multiplied by the net particle charge, $\gamma_i$, and upon summing over all mixture components we obtain

$$\frac{\partial(\Sigma_i \gamma_i C_i)}{\partial t} = \nabla \cdot (\Sigma_i D_i \gamma_i \nabla C_i) + \nabla \cdot [(\Sigma_i D_i C_i \gamma_i^2/RT)\nabla \Phi] - \nabla \cdot (\Sigma_i \gamma_i C_i v) = \nabla \cdot (\Sigma_i \gamma_i N_i) = \nabla \cdot i \tag{13}$$

where all chemical reactions have been assumed to be electrically balanced. If the medium is said to be electrically neutral, *i.e.*,

$$\Sigma_i \gamma_i C_i = 0 \tag{14}$$

then, after some rearrangement, eqn. 13 yields an expression for the electrical potential:

$$\nabla^2 \Phi = - \frac{\nabla \cdot (\Sigma_i D_i \gamma_i \nabla C_i) + [\nabla(\Sigma_i D_i C_i \gamma_i^2/RT)] \cdot \nabla \Phi}{\Sigma_i D_i C_i \gamma_i^2/RT} \tag{15}$$

The following observations can be made:

(i) For the analysis of electrochemical systems composed of ionic species only, the component continuity expression (eqn. 11) reduces to the commonly encountered multi-component diffusion equation[30], for which the distribution of migrating species in dilute solutions is expressed as

$$\frac{\partial C_i}{\partial t} + v \cdot \nabla C_i = \nabla \cdot (D_i \nabla C_i) + \nabla \cdot (u_i C_i z_i F \nabla \Phi) + r_i$$

where the fluid mixture is assumed to be incompressible and use is made of the Nernst–Einstein relationship:

$$u_i = D_i/RT$$

(ii) The second and third terms in eqn. 12 constitute contributions due to Joule heating. Under more familiar circumstances of uniform compositions and no thermal energy exchange due to chemical reactions, this energy transport equation is simplified to give

$$\varrho C_v \frac{DT}{Dt} = \nabla \cdot (k \nabla T) + \underbrace{\frac{i \cdot i}{\Sigma_i D_i C_i \gamma_i^2/RT}}_{\text{Joule heating}}$$

(iii) The charge density movement and the flowing current density induce electric and magnetic fields which, according to Maxwell's equations, follow

$$\varepsilon \frac{\partial \Phi_E}{\partial t} + i = \nabla \times B$$

$$\Phi_E = \nabla \cdot E = (1/\varepsilon) \Sigma_i \gamma_i C_i$$

If the characteristic time constants for the system are such that the electrical processes respond significantly more quickly to temporal variations relative to the co-existing mass, momentum and thermal energy processes, then a pseudo-steady-state approximation with respect to $\Phi_E$ indicates

$$\frac{\partial(\Sigma_i \gamma_i C_i)}{\partial t} = \nabla \cdot i = \nabla \cdot (\nabla \times B) = 0$$

Consequently, this procedure in general is not equivalent to invoking the electroneutrality condition as expressed by eqn. 14. In isotachophoresis with no convective currents present, this indeed holds true, as indicated by Moore[31].

(iv) It is frequently stated[30] that the electroneutrality assumption does not imply Laplace's equation for the electrical potential. This is evident from eqn. 15, which for isothermal and uniform composition systems reduces to

$$\nabla^2 \Phi = 0$$

## 5. DIMENSIONAL ANALYSIS OF ELECTROPHORETIC TRANSPORT EQUATIONS

For purposes of reducing the total number of relevant dimensional system parameters to independent subsets of dimensionless groups, the electrophoretic transport eqns. 3, 5, 11, 12 and 15 will now be simplified (to reflect the predominant processes taking place in homogeneous and heterogeneous and in continuous and discontinuous electrophoretic systems) and cast in dimensionless form.

### *5.1. Convective electrophoretic systems*

Forced-flow and continuous zone electrophoretic apparatus operate under incompressible flow conditions and with electrolytic fluid mixtures of constant viscosity. If electroosmotic effects are considered not to influence the development of the convective flow field, then, in dimensionless form, the governing transport relationships include the following:

Overall continuity:

$$\nabla \cdot v = 0$$

Motion:

$$\frac{\partial v}{\partial t} + (v \cdot \nabla)v = -\nabla p - \left(\frac{1}{Re}\right)\nabla \cdot \tau + \left(\frac{1}{Fr}\right)g$$

Component continuity:

$$\frac{\partial C_i}{\partial t} + v \cdot \nabla C_i = \left(\frac{1}{ReSc}\right)\nabla \cdot (\delta_i \nabla C_i) + \left(\frac{E_f}{ReSc}\right)\nabla \cdot [(\delta_i C_i \gamma_i / T)\nabla \Phi]$$

Energy:

$$\frac{\partial T}{\partial t} + v \cdot \nabla T = \left(\frac{1}{RePr}\right)\nabla \cdot (k \nabla T) + \left(\frac{Je}{RePr}\right)[\Sigma_i \delta_i \gamma_i \nabla C_i]\nabla \Phi + \\ + \left(\frac{JeE_f}{RePr}\right)[\Sigma_i \delta_i C_i \gamma_i^2 / T](\nabla \Phi \cdot \nabla \Phi)$$

Charge:

$$\nabla^2 \Phi = -\frac{\frac{1}{E_f}\nabla \cdot (\Sigma_i \delta_i \gamma_i \nabla C_i) + (\nabla \Sigma_i \delta_i C_i \gamma_i^2 / T) \cdot \nabla \Phi}{\Sigma_i \delta_i C_i \gamma_i^2 / T}$$

The Reynolds, Schmidt, Froude and Prandtl numbers follow standard nomenclature (see *Definitions* in Section 7)[29,30], and the remaining dimensionless parameters are defined as

$$E_f = \gamma_0 \Phi_0 / RT_0$$

and

$$Je = D_0 C_0 \gamma_0 \Phi_0 / k_0 T_0$$

$E_f$ represents the ratio of mass transport due to the externally applied electric force to mass transport due to ordinary diffusion, and $Je$ represents the ratio of thermal energy dissipated due to the externally applied electric force to thermal energy transport by conduction.

*5.2. Heterogeneous electrophoretic systems*

For electrophoretic systems with supporting media and a stagnant electrolytic solution (*i.e.*, $v = 0$), such as polyacrylamide gel electrophoresis with continuous or discontinuous buffer regions, the corresponding set of dimensionless transport relationships has the following form:

Component continuity:

$$\frac{\partial C_i}{\partial t} = \nabla \cdot (\delta_i \nabla C_i) + E_f \nabla \cdot [(\delta_i C_i \gamma_i / T)\nabla \Phi]$$

Energy:

$$\frac{\partial T}{\partial t} = Le \nabla \cdot (k \nabla T) + LeJe[\Sigma_i \delta_i \gamma_i \nabla C_i]\nabla \Phi + LeJeE_f[\Sigma_i \delta_i C_i \gamma_i^2 / T](\nabla \Phi \cdot \nabla \Phi)$$

*5.3. Electrophoretic systems with natural convection*

Homogeneous electrophoretic systems with no externally applied pressure gradients develop natural convection currents which, when included in the analysis of the electrophoretic transport relationships, give rise to the following:

Overall continuity:

$$\nabla \cdot v = 0$$

Motion:

$$\frac{\partial v}{\partial t} + (v \cdot \nabla)v = -\nabla \cdot \tau - GrgT$$

Component continuity:

$$\frac{\partial C_i}{\partial t} + v \cdot \nabla C_i = \left(\frac{1}{Sc}\right)\nabla \cdot (\delta_i \nabla C_i) + \left(\frac{E_f}{Sc}\right)\nabla[\delta_i C_i \gamma_i / T)\nabla \Phi]$$

Energy:

$$\frac{\partial T}{\partial t} + v \cdot \nabla T = \left(\frac{1}{Pr}\right)\nabla \cdot (k \nabla T) + \left(\frac{Je}{Pr}\right)\Sigma_i \delta_i \gamma_i \nabla C_i]\nabla \Phi + \\ + \left(\frac{JeE_f}{Pr}\right)[\Sigma_i \delta_i C_i \gamma_i^2 / T](\nabla \Phi \cdot \nabla \Phi)$$

It may be observed that, because of the electroneutrality condition, the charge conservation eqn. 11 is explicitly invariant to changes in fluid velocity distributions. Nevertheless, implicitly, it remains affected by such velocity distributions through the concentration gradients of the mixture components.

## 6. CONCLUSION

As large-scale preparative apparatus, electrophoretic systems have been severely limited by Joule heating and the difficulties encountered in its exchange with the system's surroundings. Rapid and highly demarcated fractionations are sought with minimal energy utilization. This, however, is necessarily accompanied by large localized voltage gradients and electrical current densities that enhance electrical energy dissipation, natural convection currents and broadening of concentration distributions. Operating parameters for optimal separations must then be obtained through an integrated analysis of the prevailing transport processes within the electrophoretic cell. To this end, mass, momentum, energy and charge conservation relationships (eqns. 3, 5, 11, 12 and 15) have been derived for electrophoretic fractionation systems. Fundamental simplifying assumptions include negligible mass transport due to thermal and pressure gradient effects, negligible thermal energy transport due to concentration gradients, molecular diffusion and thermal radiation, infinitely dilute macromolecular mixtures with no interactions among their constituents (*i.e.*, pseudo-binary solutions), ionic species considered as point charges and macromolecular species taken as non-conducting charged particles each surrounded

by a characteristic electrical double layer, and electrically neutral electrolytic mixtures.

In view of the high coupling and complexity of the derived conservation relationships, their complete solutions are seldom realized. Consequently, dimensional analysis was utilized to arrive at dimensionless parameters that can now be used to simplify such differential relationships, and to establish empirical correlations for analysis and design purposes. The resulting dimensionless parameters are $E_f$ (the ratio of mass transport due to the applied electrical force to mass transport due to ordinary diffusion) and $Je$ (the ratio of thermal energy dissipated due to the applied electrical force to thermal energy transport by conduction).

## 7. SYMBOLS

| | |
|---|---|
| $a$ | particle radius |
| $B$ | magnetic field |
| $C$ | local concentration, total concentration |
| $C_p, C_v$ | specific heat |
| $D$ | binary diffusion coefficient |
| $D_{ij}$ | multi-component diffusion coefficients |
| $\frac{\mathrm{D}}{\mathrm{D}t}$ | substantial time derivative |
| $E$ | voltage gradient |
| $E_f$ | ratio of mass transport due to externally applied electrical force to mass transport due to ordinary diffusion |
| $F$ | external force |
| $\hat{F}_i$ | applied external force per unit mass of component $i$ |
| $Fr$ | Froude number |
| $f$ | zeta potential relationship describing particle–electrical double layer forces |
| $g$ | gravitational force |
| $\bar{G}$ | partial molal Gibbs free energy |
| $Gr$ | Grashof number |
| HA | uncharged solution constituent |
| $H^+$ | hydrogen ion |
| $I$ | unit tensor |
| $i$ | current density |
| $j$ | mass flux with respect to fluid mass average velocity |
| $J$ | mass flux with respect to fluid molal average velocity |
| $Je$ | ratio of thermal energy dissipated due to externally applied electrical force to thermal energy transport by conduction |
| $k$ | thermal conductivity |
| $Le$ | Lewis number |
| $M$ | molecular weight |
| $N$ | mass flux with respect to stationary coordinate system |
| $\tilde{\mathrm{N}}$ | Avogadro's number |
| P | protein molecule; macromolecular ion |
| $\mathrm{P(HA)}_i$ | protein–uncharged solution constituent complex |
| $\mathrm{PH}_i$ | macromolecular ion |

| | |
|---|---|
| $Pr$ | Prandtl number |
| $P$ | static pressure |
| $Q$ | thermal energy source; particle charge |
| $q$ | energy flux |
| $R$ | gas law constant |
| $r$ | reaction rate |
| $Re$ | Reynolds number |
| $Sc$ | Schmidt number |
| $T$ | temperature |
| $t$ | time |
| $U$ | internal energy |
| $\overline{U}$ | partial molal internal energy |
| $u$ | Nernst–Einstein mobility |
| $\overline{V}$ | partial molal volume |
| $v$ | velocity |
| $v_x$ | bulk velocity in $x$-direction |
| $x$ | molar fraction; spatial coordinate |
| $y$ | spatial coordinate |
| $z$ | ionic charge; spatial coordinate |

*Greek symbols*

| | |
|---|---|
| $\alpha$ | temperatue coefficient |
| $\gamma$ | charge per gram-mole; charge per gram-ion |
| $\nabla^2_{e_1}$ | one-dimensional Laplacian operator in $e_1$ direction |
| $\delta$ | dimensionless binary diffusivity coefficients |
| $\varepsilon$ | dielectric constant |
| $\zeta$ | zeta potential |
| $\lambda a$ | ratio of radius of curvature to diffuse double layer thickness |
| $\lambda^{-1}$ | thickness of diffuse double layer |
| $\mu$ | mobility; fluid viscosity |
| $\nu$ | electrophoretic mobility |
| $\varrho$ | density |
| $\sigma^0_E$ | electrical conductivity at a nominal temperature $T_0$ |
| $\tau$ | stress tensor |
| $\Phi$ | electrical potential |
| $\Phi_E$ | electric flux |

*Subscripts*

| | |
|---|---|
| $E$ | electrolytic solution |
| $i$ | solution constituent |
| 0 | characteristic quantities |
| $w$ | cell wall |

*Superscripts*

| | |
|---|---|
| $(c)$ | concentration |
| $(d)$ | thermal diffusion |
| $(r)$ | radiation |
| $(T)$ | temperature, thermal |
| $(F)$ | external force |
| $(p)$ | pressure |

*Definitions*

$$\text{Reynolds number} = \frac{\text{convective forces}}{\text{viscous forces}}$$

$$\text{Froude number} = \frac{\text{convective forces}}{\text{gravitational forces}}$$

$$\text{Grashof number} = \frac{(\text{buoyancy forces})\,(\text{convective forces})}{(\text{viscous forces})^2}$$

$$(\text{Reynolds number})\,(\text{Prandtl number}) = \frac{\text{convective energy transport}}{\text{conductive energy transport}}$$

$$(\text{Reynolds number})\,(\text{Schmidt number}) = \frac{\text{convective mass transport}}{\text{ordinary diffusion mass transport}}$$

$$\text{Lewis number} = \frac{\text{conductive energy transport}}{\text{ordinary diffusion mass transport}}$$

## 8. SUMMARY

Electrophoretic fractionation systems have been used extensively in chemistry, biology, biochemistry and medicine. However, such applications have been confined to analytical-scale systems, with limited extension to preparative or large-scale engineering systems. The major difficulties encountered in the analysis and design of such systems are pointed out, and mass, momentum, energy and charge conservation relationships are derived that incorporate the effects of the composition of the electrolytic solution on the microscopic structure of the electrical double layer and the associated macroscopic concentration, velocity, temperature and electrical potential distributions. Two dimensionless parameters are found in the analysis of electrophoretic fractionation systems: the ratio of mass transport due to the applied electrical force to mass transport due to ordinary diffusion, and the ratio of thermal energy dissipated due to the applied electrical force to thermal energy transport by conduction. These, together with other normally occurring dimensionless transport parameters such as the Reynolds, Schmidt, Froude, Prandtl, Nusselt and Lewis numbers, are essential in establishing theoretical and empirical correlations for analysis and design purposes.

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# SINTERED THIN-LAYER CHROMATOGRAPHY*

TAMOTSU OKUMURA
*Shionogi Research Laboratory, Shionogi & Co. Ltd., Fukushima-ku, Osaka 553 (Japan)*
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## 1. INTRODUCTION

During the past 15 years, thin-layer chromatography (TLC) has established itself as one of the most powerful, exact and useful tools for experimental chemists. The techniques involved are relatively simple and applicable to the separation of both volatile and non-volatile substances, and the equipment required is inexpensive. TLC

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offers, in many instances, almost the only practical solution to difficult separation and purification problems.

Recently, a number of authors have reviewed the newer developments in TLC[1–5], including the combination of TLC with other techniques, for example vapour-phase chromatography.

Various kinds of pre-coated plates and sheets are now commercially available[6,7]. However, these plates become charred when sprayed with corrosive reagents or heated at high temperatures, owing to the presence of organic binders[8] such as starch, poly(vinyl alcohol), polymethacrylates and polyolefins, which are added to the thin layer to confer good abrasion resistance.

The author has devised sintered-glass TLC plates, which are acid-resistant pre-coated thin-layer plates containing inorganic adsorbents such as silica gel, alumina, Kieselguhr, Florisil, titania and magnesia, fixed with sintered soda-lime or borosilicate glass. These sintered-glass plates are highly porous and allow the developing solvents to penetrate quickly, showing that the nature of the binder does not affect the adsorption process that occurs on the surface of the adsorbents. They contain no organic binders and do not become charred under drastic conditions, such as heating at high temperatures after spraying with corrosive reagents, *e.g.*, concentraded sulphuric acid, chromic acid mixtures, trichloroacetic acid and antimony trichloride. The sprayed plates can be regenerated readily by soaking in cleaning solutions, washing with water and reactivation by heating. The author subsequently prepared polyolefin sintered sheets using, in addition to the inorganic adsorbents mentioned above, organic ion-exchange resins, cellulose ion exchangers, synthetic porous polymers, dextran gels and cellulose as the adsorbent, and microparticulate polyolefin as the binding agent. Further, in an attempt to study quantitative thin-layer stick chromatography aided by flame-ionization detection scanning, the author prepared various kinds of sintered-glass rods using silica gel and alumina as the adsorbent and soda-lime, borosilicate and ceramic glass powder as the binder. Thin-layer chromatography performed using these sintered plates, sheets and rods is termed here "sintered thin-layer chromatography".

This review describes the methods of preparing various kinds of sintered plates, sheets and rods, and presents the results of chromatographic separations on these materials of a variety of organic and inorganic compounds such as lipids, steroids, alkaloids, vitamins, amino acids, peptides, sugars, antibiotics, catecholamines, barbiturates, polychlorinated biphenyls, pesticides, tranquillizers, food dyes, organic phosphates, sulphates, nitrates, metal ions, halogen ions and oxyanions. Based on scanning electron microscopic data, the mechanism of the "welding" between the adsorbent, binder and support in the process of the formation of sintered thin layers is discussed. Also presented are data obtained from thermal analysis [thermogravimetric–differential thermal analysis (TG–DTA) and thermogravimetric–differential scanning calorimetry (TG–DSC)], scanning electron microscopy and the determination of various physical properties of the adsorbents, showing the thermal stability of porous inorganic and organic adsorbents under the welding conditions, that is, heating at 400–770° for several minutes.

## 2. FUNDAMENTAL STUDIES ON SINTERED THIN-LAYER CHROMATOGRAPHY

### *2.1. Preparation of and thin-layer chromatography on sintered-glass plates with porous inorganic adsorbents*

#### *2.1.1. Materials and apparatus*

*2.1.1.1. Glass powder*[9]. Broken soda-lime glass or borosilicate glass was ground in a ball mill, screened with a 200-mesh sieve and fractionated by sedimentation in water. This glass powder had a particle size of 1–10 $\mu$m, which is slightly smaller than that of silica gel for TLC use.

*2.1.1.2. Preparation of various sintered-glass plates.*

*2.1.1.2.1. Silica gel*[10]. A mixture of 1 part of silica gel for TLC and 2–5 parts of the prepared glass powder was suspended in a solvent such as benzene, chloroform, acetone, ethyl acetate, methanol, ethanol or water. Of these solvents, acetone was the most effective for the preparation of porous sintered plates[11]. The slurry was spread on a soda-lime glass plate in the usual manner and air dried. The layer was then heated in an electric furnace at 470–770° for several minutes to obtain a silica gel–sintered glass layer. The glass powder had to be sintered without melting the silica gel in order to protect the chromatographic activity of the layer.

*2.1.1.2.2. Alumina*[12] *and Kieselguhr*[13]. A mixture of 1 part of alumina or Kieselguhr for TLC and 1–6 parts of the glass powder was suspended in the solvents mentioned above. The welding procedure was the same as with the silica gel sintered plate.

*2.1.1.2.3. Florisil, titania, zinc oxide and magnesia*[14]. For the preparation of Florisil, titania and zinc oxide sintered plates, mixtures of 1 part of the adsorbent and 3 parts of the glass powder were used. For the magnesia sintered plate, a mixture of 1 part of magnesia and 4 parts of the glass powder was used.

#### *2.1.2. Development procedure for thin-layer chromatographic separation*

All chromatographic experiments, whether on plates or sheets, were carried out in a cylindrical or rectangular chromatographic chamber with an atmosphere saturated with the solvent. For repeated use of plates other than those of zinc oxide and magnesia, the developed plates were soaked in cleaning solutions such as a chromic acid mixture, concentrated nitric acid or organic solvents, then washed with running water and reactivated by heating at 110° for 30–60 min. Sprayed plates were heated at 400–450° for 20–30 min in an electric furnace to burn off organic compounds, including visible dyes and fluorescent or UV-absorbing substances.

#### *2.1.3. Detection*

The spots on the developed sintered plates can be made visible by most of the techniques used in conventional TLC, especially by spraying with concentrated sulphuric acid, followed by heating above 130°.

#### *2.1.4. Relationship between compound mobility and the mixing ratio of adsorbent to glass powder*

Table 1 shows the relationship between estrogen mobility and the mixing ratio of silica gel to glass powder. A ratio of 1:2 to 1:4 allowed excellent separations.

TABLE 1

$hR_F$ VALUES OF ESTROGENS ON SILICA GEL SINTERED PLATE

| *Ratio of silica gel to glass powder* | *$hR_F$ value** | | |
|---|---|---|---|
| | *Estriol* | *Estradiol* | *Estrone* |
| 1:1 | 67 | 55 | 9 |
| 1:2 | 74 | 59 | 9 |
| 1:3 | 76 | 60 | 9 |
| 1:4 | 78 | 63 | 10 |
| 1:7 | 83 | 72 | 14 |
| 1:10 | 88 | 76 | 17 |
| 1:20 | 94 | 88 | 36 |
| 1:20** | 79 | 66 | 8 |
| Silica gel | 46 | 30 | 10 |

* Mean value of five runs, using benzene–ethyl acetate (4:1) and conc. $H_2SO_4$.
** Benzene–ethyl acetate (2:1).

Similar mixing ratios gave the best separation of alkaloids on alumina sintered plates, as shown in Table 2. With Kieselguhr sintered plates, ratios of 1:1 to 1:6 gave good results with azo dyes (Table 3).

TABLE 2

$hR_F$ VALUES OF ALKALOIDS ON ALUMINA SINTERED PLATE

| *Ratio of alumina to glass powder* | *$hR_F$ value** | | | | | | |
|---|---|---|---|---|---|---|---|
| | *Codeine* | *Yohimbine* | *Reserpine* | *Ergotamine* | *Quinine* | *Cinchonine* | *Emetine* |
| 1:2 | 32 | 32 | 59 | 24 | 44 | 45 | 72 |
| 1:3 | 50 | 50 | 58 | 31 | 51 | 57 | 90 |
| 1:4 | 54 | 44 | 71 | 28 | 55 | 57 | 76 |
| 1:10 | 76 | 67 | 86 | 81 | 75 | 82** | Front |
| 1:15 | 82 | 73** | 91 | 93 | 75 | 85** | 90 |
| 1:20 | 83 | 73** | 88 | —** | 79 | 86** | 89 |
| 1:20 | 84 | 77** | 87 | —** | 87 | 95** | 90 |
| Alumina*** | 50 | 43 | 68 | 23 | 46 | 59 | 75 |

* Mean value of five runs, using benzene–chloroform–diethylamine (9:4:1) and Dragendorff reagent.
** Tailing spot.
*** Merck aluminium oxide (Type T).

*2.1.5. Reproducibility of separation on sintered plates*

Many factors affect the reproducibility of $R_F$ values in TLC[15–18] including the nature of the adsorbent, the thickness and activity of the adsorbent layer, the quality of the solvent and the degree of solvent saturation in the chromatographic chambers. Recycling the sintered thin layers eliminates factors such as the nature of the adsorbent and the thickness and activity of the layer. Table 4 gives the reproducibility of $hR_F$ values ($R_F \times 100$ values) of steroids on silica gel sintered plates that were used repeatedly.

TABLE 3

$hR_F$ VALUES OF AZO DYES ON KIESELGUHR SINTERED PLATE

| *Ratio of Kieselguhr to glass powder* | $hR_F$ *value** | | | |
|---|---|---|---|---|
| | *Sudan Red G* | *Sudan Yellow* | *Butter Yellow* | *p-Methoxy-azobenzene* |
| 1:1 | 10 | 50 | 66 | 88 |
| 1:2 | 12 | 57 | 73 | 90 |
| 1:3 | 14 | 60 | 75 | 89 |
| 1:4 | 12 | 59 | 75 | 90 |
| 1:5 | 11 | 56 | 75 | 90 |
| 1:6 | 12 | 57 | 75 | 90 |
| 1:8 | 13 | 59 | 78 | 91 |
| 1:10 | 15 | 65 | 81 | 92 |
| 1:20 | 18 | 70 | 84 | 94 |
| Kieselguhr** | 43*** | 86** | Front | Front |

* Mean value of five runs, using cyclohexane.
** Wako Kieselguhr B-O.
*** Tailing spot.

TABLE 4

REPRODUCIBILITY OF $hR_F$ VALUES ON SILICA GEL SINTERED PLATES

| *Adsorbent* | $hR_F$ *values** | | | *n*** | *Detection* | *Cleaning solution* |
|---|---|---|---|---|---|---|
| | *Estriol* | *Estradiol* | *Estrone* | | | |
| Silica gel–sintered glass powder: | | | | | | |
| 1:2 | 8 ± 1 | 51 ± 2 | 65 ± 2 | 50 | Sulphuric acid | Chromic acid mixture |
| 1:3 | 10 ± 1 | 55 ± 2 | 70 ± 3 | 50 | Sulphuric acid | Chromic acid mixture |
| 1:4 | 11 ± 1 | 59 ± 2 | 73 ± 1 | 50 | Sulphuric acid | Chromic acid mixture |
| 1:4 | 8 ± 0 | 54 ± 2 | 67 ± 1 | 10 | Ceric sulphate | Chromic acid mixture |
| 1:4 | 8 ± 1 | 54 ± 1 | 68 ± 1 | 10 | Sulphuric acid | Conc. nitric acid |
| Silica gel (laboratory prepared)*** | 4 ± 1 | 22 ± 3 | 40 ± 3 | 5 | Sulphuric acid | None |
| Silica gel (fast running)§ | 8 ± 0 | 54 ± 3 | 67 ± 3 | 5 | Sulphuric acid | None |
| | *Corti-sone*§§ | *Testo-sterone*§§ | *Proge-sterone*§§ | | | |
| Silica gel–sintered glass powder (1:3) | 36 ± 4 | 61 ± 2 | 76 ± 2 | 50 | Sulphuric acid | Chromic acid mixture |
| Silica gel (laboratory prepared)*** | 20 ± 4 | 48 ± 4 | 68 ± 3 | 5 | Sulphuric acid | None |

* Benzene–ethyl acetate (2:1).
** $n$ = Number of runs on the same plate in the case of the sintered plate. In the case of laboratory-prepared or silica gel fast-running plates, five different plates were used.
*** Merck silica gel H or HF.
§ Merck aluminium sheet, silica gel–Kieselguhr.
§§ Chloroform–acetone (4:1).

TABLE 5

REPRODUCIBILITY OF $hR_F$ VALUES ON ALUMINA SINTERED PLATE

| *Alkaloid* | *Number of recovery** | | | | *Laboratory-prepared* |
|---|---|---|---|---|---|
| | *1–32* | *33–52* | *53–77* | *78–100* | |
| Quinine | 25 ± 3 | 34 ± 3 | 31 ± 3 | 31 ± 3 | 2 |
| Codeine | 42 ± 4 | 54 ± 4 | 51 ± 6 | 51 ± 6 | 15 |
| Brucine | 59 ± 4 | 67 ± 2 | 66 ± 2 | 66 ± 2 | 15 |
| Thebaine | 80 ± 2 | 81 ± 1 | 82 ± 2 | 82 ± 2 | 50 |

* Mean value of five different plates, using benzene–chloroform–diethylamine (9:2:0.25) and Dragendorff reagent.
** Merck aluminium oxide (Type T).

Tables 5 and 6 indicate the reproducibility of the $hR_F$ values of alkaloids on alumina sintered plates and those of sugars on Kieselguhr sintered plates, respectively. In general, the standard deviation of $R_F$ values can be controlled to within less than 0.05 when sufficient care is taken with the factors that affect the reproducibility of separation. With the author's sintered plates, the variation in the standard deviation was less than 0.04. For comparison, $hR_F$ values on laboratory-prepared and other pre-coated silica gel plates are shown in Table 4.

TABLE 6

REPRODUCIBILITY OF $hR_F$ VALUES ON KIESELGUHR SINTERED PLATE

| *Sugar* | *$hR_F$ value** | | | |
|---|---|---|---|---|
| | *Sintered*** | | | *Laboratory-prepared**** |
| | *1* | *2* | *3* | |
| Rhamnose | 81 ± 3 | 81 ± 2 | 82 ± 2 | 77 |
| Glucose | 41 ± 5 | 47 ± 5 | 49 ± 4 | 43 |
| Lactose | 24 ± 6 | 25 ± 4 | 27 ± 5 | 21 |
| Diginose | | Origin | | Origin |
| Digitalose | | 83 ± 1 | | 84 |
| Digitoxose | | 91 ± 2 | | 96 |

* Buffer, 0.02 *M* sodium acetate; solvent, ethyl acetate–isopropanol–water (65:24:12); detection, conc. $H_2SO_4$.
** Mean value of ten runs on the sample plate.
*** Wako Kieselguhr B-10.

Tables 7, 8 and 9 compare the separation characteristics of estrogens, cardiac glycosides and azo dyes on silica gel, alumina and Kieselguhr sintered plates with those on other laboratory-prepared and pre-coated plates. It can be seen that the $hR_F$ value of each compound on the sintered plate is greater than that on the other types of plates, the difference ($\Delta hR_F$) between estradiol and estriol on the silica gel sintered plate (Table 7) is greater than those on the other plates, and $\Delta hR_F$ between estrone and estriol on the sintered plate is less than those on the other plates. The separation characteristics on the author's plates were similar to those on a mixed layer of silica gel–Kieselguhr or alumina–Kieselguhr (Tables 7 and 8). This indicates that the sintered plate is essentially different from the conventional TLC plates.

TABLE 7

$hR_F$ VALUES OF ESTROGENS ON VARIOUS SILICA GEL PLATES

Detection with concentrated sulphuric acid. Solvent: benzene–ethyl acetate (2:1).

| *Silica gel* | *$hR_F$ values** | | | *Development rate (min per 10 cm)* |
|---|---|---|---|---|
| | *Estriol* | *Estradiol* | *Estrone* | |
| Silica gel: | | | | |
| Laboratory-prepared | 4 ± 1 Δ18 | 22 ± 3 Δ18 | 40 ± 3 | 17 |
| Pre-coated** | 4 ± 1 Δ32 | 36 ± 2 Δ15 | 51 ± 1 | 20 |
| Silica gel–Kieselguhr (1:1): | | | | |
| Laboratory-prepared | 8 ± 2 Δ44 | 52 ± 2 Δ15 | 67 ± 2 | 20 |
| Pre-coated*** | 11 ± 2 Δ51 | 62 ± 2 Δ13 | 75 ± 3 | 12 |
| Silica gel–glass powder (1:4): | | | | |
| Laboratory-prepared | 4 ± 1 Δ51 | 55 ± 1 Δ16 | 71 ± 2 | 20 |
| Sintered | 11 ± 1 Δ47 | 58 ± 1 Δ15 | 73 ± 1 | 15 |

* Results are mean values from five different plates; Δ = differences in $hR_F$ values.
** Merck pre-coated TLC plate, silica gel.
*** Merck aluminium sheet, silica gel–Kieselguhr.

TABLE 8

COMPARISON OF SEPARATION BEHAVIOUR ON SINTERED ALUMINA, ALUMINA–KIESELGUHR AND ALUMINA–GYPSUM PLATES

Alumina = Merck aluminium oxide neutral (Type T); Kieselguhr = Merck Kieselguhr G.

| *Compound* | *$hR_F$ value* | | | | | |
|---|---|---|---|---|---|---|
| | *Alumina–10% gypsum* | *Sintered alumina (1:4)* | *Alumina–Kieselguhr* | | | |
| | | | *1:1* | *2:1* | *3:1* | *5:1* |
| *Cardiac glycoside** | | | | | | |
| Gitoxin | 30 | 50 | 59 | 50 | 47 | 43 |
| Digoxin | 45 | 57 | 60 | 55 | 50 | 47 |
| Digitoxin | 48 | 70 | 71 | 64 | 56 | 53 |
| *Cardiac genin*** | | | | | | |
| Gitoxigenin | 15 | 31 | 68 | 56 | 50 | 38 |
| Digoxigenin | 32 | 46 | 75 | 74 | 60 | 44 |
| Digitoxigenin | 57 | 69 | 86 | 78 | 74 | 69 |
| *Alkaloids* | | | | | | |
| Quinine | 2 | 25 | 21§ | 21§ | 12 | — |
| Codeine | 15 | 42 | 26§ | 26§ | 17 | — |
| Brucine | 15 | 59 | 54 | 37 | 34 | — |
| Thebaine | 50 | 80 | 75§ | 60 | 60 | — |

* Chloroform–methanol (10:1).
** Ethyl acetate–acetone (3:2).
*** Benzene–chloroform–diethylamine (9:2:0.25).
§ Tailing spot.

Compound mobility can be controlled by adding sintered glass powder to the adsorbents. By using these sintered plates containing silica gel, alumina, Kieselguhr and other porous metal oxides, the author separated various organic compounds, namely, lipids, steroids, alkaloids, vitamins, amino acids and sugars[9,19], peptides and food dyes[20], polychlorinated biphenyls[13], pesticides[14], antibiotics, catecholamines and barbiturates[21], and organic phosphates, sulphates and nitrates[22].

TABLE 9

COMPARISON OF SEPARATION BEHAVIOUR ON SINTERED KIESELGUHR AND KIESELGUHR–GYPSUM PLATES

Thin layer: 1 = Kieselguhr–sintered glass powder (1:3); 2 = Kieselguhr + 10% (Wako) or 15% (Merck) gypsum. Solvent: Cyclohexane.

| *Azo dye* | $hR_F$ *value** | | | |
|---|---|---|---|---|
| | *Merck*** | | *Wako* | |
| | *1* | *2* | *1* | *2* |
| Sudan Red G | 17 | 10 | 14 | 4 |
| Sudan Yellow | 66 | 43 | 60 | 29 |
| Butter Yellow | 77 | 64 | 75 | 44 |
| *p*-Methoxyazobenzene | 94 | 86 | 89 | 73 |
| Sudan I | 77 | 75 | 71 | 65 |
| Sudan II | 74 | 72 | 67 | 63 |
| Sudan III | 16 | 17 | 17 | 10 |
| Sudan IV | 20 | 18 | 17 | 17 |

* Mean value of five runs; the standard deviation for each $hR_F$ value was less than 2. Development rate: 30 min per 10 cm.

** Slightly tailing spots.

### *2.2. Preparation of and thin-layer chromatography on sintered sheets of organic adsorbents*[20,23]

In this instance, microparticulate polyolefins were used instead of glass powder as the binder. The adsorbents were silica gel, alumina, Kieselguhr, Florisil, synthetic porous polymers, ion-exchange resins, cellulose ion exchangers, dextran gels and cellulose.

#### *2.2.1. Microparticulate polyolefins*

Commercially available polyethylene or polypropylene (about 20 mesh) powder was recrystallized according to a known procedure[24] from an aromatic solvent such as benzene, toluene or xylene to give microparticulate material (particle size 10 $\mu$m).

#### *2.2.2. Preparation of various polyolefin sintered sheets*

A mixture of 1–5 parts of adsorbent and 1 part of microparticulate polyolefin was suspended in acetone and the slurry was spread on glass or plastic supports in the usual manner. After drying in air, the layer was heated in an electric oven at 100–180° for 10–30 min. Next, the sintered plates were soaked in a lipophilic solvent such as benzene, chloroform, diethyl ether or ethyl acetate at room temperature for several minutes. In this process, the polyolefin sintered layers peeled off from the glass or plastic supporting plates. The sintered sheets prepared in this way are self-supporting, and have satisfactory adsorption, partition and ion-exchange functions. Table 10 gives the conditions for the preparation of various polyolefin sintered sheets. Using these sheets, the author has successfully separated various organic compounds: peptides on carboxymethylcellulose sintered sheets, nucleotides on polyethyleneimine-cellulose sintered sheets and food dyes on cellulose sintered sheets. Table 11 shows

TABLE 10

PREPARATION OF VARIOUS POLYOLEFIN SINTERED PLATES

| *Adsorbent* | *Welding conditions* | | *Adsorbent to polyolefin ratio* |
|---|---|---|---|
| | *Temperature (°C)* | *Time (min)* | |
| Silica gel | 180 | 10 | 2:1 |
| Alumina | 180 | 10 | 3:1 |
| Kieselguhr | 180 | 10 | 2:1 |
| Florisil | 180 | 10 | 2:1 |
| Polyamide | 120 | 30 | 1:1 |
| Amberlite XAD | 180 | 10 | 2:1 |
| Amberlite GC-120* | 140 | 10 | 3:1 |
| Amberlite CG-50** | 140 | 10 | 3:1 |
| Amberlite CG-400*** | 180 | 10 | 3:1 |
| Lewatit MP 7080§ | 180 | 10 | 3:1 |
| CM-cellulose** | 160 | 20 | 5:1 |
| PEI-cellulose§ | 160 | 10 | 5:1 |
| ECTEOLA-cellulose§ | 160 | 10 | 5:1 |
| Cellulose | 160 | 10 | 5:1 |

* Strong acid cation exchanger.
** Weak acid cation exchanger.
*** Strong base anion exchanger.
§ Weak acid anion exchanger.

TABLE 11

TLC OF ORGANIC COMPOUNDS ON POROUS POLYMER SINTERED SHEETS

| *Porous polymer* | *Compound* | *$hR_F$ value* | *Solvent*** | *Development time (min)* | *Detection* |
|---|---|---|---|---|---|
| Polyamide | *Nitrophenols* | | BA401 | 20 | Visible |
| | 2,6-Dinitrophenol | 25 | | | |
| | 2,5-Dinitrophenol | 50 | | | |
| | *Dns-amino acids* | | BA91 | 16 | UV (365 nm), visible |
| | His | 14 | | | |
| | Gly | 30 | | | |
| | Lys | 55 | | | |
| | Pro | 80 | | | |
| XAD-2 | *vitamins* | | Ethanol | 29 | $SbCl_3$ |
| | A pal | 17 | | | |
| | A ace | 34 | | | |
| | $D_2$* | 70 | | | |
| | $D_3$* | 75 | | | |
| XAD-4 | *vitamins* | | Ethanol | 34 | $SbCl_3$ |
| | A pal | 6 | | | |
| | A ace | 13 | | | |
| | $D_2$* | 36 | | | |
| | $D_3$* | 42 | | | |

* With $AgNO_3$ impregnation.
** BA401 = benzene–acetone (40:1); BA91 = benzene–acetone (9:1).

the separation of nitrophenols and Dns-amino acids on polyamide sintered sheets and of fat-soluble vitamins on porous polymer (XAD-2, XAD-4) sintered sheets.

Table 12 shows the TLC analysis of 17 free amino acids on a strongly acidic cation-exchange resin (Amberlite CG-120) sintered sheet. The system of a strongly acidic cation exchanger and citrate buffer was better than that of a strongly basic anion exchanger (Amberlite CG-400) and pyridine–acetic acid–water.

The sintered sheet looks like a filter-paper for paper chromatography, but is mechanically stable and acid-resistant. Hence it is also suitable for the separation of radioactive compounds.

TABLE 12

TLC OF AMINO ACIDS ON ION-EXCHANGE RESIN SINTERED SHEETS

| *Amino acid* | $hR_F$ *value* | |
|---|---|---|
| | *Amberlite CG-120** | *Amberlite CG-400*** |
| Arg | 10 | 65 |
| Trp | 18 | 51 |
| His | 19 | 71 |
| Lys | 25 | 81 |
| Phe | 39 | 72 |
| Tyr | 45 | 72 |
| Pro | 53 | 72 |
| β-Ala | 64 | 85 |
| Ala | 85 | 86 |
| Gly | 84 | 88 |
| Ser | 90 | 90 |
| Cys | 91 | 90 |
| $GluNH_2$ | 95 | 75 |
| $AspNH_2$ | 96 | 71 |
| Asp | 95 | 70 |
| Glu | 95 | 76 |
| Tau | 100 | 83 |

* Eluent: citrate buffer (pH 5.30), $Na^+$ 0.35 *M*, citrate 0.117 *M*.
** Eluent: pyridine–acetic acid–water (1:10:100) (pH 3.45).

### *2.3. Consideration of the "welding" mechanism*

At the beginning of this work, the author was not certain whether solid welding would occur among heterogeneous substances such as adsorbent, binder and support, because their coefficients of expansion ($\alpha$) differ greatly from one another. For example, the coefficient of expansion of soda-lime glass powder ($\alpha = 92 \cdot 10^{-7}$ cm/cm·°C) is about 17 times greater than that of silica gel ($\alpha = 5.4 \cdot 10^{-7}$ cm/cm·°C). However, solid welding of these substances did occur to yield highly porous thin layers with intact chromatographic activity, as shown in Tables 1, 2 and 3.

The author has tried to clarify the mechanism of welding of sintered plates containing silica gel[25], alumina[26] and Kieselguhr[27] by means of scanning electron microscopy (SEM). Figs. 1, 2 and 3 are scanning electron micrographs showing the surfaces of silica gel, alumina and Kieselguhr sintered glass plates, respectively. The larger particles are silica gel, alumina and Kieselguhr, and they are not fused. The

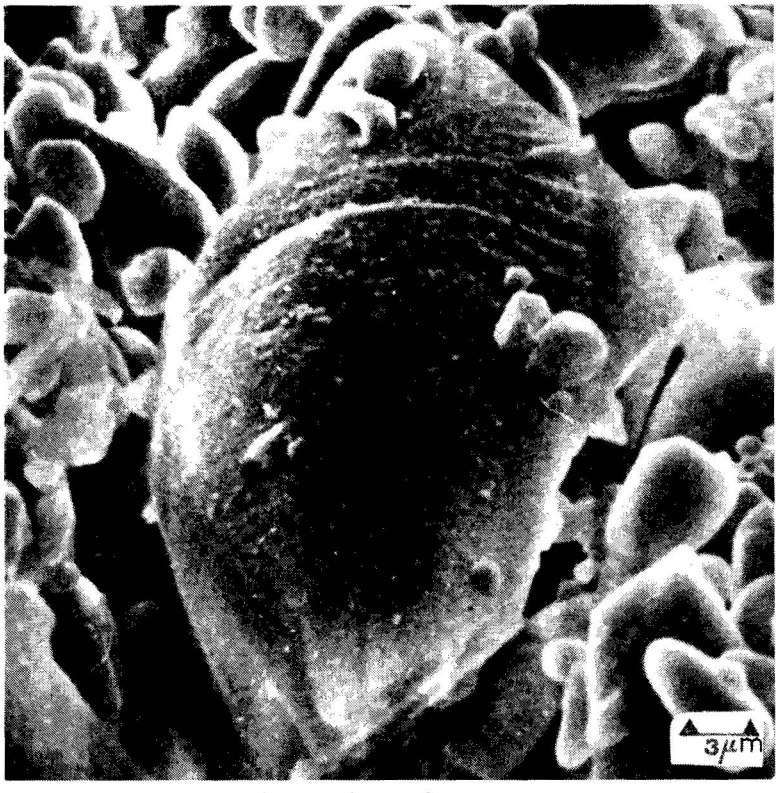



Fig. 1. SEM of silica gel sintered plate surface.

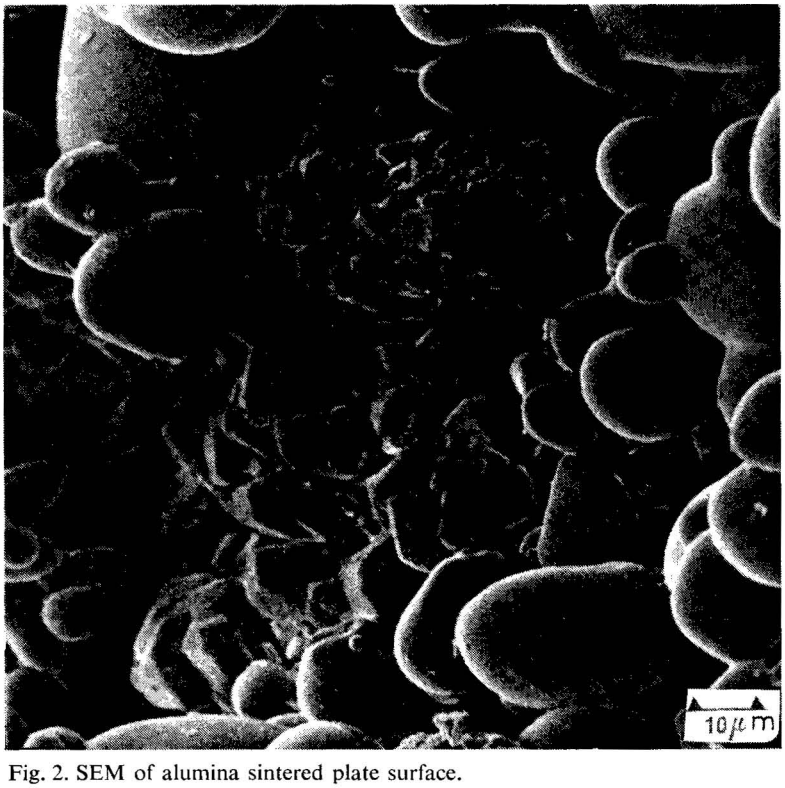



Fig. 2. SEM of alumina sintered plate surface.

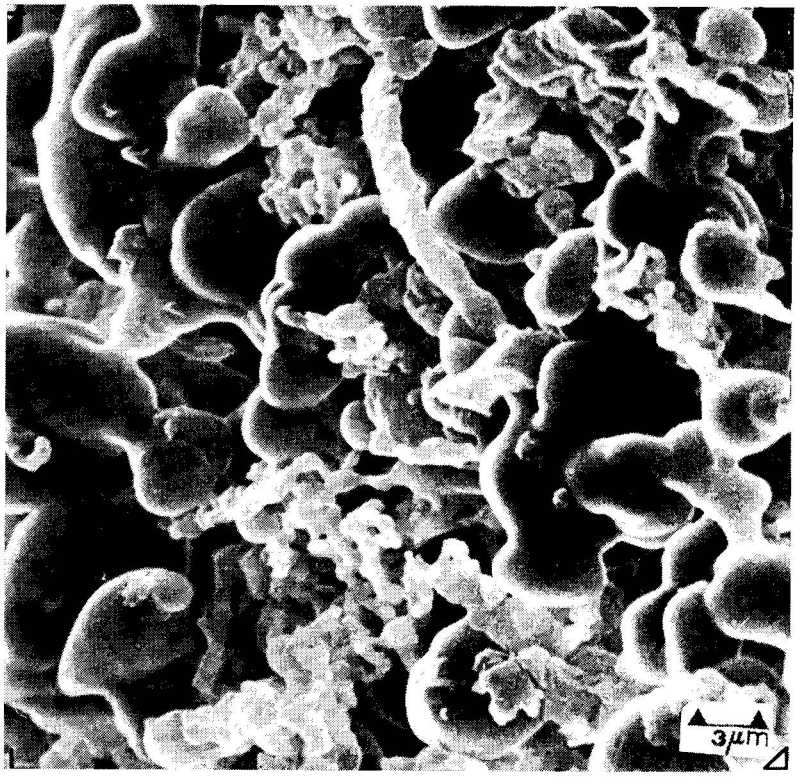



Fig. 3. SEM of Kieselguhr sintered plate surface.

smaller particles are sintered glass powder. Figs. 4, 5 and 6 show cross-sectional views of these three kinds of sintered plates. The lower layer is the glass supporting plate, and large adsorbent particles and small sintered glass particles are seen in the upper layer.

Thus, as shown in the schematic structure of the sintered glass plate (Fig. 7), these adsorbent particles are fixed, without any damage to their surface structures, in the three-dimensional space formed by the sintered-glass powder and the glass supporting plate. The sintered-glass powder plays a binding role between the adsorbent and the glass plate.

*2.3.1. Influence of pH of glass binders on formation of sintered thin layers*

Unlike soda-lime glass, borosilicate glass failed to give homogeneous thin-layers. As shown in Fig. 8, Stahl's test dyes behaved normally on the soda-lime glass sintered plate (left), but they migrated abnormally on the borosilicate glass sintered plate (right). The author thought that this phenomenon was due to the lower pH of borosilicate glass. A 10% aqueous suspension of borosilicate glass had a pH of 9.05 and that of soda-lime glass a pH of 10.35.

Fig. 9 shows the scanning electron micrograph of the homogeneous layer of silica gel on the soda-lime glass sintered plate, and Fig. 10 shows that of the hetero-

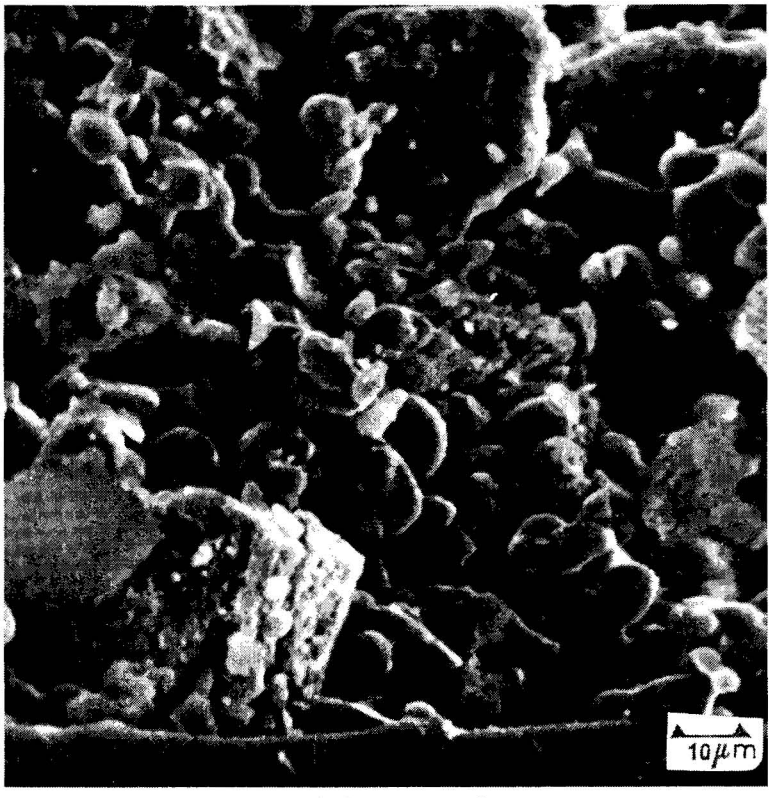



Fig. 4. SEM of silica gel sintered plate cross-section.

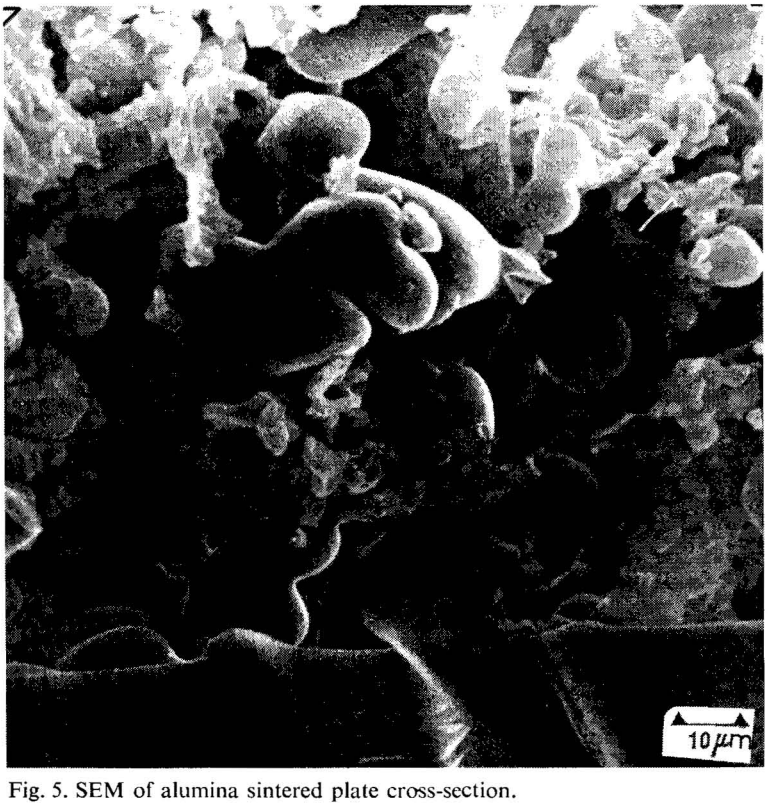



Fig. 5. SEM of alumina sintered plate cross-section.

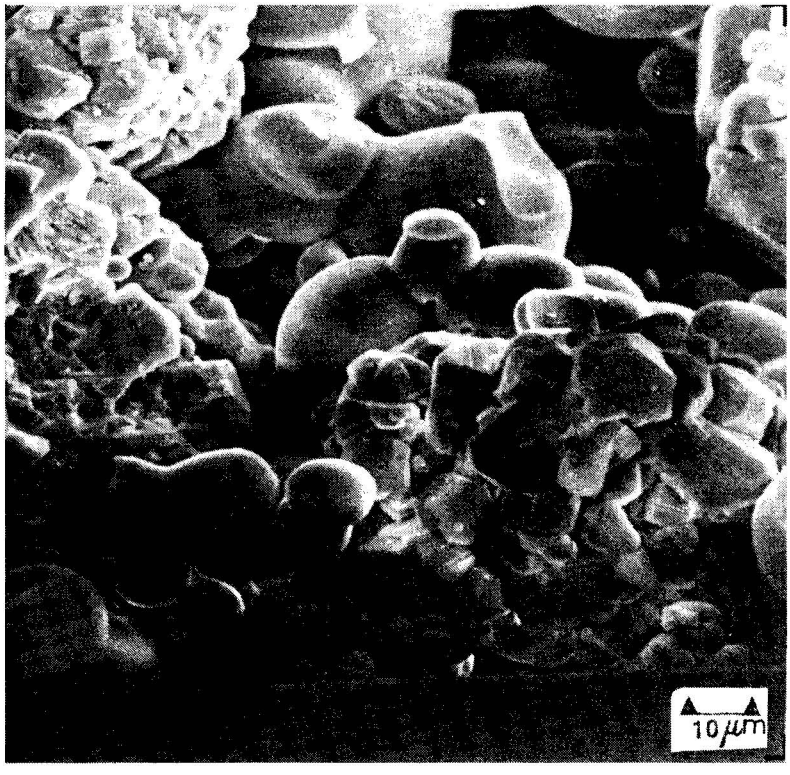



Fig. 6. SEM of Kieselguhr sintered plate cross-section.

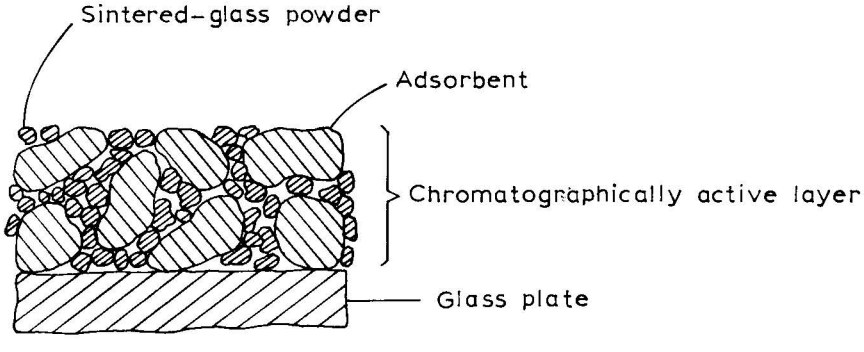



Fig. 7. Schematic diagram of structure of sintered plate.

geneous layer of silica gel on the borosilicate glass sintered plate, in which large cracks are visible.

In order to explain the different results obtained with soda-lime and borosilicate glass, their sedimentation volumes were measured. Table 13 indicates that the sedimentation volume of borosilicate glass increases about 2–2.8-fold on addition of a basic flocculant such as sodium methoxide and ammonia solution. Investigation of

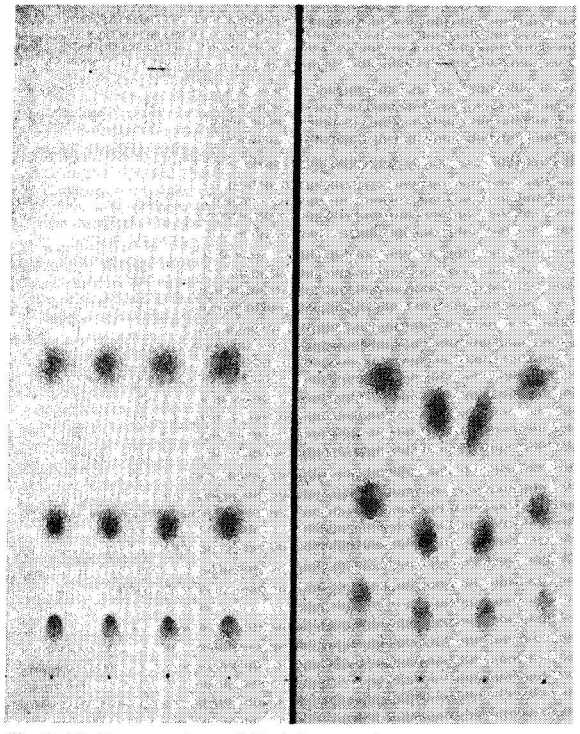


Fig. 8. TLC separation of Stahl's test dyes on silica gel sintered plate. Left, soda-lime glass; right, borosilicate glass. Dispersion medium: acetone.

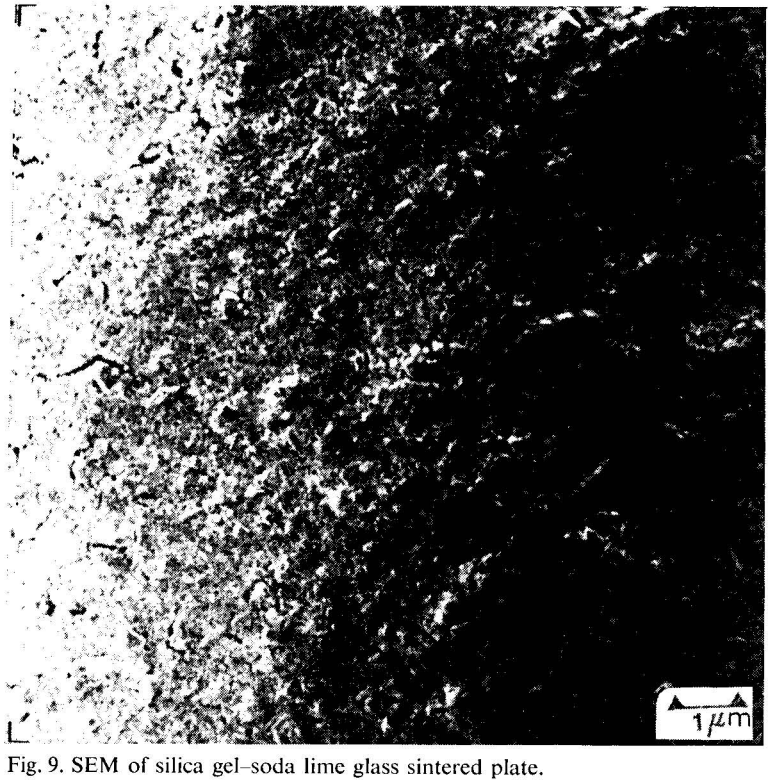



Fig. 9. SEM of silica gel–soda lime glass sintered plate.

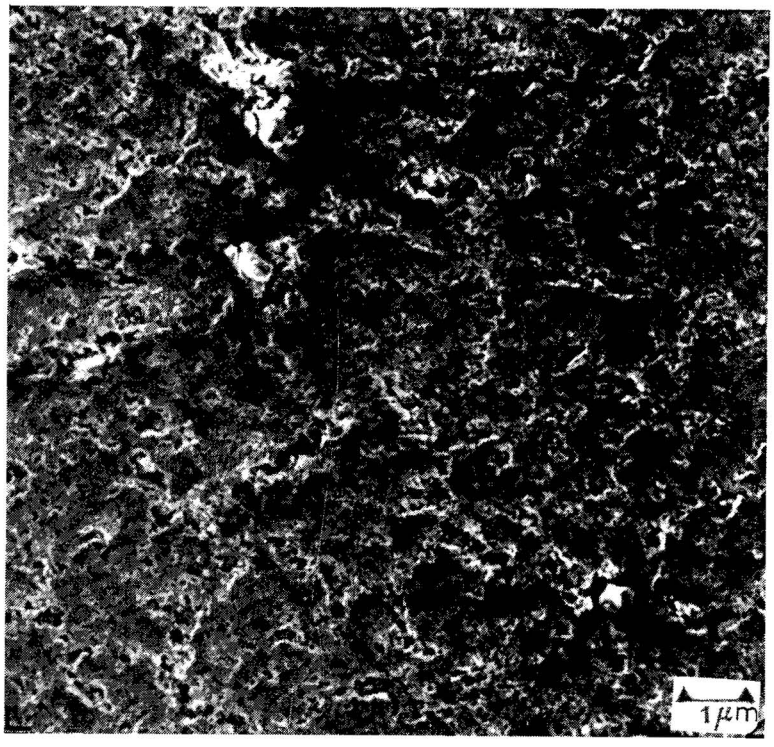



Fig. 10. SEM of silica gel–borosilicate glass sintered plate.

TABLE 13

SEDIMENTATION VOLUMES ($V_s$) OF SILICA GEL AND GLASS POWDERS USED FOR SINTERED PLATES

| *Powder* | $V_s$ *(ml)* * | | $V_s$ *with* $NaOCH_3$ / $V_s$ *without* $NaOCH_3$ |
|---|---|---|---|
| | *With* $NaOCH_3$ ** | *Without* $NaOCH_3$ | |
| Merck silica gel H*** | 15.8 | 15.2 | 1.04 |
| Soda-lime glass*** | 10.7 | 10.3 | 1.04 |
| Borosilicate glass*** | 11.0 | 4.0 | 2.75 |
| Silica gel H–soda-lime glass mixture (1:3)§ | 10.8 | 9.0 | 1.20 |
| Silica gel–borosilicate glass mixture (1:3)§ | 13.8 | 7.0 | 1.97 |

* Left to settle overnight.
** 2% (w/w) $NaOCH_3$ was added.
*** Six grams each suspended in 15 ml of acetone.
§ A mixture of 1.5 g and 4.5 g, respectively, suspended in 15 ml of acetone.

the relationship between the amount of basic flocculant added and the pH of sintered borosilicate glass–silica gel plates showed that the addition of 2–3% of sodium methoxide was necessary in order to prepare plates with the same pH as that of a soda-lime glass sintered plate (Table 14). As shown in Table 15, the addition of

TABLE 14

pH VALUES OF SILICA GEL–BOROSILICATE GLASS SINTERED PLATES

| *$NaOCH_3$ (%, w/w)* | *pH value* | |
|---|---|---|
| | *pH meter** | *pH indicator*** |
| 0 | 7.14 | 5.6 |
| 0.1 | 7.87 | 5.8 |
| 0.3 | 7.98 | 6.2 |
| 0.5 | 8.22 | 6.4 |
| 1 | 8.45 | 6.8 |
| 2 | 8.70 | 7.2 |
| 3 | 9.31 | 8.4 |
| 4 | 9.51 | 8.6 |
| 5 | 9.57 | 8.6 |
| Silica gel–soda-lime sintered plate | 8.83 | 8.4 |

* Measured with a Hitachi-Horiba F-5 pH meter.
** Measured with a Nishicator[28].

TABLE 15

*$hR_F$* VALUES OF STEROIDAL HORMONES ON SILICA GEL–BOROSILICATE GLASS SINTERED PLATES TREATED WITH SODIUM METHOXIDE

Solvent, chloroform–methanol (10:1); detection, conc. sulphuric acid.

| *$NaOCH_3$ (%, w/w)* | *$hR_F$ value* | | | *Separation* |
|---|---|---|---|---|
| | *Cortisone* | *Testosterone* | *Progesterone* | |
| 0 | 50 | 70 | 80 | Irregular |
| 0.1 | 53 | 73 | 83 | Sharp |
| 0.3 | 58 | 75 | 83 | Sharp |
| 0.5 | 57 | 77 | 87 | Sharp |
| 1 | 50 | 66 | 71 | Sharp |
| 2 | 62 | 71 | 76 | Slightly leading |
| 3 | 75 | 80 | 85 | Leading |
| 4 | 89 | 89 | 89 | Leading |
| 5 | 90 | 90 | 90 | Leading |

0.1–1% of sodium methoxide to the borosilicate glass gave normal and sharp separation of steroidal hormones. Sodium methoxide-treated borosilicate sintered plates also allowed the normal separation of estrogens, alkaloids (silica gel, Tables 16 and 17), azo dyes (alumina) and polychlorinated biphenyls (PCBs, Kieselguhr)[11].

Figs. 11 and 12 show the schematic sedimentation structure of mixtures of silica gel and borosilicate glass powder. When a mixture of silica gel and borosilicate glass powder is dispersed on a supporting plate without the addition of the basic flocculant, closely packed sedimentation results, as shown in Fig. 11a. After sintering, this closely packed layer changes into a cracked surface (Fig. 11b). The heterogeneous sintered thin layer thus formed results in the abnormal separation of various organic compounds. On the other hand, when a mixture of silica gel and borosilicate glass powder is dispersed after addition of the basic flocculant, loosely packed sedimentation is obtained, as shown in Fig. 12a. After sintering, this loosely packed layer

TABLE 16

$hR_F$ VALUES OF ESTROGENS ON SILICA GEL–BOROSILICATE GLASS SINTERED PLATES TREATED WITH SODIUM METHOXIDE

Solvent, benzene–ethyl acetate (2:1); detection, iodine vapour.

| $NaOCH_3$ (%, w/w) | $hR_F$ value | | | Separation |
|---|---|---|---|---|
| | *Estriol* | *Estradiol* | *Estrone* | |
| 0 | 9 | 46 | 60 | Irregular |
| 0.1 | 10 | 53 | 67 | Sharp |
| 0.3 | 10 | 53 | 66 | Sharp |
| 0.5 | 8 | 54 | 65 | Sharp |
| 1 | 9 | 57 | 68 | Sharp |
| 2 | 9 | 65 | 74 | Slightly leading |
| 3 | 13 | 78 | 84 | Leading |
| 4 | 26 | 93 | 97 | Leading |
| 5 | 31 | 85 | 90 | Leading |

TABLE 17

$hR_F$ VALUES OF ALKALOIDS ON SILICA GEL–BOROSILICATE GLASS SINTERED PLATES TREATED WITH SODIUM METHOXIDE

Solvent, chloroform–diethylamine (30:1); detection, Dragendorff reagent.

| $NaOCH_3$ (%, w/w) | $hR_F$ value | | | | Separation |
|---|---|---|---|---|---|
| | *Quinine* | *Codeine* | *Brucine* | *Thebaine* | |
| 0 | 10 | 29 | 35 | 39 | Irregular |
| 0.1 | 10 | 32 | 37 | 43 | Sharp |
| 0.3 | 12 | 37 | 45 | 50 | Sharp |
| 0.5 | 10 | 35 | 43 | 48 | Sharp |
| 1 | 15 | 38 | 46 | 57 | Sharp |
| 2 | 16 | 45 | 50 | 65 | Slightly leading |
| 3 | 50 | 74 | 80 | 87 | Leading |
| 4 | 80 | 90 | 95 | 100 | Leading |
| 5 | 88 | 97 | 100 | 100 | Leading |

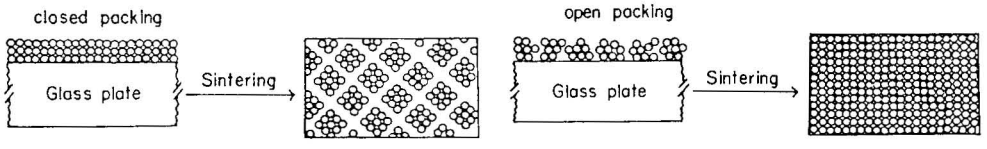



Fig. 11. Schematic diagram of structure of silica gel and borosilicate glass powder before and after sintering.

Fig. 12. Schematic diagram of structure of silica gel and borosilicate glass powder with basic flocculant before and after sintering.

changes into a homogeneous surface without cracks (Fig. 12b) which enables normal chromatographic separation. Of the several solvents tested, acetone was the most effective for increasing the sedimentation volume. The author also prepared homogeneous borosilicate alumina and Kieselguhr sintered glass plates[11].

### *2.4. Thermal stabilities of adsorbents*

Sintering of silica gel begins at 600–700° (ref. 29). However, in the preparation of sintered plates, the heating period is short and sintering of silica gel can be avoided. There is no example in the literature of the heating at high temperatures of silica gel for TLC, which has physical properties[30] such as a specific surface area of about 400 $m^2/g$, a specific pore volume of about 0.7–0.8 ml/g and a particle size distribution of 33.7% at 6 $\mu$m, 1.9% at 6–30 $\mu$m and 64.4% at 30–66 $\mu$m. Therefore, heat treatment of the silica gel was carried out under mild conditions, *i.e.*, at 470–770° for 2–7 min.

Table 18 presents some of the physical properties of silica gel for TLC. Before heat treatment, its specific surface area was about 400–500 $m^2/g$, specific pore volume 1.1–1.2 ml/g and particle size distribution 10–60 $\mu$m. After heating to 770°, these values remained the same. However, heating at 1000° for 30 min caused sintering of the silica gel powder, as indicated by the lower physical constants.

TABLE 18

PHYSICAL PROPERTIES OF SILICA GEL HEATED AT NORMAL PRESSURE

Merck silica gel H.

| *Treatment* | | *Specific surface area ($m^2/g$)* | | *Specific pore volume*** *(ml/g)* |
|---|---|---|---|---|
| *Temperature (°C)* | *Time (min)* | $N_2$* | $N_2/He$** | |
| Not heated | | 420 | 494 | 1.21 |
| 470 | 7 | 419 | 497 | 1.15 |
| 570 | 7 | 415 | 481 | 1.14 |
| 670 | 7 | 413 | 479 | 0.95 |
| 770 | 7 | 396 | 482 | 0.94 |
| 870 | 7 | 361 | 426 | 0.92 |
| 1000 | 7 | 197 | 116 | 0.43 |
| 1000 | 30 | 80 | 22 | 0.26 |

* Measured by BET method.

** Measured by continuous flow method.

*** Calculated from (1/particle density) − (1/true density).

Sintered plates were prepared from silica gel heated to 770° and tested with the separation of mixtures such as estrogens and Stahl's azo dyes. Tables 19 and 20 show the constancy of the $R_F$ values and the good separation of the test mixtures, indicating that sintering of the silica gel did not occur on heating to 770° (ref. 31). However, extreme tailing was observed in the separation of the test mixtures when silica gel powder heated at 1000° for 30 min was used. Fig. 13 is a scanning electron micrograph showing the sintering of this type of silica gel.

Tables 21 and 22 present the data on heat treatment of alumina[32] and Kieselguhr[33] for TLC, respectively. Tables 23 and 24 show the influence of the heat treatment on the TLC separation of alkaloids on the alumina and those of PCBs on the Kieselguhr, indicating that heating of these two kinds of adsorbents at 1000° for 30 min did not alter their physical properties or chromatographic activities, in contrast to the results with silica gel.

TABLE 19

$hR_F$ VALUES OF ESTROGENS ON HEATED SILICA GEL LAYERS

Solvent, benzene–ethyl acetate (2:1); detection, conc. sulphuric acid. Merck silica gel H.

| *Treatment* | | *$hR_F$ value* | | | *Separation* | *Development rate (min per 10 cm)* |
|---|---|---|---|---|---|---|
| *Temperature (°C)* | *Time (min)* | *Estriol* | *Estradiol* | *Estrone* | | |
| Not heated | | 4 | 41 | 59 | Sharp | 13 |
| 470 | 7 | 4 | 40 | 57 | Sharp | 13 |
| 570 | 7 | 4 | 39 | 57 | Sharp | 13 |
| 670 | 7 | 5 | 40 | 57 | Sharp | 11 |
| 770 | 7 | 5 | 36 | 53 | Sharp | 12 |
| 870 | 7 | 5 | 43 | 59 | Sharp | 11 |
| 1000 | 7 | 7 | 53 | 67 | Slight tailing | 11 |
| 1000 | 30 | — | — | — | Marked tailing | 8 |

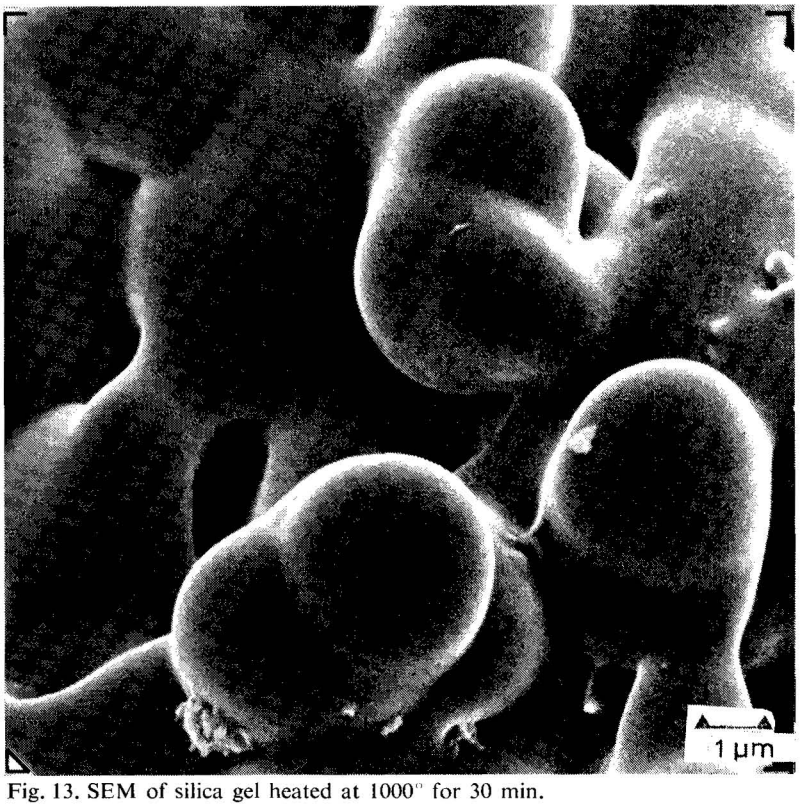



Fig. 13. SEM of silica gel heated at 1000° for 30 min.

Of the adsorbents silica gel, alumina and Kieselguhr, alumina was the most thermostable and Kieselguhr was more stable than silica gel. The author also investigated the thermal stabilities of other inorganic adsorbents[14], such as Florisil, titania, magnesia and zinc oxide, and various kinds of organic adsorbents used for the preparation of sintered sheets[20,23], employing SEM, X-ray diffraction analysis and thermal analysis (TG–DTA and TG–DSC).

TABLE 20

$hR_F$ VALUES OF STAHL'S DYES ON HEATED SILICA GEL LAYERS

Solvent, benzene. Adsorbent, Merck silica gel H.

| *Treatment* | | *$hR_F$ value* | | | *Separation* | *Development time (min per 10 cm)* |
|---|---|---|---|---|---|---|
| *Temperature (°C)* | *Time (min)* | *Indophenol* | *Sudan Red G* | *Butter Yellow* | | |
| Not heated | | 4 | 12 | 37 | Sharp | 14 |
| 470 | 7 | 4 | 11 | 34 | Sharp | 13 |
| 570 | 7 | 3 | 10 | 31 | Sharp | 14 |
| 670 | 7 | 2 | 9 | 29 | Sharp | 14 |
| 770 | 7 | 2 | 8 | 27 | Sharp | 12 |
| 870 | 7 | 2 | 8 | 28 | Sharp | 13 |
| 1000 | 7 | 5 | 20 | 47 | Slight tailing | 13 |
| 1000 | 30 | Tailing spot from original point to solvent front | | | | 7 |

TABLE 21

PHYSICAL PROPERTIES OF ALUMINA HEATED AT NORMAL PRESSURE

Merck aluminium oxide neutral (Type T).

| *Treatment* | | *Specific surface area ($m^2/g$)* | | *Specific pore volume*** (ml/g)* |
|---|---|---|---|---|
| *Temperature (°C)* | *Time (min)* | *$N_2$** | *$N_2/He$*** | |
| Not heated | | 103 | 107 | 0.51 |
| 470 | 7 | 102 | 108 | 0.51 |
| 570 | 7 | 102 | 105 | 0.52 |
| 670 | 7 | 104 | 95 | 0.49 |
| 770 | 7 | 96 | 96 | 0.51 |
| 870 | 7 | 90 | 100 | 0.50 |
| 1000 | 7 | 78 | 98 | 0.50 |
| 1000 | 30 | 65 | 66 | 0.50 |

* Measured by BET method.
** Measured by continuous flow method.
*** Calculated from (1/particle density) − (1/true density).

TABLE 22

PHYSICAL PROPERTIES OF KIESELGUHR HEATED AT NORMAL PRESSURE

Wako Kieselguhr B-0.

| *Treatment* | | *Specific surface area** ($m^2/g$)* | *True specific gravity** | *Particle specific gravity** | *Mean particle diameter** (μm)* |
|---|---|---|---|---|---|
| *Temperature (°C)* | *Time (min)* | | | | |
| Not heated | | 3.38 | 2.24 | 2.02 | 1.77 |
| 470 | 7 | 3.60 | 2.24 | 2.07 | 1.66 |
| 570 | 7 | 3.60 | 2.15 | 2.07 | 1.67 |
| 670 | 7 | 3.64 | 2.14 | 2.09 | 1.65 |
| 770 | 7 | 3.62 | 2.17 | 2.07 | 1.66 |
| 870 | 7 | 3.64 | 2.21 | 2.10 | 1.65 |
| 1000 | 7 | 3.87 | 2.23 | 2.20 | 1.55 |
| 1000 | 30 | 3.76 | 2.26 | 2.22 | 1.60 |

* Measured by air permeability method.

TABLE 23

$hR_F$ VALUES OF ALKALOIDS ON HEATED ALUMINA LAYERS

Solvent, benzene–chloroform–diethylamine (9:4:1); detection, Dragendorff reagent. Merck aluminium oxide neutral (Type T).

| *Treatment* | | *$hR_F$ value** | | | *Separation* | *Development rate (min per 10 cm)* |
|---|---|---|---|---|---|---|
| *Temperature (°C)* | *Time (min)* | *Quinine and codeine* | *Brucine* | *Thebaine* | | |
| Not heated | | 25 | 51 | 73 | Sharp | 23 |
| 470 | 7 | 27 | 57 | 77 | Sharp | 18 |
| 570 | 7 | 29 | 53 | 75 | Sharp | 20 |
| 670 | 7 | 29 | 55 | 75 | Sharp | 19 |
| 770 | 7 | 31 | 54 | 74 | Sharp | 16 |
| 870 | 7 | 30 | 58 | 78 | Sharp | 17 |
| 1000 | 7 | 34 | 60 | 78 | Sharp | 16 |
| 1000 | 30 | 53 | 71 | 85 | Sharp | 15 |

* Mean values of three runs.

TABLE 24

$hR_F$ VALUES OF PCBs ON HEATED KIESELGUHR LAYERS

| *Treatment* | | *DCB* | *Kanechlor 600* | | | *Separation* | *Development rate (min per 10 cm)* |
|---|---|---|---|---|---|---|---|
| *Temperature (°C)* | *Time (min)* | | *Spot A* | *Spot B* | *Spot C* | | |
| Not heated | | 7 | 21 | 28 | 36 | Sharp | 42 |
| 470 | 7 | 9 | 27 | 35 | 45 | Sharp | 15 |
| 570 | 7 | 7 | 24 | 30 | 39 | Sharp | 14 |
| 670 | 7 | 10 | 28 | 35 | 45 | Sharp | 14 |
| 770 | 7 | 8 | 25 | 32 | 41 | Sharp | 14 |
| 870 | 7 | 9 | 25 | 33 | 42 | Sharp | 14 |
| 1000 | 7 | 9 | 28 | 36 | 44 | Sharp | 14 |
| 1000 | 30 | 9 | 27 | 37 | 46 | Sharp | 11 |

## 3. NOVEL DETECTION METHODS FOR SINTERED THIN-LAYER CHROMATOGRAPHY

### *3.1. Fluorescence quenching detection*[34]

There are many inorganic phosphors for fluorescence quenching detection, such as zinc orthosilicate, zinc sulphide, calcium tungstate, strontium pyrophosphate and yttrium vanadate. However, they are not acid-resistant, except for yttrium vanadate[35]. Acid lability is unfavourable in TLC, because solvent systems containing strong acids are sometimes chosen as the mobile phase and spraying with concentrated sulphuric acid is a routine process in TLC. These acid-labile inorganic phosphors are dissolved or decomposed during the development or detection process.

To resolve this difficulty, the author used partially crystallized fluorescent glass, namely zinc silicate, calcium tungstate and cadmium borate glasses with partially crystallized structures, in place of the above inorganic phosphors. Partially crystallized glass is prepared by fusing a mixture of silica, basic zinc carbonate,

manganese dioxide, sodium nitrate, lead tetroxide, sodium fluorosilicate and aluminium hydroxide at 1000–1500° for 1–3 h in an electric furnace (Fig. 14a), then inducing partial crystallization on the surface by heating this glass again at 900–1100° for 30–40 min, as shown in Fig. 14b. On prolonged heating, the partially crystallized glass melted again, as shown in Fig. 14c.

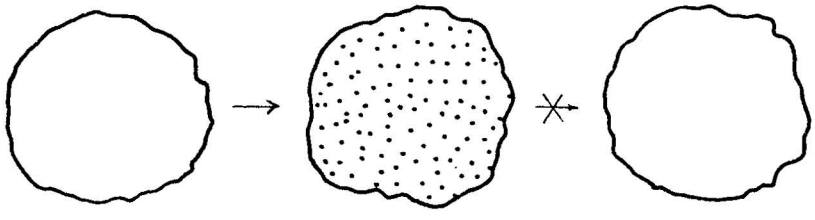



Fig. 14. Crystallized fluorescent glass.

Table 25 presents the chemical compositions of the three kinds of fluorescent glass. The X-ray diffraction analysis data shown in Fig. 15 offered proof of the partially crystallized structure of zinc silicate fluorescent glass, a scanning electron micrograph of which is shown in Fig. 16. The crude crystallized fluorescent glass thus obtained was ground in a ball mill and fractionated by water sedimentation. In this way, a fine powder of fluorescent glass with a particle distribution of 1–20 $\mu$m was obtained. Table 26 shows the properties of three kinds of fluorescent crystallized glass and some of the fluorescent materials, which indicate that in their fluorescent properties zinc silicate glass and calcium tungstate glass correspond to zinc silicate phosphor and strontium pyrophosphate phosphor, respectively.

TABLE 25

CHEMICAL COMPOSITIONS OF FLUORESCENT GLASSES

| *Fluorescent glass* | *Component (%, w/w)* | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $SiO_2$ | $Na_2O$ | $Al_2O_3$ | $CaO$ | $ZnO$ | $WO_3$ | $B_2O_3$ | $CdO$ | $MnO$ | $PbO$ |
| $Zn_2SiO_4$/Mn | 60.0 | 10.0 | 3.7 | — | 26.0 | — | — | — | 0.3 | — |
| $CaWO_4$/Mn, Pb | 56.0 | 8.0 | — | 24.0 | — | 10.7 | — | — | 0.3 | 1.0 |
| $Cd_2B_2O_5$/Mn | 10.0 | — | 2.0 | — | — | — | 26.0 | 61.7 | 0.3 | — |

Using crystallized fluorescent glass, the author prepared acid-resistant fluorescent sintered plates, for example fluorescent sintered-glass–silica gel plates, by the procedure shown in Table 27. With these plates, it was possible to separate and detect minute amounts of, for example, steroidal hormones, water-soluble vitamins and inorganic cations, without spraying with a detection reagent.

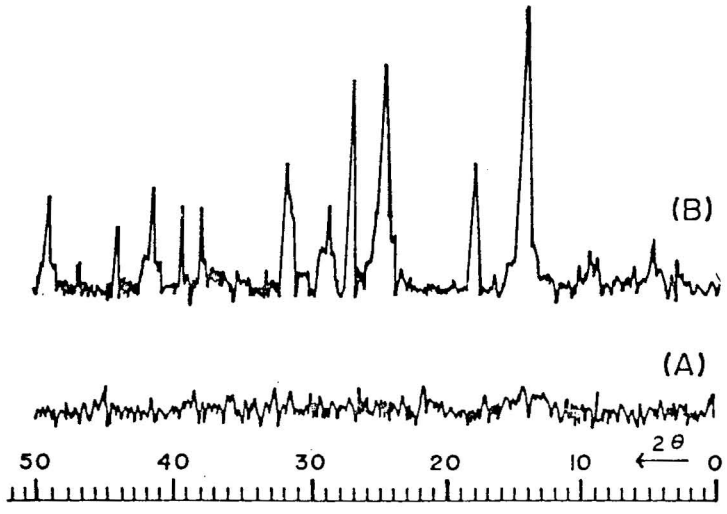



Fig. 15. X-Ray diffraction patterns of $Zn_2SiO_4$/Mn fluorescent glass: Cu K$\alpha$ radiation. (A) Before crystallization; (B) after crystallization.

TABLE 26

PROPERTIES OF FLUORESCENT SUBSTANCES

| *Fluorescent substance* | *Fluorescent properties* | | | |
|---|---|---|---|---|
| | *Excitation (max.) (nm)** | *Emission (max.) (nm)** | *Colour* | *Intensity* |
| *Glass* | | | | |
| $Zn_2SiO_4$/Mn | 276 | 527 | Green | Strong |
| $CaWO_4$/Mn, Pb | 247 | 445 | Blue-violet | Strong |
| $Cd_2B_2O_5$/Mn | 258 | 608 | Orange-red | Medium |
| Uranium | 282 | 518 | Yellow | Strong |
| Lead | 328 | 432 | Blue-white | Medium |
| *Phosphor* | | | | |
| $Zn_2SiO_4$/Mn | 284 | 528 | Green | Strong |
| $Sr_2P_2O_7$/Sn | 266 | 454 | Blue | Strong |
| $YVO_4$/Eu | 330 | 619** | Red | Strong |

* Uncorrected.
** Main peak wavelength.

TABLE 27

PREPARATION OF FOUR KINDS OF FLUORESCENT SINTERED PLATES

| *Sintered plate* | *Fluorescent glass* | *Ratio of silica gel* to fluorescent glass to UV-absorbing glass (w/w/w)* |
|---|---|---|
| A | $Zn_2SiO_4$/Mn | 2:1:6 |
| B | $CaWO_4$/Mn, Pb | 2:1:6 |
| C | $Cd_2B_2O_5$/Mn | 2:2:3 |
| D** | $Zn_2SiO_4$/Mn-$CaWO_4$/Mn, Pb-$YVO_4$/Eu*** | 1:0.4:3 |

* Merck silica gel H (Type 60).
** Mixed fluorescent sintered plate.
*** Toshiba phosphor (SPD-373B) instead of $Cd_2B_2O_5$/Mn glass.

Table 28 shows the $hR_F$ values and detection limits of steroidal hormones. The separation was excellent and the detection limit was nearly equal to that obtained with a Merck pre-coated silica gel plate.

TABLE 28

TLC OF STEROIDAL HORMONES ON ZINC SILICATE–FLUORESCENT SILICA GEL SINTERED PLATE

The limit of detection was 0.05 $\mu$g in each instance. Detection: UV (254 nm), fluorescence quenching spot.

| *n** | $hR_F$ *value*** | | |
|---|---|---|---|
| | *Cortisone* | *Testosterone* | *Progesterone* |
| 1 | 9 | 40 | 65 |
| 2 | 8 | 42 | 68 |
| 3 | 9 | 42 | 68 |
| 4 | 11 | 44 | 70 |
| 5 | 9 | 45 | 71 |
| $\bar{x} \pm$ s.d.**** | 9 ± 1 | 43 ± 2 | 68 ± 2 |
| Merck silica gel pre-coated glass plate | 5 | 28 | 52 |

* Recycled by soaking in chromic acid mixture after spraying with sulphuric acid.
** Solvent: chloroform–acetone (9:1).
*** Mean ± standard deviation.

Table 29 presents the $hR_F$ values and fluorescent quenching colours of water-soluble vitamins using the mixed fluorescent[35] silica gel sintered plates. Table 30 shows the successful separation of lead, tin, chromium, nickel, copper(II), iron(III) and bismuth cations. These cations can be located rapidly by the fluorescence quenching method even after development in solvent systems containing strong acids such as nitric and hydrochloric acids. The author confirmed that the fluorescent plates could be used repeatedly by dipping in concentrated nitric acid or chromic acid mixture, without any change in their fluorescent and chromatographic properties.

In the preparation of the acid-resistant sintered plates, a "UV-rays" glass (borosilicate glass) was used instead of soda-lime glass[36], because the former is

TABLE 29

TLC OF WATER-SOLUBLE VITAMINS ON MIXED FLUORESCENT SILICA GEL SINTERED PLATE

| *n* | *Thiamine* | | *Riboflavin* | | *Nicotinamide* | |
|---|---|---|---|---|---|---|
| | $hR_F$* | *Fluorescent colour*** | $hR_F$* | *Fluorescent colour*** | $hR_F$* | *Fluorescent colour*** |
| 1 | 16 | Red | 25 | Yellow | 76 | Reddish violet |
| 2 | 11 | Red | 23 | Yellow | 69 | Reddish violet |
| 3 | 13 | Red | 20 | Yellow | 68 | Reddish violet |
| $\bar{x} \pm$ s.d.**** | 13 ± 3 | | 23 ± 3 | | 71 ± 3 | |

* Solvent: acetone–water (9:1).
** Detection: fluorescence quenching spot observed by continuous UV wavelength.
*** Mean ± standard deviation.

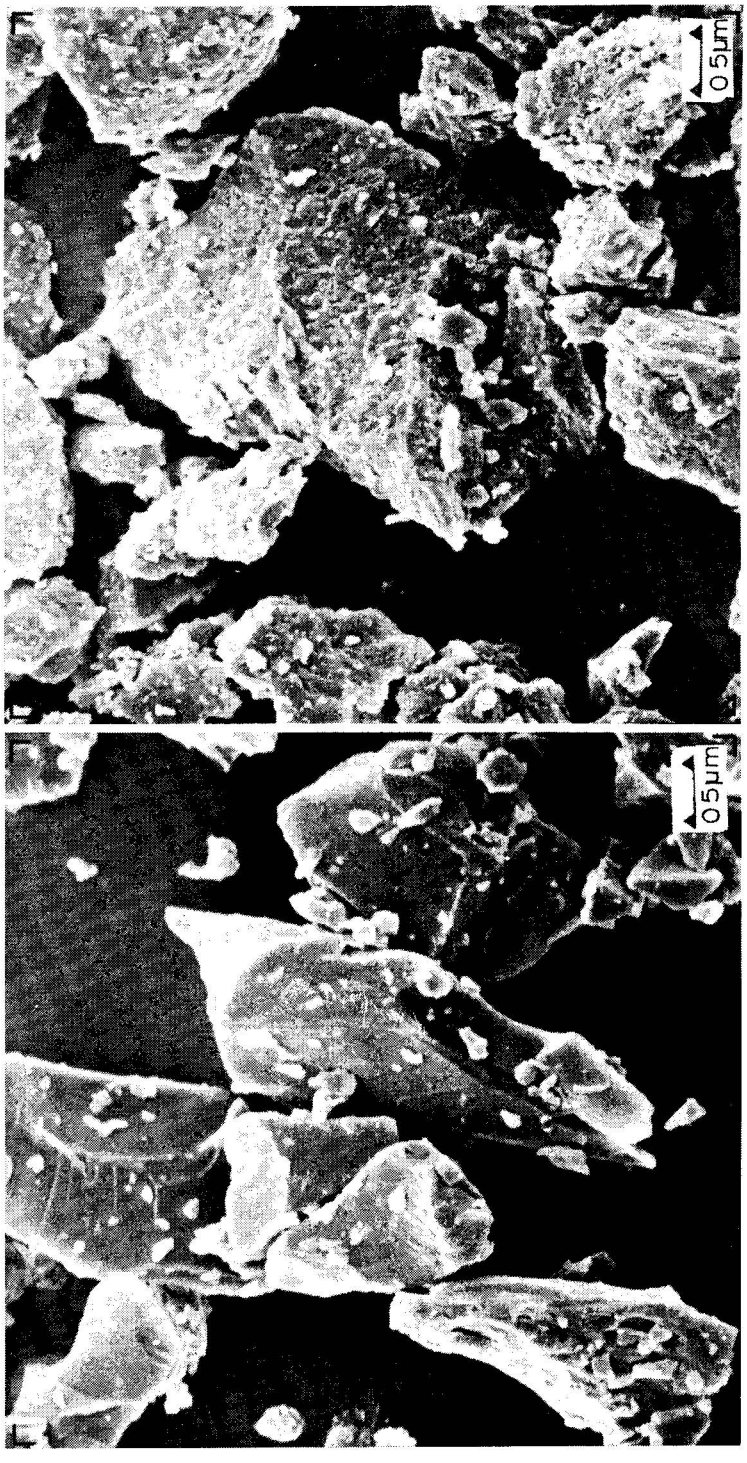



Fig. 16. SEM of $Zn_2SiO_4$/Mn fluorescent glass. Left, before crystallization; right, after crystallization.

TABLE 30

TLC OF INORGANIC CATIONS ON ZINC SILICATE–FLUORESCENT SILICA GEL SINTERED PLATE

Sample size: 20 $\mu$g per spot of each cation. Solvent: 2 *N* nitric acid–2 *N* hydrochloric acid–*n*-butanol (2:1:6, upper layer). Development time: 90 min per 10 cm. Detection: UV (254 nm) quenching spot.

| | *Inorganic cation* | | | | | | |
|---|---|---|---|---|---|---|---|
| | $Pb^{2+}$ | $Sn^{2+}$ | $Cr^{6+}$ | $Ni^{2+}$ | $Cu^{2+}$ | $Fe^{3+}$ | $Bi^{3+}$ |
| $hR_F$ value | 0 | 5 | 28 | 40 | 60 | 67 | 82 |

more sensitive than the latter to fluorescent quenching detection. Soda-lime glass absorbs UV light in the region below 300 nm. Fig. 17 compares the UV transmittance of four kinds of glasses, and Table 31 compares the detection limits between soda-lime and "UV-rays" glass plates. The author also confirmed the thermal stability of phosphors such as zinc orthosilicate, strontium pyrophosphate and yttrium vanadate under the welding conditions by determining their physical properties and fluorescent characteristics using SEM and thermal analysis[36].

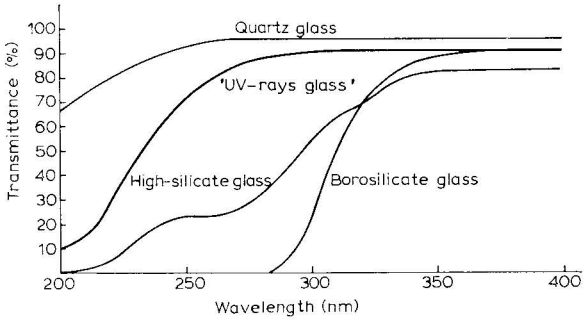



Fig. 17. Ultraviolet transmittance of various glasses. Data provided by Akagawa Glass Works and Kinmon Glass Works, Osaka, Japan.

TABLE 31

COMPARISON OF DETECTION LIMITS OF ORGANIC COMPOUNDS BETWEEN "UV-RAYS" AND SODA-LIME GLASSES AS THE BINDER

| *Fluorescent sintered plate* | *Glass* | *Compound* | *Detection limit ($\mu$g)** |
|---|---|---|---|
| Silica gel | "UV-rays" | Testosterone | 0.05 |
| | Soda-lime | | 0.2 |
| Alumina | "UV-rays" | Thebaine | 0.5 |
| | Soda-lime | | 1 |
| Kieselguhr | "UV-rays" | PCB | 0.2 |
| | Soda-lime | | 1 |

* UV quenching spot on the fluorescent background of the plate.

### *3.2. Flame-ionization detection scanning*[37,38]

Hydrogen flame-ionization detection (FID) scanning[39], when applied to TLC, is a versatile and effective method for the quantitative determination of thermo-labile or non-volatile organic compounds, which are not detectable by gas chromatography.

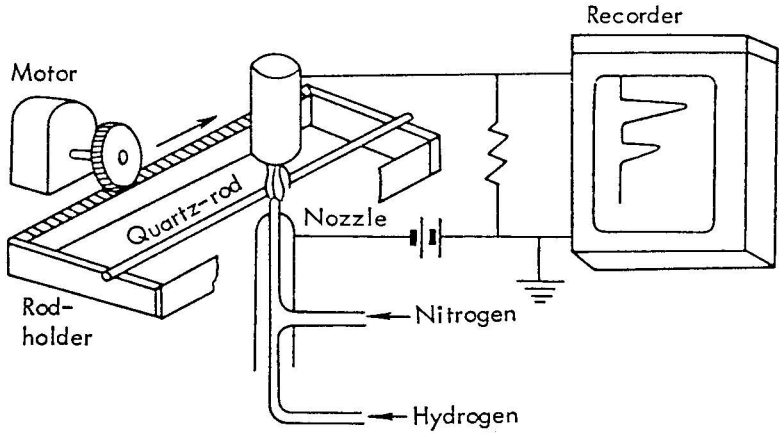



Fig. 18. Schematic diagram of flame ionization detection of TLC spots.

TABLE 32

RESPONSE CHARACTERISTICS OF SILICA GEL SINTERED RODS

Merck silica gel H–sintered glass powder (1:2). Layer thickness: 50 $\mu$m.

| *Sintered glass* | *Glass composition (%, w/w)* | | | | | | *Baseline noise* or residual response (mm)* |
|---|---|---|---|---|---|---|---|
| | $SiO_2$ | $Na_2O$ | $PbO$ | $B_2O_3$ | $Al_2O_3$ | *Others* | |
| Lead silicate | 66.0 | 3.0 | 30.0 | — | — | 1.0 | 62 |
| Soda-lime | 71.6 | 13.3 | — | — | 1.0 | 14.1 | 66 |
| Borosilicate** | 73.3 | 7.3 | — | 15.1 | 2.8 | 1.5 | 5 |
| Borosilicate*** | 80.5 | 3.8 | — | 12.9 | 2.2 | 0.6 | 3 |
| | | $ZrO_2$ | $As_2O_3$ | $Sb_2O_3$ | | | |
| Ceramic | 69.4 | 3.0 | 0.5 | 0.5 | 21.8 | 4.8 | 2 |
| Laboratory-prepared | Merck silica gel H–alumina sol (20:0.2) | | | | | | 2 |

* Maximum height from baseline.
** Akagawa Z.
*** Pyrex.

Fig. 18 gives a schematic diagram of FID scanning. The author successfully prepared silica gel– and alumina–quartz sintered rods of standard quality for use in this method. Ceramic glass was selected as a binder because it has a low baseline noise response, as shown in Table 32.

Lead silicate glass, soda-lime glass and certain borosilicate glasses showed unfavourable baseline responses to the FID, which might have resulted from the flame reaction between the FID and the glass components, disodium oxide and lead oxide. For flame-ionization detection, the author devised a compact cubic developing chamber (165 mm long × 85 mm wide × 13 mm deep). This chamber was usually lined with the silica gel sintered plates for saturation with the solvent vapour. With sintered ceramic glass–silica gel–quartz rods, a developing chamber and an FID scanner, the author was able to separate and detect minute amounts of various organic compounds such as lipids, sulphonamides, alkaloids, amino acids, water-

soluble vitamins, pesticides, polychlorinated biphenyls (PCBs), cardiac glycosides and genins, estrogens, progestins, androgens and corticoids.

The silica gel and alumina sintered rods are thermo-stable, acid resistant and can be used repeatedly without reactivation after processing in a hydrogen FID.

Fig. 19 shows the TLC separation of lipids on sintered ceramic glass–silica gel rods as located with the FID scanner. Using silica gel–sintered quartz rods, the quantitative determination of neutral lipids was achieved. The reproducibility of the response towards lipids was good. The coefficient of variation was less than 5% at three weight ratios (Table 33).

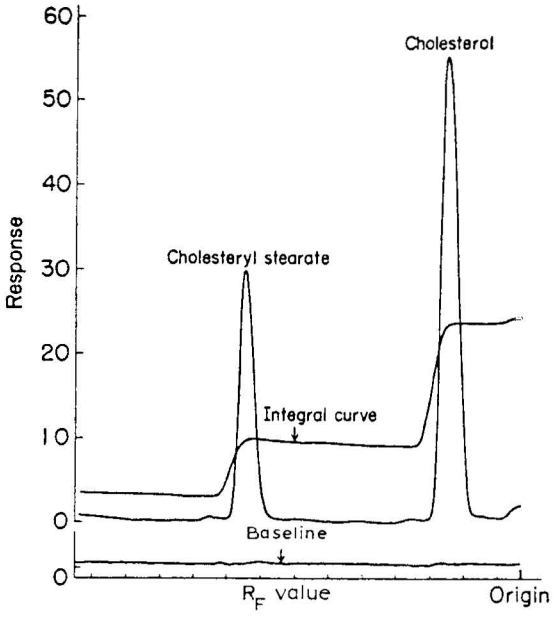



Fig. 19. TLC separation of lipids on silica gel sintered rod with FID scanner. Binder: glass ceramic.

Fig. 20 illustrates the TLC separation and determination of sulphanilic acid and sulphonamides. Alumina–sintered ceramic rods are useful for the separation and quantitation of silica gel-labile compounds.

The sintered rods, when used together with FID equipment, can facilitate both qualitative and quantitative analyses of lipids in biological fluids[40,41], heavy oil fractions in the petroleum industry[42], complex organic components in higher plants[43,44] and conjugated bile acids in bear gall[45].

## 4. THEORETICAL CONSIDERATIONS ON SINTERED THIN-LAYER CHROMATOGRAPHY

### *4.1. Inorganic anions*

Thus far, qualitative analyses of inorganic anions by paper chromatography[46] and TLC[47,48] have been reported. However, the detection of the separated anions

TABLE 33

REPRODUCIBILITY OF RESPONSE TOWARDS LIPIDS ON SILICA GEL SINTERED RODS WITH FID SCANNER

Merck silica gel H-sintered glass ceramic powder (1:4), developed with *n*-hexane–diethyl ether–acetic acid (180:30:1). Sample: mixture of 1.03 mg of cholesterol and 0.98 mg of cholesteryl stearate was dissolved in 200 $\mu$l of tetrahydrofuran.

| *Sample size ($\mu$g)* | *Run No.** | *Cholesterol* | | *Cholesteryl stearate* | |
|---|---|---|---|---|---|
| | | *Peak area* | | *Peak area* | |
| | | *mm²* | *%* | *mm²* | *%* |
| 5 | 1 | 17.5 | 60.8 | 11.3 | 39.2 |
| | 2 | 18.2 | 60.1 | 12.1 | 39.9 |
| | 3 | 16.8 | 59.0 | 11.7 | 41.0 |
| | 4 | 15.0 | 61.7 | 9.3 | 38.3 |
| | 5 | 16.3 | 59.4 | 10.9 | 40.6 |
| | $\bar{x} \pm \sigma$ | 17 ± 1 | 60 ± 1 | 11 ± 1 | 40 ± 1 |
| | C.V. | | 1.7 | | 2.5 |
| 10 | 1 | 39.4 | 57.1 | 29.6 | 42.9 |
| | 2 | 41.8 | 55.3 | 32.5 | 43.7 |
| | 3 | 41.5 | 60.1 | 27.6 | 39.9 |
| | 4 | 40.2 | 61.9 | 24.8 | 38.1 |
| | 5 | 43.8 | 58.2 | 31.4 | 41.8 |
| | $\bar{x} \pm \sigma$ | 41 ± 2 | 56 ± 2 | 30 ± 3 | 41 ± 2 |
| | C.V. | | 3.6 | | 4.4 |
| 15 | 1 | 83.5 | 60.5 | 54.5 | 39.5 |
| | 2 | 84.2 | 57.3 | 62.5 | 42.7 |
| | 3 | 76.0 | 58.9 | 53.0 | 41.1 |
| | 4 | 78.0 | 57.3 | 58.0 | 42.6 |
| | 5 | 80.9 | 59.6 | 54.7 | 40.4 |
| | $\bar{x} \pm \sigma$ | 80 ± 3 | 59 ± 1 | 57 ± 1 | 44 ± 2 |
| | C.V. | | 1.7 | | 2.5 |

* $\bar{x} \pm \sigma$ = mean ± standard deviation. C.V. = coefficient of variation.

with various colour reagents was troublesome. Some anions (iodide, bromide, chloride, thiocyanate, thiosulphate and sulphite) are known to quench the fluorescence of quinine, fluorescein and eosin, and oxyanions such as iodate, bromate and nitrate have a quenching effect on the fluorescence of anthranilic acid and naphthol[49]. Nishikawa *et al.*[50] have applied the fluorescence characteristics of morin–metal complexes to the fluorimetry of metal ions.

The author has established a simple and rapid method of detecting inorganic anions using the fluorescent aluminium–morin complex[51]. 29 inorganic anions were developed on paper and cellulose thin layers and located under UV light at 365 nm after spraying with this complex. Of these anions, 25, such as halides, halogenoxy acids, sulphur-containing anions, cyanoferrates, chromates, nitrite, nitrate, phosphate, tungstate, molybdate and vanadates, showed fluorescence quenching on the chromatograms. The remaining anions (arsenite, selenite and selenate) did not show a substantial quenching effect. Tables 34 and 35 show the separation and detection of these inorganic anions. The fluorescence quenching effect of the anions could be classified into three groups: strong (violet colour: iodo- and bromoxy

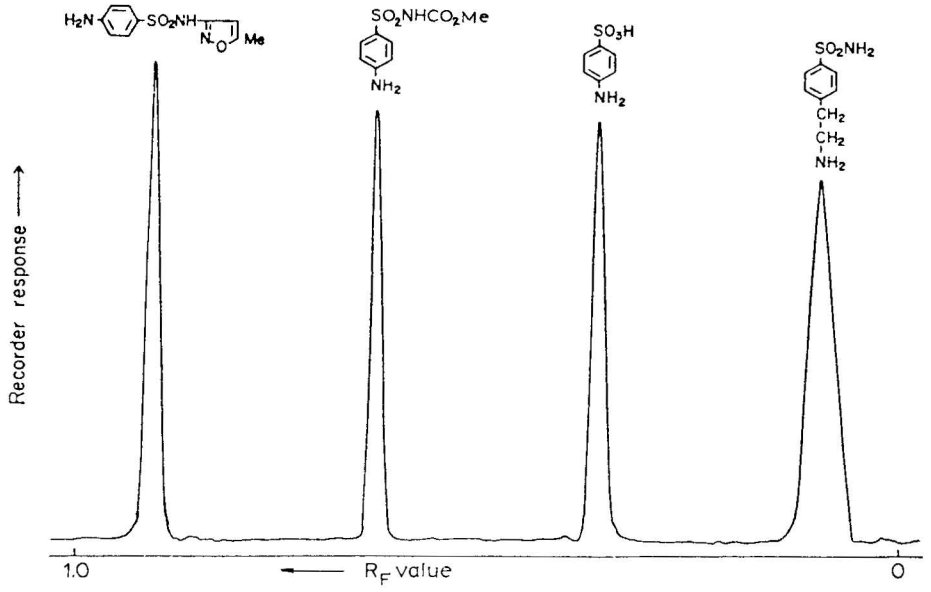



Fig. 20. TLC separation of sulphanilic acid and sulphonamides on silica gel sintered rods with FID scanner. Developer: *n*-butanol–ethanol–0.1 *N* acetic acid (3:1:1).

TABLE 34

QUALITATIVE ANALYSIS OF INORGANIC ANIONS

| *Anion* | *Cation* | *$hR_F$ value of anion** | | | *Fluorescent colour*** | *Detection* |
|---|---|---|---|---|---|---|
| | | *TLC* | | *PPC* | | |
| $F^-$ | $Na^+$ | 0 | | 0 | Blue | $AgNO_3$–UV (254 nm) |
| $Cl^-$ | $K^+$ | 38 | | 45 | Blue | |
| $Br^-$ | $Na^+$ | 60 | | 57 | Blue | |
| $I^-$ | $K^+$ | 77 | 78*** | 72 | Blue | |
| $IO_3^-$ | $Na^+$ | 13 | 30*** | 16 | Violet | KI–HCl |
| $IO_3^-$ | $K^+$ | 14 | | 19 | Violet | |
| $IO_4^-$ | $Na^+$ | 12 | 31*** | 24 | Violet | |
| $BrO_3^-$ | $K^+$ | 43 | | 57 | Violet | |
| $ClO_2^-$ | $Na^+$ | 35 | | 45 | Yellow | |
| $ClO_3^-$ | $Na^+$ | 63 | | 70 | Yellow | |
| $ClO_4^-$ | $Na^+$ | 80 | | 78 | Yellow | |
| $ClO_4^-$ | $K^+$ | 78 | | 70 | Yellow | |
| $S^{2-}$ | $Na^+$ | 10 | 33§ | 11 | Blue | $AgNO_3$ |
| $S_2O_3^{2-}$ | $Na^+$ | 8 | 30§ | 9 | Blue | $AgNO_3$ |
| $SCN^-$ | $K^+$ | 85 | | 70 | Yellow | $Fe(NO_3)_3$–$HNO_3$ |

* Solvent system: 28% ammonia solution–acetone–*n*-butanol (60:130:30). TLC: Merck precoated cellulose plate; 90 min per 10 cm. PPC: Toyo filter-paper No. 51A; 180 min per 20 cm. Sample size: 10 μg/μl.

** Under UV irradiation at 365 nm after spraying with 0.3 m*M* aluminium isopropoxide and 1 m*M* morin in ethanol.

*** Dioxane–water (3:2); 90 min per 10 cm.

§ Acetone–28% ammonia solution (3:2); 80 min per 10 cm.

TABLE 35

QUALITATIVE ANALYSIS OF INORGANIC ANIONS

| *Anion* | *Cation* | *$hR_F$ value of anion** | | *Fluorescent colour*** | *Detection* |
|---|---|---|---|---|---|
| | | *TLC* | *PPC* | | |
| $Fe(CN)_6^{4-}$ | $K^+$ | 2 | 3 | Violet | $Fe(NO_3)_3$–$HNO_3$ |
| $Fe(CN)_6^{3-}$ | $K^+$ | 32 | 20 | Violet | $Fe(NO_3)_3$–$HNO_3$ |
| $CrO_4^{2-}$ | $K^+$ | 10*** | — | Violet | $Pb(OAc)_2$ |
| $CrO_7^{2-}$ | $K^+$ | 68*** | — | Violet | $Pb(OAc)_2$ |
| $NO_2^-$ | $Na^+$ | 55 | 62 | Violet | Sulphanilic acid, etc. |
| $NO_3^-$ | $Na^+$ | 60 | 72 | Violet | Sulphanilic acid, etc. |
| $WO_4^{2-}$ | $Na^+$ | 44§ | — | Yellow | $AgNO_3$ |
| $MoO_4^{2-}$ | $Na^+$ | 36§ | — | Green | $AgNO_3$ |
| $VO_3^-$ | $Na^+$ | 20§ | — | Blue | Diaminobenzidine–KI–$SnCl_2$–HCl |
| $VO_4^{3-}$ | $Na^+$ | 16§ | — | Blue | Diaminobenzidine–KI–$SnCl_2$–HCl |
| $AsO_2^-$ | $Na^+$ | 43*** | — | Weak | Dithizone–HCl |
| $AsO_4^{3-}$ | $Na^+$ | 42*** | — | Weak | Dithiozne–HCl |
| $SeO_3^{2-}$ | $Na^+$ | 29*** | — | Weak | $SnCl_2$–HCl |
| $SeO_4^{2-}$ | $Na^+$ | 30*** | — | Weak | $SnCl_2$–HCl |

* Solvent system, TLC plate, PPC filter-paper and sample size as in Table 34.
** As in Table 34.
*** Acetone–acetic acid–water (20:1:10); 120 min per 10 cm.
§ As in Table 34..

acids, sulphite, sulphate, cyanoferrates, chromates, nitrite, nitrate and phosphate), medium (blue colour: halide, sulphide and vanadates) and weak (yellow colour: chlorooxy acids, thiocyanate and tungstate).

The mechanism of the fluorescence quenching caused by these inorganic anions might involve a reaction that converts the aluminium–morin complex into non-fluorescent aluminium salts:

$$\underset{\text{fluorescent}}{Al(C_{15}H_9O_7)_3} + 6X^- \rightarrow \underset{\text{non-fluorescent}}{AlX_6^{3-}} + 3C_{15}H_{10}O_7 \quad (1)$$

where X is a monodentate ligand.

In the separation of these inorganic anions, iodoxy, sulphate, silicate, borate, phosphate and fluoride were strongly adsorbed on paper or cellulose thin layers. Chloroxy, chlorite, nitrate, nitrite, chloride and bromide were moderately adsorbed on the stationary phase. Perchlorate and iodide were weakly adsorbed on the stationary phase when the basic, polar mobile phase 28% ammonia solution–acetone–*n*-butanol was used.

### *4.2. Organic compounds*

#### *4.2.1. Steroidal sapogenins*[52]

There are many steroidal sapogenins of natural origin[53] with the general structure shown in Fig. 21. The author conducted systematic TLC analysis of 40 kinds of steroidal sapogenins from higher plants such as *Dioscores*, *Heloniopsis*, *Metanarthecium*, *Smilax*, *Rhodea*, *Convallaria* and *Digitalis* (Table 36).

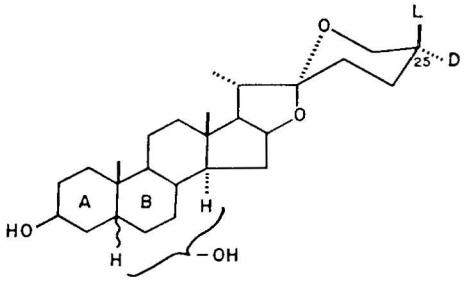



Fig. 21. Structure of steroidal sapogenins.

TABLE 36

STEROIDAL SAPOGENINS SEPARATED BY TLC ON SINTERED PLATES

| *Compound* | *M.p.(°C)* | *A/B ring* | *$C_{25}$-Me* | *OH* | *Plant source* |
|---|---|---|---|---|---|
| Chiapagenin | 249–251 | $\Delta^5$ | L | 3β,12β | *Dioscorea chiapasensis* |
| Isochiapagenin | 233–235 | $\Delta^5$ | D | 3β,12β | *Dioscorea chiapasensis* |
| Chlorogenin | 265–266 | *trans* | D | 3β,6α | Synthetic from laxogenin |
| β-Chlorgenin | 240–242 | *trans* | L | 3β,6β | Synthetic from laxogenin |
| Convallamarogenin | 253–258 | *cis* | $=CH_2$ | 1β,3β | *Convallaria majalis* |
| Digitogenin | 296 | *trans* | D | 2α,3β,15β | *Digitalis purpurea* |
| Diosgenin | 203–206 | $\Delta^5$ | D | 3β | *Dioscorea tokoro* |
| Diotigenin | 281–282 | *cis* | L | 2β,3α,4β | *Dioscorea tenuipes* |
| Isodiotigenin | 279–281 | *cis* | D | 2β,3α,4β | *Dioscorea tokoro* |
| Gentrogenin | 213–214 | $\Delta^5$ | D | 3β,12-oxo | *Heloniopsis orientalis* |
| Gitogenin | 272 | *trans* | D | 2α,3β | *Digitalis purpurea* |
| Neogitogenin | 250–254 | *trans* | L | 2α,3β | *Anemarrhena asphodeloids* |
| Hecogenin | 260 | *trans* | D | 3β,12-oxo | *Agave rigida* var. *sisalana* |
| Heloniogenin | 212–213 | $\Delta^5$ | D | 3β,12α | *Heloniopsis orientalis* |
| Kitigenin | 298 | *cis* | D | 1β,3β,4β,5β | *Reinechea cornea* |
| Kogagenin | 310 | *cis* | D | 1β,2β,3α,5β | *Dioscorea tokoro* |
| Kryptogenin | 189 | $\Delta^5$ | — | 3β,16,22-diox | *Heloniopsis orientalis* |
| Laxogenin | 210–212 | *trans* | D | 3β,6-oxo | *Smilax sieboldii* |
| Luvigenin | 183–185 | — | D | Aromatic A ring 4-Me | *Metanarthecium luteo-viride* |
| Markogenin | 149–151 | *cis* | L | 2β,3β | *Anemarrhena asphodeloides* |
| Metagenin | 264–265 | *cis* | D | 2β,3β,11α | *Metanarthecium luteo-viride* |
| Meteogenin | 157–158 | — | D | Aromatic A ring 1-Me,11α | *Metanarthecium luteo-viride* |
| Narthogenin | 214–216 | $\Delta^5$ | L | 3β,27 | *Metanarthecium luteo-viride* |
| Isonarthogenin | 238–240 | $\Delta^5$ | D | 3β,27 | *Metanarthecium luteo-viride* |
| Pennogenin | 234–235 | $\Delta^5$ | D | 3β,17α | *Heloniopsis orientalis* |
| Rhodeasapogenin | 284–285 | *cis* | L | 1β,3β | *Rohdea japonica* |
| Isorhodeasapogenin | 240–241 | *cis* | D | 1β,3β | *Convallaria keiskei* |
| Ruscogenin | 204–206 | $\Delta^5$ | D | 1β,3β | *Ruscus aculeatus* |
| Sarsasapogenin | 198 | *cis* | L | 3β | *Asparagus cochinchinensis* |
| Smilagenin | 183–188 | *cis* | D | 3β | *Allium grayi* |
| Tigogenin | 205 | *trans* | D | 3β | *Digitalis purpurea* |
| Neotigogenin | 198–199 | *trans* | L | 3β | *Smilax sieboldii* |
| Tokorogenin | 266–268 | *cis* | D | 1β,2β,3α | *Dioscorea tokoro* |
| 1α-Tokorogenin | 218–219 | *cis* | D | 1α,2β,3α | Synthetic from kogagenin |
| Neotokorogenin | 266–269 | *cis* | L | 1α,2β,3α | *Dioscorea tenuipes* |
| Yonogenin | 242–243 | *cis* | D | 2β,3α | *Dioscorea tokoro* |
| Neoyonogenin | 198–199 | *cis* | D | 2β,3α | *Dioscorea tenuipes* |

TABLE 37

TLC SEPARATION OF STEROIDAL SAPOGENIN ISOMERS ON SILICA GEL AND ALUMINA SINTERED PLATES

| *A/B cis-, trans- isomers* | | *$hR_F$ value** | | | | | |
|---|---|---|---|---|---|---|---|
| *Compound* | *cis/trans* | *Silica gel* | | | *Alumina* | | |
| | | *Sintered* | | *Merck* | *Sintered* | | *Merck* |
| Smilagenin | *cis* | 72 | $\Delta 9$ | 39 | $\Delta 9$ | 85 | $\Delta 14$ | 63 | $\Delta 11$ |
| Tigogenin | *trans* | 63 | | 30 | | 71 | | 52 | |
| Sarsasapogenin | *cis* | 71 | $\Delta 8$ | 37 | $\Delta 7$ | 87 | $\Delta 8$ | 63 | $\Delta 9$ |
| Neotigogenin | *trans* | 63 | | 30 | | 79 | | 54 | |
| 2$\alpha$-Samogenin | *cis* | 21 | $\Delta 9$ | 9 | $\Delta 1$ | 27 | $\Delta 13$ | 17 | $\Delta 8$ |
| Gitogenin | *trans* | 30 | | 10 | | 40 | | 25 | |

* Benzene–ethanol (17:1). Distance travelled: 15 cm. Detection: conc. $H_2SO_4$. $\Delta$ = Difference.

Table 37 shows the excellent resolution of stereoisomeric pairs of sapogenins. They differ from each other in the A and B ring junction: 2$\alpha$-samogenin (an A/B *cis*-isomer) and gitogenin (an A/B *trans*-isomer). Smilagenin and tigogenin, and sarsasapogenin and neotigigenin, are also A/B *cis*- and *trans*-isomers, respectively. As shown in Table 38, a good resolution was also obtained with sapogenins differing in the configuration of the hydroxyl group, such as chlorogenin (an equatorial 6$\alpha$-hydroxyl compound) and $\beta$-chlorogenin (an axial 6$\beta$-hydroxyl compound) and tokorogenin (an equatorial 1$\beta$-hydroxyl compound) and 1$\alpha$-tokorogenin (an axial 1$\alpha$-hydroxyl compound). On the other hand, separation was not possible of diastereomeric pairs differing in the configuration at the $C_{25}$ methyl group, that is, D- and L-isomers, such as gitogenin and neogitogenin, smilagenin and sarsasapogenin, tigogenin and neotigogenin, tokorogenin and neotokorogenin, and yonogenin and neoyonogenin (Table 39). As shown in Table 38, however, clear separations were obtained with

TABLE 38

TLC SEPARATION OF STEROIDAL SAPOGENIN ISOMERS ON SILICA GEL AND ALUMINA SINTERED PLATES

| *Axial equatorial epimers* | *$hR_F$ value** | | | | | |
|---|---|---|---|---|---|---|
| | *Silica gel* | | | *Alumina* | | |
| | *Sintered* | | *Merck* | *Sintered* | | *Merck* |
| Chlorogenin (6$\alpha$-OH, eq.) | 21 | $\Delta 6$ | 5 | $\Delta 2$ | 23 | $\Delta 9$ | 11 | $\Delta 4$ |
| $\beta$-Chlorogenin (6$\beta$-OH, ax.) | 27 | | 7 | | 32 | | 15 | |
| Tokorogenin (1$\beta$-OH, eq.) | 13 | $\Delta 5$ | 5 | $\Delta 2$ | 11 | $\Delta 4$ | 3 | $\Delta 1$ |
| 1$\alpha$-Tokorogenin (1$\alpha$-OH, ax.) | 18 | | 7 | | 15 | | 4 | |
| Narthogenin (27-OH, ax.)** | 60 | $\Delta 5$ | 8 | $\Delta 1$ | 53 | $\Delta 7$ | 6 | $\Delta 1$ |
| Isonarthogenin (27-OH, eq.)** | 55 | | 7 | | 46 | | 5 | |

* Benzene–ethanol (17:1). Distance travelled: 15 cm. Detection: conc. $H_2SO_4$. $\Delta$ = Difference.
** Chloroform–acetone (9:1).

TABLE 39

TLC OF STEROIDAL SAPOGENIN $C_{25}$-METHYL ISOMERS ON SILICA GEL AND ALUMINA SINTERED PLATES

| *Diastereomer* | *$hR_F$ value** | | | |
|---|---|---|---|---|
| | *Sintered* | *Silica gel* | *Sintered* | *Alumina* |
| | *A*** | *B**** | *A*** | *B**** |
| Chiapagenin (L) | 19 | 43 | 15 | 53 |
| Isochiapagenin (D) | 17 | 41 | 15 | 52 |
| Diotigenin (L) | 75§ | 6 | 35§ | 0 |
| Isodiotigenin (D) | 76§ | 6 | 36§ | 0 |
| Gitogenin (D) | 6 | 20 | 2 | 9 |
| Neogitogenin (L) | 6 | 20 | 2 | 9 |
| Rhodeasapogenin (L) | 20 | 40 | 20 | 43 |
| Isorhodeasapogenin (D) | 21 | 42 | 21 | 45 |
| Sarsasapogenin (L) | 50 | 78 | 64 | 74 |
| Smilagenin (D) | 47 | 80 | 65 | 74 |
| Tigogenin (D) | 32 | 76 | 50 | 68 |
| Neotigogenin (L) | 32 | 74 | 50 | 68 |
| Tokorogenin (D) | 81§ | 9 | 44§ | 0 |
| Neotokorogenin (L) | 82§ | 9 | 44§ | 0 |
| Yonogenin (D) | 4 | 25 | 2 | 15 |
| Neoyonogenin (L) | 4 | 25 | 2 | 15 |

* Distance travelled: 15 cm per 2 h. Detection: conc. $H_2SO_4$.
** Solvent A: benzene–ethanol (17:1).
*** Solvent B: chloroform–acetone (9:1).
§ Solvent C: chloroform–methanol (9:1).

diastereomers with a hydroxymethyl group instead of a methyl substituent at $C_{25}$, that is, narthogenin (L-form) and isonarthogenin (D-form), which resisted separation on laboratory-prepared plates and commercial pre-coated plates. This result indicates that the polarity of the substituent attached to the steroidal sapogenin nucleus influences the resolution of the diastereoisomers. Table 40 shows the excellent reproducibility of separations with various polar sapogenins.

TABLE 40

REPRODUCIBILITY OF $hR_F$ VALUES OF STEROIDAL SAPOGENINS ON SILICA GEL SINTERED PLATES

Solvent: chloroform–acetone (9:1). Detection: cinnaminaldehyde–antimony richloride.

| *Compound* | *Run No.* | | | | | | | | | | $\bar{x} \pm \sigma$* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | *1* | *2* | *3* | *4* | *5* | *6* | *7* | *8* | *9* | *10* | |
| Luvigenin | 81 | 83 | 82 | 83 | 87 | 85 | 78 | 87 | 80 | 85 | 83 ± 3 |
| Diosgenin | 59 | 62 | 62 | 62 | 66 | 65 | 58 | 66 | 60 | 65 | 63 ± 3 |
| Pennogenin | 49 | 52 | 52 | 52 | 56 | 49 | 56 | 56 | 53 | 55 | 53 ± 5 |
| Hecogenin | 35 | 42 | 42 | 42 | 46 | 46 | 40 | 45 | 41 | 44 | 43 ± 3 |
| Chiapagenin | 25 | 26 | 26 | 26 | 28 | 28 | 24 | 27 | 25 | 26 | 26 ± 1 |
| Yonogenin | 17 | 22 | 23 | 21 | 22 | 22 | 19 | 21 | 19 | 22 | 21 ± 2 |
| Gitogenin | 13 | 19 | 19 | 19 | 20 | 18 | 16 | 17 | 17 | 19 | 18 ± 2 |
| Kogagenin | 1 | 1 | 1 | 1 | 2 | 1 | 2 | 1 | 1 | 2 | 1 ± 0 |

* Mean ± standard deviation.

The application of sintered TLC to crude extracts containing steroidal sapogenins from higher plants revealed the presence of diotigenin, tokorogenin and yonogenin in the aerial part of *Dioscorea tokoro*, tokorogenin and diosgenin in its rhizome, diotigenin, yonogenin and sterol in its seedlings, ruscogenin and sterol in *Ophyopogon japonicus*, and diotigenin, tokorogenin, yonogenin, sterol and chlorophylls in *Dioscorea tenuipes*[54]. These components had not been separated by paper chromatography or TLC on laboratory-prepared and pre-coated plates.

*4.2.2. Organic phosphates, sulphates and nitrates*[22,55]

Sulphate[56,57] and phosphate[58,59] esters of organic compounds are often extracted and isolated from natural products and biological materials. Paper chromatography[60], TLC[61], column chromatography[62] and, recently, high-performance liquid chromatography[63] of these organic esters have been reported.

*4.2.2.1. Steroidal sulphates and nitrates.* Cholesteryl-3-O-sulphate [mobile phase: (a) *n*-butanol–acetic acid–water (3:1:1) and (b) benzene–acetone (9:1)] and dehydroisoandrosterone sulphate [mobile phase: (a) and (c) chloroform–acetone (4:1)] were chosen as test compounds for silica gel sintered TLC. Both esters moved from the original point in the acidic mobile phase (a), but not in the neutral mobile phases (b) and (c), presumably owing to strong adsorption on to the silica gel thin layer containing soda-lime glass powder. The $hR_F$ values were small compared with those of the free steroids [cholesterol, 70 in (a), 43 in (b); sulphate, 54 in (a), 0 in (b); dehydroisoandrosterone, 73 in (a), 54 in (c); sulphate, 37 in (a), 0 in (c)].

6-Nitroandrostenediol diacetate, a steroidal nitrate, moved smoothly even in the neutral mobile phase benzene–ethyl acetate (4:1). Its $hR_F$ value (55) was slightly lower than that of the steroid itself (61).

*4.2.2.2. Sugar phosphates and sulphates.* Table 41 shows the $hR_F$ values of hexose phosphates and free hexoses on silica gel sintered plates and Merck silica gel

TABLE 41

TLC OF SUGAR PHOSPHATES ON SILICA GEL PLATES

| *Hexose phosphate** | *$hR_F$ value*** | | | |
|---|---|---|---|---|
| | *REPLATE**** | *$\Delta hR_F$* | *MS*[§] | *$\Delta hR_F$* |
| Aldose: | | | | |
| Glucose | 73 ± 3 | | 30 ± 1 | |
| Glucose-1-phosphate | 50 ± 2 | 23 | 9 ± 2 | 21 |
| Glucose-6-phosphate | 55 ± 3 | 18 | 10 ± 1 | 20 |
| Ketose: | | | | |
| Fructose | 73 ± 2 | | 33 ± 1 | |
| Fructose-1-phosphate | 51 ± 2 | 22 | 10 ± 1 | 23 |
| Fructose-6-phosphate | 59 ± 4 | 14 | 12 ± 1 | 21 |
| Fructose-1,6-diphosphate | 35 ± 3 | 38 | 5 ± 2 | 28 |

* Products of Wako Chemicals.

** Developer, BAW311; detection, Al-morin reagent followed by conc. sulphuric acid; development rate per 10 cm, 2.0 h (REPLATE), 2.5 h (Merck silica gel–glass plate); $n = 5$.

*** REPLATE: trade-name of silica gel sintered plate distributed by Yamamoto Scientific (Tokyo, Japan).

§ Merck silica gel–glass plate.

glass plates. The $hR_F$ values on REPLATEs were larger than those on the Merck plates. The difference in $hR_F$ values ($\Delta hR_F$, 38) between fructose and its 1,6-diphosphate on the REPLATE was almost twice that (22, 14) between fructose and its 1- or 6-monophosphate, showing the additivity of $\Delta hR_F$ values for mono- and diphosphates.

Table 42 gives the $hR_F$ values of three kinds of hexoses and their sulphates on REPLATEs. Additivity of $\Delta hR_F$ values was also observed, as with hexose phosphates.

TABLE 42

TLC OF SUGAR SULPHATES ON SILICA GEL PLATES

| *Hexose sulphate** | *$hR_F$ value*** | | | |
|---|---|---|---|---|
| | *REPLATE**** | *$\Delta hR_F$* | *MS*§ | *$\Delta hR_F$* |
| *Aldose* | | | | |
| Glucose | 72 | | 27 | |
| Glucose-6-sulphate | 62 | 10 | 17 | 10 |
| Glucose-1,6-disulphate | 55 | 17 | 10 | 17 |
| Galactose | 72 | | 28 | |
| Galactose-6-sulphate | 61 | 11 | 16 | 12 |
| Galactose-1,6-disulphate | 51 | 21 | 10 | 18 |
| *Ketose* | | | | |
| Fructose | 71 | | 32 | |
| Fructose-6-sulphate | 63 | 8 | 21 | 11 |
| Fructose-1,6-disulphate | 56 | 15 | 12 | 9 |

* Synthesized by Soda's method[64].
** As in Table 41.
*** As in Table 41.
§ As in Table 41.

*4.2.2.3. Adenosine phosphates.* Table 43 shows the results of the TLC separation of adenosine 5′-mono-, -di- and -triphosphates (AMP, ADP and ATP) on polyethyleneimine (PEI)–cellulose–polyethylene sintered sheets[20]. The $hR_F$ values of adenosine phosphates decrease as the number of phosphate groups increases, in a similar

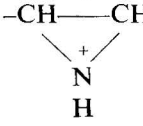
TABLE 43

TLC OF NUCLEOTIDES ON PEI–CELLULOSE SINTERED SHEET*

The cellulose sintered sheet was impregnated with 1% PEI (mol. wt. 30,000–40,000) hydrochloride aqueous solution. Detection: UV (254 nm) and Al–morin.

| *Compound* | *$hR_F$ value* | *Developer** |
|---|---|---|
| Adenosine-5′-monophosphate | 55 ($\Delta hR_F$ 25) | 1.0 *M* LiCl |
| Adenosine-5′-diphosphate | 30 ($\Delta hR_F$ 23) | 1.0 *M* LiCl |
| Adenosine-5′-triphosphate | 7 | 1.0 *M* LiCl |

* Development rate: 60 min per 10 cm.

manner to the steroidal sulphates mentioned above. As PEI–cellulose is an anion exchanger, sorption of the phosphate anion in the substrate on the stationary phase (weakly basic cation  ) is more evident than in the partition chromatography of the inorganic and organic anions cited above.

## 5. CONCLUSION

The finding of unexpected stability of inorganic and organic adsorbents under "welding" conditions led the author to the preparation of various sintered plates, rods and sheets. The TLC separation data for a variety of compounds presented here show that parallelism exists between mobilities on sintered plates and those on laboratory-prepared and pre-coated plates, indicating that the nature of the binder does not affect the adsorption process that occurs on the surface of adsorbents. In general, sintered plates and rods require developing solvents of slightly lower polarity to give mobilities comparable to those on laboratory-prepared and pre-coated plates. It is noteworthy that sintered plates almost invariably allow satisfactory separations of polar substances such as steroidal sapogenin isomers, which resist separation on laboratory-prepared or pre-coated plates. Organic phosphates and sulphates also gave clear separations only on the sintered plates, leading to the additivity rules for their differences in $hR_F$ values.

The excellent stability of the sintered plates, rods and sheets towards abrasion

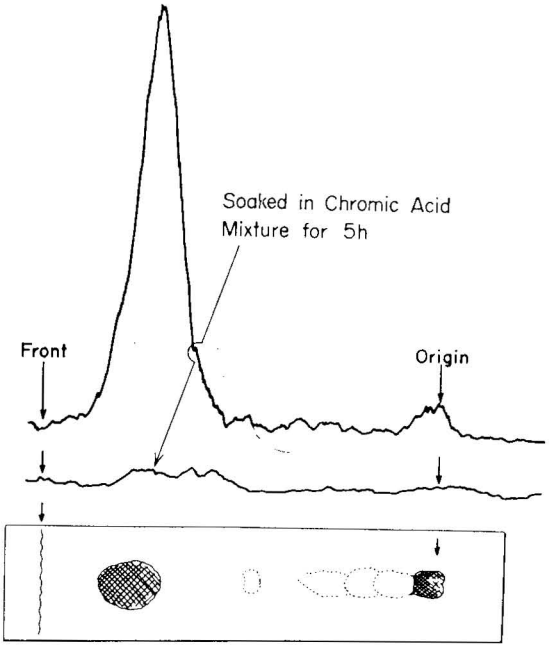



Fig. 22. Autoradiograph of $^3$H-labelled steroid on silica gel sintered plate[9].

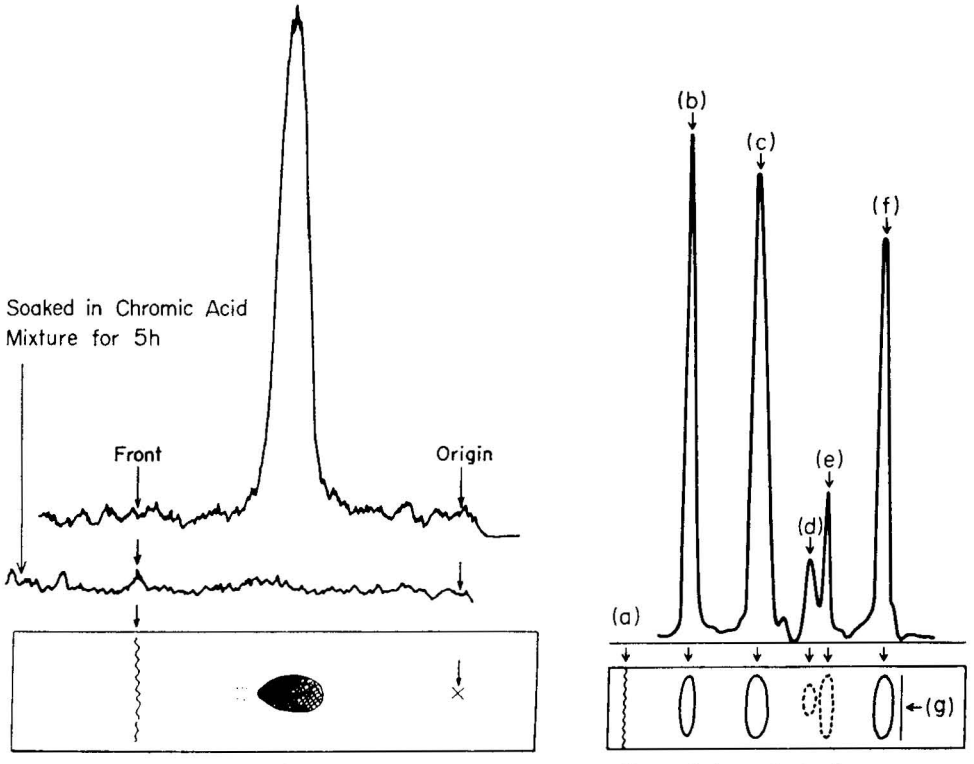



Fig. 23. Autoradiograph of $^{14}C$-labelled steroidal mixtures on silica gel sintered plate[9].

Fig. 24. Thin-layer densitometry of lipids with silica gel sintered plate[9,65]. (a) Front; (b) cholesteryl ester; (c) triglyceride; (d) diglyceride; (e) monoglyceride; (f) phospholipids; (g) origin.

heat and acids resulted in a superior reproducibility of the separation of various compounds and resolved some difficulties in current TLC techniques. For example, one can easily perform the separation and determination of radioactive compounds, the thin-layer densitometry of biological materials and the bioautography of antibiotics. Figs. 22 and 23 show autoradiograms of $^{3}H$- and $^{14}C$-radioactive steroids, and Fig. 24 shows the quantitative determination of serum lipids[66]. Fig. 25 compares a bioautogram on a silica gel sintered plate and that on a laboratory-prepared plate, showing the superiority of the sintered plate in the separation and detection of inhibition zones

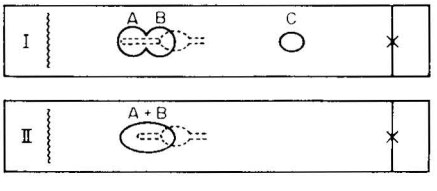



Fig. 25. Comparison of bioautography test with silica gel sintered and laboratory-prepared plates for antibacterial screening of antibiotics against *Bacillus subtilis* PCI 219. Plate I, sintered plate F; plate II, home-made plate GF. A, B and C: antibacterial spots. From ref. 9.

given by antibiotic substances. Further, the sintered plates, rods and sheets should be very useful for reversed-phase TLC, thin-layer electrophoresis, TLC with flame-ionization detection[67–69] and, probably, for many other purposes in the vast field of chromatographic analysis.

## 6. ACKNOWLEDGEMENTS

The author thanks Professor Kunio Nakagawa of Tokushima Bunri University for his continued interest and kind advice. Thanks are also due to Mr. Tetsuro Kadono of this Laboratory for help with the preparation of the sintered plates, rods and sheets.

## 7. SUMMARY

A new type of thin-layer chromatographic (TLC) plates, "sintered-glass thin-layer plates", was prepared, the layer of which consists of silica gel, alumina, Kieselguhr or another porous adsorbent, fixed with sintered glass.

These sintered plates are mechanically stable, heat- and acid resistant, and can be used in the same way as the usual laboratory-prepared or commercial TLC plates. It appears that the nature of the binder for the adsorbent particles does not essentially affect the separation process that occurs on the surface of the adsorbents. The sintered plates do not contain organic binders and are resistant to, *e.g.*, heating after being sprayed with corrosive reagents. The developed sintered plates can be regenerated readily by soaking in cleaning solutions, washing with water and reactivating by heating. The reproducibility of chromatographic separations is further improved by recycled use of the sintered plates. It was also discovered that TLC using sintered rods with flame-ionization detection is very useful for qualitative and quantitative analyses of organic compounds. The method of preparation of the sintered plates and rods, and chromatographic separation data of various organic and inorganic compounds on these sintered materials, are presented. "Welding" mechanisms among adsorbent, binder and support, and thermal stability of the adsorbent at "welding" conditions are discussed.

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CHREV. 125

# THIN-LAYER CHROMATOGRAPHIC SEPARATION OF *CINCHONA* ALKALOIDS

R. VERPOORTE, TH. MULDER-KRIEGER, J. J. TROOST and A. BAERHEIM SVENDSEN
*Department of Pharmacognosy, Gorlaeus Laboratories, Wassenaarseweg 76, P.O. Box 9502, 2300 RA Leiden (The Netherlands)*

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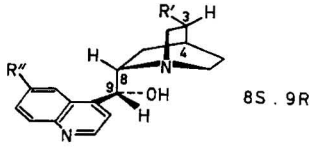
## 1. INTRODUCTION

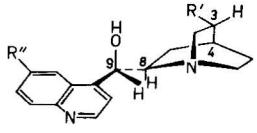
*Cinchona* alkaloids are found in the bark of *Cinchona* and *Remeijia* species of the family *Rubiaceae*. About 35 *Cinchona* alkaloids are known, of which quinine (Q) and quinidine (Qd) are the pharmaceutically most important, quinine because of its antimalarial and quinidine because of its cardiac depressant (antiarrhythmic) properties. These alkaloids have been studied by thin-layer chromatography more extensively than the other *Cinchona* alkaloids such as the stereoisomers cinchonine (C) and cinchonidine (Cd), both of which lack the methoxyl group at $C_6$, which is present in quinine and quinidine. A number of alkaloids chemically closely related to the four "parent alkaloids" mentioned are known, such as the corresponding dihydro derivatives, in which the vinyl group at $C_3$ is replaced by an ethyl group.

The dihydro alkaloids are found as common impurities in the vinyl type of alkaloids, and hence the separation of the vinyl and the corresponding dihydro derivatives has been subject of several studies. Because four asymmetric carbons are present in the parent alkaloids ($C_3$, $C_4$, $C_8$ and $C_9$), theoretically 16 isomers are possible. In nature only four are found, *e.g.*, isomers in which $C_8$ and $C_9$ are implicated: the 8*S*, 9*R* series (quinine and cinchonidine) (Fig. 1) and the 8*R*, 9*S* series (quinidine and cinchonine) (Fig. 2). In addition to the parent alkaloids, the epi series also exists, having an 8*S*, 9*S* (epiquinine, epicinchonidine) (Fig. 3) or an 8*R*, 9*R* (epiquinidine, epicinchonine) (Fig. 4) configuration.



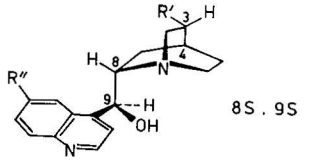

8S . 9R

Fig. 1. R′ = vinyl R″ = H cinchonidine (Cd)
R″ = OH cupreine (Cu)
R″ = $OCH_3$ quinine (Q)
R′ = ethyl R″ = H dihydrocinchonidine (HCd)
R″ = OH dihydrocupreine (HCu)
R″ = $OCH_3$ dihydroquinine (HQ)



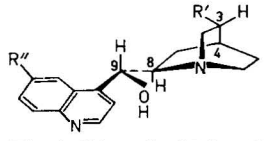

8R . 9S

Fig. 2. R′ = vinyl R″ = H cinchonine (C)
R″ = OH cupreidine (Cud)
R″ = $OCH_3$ quinidine (Qd)
R′ = ethyl R″ = H dihydrocinchonine (HC)
R″ = OH dihydrocupreidine (HCud)
R″ = $OCH_3$ dihydroquinidine (HQd)



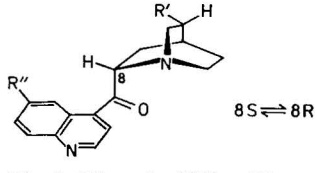

8S . 9S

Fig. 3. R′ = vinyl R″ = H epicinchonidine (epiCd)
R″ = $OCH_3$ epiquinine (epiQ)
R′ = ethyl R″ = H epidihydrocinchonidine (epiHCd)
R″ = $OCH_3$ epidihydroquinine (epiHQ)




8R . 9R

Fig. 4. R′ = vinyl R″ = H epicinchonine (epiC)
R″ = $OCH_3$ epiquinidine (epiQd)
R′ = ethyl R″ = H epidihydrocinchonine (epiHC)
R″ = $OCH_3$ epidihydroquinidine (epiHQd)




8S⇌8R

Fig. 5. R′ = vinyl R″ = H cinchoninone
R″ = $OCH_3$ quinidinone

Other minor alkaloids are cupreine and cupreidine and their dihydro derivatives. They have a hydroxyl group at $C_6$ and therefore have a more polar character than the other *Cinchona* alkaloids. Oxidation of the alkaloids can lead to the formation of N-oxides (aryl, aliphatic and di-N-oxides) and ketones (C-9, carbonyl) (Fig. 5).

Separation problems in the analysis of *Cinchona* alkaloids can be summarized as follows:

(1) separation of the parent alkaloids, Q, Qd, C and Cd;

(2) separation of the vinyl and dihydro alkaloids;

(3) separation of the epi-alkaloids from the parent alkaloids;

(4) separation of the parent alkaloid and its dihydro, epi-vinyl and epi-dihydro derivative;

(5) separation of the four possible stereoisomers of each group (Q, Qd, epiQ, epiQd and C, Cd, epiC, epiCd, for example);

(6) separation of the four compounds with the same stereochemistry (Q, HQ, Cd, HCd and Qd, HQd, C, HC, for example).

The scope of this study was to survey the literature on the thin-layer chromatographic (TLC) analysis of *Cinchona* alkaloids, to test the TLC systems described in the literature and to choose the systems most suitable for the analytical problems mentioned above.

In Table 1 the literature on the TLC of *Cinchona* alkaloids is summarized. The publications are classified into groups concerning, amongst others, the separation of the alkaloids in plant materials, in drug identification schemes, in drug of abuse screenings and in biological materials. The last two analytical fields are of interest because quinine is often found as an adulterant in drugs of abuse, and the presence of quinine in urine is often used as an indicator of the abuse of heroin.

The solvents described in the literature for the analysis of *Cinchona* alkaloids have been tested in our laboratories for the separation of the alkaloids quinine, quinidine, cinchonine, cinchonidine, dihydroquinine and dihydroquinidine. The solvents giving the best results (Tables 2 and 3) were further tested on the 24 alkaloids summarized in Table 4, leading to the choice of 18 solvents which were found to be most suitable for the different objectives already stated. Table 6 lists solvents which should be used to obtain a specific separation of some *Cinchona* alkaloids. Further, the sensitivity of a number of detection methods was tested and the results are summarized in Table 5. The colours obtained with some of these reagents are summarized in Table 7.

## 2. EXPERIMENTAL

### *2.1. Chromatography*

Solvents were of "Baker analyzed" quality. The TLC plates were 20 × 20 cm silica gel 60 $F_{254}$ pre-coated aluminium sheets, with a layer thickness of 0.2 mm (E. Merck, Darmstadt, G.F.R.). The plates were stored under normal laboratory conditions and were not activated before use.

The chromatograms were developed in normal chromatography chambers, the walls of which were lined with filter-paper. The chambers were equilibrated with the solvents for at least 30 min. The temperature was 24 ± 2° and the relative humidity in

TABLE 1

LITERATURE SURVEY OF THE TLC OF *CINCHONA* ALKALOIDS

*References dealing with the TLC separation of Cinchona alkaloids:*

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 1 | 11 | 23 | 38 | 55 | 73 | 101 | 120 |
| 2 | 12 | 25 | 39 | 56 | 74 | 112 | 123 |
| 3 | 14 | 26 | 40 | 62 | 75 | 113 | 124 |
| 5 | 17 | 30 | 47 | 64 | 76 | 114 | |
| 6 | 18 | 33 | 49 | 65 | 79 | 116 | |
| 8 | 19 | 34 | 50 | 67 | 93 | 118 | |
| 9 | 20 | 36 | 53 | 71 | 99 | 119 | |

*References dealing with the TLC separation of alkaloids in general, including some Cinchona alkaloids:*

| | | | | | |
|---|---|---|---|---|---|
| 4 | 63 | 68 | 72 | 81 | 98 |
| 48 | 65 | 69 | 77 | 97 | 100 |

*References dealing with the TLC separation of various chemical compounds, including some Cinchona alkaloids:*

| | | | | | | |
|---|---|---|---|---|---|---|
| 15 | 35 | 59 | 70 | 82 | 86 | 91 |
| 21 | 41 | 61 | 73 | 83 | 87 | 92 |
| 22 | 46 | 63 | 78 | 84 | 88 | 103 |
| 31 | 54 | 66 | 80 | 85 | 89 | 111 |

*References dealing with the TLC identification of Cinchona alkaloids (particularly quinine) in food and beverages:*

| | | | |
|---|---|---|---|
| 28 | 43 | 44 | 45 |

*References dealing with the TLC identification of Cinchona alkaloids in biological material (urine, blood, etc.):*

| | | | | | | |
|---|---|---|---|---|---|---|
| 10 | 24 | 36 | 58 | 95 | 106 | 109 |
| 13 | 27 | 42 | 60 | 104 | 107 | 115 |
| 16 | 29 | 51 | 94 | 105 | 108 | 125 |

*References dealing with the analysis of drugs of abuse, including the identification of quinine:*

| | | | | | | |
|---|---|---|---|---|---|---|
| 7 | 24 | 37 | 57 | 82 | 104 | 108 |
| 10 | 27 | 42 | 58 | 91 | 105 | 109 |
| 13 | 29 | 51 | 66 | 94 | 106 | |
| 16 | 32 | 52 | 78 | 102 | 107 | |

the laboratory was 25 ± 5%. The $hR_F$ values were calculated from at least six chromatograms. Amounts of 5–10 μg of the alkaloids were spotted 1 cm above the bottom of the plates, and the plates were developed over a distance of 10 cm.

TABLE 2

SOLVENT SYSTEMS SUITABLE FOR THE SEPARATION OF VINYL AND DIHYDRO *CINCHONA* ALKALOIDS (SEE ALSO TABLE 6)

| *Solvent system* | *Plates* |
|---|---|
| Chloroform–methanol–17% ammonia (24:6:0.5) | Silica gel, not activated |
| Acetone-benzene–diethyl ether–25% ammonia (6:4:1:0.3) | |
| Chloroform saturated with ammonia | |
| Methanol | 0.1 *M* sodium hydroxide impregnated silica gel |
| Chloroform–methanol (9:1) | |

TABLE 3

SOLVENT SYSTEMS FOR THE TLC SEPARATION OF *CINCHONA* ALKALOIDS

| *No.* | *Solvent system* | *Solvent class (Snyder[127])* | *References* |
|---|---|---|---|
| S1 | Chloroform–diethylamine (9:1) | VIII | 89, 20, 34, 64, 71, 119 |
| S2 | Chloroform–methanol–25% ammonia (85:14:1) | VIII + II | 83, 84 |
| S3 | Chloroform–acetone–diethylamine (5:4:1) | VIII + VIa | 55, 40, 70, 89, 119, 121 |
| S4 | Chloroform–acetone–(3 ml 25% ammonia + 17 ml absolute ethanol) (5:4:1) | VIII + VIa + II | 117 |
| S5 | Chloroform–acetone–methanol–25% ammonia (60:20:20:1) | VIII + VIa + II | 40 |
| S6 | Chloroform–ethyl acetate–isopropanol–diethylamine (20:70:4:6) | VIII + VIa + II | 25, 53 |
| S7 | Chloroform–dichloromethane–diethylamine (20:15:5) | VIII + V | 11 |
| S8 | Dichloromethane–diethyl ether–diethylamine (20:15:5) | V + I | 11 |
| S9 | Kerosene–acetone–diethylamine (23:9:9) | VIa | 2, 18, 39, 74, 116 |
| S10 | Acetone–25% ammonia (58:2) | VIa | 12 |
| S11 | Ethyl acetate–isopropanol–25% ammonia (45:35:5) | VIa + II | |
| S12 | Toluene*–ethyl acetate–diethylamine (7:2:1) | VIb + VIa | 17, 89 |
| S13 | Toluene*–ethyl acetate–diethylamine (10:10:3) | VIb + VIa | 68 |
| S14 | Toluene*–diethyl ether–diethylamine (20:12:5) | VIb + I | 14, 19, 33, 56, 75, 76, 122 |
| S15 | Toluene*–diethyl ether–dichloromethane–diethylamine (20:20:20:8) | VIb + I + V | 23 |
| S16 | Carbon tetrachloride–*n*-butanol–methanol–10% ammonia (12:9:9:1) | VIb + II + II | |
| S17 | Cyclohexanol–cyclohexane–*n*-hexane (1:1:1) + 5% diethylamine | II | 1, 73, 113 |
| S18 | Methanol–25% ammonia (100:1) | II | 40 |

* The original solvent described in the literature contains the more toxic benzene. Direct comparison of benzene- and toluene-containing solvents did not show any major difference.

### *2.2. Detection*

The detection limit was determined by spotting 0.01, 0.1, 1, 10 and 100 $\mu$g of the parent alkaloids in 10 $\mu$l of solution on to a TLC plate. The plates were not developed but were immediately sprayed with the spray reagents. The spray reagents were prepared according to the references mentioned in Table 5 or, if no reference is given there, according to the reagents list in ref. 126.

The alkaloids tested were kindly provided by Drs. H. B. Trijzelaar, ACF Chemiefarma, Maarssen, The Netherlands.

### *2.3. Literature survey*

#### *2.3.1. TLC systems*

On surveying the literature, it was found that some solvent systems have been utilized more often than others for the separation of the parent alkaloids. Such

TABLE 4

$hR_F$ VALUES OF *CINCHONA* ALKALOIDS IN SOLVENTS S1–S18, AS DETERMINED IN OUR LABORATORY

The $hR_F$ values found in the literature are also given.
The $hR_F$ values were calculated from at least six chromatograms run under the following conditions: plates, silica gel Si 60 F254 pre-coated aluminium sheets, 20 × 20 cm (Merck); temperature, 24 ± 2°; relative humidity, 25 ± 5%; normal chromatography chamber, saturated for 30 min before use.

| *Alkaloid* | *Solvent* | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *S1* | *Ref. 20* | *S2* | *Ref. 83* | *S3* | *Ref. 40* | *S4* | *S5* | *Ref. 40* | *S6* | *S7* | *S8* | *S9* |
| Quinine (Q) | 17 | 16 | 44 | 64 | 17 | 24 | 21 | 37 | 42 | 11 | 22 | 23 | 32 |
| Dihydro-Q | 14 | 16 | 36 | | 15 | 23 | 17 | 31 | 32 | 10 | 19 | 21 | 32 |
| Quinidine (Qd) | 28 | 36 | 44 | 66 | 26 | 45 | 26 | 41 | 46 | 21 | 34 | 35 | 41 |
| Dihydro-Qd | 24 | 29 | 35 | | 24 | 39 | 18 | 31 | 34 | 18 | 31 | 32 | 41 |
| Cinchonidine (Cd) | 25 | 29 | 38 | | 24 | 40 | 23 | 35 | 40 | 17 | 31 | 31 | 39 |
| Dihydro-Cd | 21 | 29 | 30 | | 22 | 37 | 16 | 26 | 27 | 15 | 28 | 29 | 40 |
| Cinchonine (C) | 32 | 47 | 37 | | 32 | 54 | 23 | 34 | 38 | 24 | 40 | 40 | 44 |
| Dihydro-C | 26 | 41 | 28 | | 28 | 48 | 15 | 24 | 23 | 20 | 35 | 38 | 44 |
| Epi-Q | 52 | 68 | 48 | | 42 | 64 | 32 | 37 | 41 | 26 | 56 | 46 | 39 |
| Dihydroepi-Q | 51 | | 37 | | 41 | | 24 | 26 | | 40 | 56 | 47 | 42 |
| Epi-Qd | 55 | 73 | 49 | | 44 | 68 | 33 | 39 | 43 | 31 | 59 | 50 | 42 |
| Dihydroepi-Qd | 53 | | 38 | | 43 | | 23 | 24 | | 29 | 57 | 49 | 43 |
| Epi-Cd | 54 | | 46 | | 44 | | 34 | 37 | | 31 | 57 | 49 | 43 |
| Dihydroepi-Cd | 52 | | 34 | | 44 | | 25 | 25 | | 30 | 56 | 50 | 45 |
| Epi-C | 55 | | 47 | | 45 | | 33 | 38 | | 33 | 58 | 51 | 45 |
| Dihydroepi-C | 53 | | 35 | | 43 | | 25 | 25 | | 30 | 57 | 51 | 46 |
| Cupreine (Cu) | 1 | | 19 | | 1 | | 8 | 22 | | 1 | 1 | 3 | 8 |
| Dihydro-Cu | 1 | | 15 | | 1 | | 5 | 15 | | 1 | 1 | 2 | 9 |
| Cupreidine (Cud) | 1 | | 20 | | 1 | | 7 | 21 | | 1 | 2 | 3 | 8 |
| Dihydro-Cud | 1 | | 14 | | 1 | | 4 | 12 | | 1 | 1 | 3 | 8 |
| Quinidinone | 60 | | 71 | | 53 | 76 | 53 | 65 | 74 | 41 | 63 | 57 | 49 |
| Cinchoninone | 60 | | 69 | | 52 | | 53 | 64 | | 41 | 61 | 56 | 49 |
| HQd ar-N-oxide | 12 | | 28 | | 12 | | 9 | 22 | | 6 | 16 | 14 | 16 |
| HCd ar-N-oxide | 9 | | 23 | | 11 | | 6 | 16 | | 5 | 13 | 11 | 15 |
| Development time (min/8 cm) | 15 | | 13 | | 15 | | 13 | 13 | | 13 | 12 | 11 | 15 |

solvents, all in combination with silica gel plates, are S1 and S3[89], S9[116] and S14[75] (Table 3). Systems of chloroform–methanol–DEA in different ratios have also been used by several workers[1,3,5,6,8,113]. Solvent S3 was adapted in the USP XVIII for the quality control of *Cinchona* alkaloids and solvent S14 in the British Pharmacopoeia (1973).

According to Andary[25], solvent S6 gives a complete separation of the parent alkaloids. This solvent was later used by Massa *et al.*[53] in the quantitative analysis of the parent alkaloids.

With chloroform–methanol–DEA (80:20:1) as solvent in combination with base-impregnated silica gel plates, a separation of vinyl and dihydro alkaloids is obtained[3,5]. For this separation various other solvents have also been described: S5[40,123], S10[9], S18[40], acetone–water–25% ammonia (80:20:1)[120] methyl ethyl ketone–

| *Ref. 74* | *S10* | *S11* | *S12* | *Ref. 89* | *S13* | *S14* | *Ref. 76* | *S15* | *Ref. 23* | *S16* | *S17* | *Ref. 1* | *S18* | *Ref. 40* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 35 | 32 | 49 | 12 | 17 | 18 | 18 | 25 | 20 | 19 | 67 | 41 | 56 | 45 | 50 |
| | 24 | 43 | 11 | | 17 | 17 | | 17 | | 60 | 41 | | 38 | 39 |
| 45 | 37 | 55 | 20 | 25 | 28 | 26 | 44 | 29 | 37 | 71 | 60 | 74 | 46 | 52 |
| | 27 | 49 | 18 | | 26 | 25 | 35 | 27 | | 62 | 58 | | 37 | 39 |
| 45 | 33 | 52 | 19 | | 27 | 25 | 41 | 27 | 32 | 67 | 57 | 71 | 43 | 48 |
| | 25 | 46 | 18 | | 25 | 24 | 35 | 25 | | 57 | 57 | | 35 | 35 |
| 50 | 34 | 53 | 24 | 27 | 33 | 31 | 54 | 33 | 54 | 65 | 67 | 80 | 39 | 43 |
| | 24 | 45 | 22 | | 30 | 29 | 49 | 31 | | 52 | 65 | | 29 | 30 |
| | 41 | 48 | 29 | | 35 | 30 | | 38 | | 53 | 37 | | 30 | 31 |
| | 31 | 40 | 30 | | 36 | 31 | | 39 | | 39 | 38 | | 19 | |
| | 39 | 48 | 33 | | 41 | 35 | | 42 | | 52 | 44 | | 29 | 32 |
| | 30 | 39 | 32 | | 40 | 35 | | 41 | | 39 | 41 | | 19 | |
| | 43 | 49 | 34 | | 41 | 36 | | 42 | | 53 | 46 | | 30 | |
| | 33 | 40 | 34 | | 41 | 37 | | 43 | | 39 | 45 | | 20 | |
| | 41 | 48 | 36 | | 43 | 39 | | 44 | | 51 | 49 | | 30 | |
| | 31 | 40 | 36 | | 42 | 39 | | 44 | | 39 | 47 | | 19 | |
| | 13 | 29 | 2 | 0 | 3 | 2 | | 3 | | 51 | 9 | | 43 | |
| | 8 | 22 | 2 | | 3 | 3 | | 3 | | 41 | 9 | | 34 | |
| | 11 | 29 | 2 | | 3 | 2 | | 3 | | 50 | 9 | | 42 | |
| | 7 | 21 | 2 | | 3 | 2 | | 3 | | 36 | 8 | | 29 | |
| | 59 | 59 | 44 | | 51 | 47 | | 51 | | 83 | 63 | | 54 | 64 |
| | 58 | 59 | 44 | | 50 | 47 | | 51 | | 81 | 63 | | 54 | |
| | 10 | 25 | 6 | | 10 | 6 | | 10 | | 53 | 41 | | 29 | |
| | 8 | 22 | 5 | | 8 | 5 | | 7 | | 41 | 28 | | 25 | |
| | 10 | 17 | 14 | | 12 | 11 | | 12 | | 28 | 45 | | 13 | |

ammonia (58:2)[9] and acetone–methanol–DEA (50:50:1)[20,34], all on silica gel plates. Methyl ethyl ketone–methanol–water (6:2:1)[18,19,118,119] was used in combination with 0.1 *M* sodium hydroxide impregnated silica gel plates.

Böhme and Bitsch[18] postulated that polar solvents would be able to separate the vinyl and dihydro alkaloids whereas non-polar solvents would not. Stöver[14] used the reaction of the vinyl alkaloids with mercuriacetate to separate the vinyl alkaloids from the dihydro compounds. The vinyl alkaloids give polar mercuri derivatives, which do not move in the solvent used (S14), whereas the dihydro alkaloids are not affected by mercuriacetate.

For the separation of the epi-alkaloids from the parent alkaloids, solvents S3 and S18 were proposed by Smith *et al.*[40]; Storck and co-workers[20,34] used solvent S1 for this separation.

TABLE 5

TLC DETECTION OF *CINCHONA* ALKALOIDS

| *Reagent* | *Sensitivity** | *Background colour* | *Colour with parent alkaloids* | *Reference* |
|---|---|---|---|---|
| Quenching, 254 nm | 0.1 | | | |
| Fluorescence, 366 nm (formic acid or sulphuric acid spray) | 0.01 Qd, Q, 0.1 C, Cd | | Q, Qd light blue; C, Cd dark blue | |
| Dragendorff's modification: | | | | |
| Munier–Macheboeuf | 0.1 | Yellow | Orange-red | |
| Munier | 0.01 | Light yellow | Orange-red | |
| Munier, $NaNO_2$ | 0.01 | Light yellow-white | Brown | 66 |
| Vágujfalvi | 0.1–1 | Light yellow | Orange | |
| Bregoff–Delwiche | 0.1 | Light yellow | Orange | |
| Iodine vapour | 0.1 | Yellow-white | Brown | |
| Iodine in KI | 1 | White | Brown | |
| Iodine in methanol | 0.1 | Light yellow | Brown | 87 |
| Iodine vapour, pyrrole vapour | 1 | Yellow | Brown | 83 |
| Iodine in KI and Ag acetate | 1 | | | 105 |
| Iron(III) chloride, iodine in KI | 0.1 | Light green-yellow | Brown | 85 |
| Iodoplatinate | 0.01–0.1 | Violet | Q, Qd violet; C, Cd blue | |
| Iodoplatinate, acidified | 0.1 | Dark violet | Q, Qd violet; C, Cd blue | 34 |
| Iron(III) hexacyanoferrate(III) | 10 | Light green-blue | Dark green-blue | 100 |
| Iron(III) chloride–perchloric acid | 1 | Yellow-white | Violet | |
| Methyl orange | 10 | Light orange | Orange | 104 |
| Tetraphenylborate, quercetin | 10 | | In UV: Q, Qd blue, C, Cd yellow | 110 |
| Phenothiazine, iodine vapour | 0.1 | Violet | Brown | 83 |
| Phenothiazine, bromine vapour (ammonia vapour) | 1 | Violet | Q, light brown; Qd green; C yellow; Cd red-brown | 83 |

* As tested in our laboratories for the parent alkaloids.

TABLE 6

SOLVENT SYSTEMS USED FOR THE TLC SEPARATION OF *CINCHONA* ALKALOIDS

The numbers refer to solvents S1–S18 in Table 3 which can be used to obtain a separation within a group of alkaloids or between two groups of alkaloids or between two alkaloids. Numbers in parentheses indicate that a complete baseline separation was not obtained.

| | *Parent alkaloids* | | | | *Dihydro alkaloids* | | | |
|---|---|---|---|---|---|---|---|---|
| | *Q* | *Qd* | *Cd* | *C* | *HQ* | *HQd* | *HCd* | *HC* |
| *Parent alkaloids* | 1, 3, 6–9, 12–15, 17 | | | | 2, 4, 5, 10, 11, 16, 18 | | | |
| Q | | 1, 3, (4), 6–9, (11), 12–15, 17 | 1–3, 5–10, (11), 12–15, 17 | 1–3, 5–10, (11), 12–15, 17, (18) | 2, 4, 5, (6), 10, 11, 16, 18 | 1–10, 12–18 | 1–10, (11), 12–18 | 1–10, (11), 12–18 |
| Qd | | | 2, (4), 5, 6, (7), (8), 10, (13), (16) | 1–3, (4), 5–8, (9), 10, 12–17, (18) | 1–18 | (1), 2, 4, 5, (6), 10, 11, 16, 18 | 1, 2, (3), 4–8, 10, 11, 13, (15), 16, 18 | 2, 4, 5, (8), (9), 10–14, 16–18 |
| Cd | | | | 1, 3, 6–9, 12–15, 17 | 1, 3, 4, 5, 6–15, (16), 17, 18 | 4, 5, 10, (16), 18 | 2, 4, 5, 10, 11, 16, 18 | (1), 2–14, (15), 16–18 |
| C | | | | | 1, 3, (5), 6–15, (16), 17, (18) | 1, 3, (5), 6, 7, 8, 10, (11), 12–15, (16), 17, (18) | 1–3, 5–18 | 1, 2, 5, 6, (8) 10, 11, (12), (13), (15), 16, 18 |
| *Dihydro alkaloids* | | | | | 1, 3, 6–9, 12–15, 17 | | | |
| HQ | | | | | | 1, 3, 6–9, (11), 12–15, 17 | 1–3, 5–9, 12–15, (16), 17 | 1–3, 5–9, (10), 12–18 |
| HQd | | | | | | | 2, 5, (8), (10), (11), (15), (16) | (1), 2, (3), 5, (6), (7), 8, (9), 10, (11), 12–14, (15), 16–18 |
| HCd | | | | | | | | 1, 3, 6–9, 12–15, (16), 17, 18 |

*(Continued on p. 88)*

TABLE 6 *(continued)*

| | *Epi-alkaloids* | | | | | *Epi-dihydro alkaloids* | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | *epiQ* | *epiQd* | *epiCd* | *epiC* | | *epiHQ* | *epiHQd* | *epiHCd* | *epiHC* |
| *Parent alkaloids* | 1, 3, 6–8, 12 (2×), 13–15, 18 | | | | | 1, 3, 5, 6, 7, 8, 10, 12 (2×), 13, 14, 15, 16, 18 | | | | |
| Q | | 1, (2), 3, 6–9, 12–16, 18 | 1, (2), 3, 6–9, (5), 12–18 | 1, 3, 6–10, 12–18 | 1, 3, 6–10, 12–18 | | 1, 2, 3, 5, 6, 7, 8, 9, 10, 12, 13, 14, 15, 16, 18 | 1–3, 5–10, 12–16, (17), 18 | 1–3, 5–10, 12–18 | 1–3, 5–10, 12–18 |
| Qd | | 1, (2), 3, 5–8, 11–18 | 1, (2), 3, 6–8, 11–18 | 1, 3, (4), 5–8, 11–18 | 1, 3, 5–9, 11–18 | | 1, 2, 3, (4), 5, 6, 7, 8, 10–18 | 1–3, (4), 5–8, 10–18 | 1–3, (4), 5–18 | 1–3, (4), 5–18 |
| Cd | | 1–4, 6–8, 10, (11), 12–18 | 1–8, 10, (11), 12–18 | 1–4, (5), 6–8, (9), 10, (11), 12–18 | 1–4, 5, 6–10, (11), 12–18 | | 1, 3, 5–8, 10–18 | 1, 3, 5–8, (9), 10–18 | 1, 3, 5–18 | 1, 3, 5–18 |
| C | | 1–3, (5), 6, 8–12, (13), 15–18 | 1–3, (5), 6–8, 10–18 | 1–3, 6–8, 10–18 | 1–3, 5, 6–8, 10–18 | | 1, 3, 5–8, (9), 10–13, 15–18 | 1, 3, 5–8, 10–18 | 1, 3, 5–8, 10–18 | 1, 2, 5–8, 10–18 |
| *Dihydro alkaloids* | 1, 3, 5–8, 10, 12 (2×), 13–15 | | | | | 1, 3, 6, 7, 8, 12 (2×), 13, 14, 15, 16, 18 | | | | |
| HQ | | 1–10, 12–16, (18) | 1–10, 12–17, (18) | 1–10, 12–17, (18) | 1–10, 12–17, (18) | | 1, 3, (4), 5–9, (11), 12–16, 18 | 1, 3, (4), 5–9, (11), 12–16, 18 | 1, 3, (4), 5–9, (10), (11), 12–18 | 1, 3, (4) 5–9, (11), 12–18 |
| HQd | | 1, 3–8, 10, 12–17, (18) | 1, 3–8, 10, 12–17, (18) | 1, 3–8, (9), 10, 12–17, (18) | 1, 3–10, 12–17, (18) | | 1, 3, 5–8, 11–18 | 1, 3, 5–8, (9), 11–18 | 1, 3, (4), 5–9, 11–18 | 1, 3, (4), 5–9, 11–18 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| HCd | | 1–8, 10, 12–15, (16), 17 | 1–8, (9), 10, 12–15, (16), 17 | 1–8, (9), 10, 12–15, (16), 17 | 1–10, 12–15, (16), 17 | | 1–3, (4), 6–8, (10), 11–18 | 1–3, (4), 6–8, (9), (10), 11–18 | 1–4, 6–18 | 1–4, 6–9, (10), 11–18 |
| HC | | 1–10, 12, 13, 15, (16), 17 | 1–8, 10, 12–15, 17 | 1–8, 10, 12–15, 17, (18) | 1–8, 10, 12–15, 17 | | 1–4, 6–8, 10–13, 15–18 | 1–4, 6–8, 10–18 | 1–4, 6–8, 10–18 | 1–4, 6–8, 10–18 |
| *Epi-alkaloids* | (9), 12 (2×), 13–15, 17 | | | | | 2, (3), 4, 5, 10, 11, 16, 18 | | | | |
| epiQ | | | (1), 6, 8, (9), 12–15, 17 | 6, (8), 9, (10), 12–15, 17 | 1, (3), 6, (7), 8, 9, 12–15, 17 | | 2, 4, 5, 8, (9), 10, 11, 16, 18 | (1), 2, 4, 5, 8, 9, 10–18 | (1), 2, 4–6, 8–18 | (1), 2, 4–6, 8–18 |
| epiQd | | | | (10) | (9), (10), 12, (13), 14, (15) | | 1–6, 8, 10–16, 18 | 2, 4, 5, 10, 11, 16, (17), 18 | 2, 4, 5, (9), 10, 11, 16, 18 | 2, 4, 5, 9–12, (13), 14, (15), 16, (17), 18 |
| epiCd | | | | | (1), 2, (9), (12), (13), (14), (15) | | (1), 2–6, 8, 10–14, (15), 16–18 | 2, 4, 5, 10, 11, 16, (17), 18 | 2, 4, 5, (9), 10, 11, 16, 18 | 2, 4, 5, 9–11, (12), (13), 14, (15), 16, 18 |
| epiC | | | | | | | 1–6, (7), 8, (9), 10–18 | (1), 2, 4–6, (9), 10–14, (15), 16–18 | (1), 2, 4, 5, (6), 10, 11, (12), (13), (15), 16, 18 | 2, 4, 5, (6), 10, 11, 16, 18 |
| *Epi-dihydro alkaloids* | | | | | | (9), 12 (2×), 13, 14, 15, 17 | | | | |
| epiHQ | | | | | | | | (1), (3), 8, (9), (6), 12–14, (15), 17 | (1), (3), 6, 8, (9), (10), 12–14, 15, 17 | (1), (3), 6, 8, 9, 12–15, 17 |
| epiHQd | | | | | | | | | (10), (12), (14), (17) | (9), 12, (13), 14, (15), 17 |
| epiHCd | | | | | | | | | | (12), (13), (14), (15) |
| epiHC | | | | | | | | | | |

TABLE 7

DETECTION OF *CINCHONA* ALKALOIDS: COLOURS OBTAINED AFTER SPRAYING WITH DILUTE SULPHURIC ACID AND IODOPLATINATE SPRAY REAGENT

| *Alkaloid* | *Fluorescence colour (366 nm)* | *Iodoplatinate spray reagent*[20] |
|---|---|---|
| Quinine | Light blue | Violet-brown |
| Quinidine | Light blue | Violet-brown |
| Dihydroquinine | Light blue | Violet-brown |
| Dihydroquinidine | Light blue | Violet-brown |
| Cinchonine | Dark blue | Blue-violet-brown |
| Cinchonidine | Dark blue | Blue |
| Dihydrocinchonine | Dark blue | Blue-violet |
| Dihydrocinchonidine | Dark blue | Blue-violet |
| Epiquinine | Light blue | Violet-brown |
| Epiquinidine | Light blue | Violet-brown |
| Dihydroepiquinine | Light blue | Violet-brown |
| Dihydroepiquinidine | Light blue | Violet-brown |
| Epicinchonine | Dark blue | Blue-violet-brown |
| Dihydroepicinchonine | Dark blue | Blue-violet |
| Epicinchonidine | Dark blue | Blue-violet-brown |
| Dihydroepicinchonidine | Dark blue | Blue-violet |
| Quinidinone | Yellow-green | Yellow-violet |
| Cinchoninone | Yellow-green | Yellow-violet |
| Cupreine | Orange-red | Light blue-violet |
| Dihydrocupreine | Orange-red | Light blue-violet |
| Cupreidine | Orange-red | Blue-violet-brown |
| Dihydrocupreidine | Orange-red | Blue-violet-brown |

*2.3.2. Two-dimensional chromatography*

Several workers have proposed two-dimensional chromatography for the complete separation of the four parent alkaloids. Van Severen[1] applied solvents S17 and chloroform–methanol–DEA (80:2:0.2). Kamp *et al.*[73] modified this method by first running the chromatograms in chloroform–methanol–DEA (80:20:1) followed by S17, because of difficulties in drying the plates after using S17 for the first development. Kamp *et al.* described two other combinations for two-dimensional TLC on silica gel plates: chloroform–*n*-butanol (1 + 1) saturated with 10% ammonia followed by S9 or S17. Böhme and Bitsch[18] used methyl ethyl ketone–methanol–water (6:2:1) followed by benzene–isopropanol–DEA (4:2:1) on 0.1 *M* sodium hydroxide impregnated silica gel plates. Instead of the latter solvent, Vermes[33] used benzene–diethyl ether–DEA (20:12:5) for the second run. Wyesekera *et al.*[62] described a combination of the solvents chloroform–methanol–17% ammonia (24:6:0.05) and diethyl ether–DEA (17:1) on 0.1 *M* sodium hydroxide impregnated silica gel plates for the separation of the alkaloids present in *Cinchona* bark. Pound and Sears[120] proposed the solvents acetone–water–ammonia (25%) (80:20:1) and benzene–DEA (1:1) for the separation of the parent alkaloids and the dihydro bases of quinine and quinidine.

*2.3.3. Reaction chromatography and TAS technique*

Reaction chromatography of *Cinchona* alkaloids has been described by Wilk and Brill[90]. Before developing the plates with the solvents they were exposed to iodine vapour for 18 h. After development with benzene–methanol–acetone–acetic acid

(70:20:5:5) characteristic patterns of spots were observed for the alkaloids. Kaess and Mathis[93] used several reagents in connection with the *Cinchona* alkaloids: treatment with chromic acid leads to the formation of less polar ketones, but in small yields, and treatment of quinine with potassium ethanolate (0.1 *M*) leads to the formation of a number of unidentified compounds. With acetyl chloride or acetic anhydride *Cinchona* alkaloids give less polar acetyl compounds. According to the authors, the reactions can be helpful in the identification of the alkaloids.

Jolliffe and Shellard[101] found that the results obtained with thermofractography (TAS) of *Cinchona* alkaloids from the bark were inconsistent. Investigations by Stahl and Schmitt[99,114] showed that the pure *Cinchona* alkaloids could be volatilized at temperatures above 180° without decomposition. However, from *Cinchona* bark only decomposition products, simple quinoline derivatives, were obtained. They could be used to characterize *Cinchona* bark with the TAS technique. Chmel and Chmelová-Hlavatá[118] succeeded in the thermofractography of the alkaloids present in *Cinchona* bark. They mixed 20–40 mg of bark with 20 mg of lithium hydroxide and applied a temperature of 300°. As the propellant 100 mg of $Ni(NH_3)Cl_2$ were used. As solvent for TLC they used methyl ethyl ketone–methanol–water (6:2:1) on 0.05 *M* potassium hydroxide impregnated plates.

*2.3.4. Quantitative analysis*

Oswald and Flück[75] analysed *Cinchona* alkaloids quantitatively by measuring the spot areas. Later, several workers described methods for quantitative analysis by extraction of the spots from the TLC plates followed by fluorimetric[40,69] or spectrophotometric determination[17,19,20,34,43,44]. The alkaloids were extracted with different solvents: chloroform[43,44], ethanol–acetone (1:1)[95,115], absolute ethanol[20], methanol–25% ammonia (9:1)[18,19], 0.1 *M* hydrochloric acid[17] or 0.05 *M* sulphuric acid[40,123]. Dilute hydrochloric acid or 2 *M* sulphuric acid were used to dissolve alkaloids and the stationary phase (magnesium oxide)[69,79].

For fluorimetric analysis after extraction an excitation wavelength of 350 nm has been used[40,69,115,123] and the emission measured at 450 nm[69] and 455 nm[40,123] for quinidine and quinine, respectively, in dilute acid. For indirect spectrophotometric determination wavelengths of 332 nm[18,19], 324 nm[20,34] and 366 nm[17] have been used for quinine and 316 nm for cinchonine[20,34]. Direct fluorimetric analysis of the TLC plates has been performed after acidification, with excitation wavelengths of 361 nm[45] or 345 nm[40] for quinine and 335 nm[60] or 365 nm[36] for quinidine, and emission wavelengths of 438 nm[45] or 430 nm[40] for quinine and 455 nm[36] or 430 nm[60] for quinidine. Röder *et al.*[23] described the direct fluorimetric analysis of the four parent alkaloids on TLC plates after immersion in diethyl ether–concentrated sulphuric acid (95:5). Quinine and quinidine have a fluorescence maximum at 460 nm, excitation at 365 nm. Under these conditions cinchonine and cinchonidine do not interfere. Cinchonine and cinchonidine can be determined without interference from quinine and quinidine by excitation at 313 nm and measuring the emission at 390 nm. In this way the four main alkaloids can be determined quantitatively without being separated completely. Ebel and Herold[124] used this method for the determination of quinine.

To obtain a complete separation of quinine from the other *Cinchona* alkaloids before quantitative analysis, Böhme and Bitsch[18,19] improved the separation by

first developing the plates with a polar solvent, in which quinine has a high $R_F$ value, followed by a reversed development with a less polar solvent, in which quinine has a low $R_F$ value. In this way quinine could be separated from quinidine, cinchonine, cinchonidine, dihydroquinidine and dihydroquinine.

Massa and co-workers[53,96] studied the optimal conditions for direct measurement on the plates. The four main alkaloids were separated with the solvent ethyl acetate–chloroform–isopropanol–DEA (70:20:4:6) on silica gel plates. For direct photodensitometric determination on the plate after spraying with ethanol–concentrated sulphuric acid (10%), the absorption maxima for quinine and quinidine were found to be 330 nm and for cinchonine and cinchonidine 288 nm. The detection limit was found to be 100 ng. Using fluorescence after spraying with 1% sulphuric acid in ethanol, quinine and quinidine could be determined by using an excitation wavelength of 365 nm and measuring at 450 nm. In this way the authors found the detection limit to be 1 ng. The use of an excitation wavelength of 313 nm permitted the determination of the emission of cinchonine and cinchonidine at a wavelength of 410 nm, with a detection limit of 5 ng (cinchonidine has its fluorescence maximum at 420 nm). Quinine and quinidine are also excited at 313 nm, but have their fluorescence maximum at 454 nm.

Okumura *et al.*[97] used a modified flame-ionization detection method for the quantitative analysis of a number of alkaloids, *e.g.*, quinine, on sintered silica gel or aluminium oxide on glass rods.

## 3. RESULTS AND DISCUSSION

None of the TLC systems described in the literature is able to separate all known *Cinchona* alkaloids in one run, but using solvents S6, S12, S13, S14 and S15 an optimal separation is obtained. From all the solvents and sorbents tested in our study some general conclusions can be drawn.

(1) Silica gel plates in combination with basic solvents or base-impregnated silica gel plates together with neutral solvents give the best results without tailing, except for the epi-alkaloids, which in all solvents tested show some tailing.

(2) The use of ammoniacal solvents or of base-impregnated plates usually leads to the separation of the vinyl alkaloids from the dihydro alkaloids. An increase in the pH of the mobile or stationary phase leads to an improvement in the vinyl–dihydro alkaloid separation. In addition to solvents S2, S4, S5, S10, S11, S16 and S18 found in Table 3 the solvents listed in Table 2 can be used successfully for the vinyl–dihydro alkaloid separation. On comparing ammonia-containing solvents with those containing diethylamine, it is observed that in the former both Cd and epiCd have $R_F$ values equal or higher than those of C and epiC, whereas the opposite is true in the latter. The same observation is made for Q, Cd and Qd, C. Although the differences in the $R_F$ values are smaller, a similar behaviour is observed for the epi-alkaloids.

(3) Chromatography with ammonia-containing systems leads to deterioration of the separation of the four stereoisomers of each group, such as Q, Qd, epiQ and epiQd.

(4) If diethylamine in solvent S3 is replaced with ammonia, as proposed by Puech *et al.*[117] for the analysis of tropane alkaloids (S4), an improved separation of the vinyl and dihydro alkaloids is obtained, but the separation of the parent

alkaloids and the epi-alkaloids deteriorates in comparison with the original diethylamine-containing solvent. Solvents containing diethylamine are in general suitable for the separation of the various compounds within each group of stereoisomers.

Summarizing, and considering the aims mentioned in the Introduction, the following conclusions can be drawn about the analysis of *Cinchona* alkaloids:

(1) An almost complete separation of the parent alkaloids can be achieved with solvents S6, S7, S8 and S13. Solvents S1, S3, S9, S12, S14, S15 and S17 give a nearly complete separation of three of the alkaloids and a partial separation of the fourth. None of these systems give a complete "baseline" resolution of Qd and Cd. This is achieved only in solvent S2. The solvents methanol and chloroform–methanol (9:1) on base-impregnated plates are able to separate the pair cinchonine–cinchonidine from the pair quinine–quinidine.

(2) The vinyl alkaloids can be separated from the dihydro alkaloids in solvents S2, S4, S5, S10, S11, S16 and S18 and in the solvents given in Table 2.

(3) The epi compounds usually have $R_F$ values higher than those of the parent alkaloids. The best separation from the parent alkaloids is obtained with solvents S1, S3, S6, S7, S8, S12, S13, S14, S15 and S18. The best separation of the epi-alkaloids is obtained with solvents S6, S12, S13 and S17. In solvent S17 the epi compounds have $R_F$ values between those of Q and Qd and it is therefore less suitable for the separation of the epi-alkaloids from the parent alkaloids.

(4) Separation of the parent alkaloid from its dihydro, epi-vinyl and epi-dihydro derivative is achieved with solvents S16 and S18 (not for HCd–epiCd) and partly by solvents S2 and S6. Solvents S16 and S18 give poor resolutions of Q and Qd and of C and Cd, which makes it unsuitable for the separation of the stereoisomers of each group.

(5) Separation of the four stereoisomers of each group can be obtained with solvents S1, S6, S12, S13, S14 and S15.

(6) Separation of the four derivatives of the quinine group can best be accomplished with solvents S2, S5 and S15 and partly with solvents S7, S8, S13 and S16. The quinidine group can be separated best with solvents S10 and S16 and partly with solvents S2, S5, S6, S7, S8, S13 and S18. The group of epi-quinine can be only partly resolved with solvents S9, S12, S14 and S18. The epi-quinidine group can be partly resolved with solvents S10 and S17.

The phenolic alkaloids cupreine and cupreidine were not separated in any of the solvents, nor were their dihydro derivatives separated from each other. The same was the case with quinidone and cinchoninone. The separation of the cupreine series of alkaloids, quinidone and cinchoninone from the other alkaloids does not give any problem, as can be seen from Table 4.

The keto compounds can be separated from the other alkaloids in solvents S4, S8, S11, S12, S14, S15 and S16. For the cupreine series low $R_F$ values were found in all of the systems tested, except for solvents S11 and S16. In the latter solvent the alkaloids of the cupreine series coincide with several of the other alkaloids, which makes this solvent less useful for the separation of all alkaloids.

### *3.1. Detection*

For the TLC detection of *Cinchona* alkaloids the fluorescence of these com-

pounds in acidic conditions has been widely utilized. The developed plate is sprayed with dilute sulphuric acid or 25% formic acid in water, immersed in a mixture of diethyl ether and concentrated sulphuric acid (95 + 5)[23] or exposed to formic acid vapour. The aryl-methoxyl-containing alkaloids show a strong blue fluorescence, stronger at 366 nm than 254 nm, whereas the methoxyl-free alkaloids have a weak, dark blue fluorescence (Table 7). As can be seen from Table 5, the modification of Dragendorff's reagent according to Munier and Munier and Macheboeuf is the most sensitive of the spray reagents. The iodoplatinate reagent, which gives different colours with some of the alkaloids (Table 7), is also sensitive.

## 4. SUMMARY

The TLC analysis of *Cinchona* alkaloids is reviewed. From the TLC systems described in the literature, 18 solvents were found to be most suitable for the separation of the 24 *Cinchona* alkaloids tested. The sensitivity of a number of detection methods described for *Cinchona* alkaloids is reported. Some general conclusions concerning the optimal conditions for specific separations are drawn.

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## Erratum

*J. Chromatogr.*, 165 (1979) 235–256

Page 244, 3 lines in Table 1 should read as follows:

| *Parameter* | *BS* | *ASTM* | *IUPAC* | *Notes* |
|---|---|---|---|---|
| Retention times | | | | |
| Adjusted retention time | $t'_R$ | $t'_R$ | $t'_R$ | $t'_R = t_R - t_M$ |
| Retention volumes | | | | |
| Corrected retention volume | | $V^\circ_R$ | $V^\circ_R$ | See Note 15 |
| Net retention volume | $V_N$ | $V_N$ | $V_N$ | $V_N = V'_R j = t'_R F_c j$ |

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