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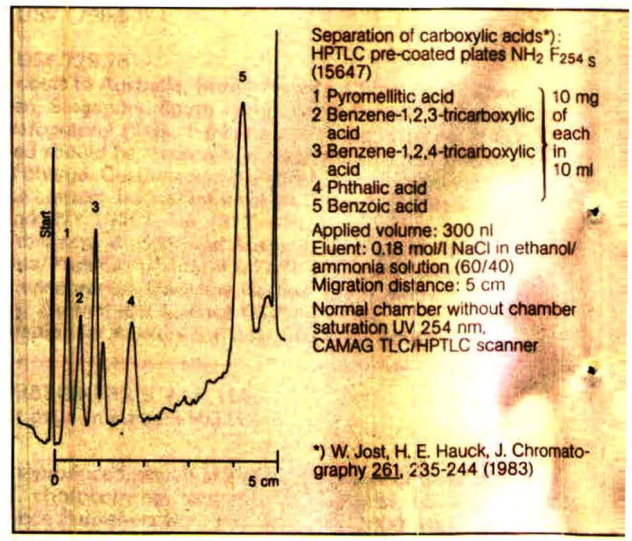

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CHREV. 195

ESTIMATION OF DEAD TIME AND CALCULATION OF KOVATS INDICES

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CONTENTS

1. INTRODUCTION

Since the experimental demonstration of gas–liquid chromatography in the early 1950s[1], the technique has achieved remarkable success. Since, that time, the growth in applications of the procedure has been phenomenal and when reliable, low-cost, easy-to-use gas chromographs became available in the mid-sixties, a proliferation of new columns, detectors and applications elevated the procedure into the most important and widely used technique in analytical chemistry today.

However, because the raw data (the chromatogram) depends on both the experimental conditions and the equipment used, it soon became apparent that a uniform system of data presentation was necessary to give the accuracy required for the comparison of data between laboratories. This requirement is even greater today with the increasing complexity of separations and the introduction of capillary columns.

Such a system should be as independent as possible of the operating conditions as well as of the experimental equipment. Unfortunately the property directly measured by the analyst, the total retention time, t_R, is the sum of two factors. One, the

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gas hold-up time (or dead time), t_M, is dependent on the system flow-rate as well as on the void volume of the experimental apparatus. The other, the adjusted retention time, t'_R, is a characteristic of the separation process

$$t'_R = t_R - t_M \tag{1}$$

and is independent of the equipment used.

Kaiser has stressed the importance of the adjusted retention time in several articles (in particular see refs. 2–4), while Parcher and Johnson[5], as well as Lin and Parcher[6], have shown its importance when determining thermodynamic properties such as Henry's law constants.

Although independent of the equipment used, adjusted retention times are still dependent on several variables such as column temperature, flow-rate, pressure drop and liquid phase. A variety of systems have therefore been introduced, which depend only on the column temperature and stationary phase used[7,8]. The most useful of these for qualitative analysis is undoubtedly the retention index system introduced by Kovats[9].

2. KOVATS INDICES

In 1958, Kovats[9] introduced a system to overcome the problem of the uniform reporting of retention data. His system used the *n*-alkanes as a series of standards and expressed the retention of other substances relative to them using

$$I = 200 \cdot \frac{\log V_R^0(\text{substance}) - \log V_R^0(n\text{P}_z)}{\log V_R^0(n\text{P}_{z+2}) - \log V_R^0(n\text{P}_z)} + 100 \cdot z \tag{2}$$

where $V_R^0(nP_z) \leqslant V_R^0(\text{substance}) \leqslant V_R^0(n\text{P}_{z+2})$, V_R^0 = retention volume and $n\text{P}_z$ = *n*-paraffin with carbon number z. This equation gives the retention index of an even-numbered normal paraffin as 100 times its carbon number. Only even-numbered *n*-paraffins were used because it was believed (incorrectly) that there would be an oscillation in the properties of successive members of the *n*-paraffin series. However, experimental results later indicated that this was not the case and thus in 1964 Kovats redefined the index as

$$I = 100 \cdot \frac{\log V_R^0(\text{substance}) - \log V_R^0(n\text{P}_z)}{\log V_R^0(n\text{P}_{z+1}) - \log V_R^0(n\text{P}_z)} + 100 \cdot z \tag{3}$$

where again $V_R^0(n\text{P}_z) \leqslant V_R^0(\text{substance}) \leqslant V_R^0(n\text{P}_{z+1})$, as related by Ettre[10]. In this report Ettre suggested that retention volumes could be replaced by adjusted retention times or corresponding distances on the chromatogram. Kovats[11] confirmed this in a later paper which introduced the more widely known formula

$$I^{\text{stat. phase}}_{\text{substance}}(T) = 100 \cdot \frac{\log t'_{Ri} - \log t'_{Rz}}{\log t'_{R(z+1)} - \log t'_{Rz}} + 100 \cdot z \tag{4}$$

where t'_{Rz} = adjusted retention time of a homologue with carbon number z and t'_{Ri} = adjusted retention time of a substance i.

It has subsequently been accepted that, under isothermal conditions, retention indices, I, can be calculated by interpolation within the linear relationship*

$$\ln t'_{Rz} = bI + c \qquad (I = 100 \cdot z) \tag{5}$$

where b and c are constants ($\log t'_{Rz} = bI + c$ is used by some authors) and where the Kovats retention indices for n-alkanes are defined as $100z$ for each liquid phase at all temperatures.

The retention index system of Kovats has now been widely accepted not only for comparing retention data, but also in the characterisation of stationary phases[13]. Mazor *et al.*[14] have shown that Kovats retention indices are independent of the carrier flow-rate. Dahlmann *et al.*[15] have shown that while the index is dependent to some degree on both the stationary phase loading and the solid support used, this can be reduced to a minimum by an appropriate choice of the solid support, chemical treatment of the support and the avoidance of very low stationary phase loadings. Finally, the temperature dependence of the index has been discussed by many authors including Wehrli and Kovats[16] who showed that a temperature dependence of less than one unit per degree applies in most instances. Novák *et al.*[17], amongst others, have reported a relationship between the specific retention volume, carbon number and temperature. A very extensive review of the Kovats retention index system published in 1983 by Budahegyi *et al.*[18] discussed these as well as many other aspects, citing almost 1400 references.

Since the introduction of the retention index system, a large number of papers have analysed its reproducibility. Among others, Takács and Králik[19] introduced the "Erdey" equation, a theoretical equation giving the error of determination of retention indices. Other authors discussed individual contributions to the error and have suggested methods to minimise such errors[4,8,20–24]. The paper by Schomburg and Dielmann[8], which is a particularly useful discussion on sources of error, suggests an interlaboratory reproducibility of about one unit.

Except a few theoretical methods of calculating retention indices[25,26], it can be seen from eqns. 1, 4 and 5 that a knowledge of the adjusted retention times and thus the column dead time is essential.

3. COLUMN DEAD TIME

There has been increasing discussion in recent years regarding the merits of the various methods for determining dead time. In a number of papers several of these methods have been compared in an attempt to find a simple, accurate method[18,27–32]. Here we update and extend the review carried out by two of us in an earlier publication[32].

The different techniques can broadly be classified into eight categories.

3.1. Theoretical

Evans and Smith[33] suggested in 1962 that the static column interstitial volume could be calculated from

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* We have used the nomenclature recommended by ASTM E 355[12] where possible.

$$d_0 = \frac{2.54\ SV}{60\ F}\left[1 - \frac{B_p}{D_p} - \frac{PB_p}{D_L(100 - P)}\right] \tag{6}$$

where d_0 = distance (cm) from the injection point to the dead volume point on the chart, S = the recorder chart speed (in./h), V = volume of the column packed, F = carrier gas flow-rate (cm³/min) at the average column pressure, B_p = bulk density of the support, D_p = true density of the support, P = percentage (w/w) of stationary phase and D_L = density of stationary phase. This is subtracted from the observed retention distances for the *n*-alkanes and the adjusted retention distances are plotted against carbon number on log-linear graph paper. A further correction, δa, to account for the difference between static and dynamic column dead volumes is then applied

$$\delta a = \frac{2.303 \cdot \Sigma\ \delta \log R}{\Sigma\ 1/R_{\text{ext.}}} \tag{7}$$

where $\delta \log R = \log R - \log R_{\text{ext.}}$, R = retention distance obtained from the assumed retention volume and $R_{\text{ext.}}$ = value obtained from extrapolation on the linear part of the graph. The resulting adjusted retention distances are then replotted and generally give a straight live. Evans and Smith reported that occasionally a second correction (apparently trial and error) to linearise the "log plot" may be necessary.

3.2. Indirect measurement

Kaiser[27] suggested a method which involved the measurement of the total gas volume of the system using an azotometer and the calculation of the hold-up time, t_M (min), from that total gas volume, V_{GC} (cm³). He described the experimental system in detail and used eqns. 8 and 9 to calculate t_M

$$V_{GC} = V \cdot \frac{273.2\ p_M}{(273.2 + T_M)\ 760} + V_D \tag{8}$$

$$t_M = \frac{V_{GC}(273.2 + T_M)\ 2\ [(p_i/p_0)^2 + (p_i/p_0) + 1]}{273.2\ F\ p_M \cdot 3\ [(p_i/p_0) + 1]} \tag{9}$$

where V = azotometer reading (cm³), p_M = gas pressure (Torr) in the azotometer and in the room, T_M = gas temperature (°C) in the azotometer and in the room, V_D = volume (cm³) of apparatus not registered during purging with CO_2, p_i = pressure (Torr) in dosing system, p_0 = pressure (Torr) in the detector and F = carrier gas flow-rate (cm³/min) at T_M and p_M. However, as Kaiser pointed out, this procedure is time-consuming, liable to error and requires much experience. Also its application with modern equipment may require extensive modifications.

3.3. Direct measurement

In an ideal system the dead time is considered to be the time an infinitesimal

amount of non-adsorbed gas takes to pass through the chromatographic system under identical conditions to those for the sample being analysed. James and Martin[1] designated the air peak as the dead time and measured the retention times of substances being analysed as the distances from this peak. Since then controversy has developed over the question of the best substance to use for dead-time measurement. The problem is especially acute with flame ionisation detectors which do not normally respond to air or inert gases. Thus methane has been suggested as a substitute when using such detectors[34].

Many authors have supported the use of air and/or methane as an indication of dead time, especially in comparison to some of the early mathematical methods of calculating this parameter[5,8,22,23,35–38]. In addition several unique methods have been developed for use with flame ionisation detectors. Hilmi[39] outlined a method in which the carrier gas is presaturated with a low volatility organic solvent, thus producing a negative air peak with a flame ionisation detector. By measuring the retention time of the air peak and making allowance for the vapour pressure of the solvent, he determined the column dead time. 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.

Riedmann[40] developed a method in which the flow-rate of hydrogen to a flame ionisation detector is reduced to 3–5 cm^3/min. He found that the injection of a hydrogen sample of about 1 cm^3 and a splitting ratio of 1:100 produced a recorder-pen deflection and could be used as a measure of the system's dead time. Cramers *et al.*[20] described a similar method. In addition, Riedmann described a method suitable for Hewlett-Packard Series 5700 gas chromatographs (or any other chromatograph with a flame ionisation detector in which the ionisation current varies with the air flow). With the detector operating within the recommended air and hydrogen ranges, an injection of $\geqslant 1$ mm^3 of air will generate a detectable peak which can then be used as a measure of the column dead time.

Guberska[36,37] investigated the use of methane to measure column dead time at a series of temperatures and compared the results with the method developed by Hansen and Andresen[41]. Guberska found that although the retention time of methane was greater than the dead time obtained by the method of Hansen and Andresen, the standard deviation did not change with temperature, while the standard deviation of the dead time increases with an increase in temperature. He therefore proposed that the retention time of methane, adjusted according to

$$t_M = t_{CH_4} - (t_{CH_4}A)/163 \tag{10}$$

where A = amount (%, w/w) of stationary phase in the column, should be used as a measurement of the column dead time.

However, the use of air or methane not only creates experimental difficulties but evidence of a net retention has been presented by many authors. In a series of papers, experimenters from the University of New South Wales have shown that both air and methane show significant retention under the experimental conditions used, with methane showing retention on a wide range of column packings[30,32,42–47], Garcia Dominguez *et al.*[48] also reported that methane was retained by normal liquid phases at temperatures as high as 180°C. Kaiser[27] warned against the use of several

gases including air and methane. Ettre[13] reported errors when methane was used below 70°C, while Becerra *et al.*[49] reported that methane shows significant retention.

Further, if one accepts the evidence that the use of any homologous series provides the same estimate of dead time[7,50,51], then one is left with the uncomfortable conclusion that the retention of any C_1 compound should be a good estimate of the column dead time.

The use of other inert gases has been investigated by several authors. Riedo *et al.*[52] presented extensive data on a series of permanent gases and light hydrocarbons at temperatures between 30 and 230°C on a tailor-made C_{87} hydrocarbon stationary phase. Neon was found to have the smallest gross retention volume. Nitrogen, hydrogen and argon had the next smallest, with all other substances having much larger gross retention volumes (note that as helium was the carrier gas it could not be tested). Parcher and Johnson[5] compared the dead time estimated using four inert gases with that calculated using C_1–C_5 hydrocarbons. Their study confirmed that neon had the lowest retention time (again helium was not tested) and that methane had a small but measureable net retention time. Finally, a study by Ezrets and Vigdergauz[23] confirmed that methane has a small net retention compared to an inert gas (helium and hydrogen), but concluded that it was smaller than that introduced by a mathematical method such as that of Peterson and Hirsch[53].

We believe that the explanation of the wide divergence of opinion on the use of air and especially methane as a dead-time indicator lies in three areas, the experimental conditions, the accuracy of retention-time measurements and the use to which the retention data are put. The retention of methane in particular is greatly affected by experimental conditions and is larger at low temperatures, in gas–solid chromatography, and at high pressures. The accuracy of measuring retention times becomes of importance under conditions of low methane retention and/or small gross retention times. Finally, the use of methane under conditions of low retention for the calculation of Kovats indices above 400 is unlikely to introduce large errors. On the other hand, its use at low temperatures on porous polymers may introduce significant errors for all but very strongly retained substances. A final problem lies in the comparison of classical methods of dead-time calculation using methane. As will be pointed out later, such methods can also lead to very significant errors.

Therefore methane should only be used with extreme caution as an indication of the column dead time and the results should be verified by another method whenever the experimental conditions are changed.

From the above discussion and the fact that flame ionisation detectors do not respond to permanent gases such as neon, it is clear that there is a need for a mathematical method of dead-time calculation.

3.4. Graphical

This method involves plotting the logarithm of the adjusted retention times of the *n*-alkanes against their carbon numbers and linearisation of this plot by trial-and-error variation of the dead time. First reported by Evans and Smith[33] as well as Castello and Parodi[54], it is tedious and inaccurate and was soon superseded by various mathematical treatments of the data.

3.5. *Classical*

These methods use a limited number of homologues and generally require the solution of a small number (often one) of simple equations. The most widely used of the methods are described below.

(a) The method of Peterson and Hirsch[53] requires three evenly spaced homologues. It is based on the assignment of an arbitrary carrier-gas front (column dead time), x_0, as shown in Fig. 1 which uses three evenly spaced peaks to illustrate the method. All distances are then measured from this point which differs from the true dead time by δ. If x_0 corresponds to the true column dead time then $\delta = 0$. In general, however, this is not the case and the distance to a given peak, t^*_{Rz}, is not measured from the true column dead time but from x_0:

$$t'_{Rz} = t^*_{Rz} + \delta \tag{11}$$

The linear relationship between retention time and carbon number can now be expressed as

$$\log (t^*_{Rz} + \delta) \propto z \tag{12}$$

and when three evenly spaced peaks are used

$$\frac{t^*_{Rz+i} + \delta}{t^*_{Rz} + \delta} = \frac{t^*_{Rz+2i} + \delta}{t^*_{Rz+i} + \delta} \tag{13}$$

or

$$\delta = \frac{t^{*2}_{Rz+i} - t^*_{Rz} t^*_{Rz+2i}}{t^*_{Rz+2i} + t^*_{Rz} - 2t^*_{Rz+i}} \tag{14}$$

By measuring from any arbitrary point, x_0, to the peaks of three evenly spaced homologues, eqn. 13 can be solved for δ which is positive if t_M precedes x_0 and negative if t_M follows x_0. The column dead time can then be determined by measuring a distance δ from the arbitrary point x_0.

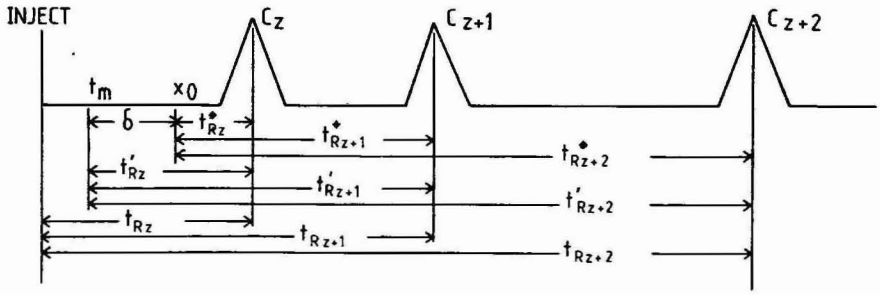

Fig. 1. Schematic chromatogram. t_R = Unadjusted retention time; t^*_R = retention time relative to x_0; t'_R = adjusted retention time.

Peterson and Hirsch suggested a further simplification. By designation x_0 as the peak of the second homologue, $t'_{Rz+i} = 0$ and the distance measured between the peaks of homologues 2 and 1 multiplied by the distance between homologues 2 and 3, divided by their sum (t'_{Rz} is negative in this formulation), is the distance from homologue 2 to t_M.

(b) Gold[55] improved upon the method of Peterson and Hirsch by developing one which did not require the three homologues to be equally spaced. The method is based on the fact that log $(t_{Rz} - t_M)$ is proportional to the carbon number, z:

$$z = m \log (t_{Rz} - t_M) + k \tag{15}$$

where m and k are constants. Letting the difference between carbon numbers be $z_2 - z_1 = \Delta z_{1,2}$, then

$$\Delta z_{1,2} = m [\log (t_{Rz_2} - t_M) - \log (t_{Rz_1} - t_M)] \tag{16}$$

and

$$m = \frac{\Delta z_{1,2}}{\log [(t_{Rz_2} - t_M)/(t_{Rz_1} - t_M)]} \tag{17}$$

Rewriting in terms of $\Delta z_{1,3}$ and solving for t_M gives:

$$t_M = \frac{t_{Rz_3} - 10^{(\Delta z_{1,3}/m)} \cdot t_{Rz_1}}{1 - 10^{(\Delta z_{1,3}/m)}} \tag{18}$$

Eqns. 17 and 18 are simultaneous and can be solved by the method of successive approximations.

Gold also suggested a graphical procedure in which eqn. 17 is rewritten in terms of $\Delta z_{1,3}$, giving two equations for m. The lines for these two equations are plotted by assuming values for t_M and calculating the resulting values of m with each of the two equations. These sets of points are plotted and their common solution is the point at which the two lines intersect.

Both these methods are tedious and have little to recommend them.

(c) Kaiser[56] developed a program for the HP-65 programmable calculator which calculated the dead time by the method of Gold. In the same paper, another program for the HP-65 was presented which calculated the constants in the log t'_R *vs.* I relationship once the dead time was known. This program was subsequently reprinted with a correction by Ebel and Kaiser[57]. Although this eliminated the tedium from Gold's method, it did not become widely used. This was probably due to the lack of availability of programmable calculators in 1974 and the fact that the advantages of the method over contemporary methods was relatively small.

(d) Hafferkamp[58] as well as Hansen and Andresen[41] developed a method which used three equally spaced homologues as proposed by Petersen and Hirsch. The advantage of their method was that it eliminated the use of an artificial dead time and used a single calculation to determine the column dead time. Their equation can be expressed as follows:

$$t_M = \frac{t_{Rz+i}^2 - t_{Rz}t_{Rz+2i}}{2t_{Rz+2i} - t_{Rz} - t_{Rz+i}} \tag{19}$$

Ettre[31] pointed out that this equation is in fact only a rearrangement of the simplified equations derived by Peterson and Hirsch[53] (last paragraph in section 3.5a). He then proceeded to generalise this approach to the situation where the homologues are separated by unequal distances. Thus $t_{Rz_3} - t_{Rz_2} = n(t_{Rz_2} - t_{Rz_1})$. Unfortunately this leads to the complication of solving higher-degree equations. For $n = 2$ (the only case quoted) a quadratic must be solved.

(e) In 1979 a method with some similarities to that of Gold and partially based on a method developed by Ševčík and Löwentap[29] was described by Al-Thamir *et al.*[59]. It begins with the linear relationship between the adjusted retention time and the carbon number and shows that this leads to a constant ratio of successive differences in retention times

$$\frac{\Delta t_{Rz+1,z+2}}{\Delta t_{Rz,z+1}} = e^b \tag{20}$$

where b = the slope of the plot of eqn. 5. Hence:

$$b = \ln (\Delta t_{Rz+1,z+2}/t_{Rz,z+1}) \tag{21}$$

Having evaluated b, c is calculated as:

$$c = \ln \left(\frac{\Delta t_{Rz,z+1}}{e^b - 1} \right)^{-bz} \tag{22}$$

This is eqn. 4 of Al-Thamir *et al.*[59] rewritten in a more convenient form. Then t_M is calculated from:

$$t_M = t_{Rz} - e^{(bz+c)} \tag{23}$$

Now t_M can be calculated for all homologues employed and a consistent result shows its validity.

A comparison of the method with that of Hansen and Andresen[41] (eqn. 19) shows that although the method involves more calculation, it provides no advantage over Hansen and Andresen's method.

Ettre[31] pointed out that the method is equally valid for homologues which differ by any distance, i, and thus is not restricted to consecutive homologues. Thus the method can be used for homologues with retention times t_{Rz}, t_{Rz+i} and t_{Rz+2i}. In addition, he showed how the method could be generalised even further to the case where $t_{Rz_3} - t_{Rz_2} = n(t_{Rz_2} - t_{Rz_1})$. Unfortunately this leads to the need to solve higher order equations ($n = 2$ leads to a quadratic) and thus the method becomes very difficult for all but the simplest extensions.

However, Ettre[31] criticised the approach because of the "danger of 'linearizing' the t_{Ri} *vs.* c_i relationship even where it is not valid". He also pointed out that "individual gas hold-up time values are obtained for the individual homologues which,

of course, is absurd since one can only have one single gas hold-up time for one chromatogram".

(f) In a recent paper Tóth and Zala[60] extended the method of Al-Thamir *et al.*[59] by deriving an expression for b which takes into account all possible ratios of the differences in retention times of neighbouring members of an homologous series. Expressions were also presented for c and t_M which take into consideration the retention values of all homologues of a consecutive series.

According to Al-Thamir *et al.*[59] (eqn. 21) the ratio between two neighbouring retention-time differences can be expressed as

$$b = \ln (\Delta_z/\Delta_{z-1}) \tag{24}$$

where Δ_z and Δ_{z-1} are consecutive retention differences and $z > 1$. Thus b can be more generally expressed by not necessarily using consecutive differences, giving

$$b_{z/z-n} = \frac{1}{n} \cdot \ln (\Delta_z/\Delta_{z-n}) \qquad (1 \leqslant n \leqslant z - 1) \tag{25}$$

where $b_{z/z-n}$ = b value obtained using the ratio of the zth and $(z - n)$th retention differences.

For an homologous series with N members, $N - 1$ Δ values are obtained. By combining these differences in pairs, the total number of pairs can be expressed as:

$$\frac{(N - 1)!}{2[(N - 1) - 2]!} \tag{26}$$

Therefore $N - 2$ values of b can be calculated from an homologous series having N members ($N - 1$ Δ values), using only neighbouring Δ values (in this case $n = 1$):

$$\sum_{z=2}^{N-1} b_{z/z-1} = \ln \left(\frac{\Delta_2\Delta_3 \ldots\ldots\ldots \Delta_{N-2}\Delta_{N-1}}{\Delta_1\Delta_2 \ldots\ldots\ldots \Delta_{N-3}\Delta_{N-2}} \right) \tag{27}$$

Also $N - 3$ values are obtained using the ratio of those b values which follow each other by the omission of one Δ (in this case $n = 2$). The sum can be expressed as:

$$\sum_{z=3}^{N-1} b_{z/z-2} = \tfrac{1}{2} \ln \left(\frac{\Delta_3\Delta_4 \ldots\ldots \Delta_{N-2}\Delta_{N-1}}{\Delta_1\Delta_2 \ldots\ldots \Delta_{N-4}\Delta_{N-3}} \right) \tag{28}$$

By increasing the value of n the ratio of the first and last b value is obtained:

$$b_{z/z-(N-2)} = \frac{1}{N - 2} \cdot \ln (\Delta_{N-1}/\Delta_1) \tag{29}$$

Now the sum of all b values expressed by all possible Δ values is given by

$$\Sigma b_{z/z-n} = \ln \prod_{z=1}^{L} (\Delta_{N-z}/\Delta_z)^{1/z+1/(z+1)+\ldots\ldots.1/(N-z-1)} \tag{30}$$

where $L = (N - 1)/2$ if N is odd and $L = (N/2) - 1$ if N is even.

Taking into account the total number of pairs of Δ values (eqn. 26), the mean value of b can be expressed as:

$$\bar{b} = \frac{2[(N-1)-2]!}{(N-1)!} \cdot \ln \prod_{z=1}^{L} (\Delta_{N-1}/\Delta_z)^{1/z+1/(z+1)+\ldots.1/(N-z-1)} \tag{31}$$

After calculating the mean value of b, the values of c and t_M can be obtained from:

$$c = \ln\left(\frac{t_{Rz+n} - t_{Rz}}{\exp[b(z+n)] - \exp[bz]}\right) \qquad (z + n > z) \tag{32}$$

$$t_M = t_{Rz} - \exp(bz + c) \tag{33}$$

Now these equations can be used to express the mean values of c and t_M as follows:

$$\bar{c} = \ln\left[\frac{(\Delta_1\Delta_2\ldots\Delta_{N-1})^{1/(N-1)}}{(\exp\bar{b}) - 1}\right] - \frac{\bar{b}(2z_1 + N - 2)}{2} \tag{34}$$

$$\bar{t}_M = \frac{\Sigma t_{Rz}}{N} - \frac{\exp[z_1\bar{b} + c][\exp(\bar{b}N) - 1]}{N[\exp(\bar{b}) - 1]} \tag{35}$$

Tóth and Zala[60] went on to show that their method gave similar results to both the statistical method of Grobler and Balizs[61] and their earlier iterative method (see section 3.7e).

(g) Garcia Dominguez *et al.*[48] described a method ("approximate series method") using any three homologues to calculate approximate retention times (and therefore the dead time). Two homologues are used to determine the approximate slope from:

$$b \approx [\log(t_{Rz_3}/t_{Rz_2})]/(z_3 - z_2) \tag{36}$$

A first approximation to the adjusted retention time of the first homologue can then be calculated from:

$$t'_{Rz_1^1} = (t_{Rz_2} - t_{Rz_1})/\{10^{[(z_2-z_1)\log(t_{Rz_3}/t_{Rz_2})/(z_3-z_2)]} - 1\} \tag{37}$$

The accuracy of $t'_{Rz_1^1}$ will increase as the difference $z_3 - z_1$ increases and for lines with higher slopes. For increased accuracy the process can be repreated to obtain a better estimate of t'_{Rz_1}. If three estimates are obtained, a better approximation can be made using

$$t'_{Rz_1} = t_{Rz_1^1} + d_1/(1 - d_2/d_1) \tag{38}$$

where $t'_{Rz_1^1}$ is the first approximation of t'_{Rz_1} and d_1, d_2 are the differences between the first and second and the second and third approximations of t'_{Rz_1}.

The dead time can then be calculated by subtracting the value found using eqn. 38 from the experimentally measured total retention time. However, the method shows no significant improvement over other classical methods and involves more effort in calculation than most. A second approximate method for use with only two homologues ("slope method"), presented in the same paper, is even less accurate and thus has little to recommend it.

(h) Recently Tóth and Zala[62] introduced a method based on three non-successive *n*-alkanes. They developed a series of equations for calculating the slope, *b*, given different combinations of known and unknown *n*-alkanes within a group of either four or five consecutive *n*-alkanes. The dead time, t_M, as well as *c* are then calculated using simple formulae. Unfortunately a different equation is required for the calculation of *b* in each case. In addition, no indication was given as to whether the method can be extended to the more general case where the three homologues are separated by random distances, *e.g.*, homologues with carbon numbers *z*, $z + x$ and $z + y$ where *x* and *y* are random integers. The method is more complex than other classical methods and therefore does not offer any advantage over other less complex methods.

3.6. Statistical

The previous classical methods used a limited number of homologues and thus their accuracy was poor (see section 5 for a more detailed discussion). In addition some used graphical or numerical trial and error which decreased their usefulness. As the use of programmable calculators and microcomputers increased it became clear that far more sophisticated methods were not only feasible, but were a necessity if consistently reliable results were to be obtained.

(a) Grobler and Balizs[61] developed a method which relied on the use of two linear regressions. The derivation, which is fully described in a previous paper by two of us[32] (not in Grobler and Balizs' paper), starts with eqn. 5. From this the following relationship between the difference in retention times and the carbon number can be derived

$$\ln (t_{Rz+1} - t_{Rz}) = \ln A + bz \tag{39}$$

where $A = e^c(e^b - 1)$, and therefore a linear regression on eqn. 39 of $\log (t_{Rz+1} - t_{Rz})$ against *z* will give *b* as the slope. Therefore:

$$b = \frac{(n-1)\sum_{i=z}^{z+n-1} i \cdot \ln [t_{R(i+1)} - t_{Ri}] - \sum_{i=z}^{z+n-1} i \cdot \sum_{i=z}^{z+n-1} \ln [t_{R(i+1)} - t_{Ri}]}{(n-1)\sum_{i=z}^{z+n-1} i^2 - \left(\sum_{i=z}^{z+n-1} i\right)^2} \tag{40}$$

Now

$$t_{Rz} = t_M + Aq^z \tag{41}$$

where $A = e^c$ and $q = e^b$. Therefore a further linear regression can be carried out on eqn. 41 of ln t_{Rz} against z. This gives

$$t_M = \frac{\sum_{i=z}^{z+n} q^i \cdot \sum_{i=z}^{z+n} t_{Ri}q^i - \sum_{i=z}^{z+n} q^{2i} \cdot \sum_{i=z}^{z+n} t_{Ri}}{\left(\sum_{i=z}^{z+n-1} q^i\right)^2 - n \sum_{i=z}^{z+n-1} q^{2i}} \tag{42}$$

and

$$c = \sum_{i=z}^{z+n} \ln t'_{Ri} - \left(b \sum_{i=z}^{z+n} i\right) \Big/ n \tag{43}$$

Eqns. 40, 42 and 43 allow the retention index of any compound to be calculated from:

$$I = 100\ (\ln t'_R - c)/b \tag{44}$$

(b) This method has been extended by Van Tulder *et al.*[63]. In addition they carried out an extensive evaluation of the method and produced a series of criteria to allow the best selection of homologues to be used in the two linear regressions.

They proposed an extension in which homologues with equidistant carbon numbers were used instead of consecutive homologues. Thus the method starts by assuming a set of n consecutive homologues, starting with homologue z^0 as follows*:

$$(t_{Rz}, t_{Rz+1}, \ldots\ldots\ldots t_{Rz+n-1})$$

or

$$(t_{Ri} \text{ where } i = z \text{ to } z + n - 1) \tag{45}$$

Now a subset of n^1 homologues are selected with carbon numbers at equal distance, m, from each other, starting with z^1. This subset can be characterised by:

$$[t_{Rj} \text{ where } j = z^1 \text{ to } z^1 + m(n^1 - 1)] \tag{46}$$

Therefore eqn. 5 can be rewritten as

$$\ln (t_{Rj} - t_M) = b(z^0 + j) + c \tag{47}$$

---

* Note that in the paper of Van Tulder *et al.*[63] i and j are the sequence numbers of the homologues (starting with 1), while in our review they are the true homologue numbers.

where z^0 represents the carbon number of the basic group and j is the homologue number (z^0 will be set to 0 for the remainder of this review).

Now eqn. 39 can be rewritten as

$$\ln (t_{Rj+m} - t_{Rj}) = \ln A^1 + bj \tag{48}$$

where $A^1 = e^c(e^{mb} - 1)$. Therefore a linear regression on eqn. 48 produces

$$b = \frac{(n^1-1)\sum_{j=z^1}^{Z-m} j \cdot \ln [t_{R(j+1)} - t_{Rj}] - \sum_{j=z^1}^{Z-m} j \cdot \sum_{j=z^1}^{Z-m} \ln [t_{R(j+1)} - t_{Rj}]}{(n^1-1)\sum_{j=z^1}^{Z-m} j^2 - \left(\sum_{j=z^1}^{Z-m} j\right)^2} \tag{49}$$

where $Z = z^1 + mn^1$ and thus:

$$t_{Rj} = t_M + A^1 q^j \tag{50}$$

A second linear regression on eqn. 38 gives

$$t_M = \frac{\sum_{j=z^1}^{Z} q^j \sum_{j=z^1}^{Z} t_{Rj}q^j - \sum_{j=z^1}^{Z} q^{2j} \cdot \sum_{j=z^1}^{Z} t_{Rj}}{\left(\sum_{j=z^1}^{Z-m} q^j\right)^2 - n\sum_{j=z^1}^{Z-m} q^{2j}} \tag{51}$$

and

$$c = \frac{\sum_{j=z^1}^{Z} \ln t'_{Rj} - b\sum_{j=z^1}^{Z} j}{n^1} \tag{52}$$

Van Tulder *et al.*[63] carried out an extensive computer simulation to investigate the influence of statistical error on the relative standard deviation of the dead time, R.S.D. (t_M). The study indicated that the minimum number of homologues required depended on the slope b. From a large number of computer simulations, the following empirical equation was developed

$$N_0 = (3/b) + 1 \tag{53}$$

where minimum N_0 is 3 and N_0 = the optimum number of homologues. In addition, surprisingly it was found that three homologues with a separation distance, m, of $1.5/b$ gave the lowest R.S.D. (t_M) values. Hence the following procedure to reduce the statistical error in dead-time calculation to a minimum was proposed.

First an initial calculation should be preformed with as many consecutive homologues as possible, excluding any lower members which show non-linear behaviour. From this calculation a value for b is obtained and used to calculate the distance, m, between homologues to obtain a slope factor $\geqslant 1.5$. Finally the t_M calculation is repeated with the properly selected homologues.

Although extensive experience in our laboratory has shown that in general this method selects homologues which give a good estimate of dead time, two problems are apparent. The first is the authors' use of R^2 as an indication of linearity. In fact, R^2 is particularly insensitive in this application and is of no use when using only three homologues. A better test of linearity is needed. The second problem, which is discussed in more detail in our recent paper[64], is that the method only ensures that over a large number of trials it will give the lowest R.S.D. (t_M). However there is no guarantee that, given a particular set of retention times, the method will produce an acceptable estimate of the dead time. Our experience has been that in some cases the method can lead to very poor results. The reason is not difficult to understand. While the method provides the range of homologues needed to obtain a good estimate, the use of such a limited number of homologues for the actual dead-time calculation means that errors in the retention times of the chosen homologues can, under certain conditions, be magnified in the final dead time[28,32]. We therefore recommend caution in the use of this section of the method.

(c) Ambrus[76] recently presented a method which determines both the column dead time, t_M, and the slope in a single linear regression. Where Kovats indices are required, c can then be calculated either from a second regression or as the mean of a set of c values calculated using each adjusted retention time.

Starting with the usual assumption of linearity of the log plot, an expression for relative retention can be calculated as follows:

$$\ln (t_{Rz} - t_M) = bz + c \qquad \text{(eqn. 5)}$$

$$t_{Rz} - t_M = e^{bz+c} = e^{bz}e^{c} \tag{54}$$

and

$$t_{R(z+1)} - t_M = b(z + 1) + c = e^{bz}e^{b}e^{c} \tag{55}$$

thus

$$\frac{t_{R(z+1)} - t_M}{t_{Rz} - t_M} = \frac{e^{bz}e^{b}e^{c}}{e^{bz}e^{c}} = e^{b}, \tag{56}$$

which is a constant. Letting the constant be q gives

$$t_{R(z+1)} - t_M = qt_{Rz} - qt_M \tag{57}$$

and thus

$$t_{R(z+1)} = qt_{Rz} - B \tag{58}$$

where $B = t_M(q - 1)$. Therefore a regression of $t_{R(z+1)}$ against t_{Rz} will give q as the slope (from which b can be calculated as ln q and B as the intercept [from which t_M equals $B/(q - 1)$].

For the calculation of Kovats indices, c can now be calculated from eqn. 5 by a further regression of ln t'_{Rz} against I. Alternatively, a series of values of c can be obtained by substituting each retention time into eqn. 5, and the mean of these values used as an estimate of c.

Ambrus' methods[76] can also be extended to homologues with equidistant carbon numbers by the technique of Van Tulder *et al.*[63] (section 3.6b).

3.7. Iterative

(a) Guardino *et al.*[24] presented a method in which an iteration is carried out on t_M, with b and c calculated using a least-squares fit. The optimum values of t_M, b and c are determined by minimising the sum of squares of the differences between the known and calculated retention-index values. A flow chart of the method is shown in Fig. 2, where UPLIM and LOWLIM are the upper and lower limits, respectively of the sum of squares of the deviation, TM is the dead time, INC is the increment in the dead time, IC is the calculated Kovats retention index, SUM is the sum of squares of the deviations, TR is the unadjusted retention times of the homologues, I is the known Kovats retention index (100z where z is the carbon number) and PREC is the precision to which the answer is required.

The method starts with an initial estimate of the dead time, which is used to determine adjusted retention times. A linear regression then allows b and c to be calculated and thus retention indices can be determined. Subtracting these from the known values gives a sum of differences which is compared to the upper and lower limits. If the estimate of t_M is below the lower limit, the limits are reduced and the estimate of t_M is increased. When this estimate increases above the lower limit, it is decreased and the increment is lowered by a factor of 10. The whole process is repeated until the increment is less than the required precision (PREC).

A very important point when using this method (not mentioned in the paper) is that the initial estimate of dead time must be less than the true mathematical dead time or the method will fail. Guardino *et al.* suggested using 98% of the value determined by the method of Haferkamp[58] as the initial estimate for their method. However, due to the occasional large errors that can occur when using any of the classical methods of estimating dead time, there is the possibility that this estimate may lead to an incorrect dead time. The best way of ensuring this does not happen is to monitor the difference in consecutive sums of squares and ensure that a change of sign occurs before the sum of squares becomes larger than the lower limit and that the program does not stop within the first two iterations.

(b) Bellas[65] also reported an iterative method of calculating dead time. The program, written in FORTRAN, makes an initial estimate for the dead time of one eighth of the minimum retention time. A linear regression is then carried out and the sum of squares of the deviations of the indices calculated for the reference compounds is found. The dead time is increased incremently until a minimum is passed in the sum of squares. The increment is then halved and changed in sign and the fitting continued. Thus, after the first passage the increment adopts successive values of

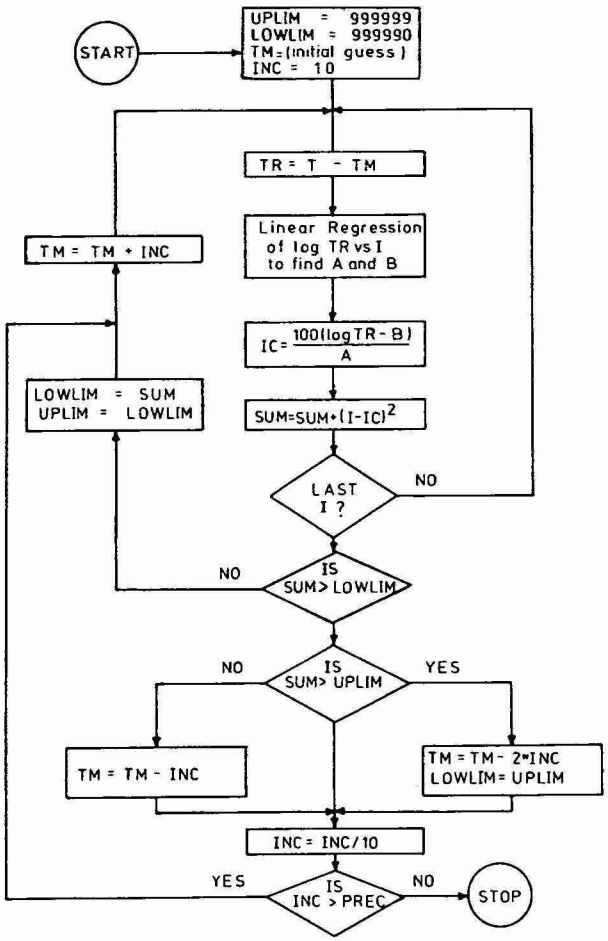

Fig. 2. Flow chart for calculation of mathematical dead time by the method of Guardino *et al.*[24].

$(-1)^n 2^{-n}$ · (initial increment). Twelve is set as the maximum value of n. In addition, limits are placed on the permissible values for t_M, with negative values and values greater than the minimum retention time being rejected.

Bellas also reported that the program had been extended to calculate the index for data from linear temperature-programmed gas chromatography by fitting a polynomial of degree equal to the number of data points.

Because of the similarity to the method of Guardino *et al.*[24] (the data used by Bellas give vertually identical results when used in the method of Guardino *et al.*), it will be assumed that the methods are equivalent and only the method of Guardino *et al.* will be discussed further.

(c) Garcia Dominguez et al.[48] reported an iterative method ("exact calculator method") in a paper which introduced several new methods (section 3.5g). A flow chart of the method is shown in Fig. 3.

The method initially uses the retention times of substances with known Kovats

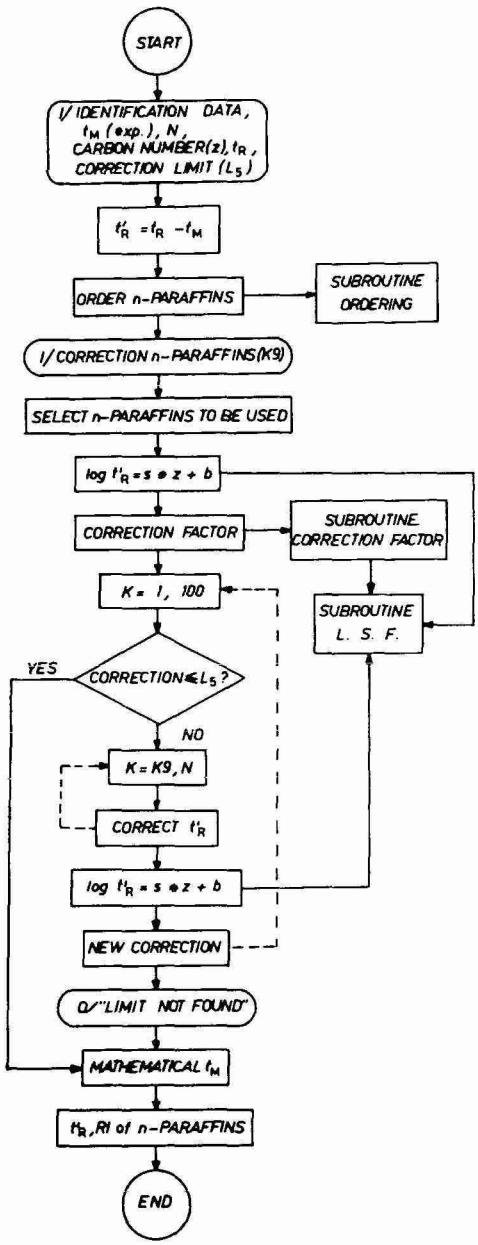

Fig. 3. Flow chart for the "exact calculator method" of Garcia Dominguez *et al.*[48].

indices (such as the *n*-alkane homologues), measured from any point on the chromatogram, in a linear regression. Letting the initial retention time of the alkane with the least retention be $t_{Rz_0^1}$, the coefficients determined from the regression can be used to determine a new retention time for that alkane of $t_{Rz_1^1}$. A correction factor is then calculated:

$$d_1 = t_{Rz_1^1} - t_{Rz_0^1} \tag{59}$$

This correction, which can be either positive or negative depending on whether the initial retention time of the first alkane, $t_{Rz_0^1}$, is shorter or longer than the true retention, is now applied to all peaks, producing a second set of retention times. These times are subsequently used in another linear regression and a second correction is calculated from the new retention of the alkane which initially had the lowest retention time:

$$d_2 = t_{Rz_2^1} - t_{Rz_1^0} \tag{60}$$

This equation is equivalent to eqn. 3 of Garcia Dominguez *et al.*[48], except for a correction. The original equation implies that the correction is always the difference between the latest two values of the retention time of the lowest alkane. In fact, for the method to converge, the correction must be the difference between the latest value of the retention time and the originally measured retention time.

After a sufficient number of cycles a final set of adjusted retention values is obtained. A refinement to the program is that the actual correction applied to each set of retention times can be multiplied by a "correction factor" to reduce the number of iterations required by the program. Fig. 4 shows the effect the process has on the linearity of the lot plot; the curvature of the log plot is reduced as the adjusted retention times approach their true values.

(d) Tóth and Zala[62] mentioned an iterative method and also introduced a classical method (see section 3.5f). The method calculates the parameters in eqn. 5 by first determining t_M. On the basis of data pairs (t_{Rz},I) obtained by measurements on the *n*-alkanes, the coefficient of determination, R^2, between the quantities I and $\ln (t_{Rz} - t_M)$ as a function of t_M can be expressed as:

$$R^2 = R^2(t_M) = \frac{\left\{\left[\sum_{i=z}^{z+n} \ln (t_{Ri} - t_M) I_i\right] - \frac{\sum_{i=z}^{z+n} I_i \cdot \sum_{i=z}^{z+n} \ln (t_{Ri} - t_M)}{n}\right\}^2}{\left\{\sum_{i=z}^{z+n} I_i^2 - \frac{\left(\sum_{i=z}^{z+n} I_i\right)^2}{n}\right\}\left\{\sum_{i=z}^{z+n} [\ln (t_{Ri} - t_M)]^2 - \frac{\left[\sum_{i=z}^{z+n} \ln (t_{Ri} - t_M)\right]^2}{n}\right\}} \tag{61}$$

The dead time can be calculated from the value at which $R^2(t_M)$ is a maximum. This value of t_M can be obtained by solving the non-linear equation

$$dR^2/dt_M = 0 \tag{62}$$

by an iterative technique. With a knowledge of t_M, estimates of b and c can be computed by linear regression of I *versus* $\ln (t_{Rz} - t_M)$.

Although no method was given for the iterative solution of $dR^2/dt_M = 0$,

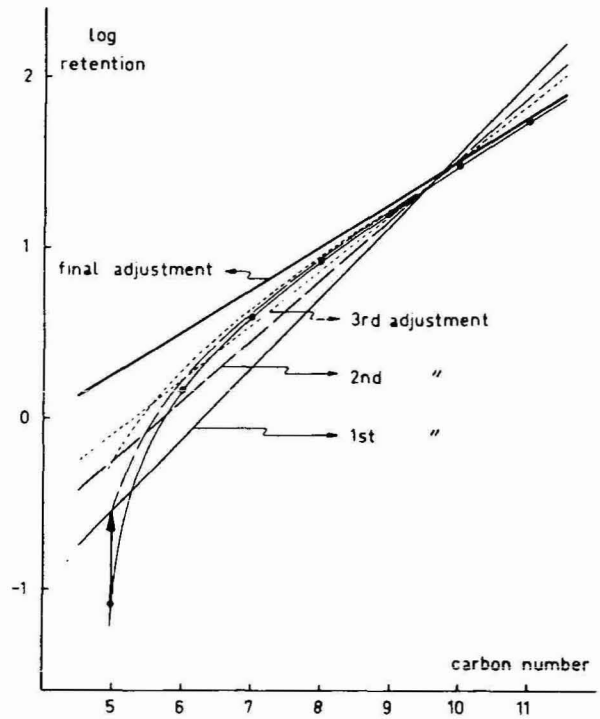

Fig. 4. Schematic drawing of the "exact calculator method" of Dominguez *et al.*[48]. The points represent initial retention values. The arrow shows the first correction applied.

several methods of numerical differentiation are given in a book by Dorn and McCracken[66]. It should be noted that numerical differentiation suffers from subtractive cancellation, which can lead to very large errors. Because of the difficulty of overcoming this problem, numerical differentiation is not normally recommended if there are other methods available to solve the problem.

An alternative solution which is not only faster, but also does not involve the problems associated with numerical differentiation is to minimise $1 - R^2$ using an appropriate iterative technique.

(e) One of the most versatile methods of dead-time estimation is undoubtedly the simultaneous non-linear least-squares estimation of t_M, b and c[28,30] by the use of numerical minimisation[67]. When using this method it is necessary to define the objective function which is to be minimised. This should be done very carefully because different objective functions will lead to different estimations of t_M, b and c as reported by us[30]. For the calculation of Kovats indices, the sum of squares of the differences between the known and calculated I values is a suitable objective function.

However, this method suffers from three problems. First it is very complex to program. Secondly, the execution time is much longer than in non-iterative methods and it can take as long as half an hour to achieve maximum accuracy on a microcomputer. Finally the method may converge to a local minimum rather than the global minimum. This is especially true with complex functions such as higher-degree

polynomials. The method thus requires good initial estimates of t_M, b and c as well as a critical evaluation of the output of the program.

Therefore the method is not suitable for routine use and is only useful in the evaluation of other methods of calculating dead time.

3.8. Others

(a) A method was reported by Ševčík[68] and later extended by Ševčík and Löwentap[29] which does not require calculation of the column dead time. Later[69] the method was used for the classification of stationary phases. That paper showed that A was independent of the carrier gas flow-rate and pressure as well as a high degree of reproducibility with time. The method, which can be best understood by reference to Fig. 5, uses the ratio of the differences in retention times between consecutive *n*-alkanes, A, to calculate adjusted retention times.

Defining the difference between consecutive gross retention times as Δ gives:

$$\Delta_z = t_{Rz} - t_{R(z-1)} \tag{63}$$

Now, from Fig. 4, the adjusted retention time of a peak can be expressed as

$$t'_{Rz} = \Delta_1 + \Delta_2 + \Delta_3 + \ldots\ldots \Delta_{z-1} + \Delta_z \tag{64}$$

where Δ_1 is the time difference between the elution of a substance with $I = 0$ and one with $I = 100$, etc.; Δ_1 actually equals the adjusted retention time of a homologue with a carbon number of one, *i.e.*, $\Delta_1 = t'_{R_1}$.

Experimentally, Ševčík and Löwentap[29] found that the ratio of successive time differences in an homologous series was constant. Therefore:

$$\Delta_z = A\,\Delta_{z-1} \tag{65}$$

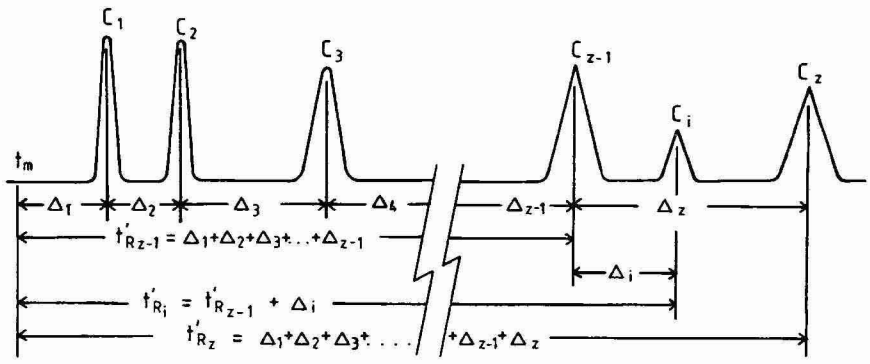

Fig. 5. Schematic chromatogram illustrating the method of Ševčík and Löwentap[29]. t'_R = Adjusted retention time; Δ_z = time difference between consecutive homologues; Δ_i = time difference between compound i and the nearest homologue.

Thus

$$\begin{aligned}
\Delta_{z-1} &= A^{-1}\Delta_z \\
\Delta_{z-2} &= A^{-1}\Delta_{z-1} = A^{-2}\Delta_z \\
\Delta_{z-3} &= A^{-1}\Delta_{z-2} = A^{-3}\Delta_z \\
&\vdots \qquad\quad \vdots \qquad\quad \vdots \\
\Delta_2 &= A^{-1}\Delta_3 = A^{2-z}\Delta_z \\
\Delta_1 &= A^{-1}\Delta_2 = A^{1-z}\Delta_z
\end{aligned}$$

and eqn. 64 can be written as:

$$\begin{aligned}
t'_{Rz} &= \Delta_z + \Delta_{z-1} + \Delta_{z-2} + \ldots\ldots \Delta_2 + \Delta_1 \\
&= \Delta_z + A^{-1}\Delta_z + A^{-2}\Delta_z + \ldots\ldots A^{2-z}\Delta_z + A^{1-z}\Delta_z \\
&= \Delta_z(1 + A^{-1} + A^{-2} + \ldots\ldots A^{2-z} + A^{1-z})
\end{aligned} \tag{66}$$

This is a geometric series and can be summed as follows:

$$t'_{Rz} = \frac{\Delta_z[1 - (1/A)^z]}{1 - (1/A)} = \frac{\Delta_z(A^{z+1} - A)}{A^{z+1} - A^z} \tag{67}$$

Therefore the method allows the calculation of the adjusted retention time of any homologue without reference to the dead time. The adjusted retention of other substances can be calculated by adding the difference in retention times between the substance and any alkane to the adjusted retention time of the alkane as calculated by eqn. 64 or 67. Therefore in Fig. 5

$$t'_{Ri} = t'_{Rz-1} + \Delta_i$$

where t'_{Rz-1} is calculated by eqn. 64 or 67 and Δ_i is the difference in retention times between a substance i and a standard $z - 1$.

The method can also be used for extrapolation by noting that eqn. 65 means that the dependence of the logarithm of the difference in times, ln Δ_z, *versus* z is linear. Thus:

$$\ln \Delta_z = dz + e \tag{68}$$

where d and e are constants.

Ševčík and Löwentap[29] also showed that their method leads to the conclusion that relative retention is dependent on the carbon number. Eqn. 64 can be expressed as:

$$\begin{aligned}
t'_{Rz+1} &= \Delta_{z+1} + \Delta_z + \Delta_{z-1} + \ldots\ldots \Delta_3 + \Delta_2 + \Delta_1 \\
&= \Delta_{z+1} + \frac{\Delta_z(A^{z+1} - A)}{A^{z+1} - A^z}
\end{aligned} \tag{69}$$

Combining eqn. 69 with eqn. 65 gives:

$$t'_{Rz+1} = A\Delta_z + \frac{\Delta_z(A^{z+1} - A)}{A^{z+1} - A^z}$$
$$= \frac{\Delta_z[A(A^{z+1} - A^z) + (A^{z+1} - A)]}{A^{z+1} - A^z}$$
$$= \frac{\Delta_z(A^{z+2} - A)}{A^{z+1} - A^z} \tag{70}$$

Dividing eqn. 70 by eqn. 67 gives:

$$\frac{t'_{Rz+1}}{t'_{Rz}} = \frac{\Delta_z(A^{z+2} - A)}{A^{z+1} - A^z} \cdot \frac{1}{\Delta_z} \cdot \frac{A^{z+1} - A^z}{A^{z+1} - A}$$
$$= \frac{A^{z+2} - A}{A^{z+1} - A} \tag{72}$$

Rearranging eqn. 72 gives:

$$\frac{t'_{Rz+1}}{t'_{Rz}} = \frac{A - (1/A^z)}{1 - (1/A^z)} \tag{73}$$

From this expression it can be seen that as $z \to \infty$ then $t'_{Rz+1}/t'_{Rz} \to A$. Thus the method predicts that relative retention will only be constant at high values of z.

Unfortunately a similar analysis of eqn. 5 gives a very different result

$$\frac{t'_{Rz+1}}{t'_{Rz}} = A \tag{74}$$

where $A = e^b$ (see eqn. 56). This discrepancy leads to one of two difficulties with the method. Because the method has no independent procedure for calculating Kovats indices of substances other than the homologues, it is left without a consistent procedure for calculating Kovats indices, as the usual formula (eqn. 5) is no longer valid and their relationship (eqn. 68) is only suitable for calculating adjusted retention times. It is not suitable for the reverse process of calculating z numbers (and thus I values) from the adjusted retention time of some unknown substance.

The second problem arises because the method leads to different dead times for each homologue. Although the authors assume that this is due to errors in reported retention times, their own data show a disturbing variation in dead time between the measured data (high z) and the extrapolated data (low z) as shown in Table 1. Ettre[31] has discussed this particular problem with specific reference to the method of Al-Thamir *et al.*[59]. He does, however, point out that the problem exists in all methods which calculate adjusted retention times without first estimating a gas hold-up time.

Finally it is worth pointing out that workers from the University of New South Wales have found that the method is a very sensitive indicator of linearity and have published a series of papers[43–47] in which it is used to test the linearity of a range of homologues on a wide selection of columns.

TABLE 1

DATA FROM ŠEVČÍK AND LÖWENTAP[29] SHOWING VARIATION IN DEAD TIME

z	*Measured* t_{Rz} *(mm)*	Δ_z *(mm)*	A	Δ_z *(mm) (calc.)*	t'_{Rz}	$r = \frac{t'_{Rz+1}}{t'_{Rz}}$	t_M* *(mm)*
20	2598				2564.46		33.5
		704		703.56		1.3781	
19	1894		1.3804		1860.90		33.1
		510		510.86		1.3784	
18	1384		1.3747		1350.04		34.0
		371		370.94		1.3789	
17	1013		1.3792		979.10		33.9
		269		269.34		1.3795	
16	744		1.3724		709.76		34.2
		196		195.57		1.3804	
15	548		1.3803		514.19		33.8
		142		142.00		1.3815	
14	406		1.3786		372.19		33.8
		103		103.11		1.3832	
13	303		1.3377		269.08		33.9
		77		74.87		1.3856	
12	226		1.3750		194.21		31.8
		56		54.36		1.3888	
11	170		1.4000		139.85		30.2
		40		39.47		1.3931	
10	130		1.3793		100.38		29.6
		29		28.66		1.3996	
9	101			20.81	71.72	1.4090	29.3
8	(80.19)**			15.11	50.91	1.4222	29.3
7	(65.08)			10.97	35.80	1.4414	29.3
6	(54.11)			7.97	24.83	1.4745	29.3
5	(46.14)			5.78	16.85	1.5212	29.3
4	(40.36)			4.20	11.07	1.6114	29.3
3	(36.16)			3.05	6.87	1.7984	29.3
2	(33.11)			2.21	3.82	2.3727	29.3
1	(30.90)			1.61	1.61		29.3
0	(29.29)						

* Estimated column dead time, $t_M = t_{Rz} - t'_{Rz}$.

** Figures in brackets are calculated gross retention times, $t_{Rz} = t_{Rz+1} - \Delta_z$.

(b) All methods discussed so far have relied on the accepted linear relationship between $\ln (t_{Rz} - t_M)$ and I, even though its linearity for homologues with low carbon numbers has been questioned (see section 4). However, a new method based on a cubic relationship between $\log t'_{Rz}$ and I has been reported by Heeg *et al.*[70]. The assumption of non-linearity was based on reports in the literature of experimental results[74] as well as on thermodynamic considerations[71].

Heeg *et al.*[70] compared several traditional methods of calculating retention indices with methods based on a quadratic and a cubic fit:

$$\log t'_{Rz} = az^3 + bz^2 + cz + d \tag{75}$$

For Kovats index calculation, eqn. 75 can be more conveniently expressed in the form:

$$I = a'(\log t'_{Rz})^3 + b'(\log t'_{Rz})^2 + c'(\log t'_{Rz}) + d' \tag{76}$$

This comparison led to the conclusion that a quadratic fit was inadequate and that a cubic fit (eqns. 75 and 76) was necessary to obtain good accuracy.

However, the method presented a problem because the authors were not able to manipulate the cubic equation in such a way that would allow it to be solved for

t_M. Thus they recommended that t_M be first calculated using two linear regressions as outlined by Grobler and Balizs[61]. Having calculated t_M, the constants a, b, c and d can be found by using one of several methods of fitting data by a cubic equation.

All methods designed to fit cubic and higher-degree polynomials are based on matrix manipulations. Such methods are relatively complex and suffer from round-off and subtractive cancellation errors. It is therefore essential that the maximum number of significant figures be carried throughout the calculations. We have found that the method of Gaussian elimination with partial pivotal condensation[66] using double precision arithmetic (with 16 digits accuracy) gives acceptable results.

A final problem with this method is its use of a dead-time value calculated by a linear method when the data are actually being fitted by a cubic polynomial. This is mathematically undesirable and likely to produce a less than optimum fit. A way of overcoming this problem is to modify one of the iterative methods to allow a cubic fit instead of a linear fit. Such an approach sacrifices speed in an attempt to improve the fit.

4. LINEARITY OF THE PLOT OF LOG ADJUSTED RETENTION TIME *VS.* CARBON NUMBER

Almost all methods of calculating Kovats indices are based on eqn. 5 which states that the relationship between the log of the adjusted retention time and carbon number is linear. Although some authors have accepted that this relationship is linear over the entire range of carbon numbers[72,73], an increasing amount of evidence has accumulated that the relationship is non-linear for low carbon numbers. This evidence is both experimental and theoretical.

4.1. The experimental evidence

In a large number of papers experimental evidence has been presented as to the non-linearity of the log relationship for homologues with low carbon numbers. The most extensive evidence has been presented by Haken, Wainwright and Srisukh from the University of New South Wales[42–47]. Although their first paper[42] (which is more concerned with mathematical dead time *versus* methane retention than the question of linearity) present evidence for the linearity of this relationship for C_1–C_9 alkanes on both SE-30 and OV-25, a close examination of the results in conjunction with results presented in a later paper[43] strongly indicates that the apparent linearity was a result of the relative high temperatures used in the study.

First the results on OV-25 show a calculated methane retention time which is consistently 2–3 sec less than the measured retention time, thus indicating a slight non-linearity. The retention times, which were calculated using the method of Grobler and Balizs[61], are confirmed in Table 2, where the retention time of methane has been recalculated for five sets of conditions using the method developed by Ševčík and Löwentap[29]. Although the discrepancy is small it translates into Kovats indices which range from approximately 180 to 250. Secondly, although the SE-30 data show linearity at temperatures of 120–140°C, a later paper[43] shows non-linearity for C_1–C_4 alkanes on both SE-30 and OV-25 at 30°C.

The other papers by these workers provide evidence for non-linearity of the relationship for lower members of *n*-alkanes, *n*-alcohols, *n*-aldehydes, acetates and

TABLE 2

DATA FROM REF. 42. RECALCULATED USING THE METHOD OF ŠEVČÍK AND LÖWENTAP[29]

Carbon number	*Retention time**	*Δ (sec)*	*A*	*Retention time**	*Δ (sec)*	*A*	*Retention time**	*Δ (sec)*	*A*
SE-30	*(120°C, 30 ml/min)*			*(130°C, 40 ml/min)*			*(140°C, 45 ml/min)*		
1	116.0 (116.5)**	(2.45)		96.2 (96.8)**	(1.53)		80.7 (81.3)**	(1.35)	
2	(118.95)	(4.33)	(1.77)	(98.30)	(2.70)	(1.76)	(82.66)	(2.28)	(1.69)
3	(123.28)	(7.66)	(1.77)	(101.20)	(4.75)	(1.76)	(84.94)	(3.85)	(1.69)
4	(130.94)	(13.56)	(1.77)	(105.95)	(8.35)	(1.76)	(88.79)	(6.51)	(1.69)
5	144.5	24.0	(1.77)	114.3	14.7	(1.76)	95.3	11.0	(1.69)
6	168.5	43.40	1.81	129.8	27.5	1.87	106.3	18.9	1.72
7	211.9	76.1	1.75	157.3	46.4	1.69	125.2	31.5	1.69
8	288.0	134.1	1.76	203.7	79.5	1.71	156.7	52.0	1.65
9	422.1			283.2			208.7		
OV-25	*(120°C, 45 ml/min)*			*(130°C, 45 ml/min)*					
1	197.2 (194.9)**	(2.03)		191.1 (189.4)**	(1.72)				
2	(196.94)	(3.53)	(1.74)	(191.12)	(2.93)	(1.70)			
3	(200.47)	(6.14)	(1.74)	(194.05)	(4.98)	(1.70)			
4	(206.61)	(10.69)	(1.74)	(199.03)	(8.47)	(1.70)			
5	217.3	18.6	(1.74)	207.5	14.4	(1.70)			
6	235.9	31.9	1.72	221.9	25.8	1.79			
7	267.8	55.7	1.75	247.7	42.1	1.63			
8	323.5	96.9	1.74	289.8	71.0	1.69			
9	420.4			360.8					

* Measured retention times (sec) are the average values from two or three determinations.
** Calculated values are shown in brackets.

methyl ketones on a series of porous polymers as well as for *n*-alkanes on porous silica.

Heeg *et al.*[70] found that the log relationship was non-linear and developed a method based on a cubic fit to determine retention indices. In addition, Heldt and Köser[51] presented evidence in their 1980 paper that the relationship is non-linear.

Parcher and Johnson[5] as well as Lin and Parcher[6] presented evidence for the non-linear behaviour of methane, and suggested that an effective carbon number of 0.5 should be assigned to it in order to linearise the log relationship. This approach has been discussed by Wainwright and Haken[47] who pointed out several problems, including their findings that the effective carbon number of methane varied with the column packing, that it was closer to 1.0 than 0.5 and that other *n*-alkanes showed non-linear behaviour. There is also a fundamental problem with changing the carbon number of methane. This relates to the fact that the retention index system is based on the definition that the retention index of an *n*-alkane is 100 times its carbon number. The setting of the effective carbon number of methane to anything other than 1 is theoretically undesirable. Even if the practice led to accurate dead times, it would lead to difficulties in the definition and calculation of retention indices.

Riedo *et al.*[52] carried out an extensive study during an 8-month period. About

1500 retention values for *n*-alkanes from pentane to pentadecane were determined on two columns at temperatures from 30 to 250°C. The results were used to calculate thermodynamic properties which were then plotted, together with the results for the light hydrocarbons (C_1–C_4), as a function of the carbon number of the *n*-alkane. They concluded that "this plot is not linear. Therefore, the determination of the starting point of the chromatogram is not justified by linearization of the logarithms of the retention data of *n*-alkanes".

4.2. The theoretical evidence

Rohrschneider[74] was one of the first to discuss the linearity of the log plot in relation to basic thermodynamic parameters. He reported that the slope of the *n*-alkane curves depended on the ratio of the vapour pressure, p, and activity coefficient, γ:

$$b = \log \frac{t'_{Rz+1}}{t'_{Rz}} = \log \frac{p_z}{p_{z+1}} + \log \frac{\gamma_z}{\gamma_{z+1}} \tag{77}$$

By calculating this value for C_2 through C_{10} he was able to show that *n*-alkane curves show a definite deviation from linearity caused above all by the non-linear vapour-pressure dependence.

Sojak *et al.*[75] proposed a relationship between the retention index, boiling point and the activity coefficient of a substance. Starting with the same equation as did Rohrschneider[74] (eqn. 77), they extended the relationship to boiling points using a relation derived by Purnell for a compound at a given temperature:

$$\log p^0 = k_1 + k_2 T_b \tag{78}$$

where k_1 and k_2 are constants. Thus:

$$\log (p_2^0/p_1^0) = k_2(T_{b,2} - T_{b,1}) \tag{79}$$

Combining eqn. 79 with eqn. 77 gives:

$$\log \frac{t'_{R_2}}{t'_{R_1}} = -k_2(T_{b,2} - T_{b,1}) - \frac{\log \gamma_2}{\gamma_1} \tag{80}$$

Although eqns. 78–80 are only approximations and are not valid for substances capable of hydrogen bonding, the general derivation supports Rohrschneider's work in this area.

Bach *et al.*[71] discussed, using basic thermodynamics, the dependence of retention times on boiling points. By using an equation derived by Klages, in which the square of the boiling temperature of an organic substance is represented as an additive quality of the molecule, they established an analytical relationship between retention times and carbon numbers. Simplifying approximations enabled the relationship to be linearised and thus the Kovats system could be introduced. The range of linearity was found to commence approximately at pentane.

Therefore, the theoretical evidence supports the experimental observations of the non-linearity of the log plot at low carbon numbers. The reason that this non-linearity has not been uniformly recognised is a result of several factors. First the non-linearity is minimised for homologues with large carbon numbers, at high temperatures and on non-polar stationary phases. Secondly, it can be obscured (if not large) by the mathematical treatment of the data. This problem has been discussed by both Rohrschneider[74] and Ettre[31].

The data in Table 3 show the problems that can arise. The retention data in column 1 were generated assuming a dead time of 100 sec and adjusted retention times conforming to eqn. 79:

$$\ln t'_{Rz} = 0.5z + 0.3 \tag{81}$$

Column 2 shows the inaccuracies introduced purely by rounding off the data to the nearest 0.1 sec. It should be noted that already an error of up to 1.3 units in Kovats retention indices has resulted, even though the coefficient of determination, R^2, is 0.99998. Column 3 adds a non-linearity for C_1–C_3; their effective carbon numbers are shown in brackets. The unexpected finding is that even though the curvature is significant, the accuracy of fit as shown by both R^2 and the individual index values is almost identical to that in column 2. Column 4 introduces an even larger non-

TABLE 3

HYPOTHETICAL COMPARISON OF THE EFFECTS OF A NON-LINEAR BEHAVIOUR OF THE LOWER ALKANES WITH SMALL RANDOM DEVIATIONS

z	t_{R_1} *(correct)*	t_{R_2} *(rounded off)*	t_{R_3} *(non-linear)*	t_{R_4} *(non-linear)*	t_{R_5} *(t_{R_2} + deviat.)*	t_{R_6} *(t_{R_4} + deviat.)*
1	102.23	102.2	101.9 [0.7]*	101.7 [0.5]*	102.1	101.6
2	103.67	103.7	103.4 [1.8]*	103.2 [1.7]*	103.8	103.3
3	106.05	106.1	105.9 [2.9]*	105.5 [2.8]*	106.2	105.6
4	109.97	110.0	110.0	109.5 [3.9]*	110.0	109.5
5	116.44	116.4	116.4	116.4	116.3	116.3
6	127.11	127.1	127.1	127.1	127.0	127.0
7	144.70	144.7	144.7	144.7	144.7	144.7
t_M**	100.0	99.9	99.5	99.5	99.6	99.2
b**	0.500	0.495	0.488	0.502	0.480	0.486
c**	0.297	0.336	0.396	0.306	0.435	0.408
R^2**	1.00000	0.99998	0.99998	0.99992	0.99967	0.99973
Kovats retention indices						
1	100.0	99.3	100.3	99.7	96.8	97.3
2	200.0	201.3	199.1	201.9	206.4	207.0
3	300.0	300.6	300.2	297.4	301.5	298.3
4	400.0	399.4	401.5	398.7	396.8	396.0
5	500.0	498.7	498.9	503.0	495.9	500.1
6	600.0	599.9	599.4	600.6	599.3	600.0
7	700.0	700.8	700.5	698.8	703.4	701.3

* Effective carbon number.

** Calculated by the method of Guardino *et al.*[24].

linearity into the C_1–C_4 data. This time the fit is not as good with a maximum error in the Kovats indices of 3. However, R^2 is still a respectable 0.99992. An important feature of this analysis is that it is impossible to assign the less than perfect fit to its true cause, the non-linearity. The variation in Kovats indices appears to be completely random. This problem is emphasised by the data in columns 5 and 6. These columns have had an identical deviation of ± 0.1 sec added both to the "correct" data and the most non-linear data. The result is that the data from both columns give fits of almost equal accuracy, showing that the non-linearity has been "eliminated" by mathematical manipulation.

Therefore this analysis is in agreement with those authors[28,30,31,74] who have argued both that random deviations can introduce significant errors into the calculation of mathematical dead time and that the actual treatment of the data can obscure a non-linearity. In particular, Ettre[31] was critical of the use of the full range of homologues for this very reason, suggesting that only the linear part of the curve should be used. However, as pointed out in our recent paper[64], this is not really a problem when calculating Kovats indices where the real requirement is accurate and reproducible index values. The exclusion of some of the data from the analysis is likely to lead to significant errors in those regions which have been excluded.

On the other hand, the problem of random deviations is a real one and thus a higher accuracy than has generally be recognised is required to obtain reproducible results. Retention times should be measured to at least the nearest 0.1 sec unless very long times are involved, in which case a lower accuracy may be acceptable.

5. COMPARISON OF METHODS

Several papers[18,27,28,30,32] have previously reviewed the calculation of dead time in general, while a larger number of papers have compared two or three specific methods (see for example refs. 31, 36–38). In this section we will summarise the conclusions that have been drawn in previous sections of this paper as well as presenting results from our recent paper[64] in which we carried out an extensive comparison of several statistical and iterative methods.

The use of theoretical and graphical methods as well as methods involving indirect measurement was discussed in section 3. As these are considered complex, time-consuming and of very limited usefulness, they will not be discussed further. Direct-measurement techniques were extensively covered in section 3.3. In general such methods are either experimentally difficult or likely to introduce significant errors into dead-time and retention-index calculations. The use of methane in particular was shown to lead to large errors in many cases and should be discouraged. The only method which was shown to lead to accurate results under a wide variety of conditions was the use of neon or possibly helium together with the appropriate treatment of the data. In fact, the use of multiple analyses using neon is, in our opinion, the most reliable and accurate method of determining system dead time and is the method of choice when determining absolute data such as Henry's law values or other thermodynamic data. However, due to the difficulty of using this method (gas chromatography–mass spectrometry has been recommended for the determination), a mathematical determination is more useful for the vast majority of analyses, where the final data are comparative, such as in the calculation of Kovats indices.

Of the various mathematical methods, several authors have been critical of the use of the classical methods, especially when compared to the use of methane. In particular, the results of Guberska[36,37], Sharples and Vernon[38] as well as our own results reported in an earlier paper[30] show that large errors can result from the use of such methods. This problem is especially acute when inaccurate timing methods are used, as reported by Sharples and Vernon. We therefore believe that the inaccuracies inherent in these methods, together with the availability of inexpensive programmable calculators and notebook-size computers, render them of limited use other than to provide an initial estimate for a more accurate iterative method.

Of the statistical and iterative methods, the method of Ševčík and Löwentap[29] was discussed in section 3.8a. Because of the problems discussed in that section, including Ettre's[31] comments on a similar method, we believe that the method offers no advantage over other methods. However, it should be noted that several workers in our laboratories[42–47] have reported that the method is a sensitive indicator of linearity. This also means that it is a good indicator of the degree of scatter of the data and therefore holds merit as an initial test of the accuracy of a new set of retention data.

This leaves the following statistical and iterative methods.

(1) The method of Grobler and Balizs[61] as extended by the technique of Van Tulder *et al.*[63]

(2) The method van Guardino *et al.*[24]

(3) The "exact calculator method" of Garcia Dominguez *et al.*[48]

(4) The emthod of Ambrus[76], also extended by the technique of Van Tulder *et al.*[63]

(5) The iterative method mentioned by Tóth and Zala[62]

(6) The flexible Simplex method[67]

(7) The method of Heeg *et al.*[70]

These seven methods, together with two modified approaches allowing the optimisation of t_M while simultaneous fitting a cubic or fifth-degree polynomial to the data, have been compared in our recent paper[64]. That paper showed that the linear methods (methods 1–6 above) gave similar estimates of the mathematical dead time in most cases. In fact, the methods of Tóth and Zala[62], Guardino *et al.*[24] and flexible Simplex[67] gave identical results in all cases.

The paper also showed that for the calculation of Kovats indices the method of Guardino *et al.*[24] was not only the fastest, but the most accurate. This conclusion, while differing from those expressed in earlier papers, results from the recently recognised instability inherent in methods such as those of Ambrus[76] or Grobler and Balizs[61]. These methods, while providing an acceptable estimate of the equation parameters in the majority of cases, can lead to large errors in other cases. This means that all homologue combinations must be checked to reduce the likelihood of introducing significant errors into the calculation. However, this procedure increases both the program's complexity and its running time.

With regard to the polynomial methods, the paper concluded that in general they offer only a small improvement over the linear methods. Therefore, since these methods involve greater complexity, including the need to carry a large number of significant figures throughout the calculations, and can be difficult to interpret, they

should only be used when extreme accuracy is required or where highly variable data are involved. In this case, an improvement in the experimental technique is more likely to increase accuracy than the use of a more complex method.

In addition these methods do not give an estimate of the mathematical dead time. The parameter (t_M), which in the linear methods is accepted as an estimate of the system dead time, shows no relation to the dead time in polynomial methods and thus should be treated in the same manner as the other equation parameters, with the single exception that its value must be less than the retention time of the homologue with the lowest retention time.

Finally the paper investigated the problems associated with extrapolation, particularly with the polynomial methods. It concluded that this technique should be avoided where good accuracy is required.

6. CONCLUSIONS AND RECOMMENDATIONS

In choosing a method to analyse the raw data from a chromatographic analysis, several factors should be taken into account. These include the accuracy of the original data, the required accuracy of the final result and the purpose to which the data are to be put.

When the resultant data are to be used to calculate some property of the column or stationary phase such as Henry's law constants, the system dead time should, where possible, be determined experimentally using an inert gas such as neon. On the other hand, where the data are to be used in a comparative method, such as the calculation of Kovats indices, a mathematical method is more appropriate.

With the availability of inexpensive programmable calculators, the method of choice is an iterative one such as that of Guardino *et al.* This method, while more time-consuming than the classical methods, overcomes the problems with accuracy inherent not only in the classical methods, but to a lesser degree in the statistical methods.

For maximum accuracy a polynomial method can be employed. However, not only must double precision be used throughout these methods, but all results should be critically evaluated before being accepted. In addition such methods cannot be used for the estimation of the system dead time.

The reproducibility of Kovats indices is dependent not only on the mathematical method used to manipulate the raw data, but also on the original data. The combination of a well designed experimental technique, an accurate timing mechanism (nearest tenth of a second) and an appropriate mathematical method will give an interlaboratory reproducibility of one unit for larger values of Kovats indices and two units for indices below approximately 400 (ref. 64).

7. SUMMARY

To increase the usefulness of gas chromatography, a method of data presentation is required that removes interlaboratory variations. All such methods require the determination of the system dead time, with the most useful being the retention index system of Kovats. This paper reviews the many theoretical, experimental and mathematical methods of estimating system dead time and evaluates their usefulness,

not only in the estimation of dead time, but also in the subsequent calculation of Kovats indices. Recommendations are made as to the appropriate method to be used, and the expected accuracy in retention index values is estimated.

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CHREV 194

ON-COLUMN INJECTION OF LARGE SAMPLE VOLUMES USING THE RETENTION GAP TECHNIQUE IN CAPILLARY GAS CHROMATOGRAPHY

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CONTENTS

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1. INTRODUCTION

The use of long, uncoated column inlets as retention gaps[1] allows the on-column injection of much larger sample volumes than are routinely used today. The injection of volumes up to 100 μl is easy. The injection of several times larger sample volumes is also possible, but the technique becomes cumbersome and the gain in sensitivity is no longer dramatic. The injection of large sample volumes has important advantages, as discussed below.

1.1. Advantages of injecting large sample volumes

1.1.1. Increased sensitivity for analysis of volatile solutes

The injection of large sample volumes is a particularly valuable method for increasing sensitivity if the sample cannot be reconcentrated by the classical methods of solvent evaporation. If present in trace concentrations, solutes of surprisingly high boiling points are co-evaporated with the solvent. On evaporating, *e.g.*, *n*-pentane, the far less volatile naphthalene or alkanes such as *n*-hexadecane are lost to a considerable extent, even if the *n*-pentane is not evaporated to dryness.

Solvent evaporation in the column inlet prevents losses of solute material because all the injected material arrives at the detector. However, there are analogous phenomena of co-evaporation: solutes co-evaporating with the solvent form broad, distorted bands (partial solvent trapping[2]) that do not allow reasonable quantitation. The analogue of solute loss in solvent evaporation before injection is in fact peak distortion. However, co-evaporation in the column inlet occurs only for a far narrower range of solute volatilities; for instance, *n*-octane is completely retained in a layer of *n*-hexane until the latter is fully evaporated, whereas with conventional methods of solvent evaporation *n*-octane would be nearly completely lost. Further, if co-evaporation occurs (*e.g.*, for *n*-octane in *n*-heptane), the solute material can often be reconcentrated to a sharp band by the phase soaking effect[3]. Hence, mastering the solvent effects, *n*-octane can be determined quantitatively in *n*-heptane, although the two have boiling points that differ by only 25°C. In other words, solvent evaporation in the column inlet is far more selective than the classical methods of evaporation, as pointed out by Roeraade and Blomberg[4].

1.1.2. Handling of the complete sample

In trace analysis, it is often desirable to inject the whole sample in a quantitative manner. However, this is virtually impossible if conventional sample volumes are injected because the sample cannot be picked up by, *e.g.*, 2 μl of solvent. Therefore, it is common practice to work up an excessively large sample volume. After removal of the solvent used for the sample preparation (extraction, column liquid chromatography, etc.), the sample is rinsed from the walls of the round flask using 0.1–1 ml of solvent and transferred into a smaller vial. There the solvent is evaporated

again and the sample material washed from the walls, this time using perhaps only 20 μl of solvent. A 2-μl aliquot of this is injected; hence 90% of the sample is wasted. If the whole sample liquid can be handled, either ten times less sample material is needed to obtain the same sensitivity or a ten-fold increased sensitivity is achieved. The volume of solvent required for quantitatively picking up a sample from a flask is of the order of 30–100 μl, *i.e.*, in a range that can easily be handled by on-column injection into a retention gap of suitable length.

1.1.3. Convenience for sample preparation

The possibility of injecting large sample volumes often renders sample preparation more convenient. It may simply become unnecessary to reconcentrate the sample by solvent evaporation before the analysis (a step that frequently also introduces artefacts). In other instances extraction may be carried out with more solvent, improving the extraction efficiency, but primarily facilitating the separation of the extract from the extracted liquid or solid, *e.g.*, if the separation is hindered by the

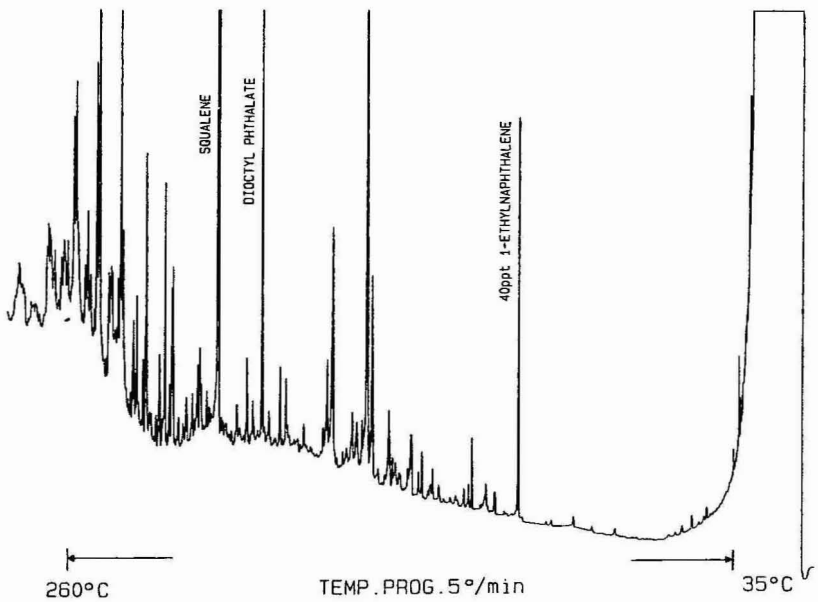

Fig. 1. Analysis of higher boiling trace components in drinking water (Zürich tap water). A 1-l volume of water was extracted with 0.6 ml of pentane in a measuring flask[5]. A third of the recovered extract, *i.e.*, 50 out of 150 μl, was injected on to a 15 m × 0.31 mm I.D. glass retention gap deactivated by trimethylsilylation (well wetted by pentane). This pre-column was attached to the separation column by fusing it to an intermediate piece of fused silica with an outer diameter (without polyimide coating) fitting into the butts of the retention gap and the separation column (see section 3.5). The sample was separated on a 10 m × 0.32 mm I.D. glass capillary column coated with SE-54 of 0.3-μm film thickness. Carrier gas inlet pressure, 0.6 atm (H_2); injection at 25°C, followed by solvent evaporation at 35°C. Owing to the high carrier gas flow-rate (7 ml/min), the solvent peak has a width of only 4 min. A 40 ppt (40 ng) amount of 1-ethylnaphthalene was added to the water as an internal standard before the extraction. Injection of a 50-μl volume increased the sensitivity by a factor of 20 compared with earlier analyses carried out by the same method. From the point of view of solute volatility, reconcentration by solvent evaporation would have been possible. However, it is technically difficult to evaporate the solvent down to about 6 μl, of which 2 μl were injected.

formation of foam or emulsions. Another application in which the injection of large sample volumes is attractive is extraction of adsorbents or filters, particularly if used for trapping volatile components.

Fig. 1 shows an application involving a 50-μl injection of an aqueous extract.

1.2. Methods described in the literature

Vogt *et al.*[6] injected up to 250-μl volumes of liquid samples into packed inserts of a cold injector. The solvent was evaporated and vented through the split exit, then the injector was rapidly heated for elution of the solute material into the column. This technique, later termed "programmed temperature vaporizing" (PTV) injection[7] or "cold injection"[8], used in the "solvent split mode", provides a simple method of introducing large sample volumes, provided that the injector insert is sufficiently large to keep the sample. However, as in other solid injection techniques (*e.g.*, moving needle injection), volatile solutes are at least partly lost owing to co-evaporation with the vented solvent, whereby losses are known to affect solutes up to *n*-octadecane or the C_{12} methyl ester. Hence, the range of applications for such injection techniques is restricted to samples where reconcentration by conventional solvent evaporation before gas chromatographic (GC) analysis is possible also. Kirschmer and Oehme[9] described the use of a thermal desorption unit for injection of liquid samples of volume *ca.* 100 μl. The concept resembles that of PTV injection, whereby an intermediate cold trap was used for reconcentration of the solute bands.

Zlatkis and co-workers described the on-column injection of large volumes involving a trapping step and reversal of the column[10] or using a two-column system with a cold trap between them[11]. More elegantly, Fogelqvist and Larsson[12] injected up to 250 μl of a pentane extract of sea water for the analysis of C_1 and C_2 halocarbons, using conventional on-column injection into a fully coated (long) column. They were successful because they did not increase the column temperature for the elution of the solutes (no band broadening in space), and because they obtained a highly efficient phase soaking effect for reconcentration of solute bands distorted by partial solvent trapping.

Miller and Barringer[13] introduced extremely large sample volumes very slowly into a column kept at a temperature well above the boiling point of the solvent. Owing to the violent solvent evaporation in the hot column inlet, most of the sample liquid was pushed backwards out of the column inlet into the injector. The major part of the solvent evaporated there and the resulting vapours were blown through the injector head. However, the method does not seem highly reliable.

Etzweiler[14] described an injection device resembling a a very short retention gap. It contains a bulb volume of 80–200 μl into which the sample liquid flows from the bottom. The liquid cannot leave this bulb at the top, accumulates there and the solvent evaporates on bubbling carrier gas through it. Up to 100-μl volumes could be injected using this device.

1.3. Advantages of the method involving retention gaps

The method described below, involving the use of uncoated column inlets of length 10–20 m as retention gaps, has a number of advantages:

(a) It is simple in terms of instrumentation. Once the pre-column is attached to a suitable separation column, injections are carried out as usual. No special equipment other than an ordinary on-column injector is needed.

(b) The method is applicable to all types of samples. Volatile solutes are not lost during solvent evaporation as in solvent split techniques, nor are solutes eluted at elevated temperatures broadened as a result of band broadening in space.

(c) The method provides results with the same accuracy and precision as on-column injection.

(d) The background of the technique has been thoroughly investigated. Hence, its limitations and sources of problems are relatively well known and can be explained in a straightforward way.

This review discusses the important aspects of the technique and provides further guidelines on the preparation of a system suitable for the injection of large sample volumes and on the chromatographic conditions required. It places considerable emphasis on possible problems, not to discourage but to help the user to recognize symptoms if problems really occur.

2. CONCEPT OF THE METHOD

First, it is essential to realize that since the introduction of split injection, most newly developed injection techniques for capillary GC violate the basic principles of chromatography concerning the initial band widths. The injection of large sample volumes violates them considerably. The initial band widths, measured in terms of column length (band broadening in space) and of time (duration of solute introduction into the column), must not contribute significantly to the width of the eluted band. Further, the sample should not influence its own chromatography (overloading effect). However, the situation on injection of large sample volumes is as follows:

(a) The flow of sample liquid in the column inlet spreads the solute material easily over half of the column length.

(b) The volume of vapour resulting from sample evaporation may exceed 50 ml, corresponding to several times the internal column volume. Hence, if co-evaporation of solute with the sample solvent occurs, extremely broad initial solute bands (easily exceeding 10 min) are formed.

(c) The amount of sample liquid introduced exceeds that of the stationary phase in the whole column by factors of up to 100. Hence, in the presence of condensed solvent, the sample liquid is by far the predominant stationary phase.

Nevertheless, there is no reason to be too concerned, as the resulting problems are known and can be mastered with mostly little effort. The subjects involved are the band broadening in space and the solvent effects, the essence of which is summarized below.

2.1. Solvent effects

The introduction of the sample into the column is relatively rapid. Hence, the injection time does not affect the initial band widths (with the exception of a few rather special cases). However, the problematic step just follows the injection, *viz.*, evaporation of the sample in the column inlet. The sample liquid forms a layer on

the column wall several metres long. The solvent evaporates from the rear to the front of this layer[15], which usually requires several minutes. Co-evaporation of solute material with the solvent is undesirable, as it produces initial band widths that are mostly equal to the solvent evaporation time. The bands have a "stool" or a "chair" shape, with the "back" of the "chair" eluted last. This phenomenon of "partial solvent trapping" was discussed in detail in previous papers[16].

The problem of peak distortion due to partial solvent trapping may be solved by the selection of a better solvent that retains the solute material more strongly in the sample layer and releases it only at the end of the solvent evaporation. This release of the "fully trapped" solute material is very rapid and ensures a sharp initial band (Fig. 2). Achievement of full trapping is surprisingly easy if the solutes have a clearly higher boiling point than the solvent, but creates problems if the boiling point of the solute is less than about 30°C above that of the solvent or if there is a polarity mismatch between the solvent and solutes[17]. Full trapping requires a volatile solvent with a polarity that provides a high retention power for the solute (which does not necessarily mean same polarity as that of the solvent).

If full solvent trapping cannot be achieved, there remains the possibility of reconcentrating solute bands distorted by partial solvent trapping by use of the phase soaking effect[18]. This second solvent effect is created by swelling the stationary phase film with solvent. The swollen stationary phase has a several-fold increased film thickness and an accordingly increased retention power. During a dynamic process, solute

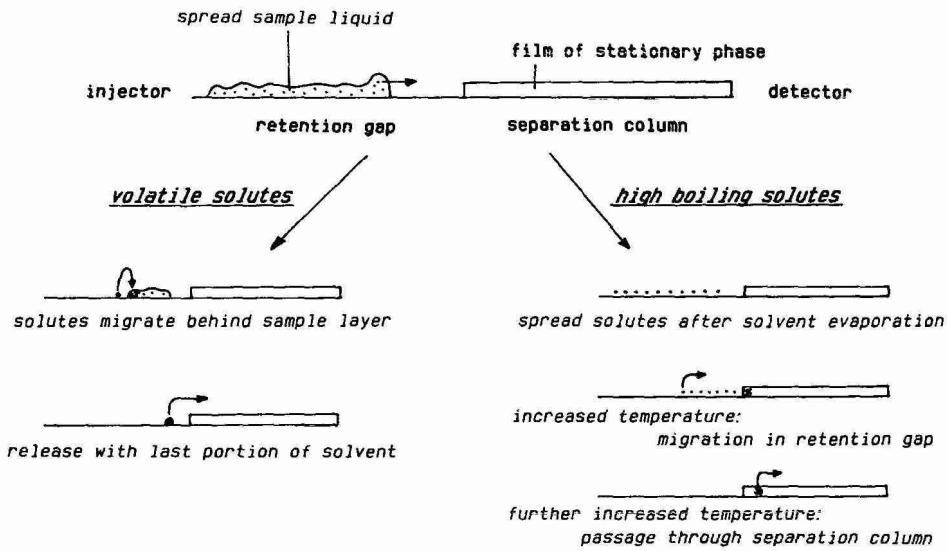

Fig. 2. Effects involved in the reduction of the excessively long (broad) initial bands: injection forms a sample film in the uncoated column inlet (retention gap, one wall of which is shown schematically). Volatile and high-boiling sample constituents behave fundamentally differently: the volatile components are reconcentrated by the solvent effects, of which the solvent trapping is shown on the left. The solvent evaporates from the rear to the front of the sample layer. The fully trapped solutes do not migrate noticeably within the solvent-coated inlet section, but they follow the rear of the layer and are reconcentrated at the point where the last portion of solvent evaporates. High-boiling solutes remain spread throughout the flooded zone. However, as the temperature of the column is increased, they pass the retention gap much earlier than the separation column and are reconcentrated at the beginning of the separation column.

band widths of several minutes were observed to be reconcentrated to a width less than 0.5 sec. The most effective reconcentration by phase soaking is achieved by using solvents that strongly retard the solute migration in the soaked stationary phase, either by strong swelling of the stationary phase film or by a shift of the polarity of the solvent–stationary phase mixture in the direction of producing a higher solute retention[18].

2.2. Band broadening in space

Sample liquid introduced by on-column injection forms a plug that closes the bore of the column. The plug is pushed into the column by the carrier gas, leaving a layer of liquid behind on the column wall[19]. It continues to move until all the sample liquid is spread as a film. This sample film is mostly too thick to be mechanically stable, and the liquid continues to flow along the wall further into the column, although with decreasing speed, until the sample solvent is evaporated (proceeding from the rear to the front of the sample layer). The volatile sample constituents follow the rear of the evaporating sample layer and are reconcentrated at the point where the last portion of solvent evaporates (Fig. 2). Solutes that do not migrate noticeably at the injection temperature, however, remain spread throughout the flooded zone. The phenomenon is called "band broadening in space" because all except volatile solutes have the same initial band width in terms of column length or column volume.

The length of the flooded zone in a coated column, and therefore of the initial solute bands, is of the order of 25 cm per microlitre sample volume injected. The maximum tolerable initial band length is between 5 and 30 cm, depending on the column length and the separation efficiency obtained. Hence the initial bands created by injection of large sample volumes must be shortened by factors up to 100.

If small sample volumes are injected, there are two possibilities of avoiding peak broadening by band broadening in space: the use of retention gaps or injection at column temperatures above the boiling point of the solvent[20]. However, for injection of sample volumes exceeding about 5 μl, there only remains the retention gap technique. This method involves the use of uncoated column inlets or pre-columns in which the sample is allowed to spread. The far lower retention power within this inlet accelerates solute migration, resulting in reconcentration of the initial bands at the beginning of the coated column part. The mechanism involved in the reconcentration effect is shown schematically in Fig. 2 as being chromatography in two steps. The solute material passes through the inlet of low retention power at a low temperature. It is stopped at the beginning of the stationary phase film, where the solute material is recombined to a sharp band. Chromatography of the solute continues only when the column temperature is further increased to give the solute the required volatility over the stationary phase. This simplified model requires some refinement, as indicated below.

The flooded zone must be restricted to the uncoated column inlet. If the sample liquid flows into the separation column, the extractable parts of the stationary phase coating (present event if the film is immobilized) are carried towards the front of the flooded zone, causing phase stripping. However, more important, the solute material spread in the separation column is not reconcentrated. Fig. 3 shows a chromatogram

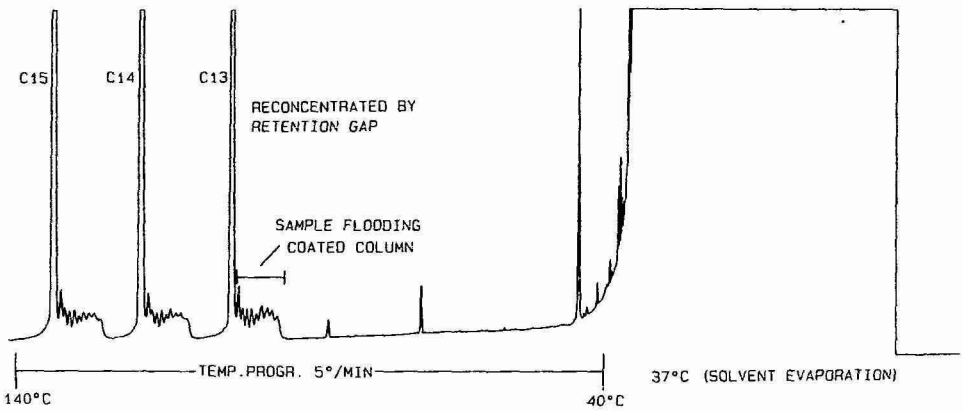

Fig. 3. Effect of using retention gaps of insufficient length: sample liquid penetrates into the separation column and spreads the dissolved solute material there. This material elutes earlier than the bulk of the material deposited in the retention gap because it starts chromatography from an advanced position. The reason why the early eluted material forms a band with small maxima with a constant distance between each other is discussed in the text. The finally eluted large peaks represent the solute material originally spread in the retention gap, then reconcentrated at the beginning of the separation column. A 15 m × 0.30 mm I.D. glass capillary column coated with SE-54 of 0.3-μm film thickness was coupled by means of a butt connector (Carlo Erba) to a wide-bore, 10 m × 0.50 mm I.D. fused-silica retention gap with a trimethylsilylated internal surface; inlet pressure (hydrogen), 0.3 atm. The coupling is not perfect as the baseline after the solvent peak does not return to the level before injection. A rapid increase in the oven temperature shortly after the solvent peak would have resulted in a "hump" of the type shown in Fig. 5.

obtained by injection of 120 μl of a pentane solution on to a 10 m × 0.50 mm I.D. retention gap. This wide-bore retention gap was too short to keep the large volume of sample liquid from the separation column. The solute material remaining in the retention gap was reconcentrated at the entrance of the separation column and eluted as sharp peaks. The earlier eluted pre-peaks are due to solute material deposited ahead of the material forming the large peaks, *i.e.*, deposited in the separation column by the flowing sample liquid. The maxima of the pre-peaks represent material deposited in those parts of the glass capillary coils where the sample liquid tended to flow backwards against the stream of carrier gas (at those points more solvent is evaporated per unit column length, which also means deposition of more solute material). From the fact that there are nine maxima, it may be concluded that the sample material penetrated 3.5 m into the separation column or that the sample volume injected was too large by about 20 μl. In Fig. 3 the pre-peaks are obvious. However, in a complex mixture and for smaller peaks they would probably be overlooked. Hence care is required in order to detect such deficiencies.

3. TECHNICAL ASPECTS TO BE CONSIDERED

3.1. Wettability of the retention gap surface

The formation of a sample film in the retention gap is a prerequisite, which requires that the retention gap surface be wettable by the sample liquid. Samples that do not wet the internal wall of the retention gap unregularly leave droplets behind

the moving sample plug and easily flow 10–20 times further into the column than liquids that form a film (4–5 m per microlitre of liquid).

The most commonly applied methods of deactivating the support surface for non-polar capillary columns result in trimethylsilylation of the silanol groups. Such surfaces are wetted only by alkanes or ethers as solvent, but not, *e.g.*, by dichloromethane, benzene or acetone. Phenyldimethylsilylated surfaces, however, are wetted by virtually all commonly used solvents, excluding water. Methanol is spread over an elongated flooded zone, indicating critical wettability. The even more polar diphenylmethylsilylated surfaces are wetted by the same range of solvents. As their inertness is usually inferior to that of phenyldimethylsilylated surfaces, this type of deactivating retention gap is not attractive for our purpose[21].

Methanol and water are the most difficult solvents regarding wettability of retention gap surfaces. Silylated surfaces are not wetted by water or aqueous alcohols. Carbowax-deactivated capillary tubes would be expected to exhibit better wettability. However, methanol and water wash out the deactivating layer of Carbowax. Finally, it must be noted that the problem is of a fundamental nature: surfaces with a sufficiently high activity to be wetted by polar solvents tend to be active also towards the solutes in the sense of adsorptivity.

3.2. Required length of the retention gap

The uncoated column inlet must be long enough to prevent flow of sample liquid into the separation column. On the other hand, excessively long retention gaps slow the analysis and possibly contribute to peak broadening. Therefore, some guidelines are required to help to estimate the length of the flooded zone.

The length of the flooded zone is strictly proportional to the volume of injected sample liquid[22]. This allows the length of the flooded zone to be expressed as length per unit sample volume injected (cm/μl).

3.2.1. Factors affecting the length of the flooded zone

The length of the flooded zone per unit sample volume depends primarily on three factors. First, it may depend on the speed of the sample plug in the column inlet. As is well known from the dynamic coating of capillary columns with stationary phases, a slowly moving plug leaves a thin layer behind it. Accordingly it moves further than a rapidly moving plug until all its liquid is spread on the column wall, *i.e.*, the flooded zone is elongated[21]. However, above a low, critical plug velocity the thickness of the final sample layer (and hence also of its length) becomes independent of the plug velocity because the layer approaches the maximum of what is mechanically stable. In order to avoid elongated flooded zones, plug velocities below 10–15 cm/sec should be avoided.

Unfortunately, there is no straightforward relationship between the carrier gas velocity and the velocity of the sample plug. The two are not equal because of the resistance to the movement of the sample liquid and the resulting pressure drop over the plug. This pressure drop over the sample plug depends on the length of the plug, the number of included gas bubles, the nature of the sample liquid and the plug velocity,but easily reaches 0.2–0.3 atm. In order to avoid a considerable reduction in the flow-rate during sample introduction or even complete stoppage of the sample plug, elevated column head pressures should be used (see section 3.9.2).

TABLE 1

EXPECTED LENGTHS OF THE FLOODED ZONE AT VARIOUS INJECTION TEMPERATURES RELATED TO THE BOILING POINT OF THE SOLVENT

0.3 mm I.D. retention gaps, wetted by the sample liquid; conditions resulting in a plug velocity of at least 10 cm/sec are used.

Injection temperature	*Length of flooded zone (cm/μl)*
> 20°C below b.p.	30
5–20°C below b.p.	25
Up to 10°C above b.p.	20

Second, the length of the flooded zone depends on the inner diameter of the retention gap or rather the internal surface area per unit length of the uncoated inlet. In a wide-bore (*e.g.*, 0.5 mm I.D.) retention gap the length of the flooded zone is markedly reduced.

Third, and most important, the length of the flooded zone is influenced by the column temperature in relation to the boiling point of the solvent. At relatively high column temperatures the flooded zone is shortened owing to the accelerated solvent evaporation and the accordingly reduced time available for the flow of liquid.

3.2.2. Guidelines

Table 1 gives some values recommended as guidelines for estimating the length of the flooded zone as a function of the column temperature relative to the boiling point of the solvent. The values actually measured are usually lower, and hence the guidelines are on the safe side. Accordingly, a 20 m × 0.30 mm I.D. retention gap allows the injection of 100-μl sample volumes if solvent evaporation occurs at or slightly above the boiling point of the solvent, of 80 μl at up to 20°C below the boiling point of the solvent and of 70 μl at even lower column temperatures. However, volumes of more than 150 μl were repeatedly injected into such retention gaps using an inlet pressure of about 1.5 atm, flow-rates of around 7 ml/min and solvent evaporation temperatures 20°C above the boiling point of the solvent.

3.2.3 Visual control?

It is helpful to have direct visual control over the length of the flooded zone rather than being blindly dependent on data from the literature. Visual control, however, is possible only if glass capillary retention gaps are used. In order to permit observations at elevated oven temperatures, the oven door is left open and the oven is closed by a pane of glass. Window glass panes can be used up to around 80–100°C. At higher oven temperatures, panes of borosilicate glass are required.

3.3. Retention power in the retention gap

The reduction of the initial band length by use of retention gaps is the result of the different retention powers in the retention gap and the separation column, although the chromatographic conditions applied are also of importance (see below).

The lower the retention power in the retention gap, the more efficiently are the bands reconcentrated during the transition from the retention gap into the separation column.

The retention power in an uncoated capillary tube is obviously not zero. It depends on the pretreatment (deactivation) of the internal wall and the polarity of the solutes of interest. Such retention powers have been measured for various types of retention gaps[23], expressing retention power in terms of "apparent film thickness of an apolar stationary phase", *i.e.*, assuming that the retention power is due to a film of a non-polar stationary phase. This was preferred to the more commonly used phase ratio, β, because the latter also depends on the bore of the capillary tube, whereas it was required to describe a surface property only. Further, "apparent film thickness" allows an easy comparison with the retention power in the separation column, as the latter is usually also expressed in terms of film thickness. If polar stationary phases are involved, the increase or decrease in the retention power compared with non-polar stationary phases must be estimated, which is not too difficult if it is considered that only rough estimations are required for combining retention gaps with suitable separation columns.

Table 2 lists some approximate retention powers typically found for retention gaps deactivated by silylation with hexamethyldisilazane (HMDS) or diphenyltetramethyldisilazane (DPTMDS) or by treatment with Carbowax. Deactivation by Carbowax is simple and rapid, but the resulting retention powers are very high and the reconcentrations obtained are sufficient only if the flooded zones are relatively short. Silylated surfaces exhibit lower retention powers than the untreated surfaces whereby the trimethylsilylated (HMDS-treated) surfaces have 2–4 times lower retention powers than the phenyldimethylsilylated (DPTMDS-treated) surfaces. On the other hand, the phenyl groups may be needed in order to obtain a satisfactory wettability of the surface (see above). The values shown in Table 2 represent averages also between glass and fused-silica capillaries; the retention powers in fused-silica retention gaps tend to be clearly (up to a factor of 2) below those of glass retention gaps.

3.4. Film thickness in the separation column

The retention power in the retention gap is more or less a given quantity. If very long flooded zones (resulting from the injection of very large sample volumes) require high reconcentration factors, it may become necessary to use separation col-

TABLE 2

TYPICAL RETENTION POWERS (APPARENT FILM THICKNESSES OF A NON-POLAR STATIONARY PHASE) IN DIFFERENTLY PRE-TREATED RETENTION GAPS, MEASURED FOR DIFFERENT CLASSES OF SOLUTES

Pre-treatment	*Apparent film thickness (nm)*			
	Alkanes	*Aromatics*	*Esters*	*Alcohols*
Silylated with HMDS	0.5	0.5	0.5	1.5
Silylated with DPTMDS	2	1	2	3
Treated with Carbowax	4	8	7	27

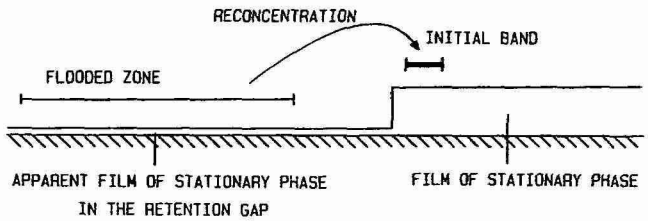

Fig. 4. Reconcentration of solute bands spread within the flooded zone occurring during the transition of the material from the uncoated inlet of low retention power (the apparent film thickness of stationary phase is a calculated unit for expressing the retention power in the inlet) to the more strongly retaining separation column. The reconcentration efficiency is related to the difference in retention powers in the two column parts.

umns of increased retention power (*i.e.*, of elevated film thickness) in order to achieve a sufficient difference in the retention powers in the two column parts. This compels us to consider more closely the required reconcentration factor and the consequences for the selection of the separation column.

3.4.1. Maximum tolerable residual initial band length

The reconcentration obtained by the transition from an inlet of low retention power into a separation column of high retention power (Fig. 4) must be sufficient to result in a residual initial band length in the separation column that does not broaden the solute peaks excessively. The maximum tolerable residual initial band length is estimated on the basis of the terminal band length[24], the length of the solute band when passing from the separation column into the detector, under conditions ensuring ideally sharp initial bands. For most applications, the initial band length may correspond to 30–60% of the terminal band length[25].

Usually, initial band lengths of around 20 cm can be accepted in the separation column. Band lengths of only 10 cm can be accepted if the ultimate of the separation power must be exploited or if the separation column is very short. On the other hand, residual initial band lengths between 50 cm and 1 m can be tolerated if the separation efficiency is not critical, if long separation columns are used or if the separation efficiency of the column is poor (*e.g.*, if wide-bore separation columns are involved).

3.4.2. Required reconcentration factor

Division of the length of the flooded zone by the tolerated residual initial band length yields the required reconcentration factor. For instance, if injections of 200-μl volumes create flooded zones of 40 m length and if the acceptable band length in the inlet of the separation column is 20 cm, the required reconcentration factor is 200.

3.4.3. Achievement of reconcentration

The mechanism of reconcentration during the passage of the solute material from the retention gap into the separation column is discussed in detail in ref. 25. As an approximation, the reconcentration may be considered as a phase-ratio focusing (a term suggested by Takeoka and Jennings[26]), assuming a reconcentration that is equal to the difference in retention powers in the two column parts[1]. This model

neglects the dead time (gas hold-up time) of the flooded zone, *i.e.*, the fact that the rear molecules need a considerable amount of time to cover the distance to the front, even if the retention gap exerts no retention power at all. Owing to this neglect, the model of phase-ratio focusing is optimistic; the real reconcentration is less effective, although the difference becomes small if the chromatographic conditions allow the solute material to pass the retention gap far below the elution temperature (see section 3.12).

Applied to the example above, the required reconcentration by a factor of 200 calls for a separation column with a retention power exceeding that of the retention gap by a factor of 200. If we further assume that the sample is dissolved in a solvent of intermediate polarity, compelling us to use a phenyldimethylsilylated retention gap with a retention power for polar solutes corresponding to an apparent film thickness of 3 nm, a non-polar separation column must be coated with a 0.6-μm thick film (possibly only 0.2 μm if a polar separation column is used). On the other hand, a sample of hydrocarbons dissolved in an aliphatic solvent could be introduced into a trimethylsilylated retention gap with a six-fold lower retention power. In this instance, the minimal film thickness in the separation column required for a sufficient reconcentration would be only 0.1 μm.

The example discussed above indicates that the retention power (film thickness) in the separation column requires special attention only if long flooded zones are involved. Solutes spread in flooded zones of length 10 m (resulting from injections ranging between 30 and 50 μl) are sufficiently reconcentrated under virtually all conditions.

The use of pre-columns with inner diameters exceeding that of the separation column presupposes enhanced reconcentration factors due to the elongation of the vapour plug when entering the narrower bore separation column. The required increase in reconcentration is equal to the ratio of the inner diameters of the two column parts[27]. However, the flooded zone per unit volume of sample is shortened when using wide-bore inlets, with the effect that there is no increase in the required reconcentration efficiency per unit sample volume injected.

A mathematical treatment[27] allows calculation of the minimal film thickness in the separation column for a given tolerance in peak broadening as a function of the length of the flooded zone, the retention power in the retention gap and the dimensions of the two column parts. However, in practice, rough estimations according to the above guidelines are sufficient.

3.5. Coupling the pre-column to the separation column

The best column equipped with a retention gap is that with the uncoated inlet section built into the same piece of capillary tube as the separation column, circumventing the need for a coupling. A relatively long piece of capillary tube (*e.g.*, 40 m) is deactivated by the appropriate silylation method (considering the wettability of the surface for the samples of interest), but only part (*e.g.*, 15 m) of this capillary is actually coated with stationary phase. This approach presupposes that the analyst prepares his own columns, which is easy, rapid and economical with today's column preparation methods.

It is impossible to discuss all the techniques for connecting pre-columns to

separation columns as proposed in recent years. We therefore only summarize our own experiences.

Shrinkable PTFE tubing still allows the easiest preparation of joints between pre-columns and separation columns; the resulting connections have the additional advantage that the pre-column may be disconnected and reconnected to the separation column without demounting the column from the instrument, just by slipping the butt out of the sleeve. This is particularly useful if the pre-column requires frequent rinsing with solvent (see below). The drawbacks of PTFE connections were discussed in ref. 28, and are related to restricted thermal stability (220°C to be safe), tailing solvent peaks, owing to solvent penetrating into and slowly returning from the PTFE, and also significant losses of solute material above about 150°C. Oxygen diffusing through the PTFE into the column is a problem only for oxygen-sensitive stationary phases such as the polyglycols. The poor mechanical stability (particularly at elevated temperatures) can easily be overcome by strengthening the joint by means of a tube, such as a glass tube taken from the tip of a Pasteur pipette.

Butt connectors, consisting of unions containing one or several ferrules, have been used successfully in many applications by many laboratories. The resulting joints are as thermostable as the columns; losses of solute material by adsorption on the ferrule material are surprisingly small. Solvent peaks are mostly acceptable although not perfect. However, the cost of such devices is still very high.

A very promising method was recently proposed by Etzweiler[29]. He melted glass capillary butts on to bare fused-silica butts, mechanically stabilizing it with a polyimide coating. The resulting connections are thermostable and have nearly perfect chromatographic properties; there is virtually no dead volume, and contact between solvent or solute material and plastic seals is excluded.

The commonly observed phenomena created by poor connections are shown schematically in Fig. 5. The solvent peak may be broadened, but often this broad-

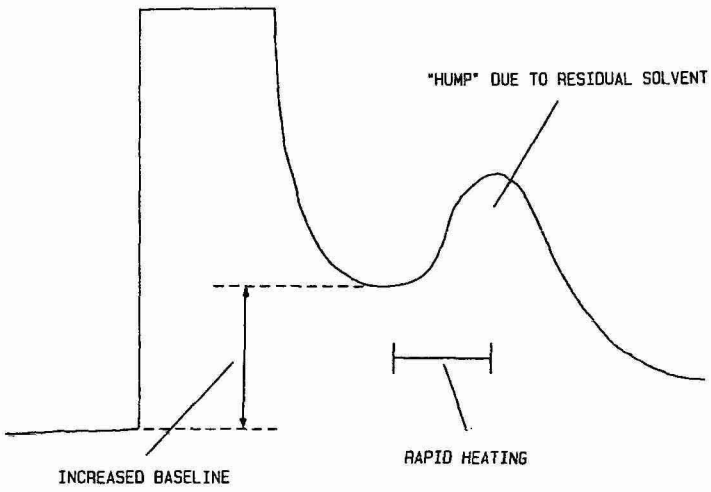

Fig. 5. Symptoms of poor connections between the pre-column and the separation column: baseline not returning to the level before injection and a "hump" created by the rapid increase in the oven temperature. Such deficiencies become more pronounced if large sample volumes are injected, presumably owing to the prolonged contact time between the solvent vapour and the adsorbing plastic material in the connection.

ening is not important. Tailing of the solvent peak also is mostly modest. However, the baseline does not return to the level before elution of the solvent peak. This is due to solvent slowly leaving a dead volume or being desorbed from plastic material (ferrule, adhesive or PTFE tubing). When the coupling is heated specifically or by heating the GC oven, a "hump" is produced owing to accelerated elution of solvent vapour from the connection. Such phenomena are virtually absent if fused couplings are used such as those proposed by Etzweiler[29]. On the other hand, it must be added that the increased baseline after the solvent elution and the "hump" created by a rapid increase in oven temperature are to a lesser extent also visible when using columns without any connections.

3.6. Cleaning of retention gaps

Involatile sample by-products remain spread within the flooded zone in the retention gap and mostly increase the retention power (and/or adsorptivity) of this section. An increased retention power, however, results in reduced reconcentration of the solute bands broadened in space at the beginning of the separation column. Solute peaks start to become broad and to tail, mostly combined with a reduction in the peak areas.

Cleaning of retention gaps by rinsing with solvent is easier than cleaning coated columns because there is no film of stationary phase preventing contact between the extraction solvent and the dirt to be removed, being adsorbed on the support surface underneath the stationary phase. It is recommended to rinse pre-columns with methanol, dichloromethane and pentane, pushing plugs of the corresponding solvents (0.5–1 ml each) one behind the other relatively slowly through the capillary tube. If washing with solvent is not successful, silylation analogous to resilylation of coated columns[30] may facilitate the dissolution and removal of the hindering material by the subsequent rinsing with solvent.

3.7. Choice of sample solvent

The choice of the sample solvent should take into account the following implications for the analysis:

(a) If solutes are analysed that elute near to the injection temperature, the solvent must be suitable for the required solvent effects, *i.e.*, providing full solvent trapping of the solutes of interest or strong phase soaking if full solvent trapping cannot be achieved (see section 2.1.).

(b) The solvent must wet the internal surface of the uncoated column inlet.

(c) Depending on the length of the uncoated column inlet, injection should be carried out near to the boiling point of the solvent in order to keep the flooded zone relatively short.

(d) Solvent evaporation in a column kept near the boiling point of the solvent accelerates volatilization of the solvent and reduces the width of the solvent peak. Injection and solvent evaporation near the boiling point of the solvent does not improve the separation of early eluted solute peaks from the solvent peak, but shortens the isothermal period required for solvent evaporation. The latter argument is not unimportant if large sample volumes are injected: at column temperatures far

below the boiling point of the solvent the solvent peak has a width that rapidly exceeds 30 min!

(e) The solvent must be acceptable for the detector. Use of benzene or toluene cannot be recommended for flame-ionization detection because of the formation of thick, black smoke. Other solvents (*e.g.*, chlorinated solvents) may create a health hazard, calling for pumping off the gases leaving the detector. Switching off the detector may be a solution to some problems. However, especially for specific detectors, one should consider means of bypassing the solvent vapour.

3.8. On-column syringes for injection of large volumes

On-column syringes suitable for on-column injection of up to 100-μl volumes are available from Hamilton, whereby the fused-silica needle and the ferrule system of a 10-μl on-column syringe (701 RN FS) must be transferred to a 100-μl syringe, replacing the standard removable steel needle. Use of 0.23 mm O.D. fused-silica needles is preferable to the 0.17 mm O.D. needles because the wider bore facilitates the picking up of large volumes of liquids.

On-column syringes for injection of larger volumes are not available commercially. However, they can easily be prepared using a standard 500-μl syringe and fitting a fused-silica needle into the exit of the steel needle with adhesive.

A major problem with all syringes with removable fused-silica needles is the memory effect: sample material from previous injections returns from dead volumes or is desorbed from the plastic ferrule in the needle attachment part into the following sample. Rinsing of syringes is inefficient in cleaning dead volumes or extracting ferrules. Memory effects can be avoided by placing pure solvent in the needle attachment zone immediately after an injection. The pure solvent extracts the problematic parts, but first of all prevents sample material from diffusing into the ferrule and the dead volumes during the possibly long periods between two injections.

3.9. Injection speed

The injection speed must be adjusted to the capability of the carrier gas to carry sample liquid away from the injection point. If sample introduction is forced at excessively high speeds, sample liquid is pushed backwards out of the capillary inlet into the injector.

3.9.1. Effects due to excessively rapid injection

If sample liquid returns into the injector, three effects must be expected. The first is concerned with the solvent peak: a substantial amount of sample liquid remains in the dead volumes or as a film on the surfaces in the injector. The solvent evaporates from there and is rinsed back into the column during an extended period of time, creating solvent peaks of the type shown in Fig. 6. The solvent peak is broadened to a width easily exceeding 30 min, and the pen returns only slowly, resulting in broad tailing. If the carrier gas supply enters the injector below the column inlet, the zone creating problems is located above the column entrance including the valve area. In this event, return of the pen to the baseline is achieved by opening the valve, creating a modest leak that causes the solvent residues to be purged from

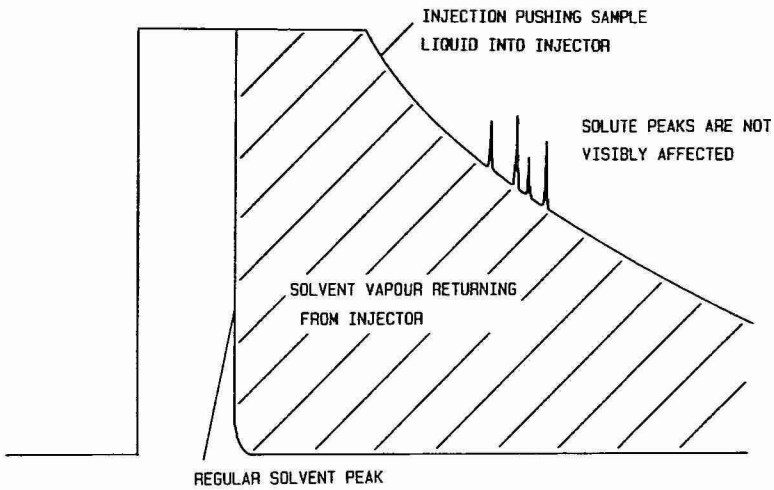

Fig. 6. Solvent peak after an excessively rapid injection: if the sample liquid is introduced more rapidly than the carrier gas is able to carry away liquid further into the column, some of the liquid is pushed through the narrow space between the needle and the column wall backwards out of the column inlet into the injector. If the injector is not equipped with a built-in purge, such a back-flow is immediately detected by the shape of the solvent peak, which becomes broad and tailing.

the injector rather than allowing them to diffuse into the carrier gas stream entering the column. Often a small purge flow-rate must be maintained over a considerable period of time until a low and stable baseline is restored. The effects of returning sample liquid on the solvent peak are drastic because only the (tailing) base of the solvent peak is shown on the chart paper. Volatile solutes behave in the same manner, but owing to their much smaller size, peak distortion is not visible.

The two other effects caused by sample liquid pushed backwards into the injector are related to losses of higher boiling solute material in the cooled injector. This material is lost from the analysis, resulting in smaller solute peaks but also in discrimination of the later eluted components if mixtures are analysed that contain solutes with a wide range of volatilities. However, such material is not lost for ever: it is collected by the subsequent injections bringing sample liquid into the injector. As some of the liquid returns into the column, high-boiling material is rinsed back into the separation column. As this washing effect is inefficient, the memory effects are observed over many injections. Solutes of intermediate volatility (typically eluting between 150 and 230°C from standard non-polar columns) also return via the gas phase.

Memory effects due to transport through the gas phase can be reduced by heating the oven (and the injector) to an elevated temperature and keeping the injector opened. More efficient, however, is rinsing with solvent: the capillary column and the ferrule of the column attachement are dismounted. A small carrier gas flow-rate from the regulator into the injector precludes solvent penetrating into the gas supply line and the manometer. Then several millilitres of solvent, *e.g.*, dichloromethane, are passed through the injector.

3.9.2. Resistance to the movement of the sample plugs

For a better understanding of the background determining the suitable injection speed, the events during the injection process must be considered more carefully (Fig. 7). A 50-μl volume of liquid forming a single plug fills a 0.3 mm I.D. capillary to a length of about 70 cm. However, in practice this sample liquid is split into many smaller plugs: in order to rule out back-flow into the injector, the flow of sample liquid leaving the syringe needle must be below the flow rate leaving the injection point. Therefore, the sample plug is interrupted by numerous gas bubbles of various sizes. The first plugs of the sample liquid still move at a speed near to the linear velocity of the carrier gas, but on continuing the sample introduction, this speed is reduced as it becomes more difficult for the carrier gas to push the longer train of liquid plugs and air bubbles further into the column.

At a carrier gas flow-rate (before injection) of 3 ml/min, a gas volume of 50 μl passes the exit of the syringe needle per second. If this flow-rate could be maintained, a 50-μl volume of liquid could be injected within 1 sec. However, this speed is possible at best during the first moments of the injection.

No specific data can be given on the reduction of the flow-rate at the injection point as a function of the sample volume injected, the column inner diameter and the previously adjusted carrier gas flow-rate. As a simplification, it may be assumed that the speed of a long sequence of sample plugs depends on the carrier gas inlet pressure rather than its flow-rate. The higher the inlet pressure, the greater may be the pressure drop over the plugs of liquid, still leaving a pressure drop over the

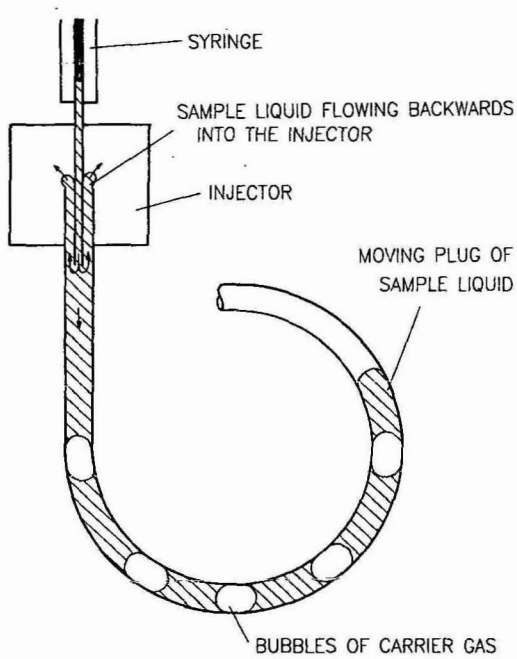

Fig. 7. Return of sample liquid into the injector upon excessively rapid injection. The flow of sample liquid leaving the syringe needle must be restricted to that which the carrier gas can move further into the column.

remainder of the column, which ensures that the carrier gas ahead of the sample plugs continues to flow through the column. In fact, if the carrier gas inlet pressure is low, the plugs of liquid may come to a complete stop, regardless of the carrier gas flow-rate before injection. On the other hand, the flow-rate is only slightly reduced if the inlet pressure is high.

Injection must be extremely slow to create some negative effects. There is no band broadening due to slow injection because of the solvent effects. Furthermore, sample evaporation on the needle tip, resulting in deposition of high-boiling solute material on the needle and their removal from the column inlet[31], becomes important only if extremely slow injection is combined with a high gas flow-rate and a column temperature near the boiling point of the solvent.

Injection of large sample volumes always results in return of the sample liquid to the needle tip and liquid being sucked into the narrow space between the needle and the column wall above the injection point[32]. However, losses of solute material are negligible, and memory effects are also small.

3.9.3. Practical recommendations

As a rule of thumb stemming from practical experience, the carrier gas inlet pressure for a 50-μl injection should be at least 0.5 atm and for a 100-μl injection at least 0.8 atm. At the beginning of the injection the flow of sample liquid released from the syringe needle may approach the carrier gas flow-rate before injection, corresponding to several tens of microlitres per second. Later, the speed must be reduced to around 5–10 μl/sec. It is important to depress the plunger in a smooth fashion. Steps introducing more than about 5 μl at a time easily cause back-flow of liquid into the injector.

On-column injectors equipped with a well designed, permanent injector purge allow perfectly shaped solvent peaks to be produced under nearly all circumstances. However, their effect is "cosmetic". At least for some injections it is advisable to close the purge exit (and to leave it closed until the solvent peak is fully eluted) in order to ensure that the injections are carried out correctly.

3.10. Injection temperature

The flooded zone is the shorter the higher is the column temperature during the flooding process (see section 3.2.1), which allows a further increase in the sample volume injected or the use of a shorter retention gap. Further, high temperatures accelerate evaporation of the solvent, reducing the width of the solvent peak. However, what is the maximum tolerable injection temperature?

We distinguish between the injection temperature, *i.e.*, the temperature during the sample introduction, and the solvent evaporation temperature because their selection is guided by somewhat different arguments.

At injection temperatures considerably below the boiling point of the solvent the injected sample liquid must replace an equivalent volume of carrier gas (which forces one to inject at a reduced speed). At injection temperatures near the boiling point of the solvent, the volume of carrier gas to be replaced increases by the volume of solvent vapour diffusing into the carrier gas at the front and rear of each plug of sample liquid. This solvent vapour diffusing into the bubbles of gas between the plugs

of liquid elongate the latter and compel one to inject more slowly to allow the extra volume to run off the front of the flooded zone. On approaching the boiling point of the solvent at the column inlet pressure (which is above the boiling point of the solvent at ambient pressure), the vapour pressure of the solvent becomes large and renders injection very difficult. Above the column temperature corresponding to the actual boiling point of the solvent under the carrier gas inlet pressure, injection becomes impossible because the gas bubbles continuously expand and push the injected sample liquid backwards out of the column inlet. If large sample volumes are injected, secondary cooling or other systems for independently cooling a section of the column inlet are no longer of any help: the injection temperature must be below the boiling point of the sample at the gas inlet pressure[33].

The column temperature during the introduction of the sample liquid should be at least 20°C below the actual boiling point of the sample (solvent). This means keeping the column about 15°C below the boiling point of the solvent at ambient pressure if the carrier gas inlet pressure is relatively low and to keep it near the boiling point of the solvent if the inlet pressure is at least 2 atm.

3.11. Solvent evaporation

An 80-μl volume of solvent produces about 20 ml of vapour (about 50 ml for a low-molecular-weight solvent such as methanol). If the gas flow-rate through the column is assumed to be 5 ml/min and if the solvent vapour were to replace the carrier gas in the column completely, the solvent evaporation would take 4–10 min. On the other hand, if the solvent vapour constitutes only 20% of the gas passing through the column and if the carrier gas flow-rate is only 1 ml/min, solvent evaporation would take 100–250 min. This demonstrates that the conditions used during solvent evaporation deserve special attention.

The rate of evaporation of a given solvent in the column inlet depends on the flow-rate through the column (which may differ considerably from the carrier gas flow-rate adjusted previously owing to the changed viscosity of the gas–vapour mixture). Further, it depends on the proportion of solvent vapour in the gas passing through the column, this proportion corresponding to the partial vapour pressure of the solvent at the actual column temperature. Theoretically, the solvent vapour could

TABLE 3

RECOMMENDED MAXIMUM COLUMN TEMPERATURE DURING SOLVENT EVAPORATION IN THE COLUMN INLET AS A FUNCTION OF THE CARRIER GAS INLET PRESSURE: DIFFERENCES BETWEEN THE SOLVENT EVAPORATION TEMPERATURE AND THE BOILING POINT OF THE SOLVENT AT AMBIENT PRESSURE

Inlet pressure (atm)	*Temperature difference (°C)*
0.5	0
1.0	+ 8
1.5	+15
2.0	+20
3.0	+30

completely replace the carrier gas. Undiluted solvent vapour would pass through the column if the vapour pressure of the solvent is equal to the inlet pressure of the carrier gas, *i.e.*, if the column temperature corresponds to the actual boiling point of the sample (solvent). However, this is not realistic: the vapour pressure of the solvent must remain below the inlet pressure of the gas in order to prevent back-flow of sample material into the injector.

Recommended differences between the evaporation temperature of the solvent and the boiling point of the solvent at ambient pressure are listed in Table 3 as a function of the carrier gas inlet pressure. This solvent evaporation temperature must be maintained until the solvent is completely evaporated. As the end of the solvent evaporation cannot be easily detected, it is recommended that the temperature is maintained until the solvent peak is completely eluted. This period of time is in excess of what is required by the dead time of the non-flooded part of the column and the width of the solvent peak related to the phase soaking process of the last solvent passing through the column. However, this extra time is comparatively small.

3.12. Rate of temperature increase

This section is of interest only if long retention gaps (exceeding 10–20 m) are used and the carrier gas velocities applied in the retention gap are below about 30 cm/sec.

In section 3.4 it was mentioned that the reconcentration obtained by using retention gaps also depends on the chromatographic conditions applied. This dependence on the operational parameters is related to the dead time of the flooded column inlet section, *i.e.*, the time required by the rear solute material to cover the distance to the front of the flooded zone, even if the solute is not retained by the surface of the uncoated pre-column. This dead time, which easily exceeds 1 min if long flooded zones and low carrier gas velocities are involved, creates a corresponding peak broadening unless the band is reconcentrated by a type of column-internal cold trapping effect. It was shown[34] that the peak broadening due to the dead time of the flooded column inlet section becomes negligible if the solute material is transferred to the entrance of the separation column at least about 80–120°C below the elution temperature. This, however, may compel us to adjust the temperature increase in analytical runs to the speed of the solute migration through the retention gap.

As an example, a solute is assumed to be eluted from the separation column at 250°C and solvent evaporation is carried out at 50°C. The most rapid analysis of this solute would involve ballistic heating from the solvent evaporation temperature to the elution temperature immediately after completion of solvent evaporation in the column inlet. However, when at what temperature is the solute passed through the retention gap?

At low temperatures the solute does not move within the retention gap because, despite of the absence of stationary phase, the retention by the internal walls is excessive. It is realistic to assume that the solute is chromatographed through the uncoated inlet with a capacity ratio, k, between 2 and 5 at a temperature of about 150°C. If the retention gap is assumed to be 40 m long and if the average linear gas velocity within this inlet is 40 cm/sec, several minutes are required to pass the solute through the inlet. However, during ballistic heating (40°C/min for a powerful oven),

the column is at about 150°C only for a few tens of seconds. As a result, the last solute material arrives at the entrance of the separation column near to (in more extreme cases even at) the elution temperature (250°C). At these high temperatures the most advanced solute material (from the front of the flooded zone) migrates into the separation column instead of "waiting" for the rear solute material at the entrance of the separation column, and the resulting peak is broadened.

If the retention gap is only 20 m long and the carrier gas velocity is 80 cm/sec, the solute passes through the uncoated inlet within about 1 min (again assuming a temperature at which there is some retention in the inlet). This allows transfer of all the solute material to the entrance of the separation column at sufficiently low temperatures even if ballistic heating is applied.

The transfer of solute through the uncoated inlet requires special consideration if the inlet is long, if its inner diameter exceeds that of the separation column (resulting in a low carrier gas velocity in the inlet) and if the carrier gas flow-rate is low. The problem can be solved in two ways. First, a temperature programme is applied, starting at the solvent evaporation temperature or at least 120°C below the elution temperature; the programme rate must be adjusted to the longer of the two column parts. In this way the solute selects a suitable temperature for passing through the retention gap by itself. Second, the solute may be transferred to the entrance of the separation column by an intermediate isothermal step at a temperature 80–120°C below the elution temperature. The latter method results in shorter analysis times because heating may be carried out ballistically, but the determination of a suitable intermediate temperature and of the duration of this step is more demanding, usually requiring some experimentation. Further, this method is only suitable for samples containing solutes with a narrow range of elution temperatures.

3.13. Carrier gas flow-rate

The rate of evaporation of the solvent in the column inlet is proportional to the carrier gas flow-rate. In fact, at low carrier gas flow-rates solvent evaporation may become unreasonably time consuming. Further, the dead time of the flooded zone becomes a hindering factor if the solutes are slowly transferred through the uncoated inlet. Hence, there is considerable interest in a high carrier gas flow-rate.

The most important factor determining the applicable carrier gas flow-rate is the type of carrier gas used. Hydrogen is much preferred to helium or nitrogen, first because the flow-rate providing the maximum separation efficiency is twice or three times that of the alternative gases, and second an increase in the flow-rate beyond the optimum results in the smallest reduction in the separation efficiency if hydrogen is used. For 0.3 mm I.D. capillaries, carrier gas velocities of up to several metres per second can be used without sacrificing much in terms of column efficiency.

The applicable carrier gas flow-rate also increases if the column inner diameter is increased, but not linearly. The optimal flow-rate through a 0.5 mm I.D. column is not much above that of a 0.3 mm I.D. column, and excessive flow-rates cause a particularly rapid reduction in column efficiency if wide-bore columns are used. If flow-rates, of, *e.g.*, 10 ml/min are required, 0.3 mm I.D. capillaries are still preferable to the 0.5 mm I.D. tubes.

The 0.3 mm I.D. capillaries are optimal for our purpose also because of the

pressure drop along the column: they create the desirable high pressure drop for the fairly high flow-rate required. For instance, 20-m separation columns equipped with 20–30-m retention gaps can be used with a 2 atm inlet pressure (hydrogen).

3.14. Flow-regulated carrier gas supply?

The main advantage of a flow-regulated carrier gas supply is facilitated solvent evaporation. First, flow regulation precludes a back-flow of sample material upon excessive temperature increase after completion of the sample introduction causing (which may occur, *e.g.*, if the boiling point of the sample is considerably below that of the pure solvent). The flow regulator reacts to high solvent vapour pressures by an increase in the inlet pressure.

Second, flow regulation allows accelerated solvent evaporation owing to the possiblity of using high solvent evaporation temperatures. The regulated flow of carrier gas may force a large volume of solvent vapour through the capillary, provided that the gas pressure delivered to the instrument allows the flow regulator to supply the necessary inlet pressure. Experiments with strongly forced conditions, however, indicated that exaggerated conditions may affect the solvent effects. Third, flow regulation allows reasonably short solvent evaporation times (narrow solvent peaks) to be achieved even with relatively low carrier gas flow-rates, as a large volume of solvent vapour may be moved with a small carrier gas flow-rate.

However, flow regulation is not capable of preventing a back-flow of sample liquid into the injector upon injection at excessively high column temperature or at excessive speed because it reacts too slowly. Injection still requires column temperatures clearly below the boiling point of the solvent and the injection speed cannot be substantially increased. After completion of the sample introduction, the column temperature is increased to the solvent evaporation temperature by programming at 10–30°C/min, adjusted to the capability of the flow regulator to fill the gas volume behind the column inlet with the necessary gas pressure (thus depending on the regulated flow-rate and the gas volume between the regulator and the column entrance).

4. SUMMARY OF RECOMMENDATIONS

The above sections explain the background of the technique and of the guidelines concerning the conditions to be adopted. Below we summarize these guidelines by suggesting a set-up to start with.

A 15–20 m × 0.30–0.35 mm I.D. pre-column of glass or fused silica, deactivated by a phenyldimethylsilylating reagent, is coupled by a butt connector to a separation column of about 0.3 mm I.D. and with a length that can vary between 10 and 60 m, coated with any stationary phase of at least 0.4-μm film thickness. Hydrogen is used as the carrier gas at an inlet pressure of 0.8 atm if the separation column has a length of 10 m and up to 2.5 atm if the separation column is very long.

The 15-m retention gap allows the injection of up to 50-μl volumes of any samples except solutions in methanol and water at any temperature below the boiling point of the solvent. For solutions in methanol the maximum sample volume is only 30 μl; aqueous solutions cannot be handled in this way. If the sample is introduced at a temperature 5°C below the boiling point of the solvent at ambient pressure using

a 0.8-atm inlet pressure, or 10°C above the boiling point of the solvent at a 1.5–2.5-atm pressure, the maximum sample volume can be increased to 80 μl. For 20-m retention gaps the corresponding sample volumes are 70 and 100 μl. This does not mean that this composite column can only be used for injections of large sample volumes —even split injection is possible.

For the introduction of 50–100-μl volumes of liquid, one starts with an injection speed of at most 20 μl/sec and reduces it to 5 μl/sec towards the end of the injection. After completion of the sample introduction the oven temperature may be increased by 10°C above the the maximum injection temperature given above. This temperature must be maintained until the pen of the recordeer returns from the solvent peak.

If dirty samples are analysed, the pre-column requires frequent rinsing.

The above guidelines contain considerable safety margins, which should ensure the success of the experiments.

5. CONCLUSION

There is no natural law which states that the ideal sample volumes of on-column injections are between 0.5 and 3 μl. On the one hand, even these amounts of sample liquid severely overload capillary columns, compelling us to rely on reconcentration by solvent effects or measures against peak broadening due to band broadening in space. On the other hand, as efficient techniques are available for solving

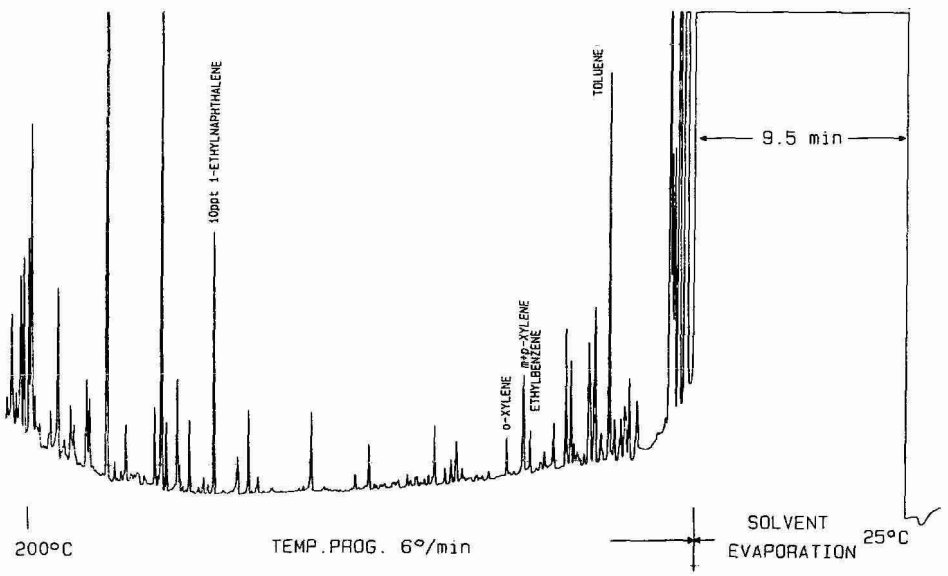

Fig. 8. Pentane extract of Zürich drinking water prepared as the extract in Fig. 1. The water, containing less than 20 ppt of gasoline, was spiked with 100 ppt (100 ng/l) of gasoline and 10 ppt of internal standard (1-ethylnaphthalene); 80 μl were injected on to a 20 m × 0.32 mm I.D. glass capillary coated with 1.5 μm of immobilized PS-255 (a methylsilicone), equipped with a 20 m × 0.32 mm I.D. glass retention gap, leached and silylated with HMDS as described previously[35]. The two column parts were coupled by fusing their butts on to a 10 cm × 0.20 mm I.D. fused-silica capillary. Inlet pressure (hydrogen), 0.8 atm.

these problems, why should one not exploit them much better than is currently done? If we could lose the habit of injecting sample volumes of around 1 μl, how many analyses could be facilitated by injecting 10–50 times larger sample volumes?

Fig. 8. shows the analysis of an extract of drinking water spiked with 100 ppt (100 ng/l) of gasoline and 10 ppt of 1-ethylnaphthalene (internal standard). The injection of large volumes increased the sensitivity obtained by a factor of 40 and provided a very rapid method for determinations of trace concentrations of gasoline in ground water.

5.1. Broad solvent peaks obscuring solute peaks?

Solvent peaks become considerably broadened on injection of large sample volumes, owing to the necessity to transfer all the solvent through the column to obtain the required solvent effects. Such broad solvent peaks may create the impression that many solute peaks may be obscured. However, at least for fully trapped solutes (the majority of solutes) this is not true, as shown in Fig. 9, where the early peaks of a 100-μl on-column injection are at least as well separated from the solvent peak as by split injection of a 3000-fold more concentrated solution using the separation column without the uncoated pre-column. Peaks of solutes that are only partially trapped and reconcentrated in the separation column by the phase soaking effect are not as efficiently shifted to higher retention times. However, they are still not affected by the broad solvent peak.

Another problem that must be faced is the effect of the sample on the absolute

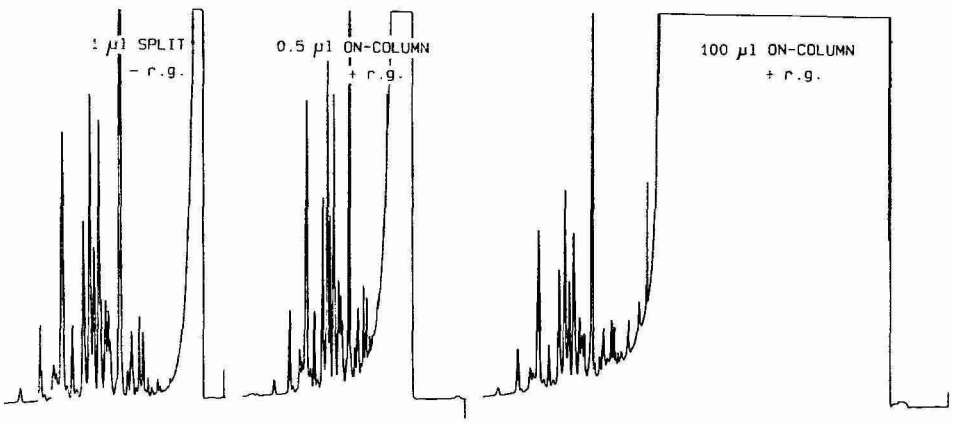

Fig. 9. Do broad solvent peaks obscure early solute peaks? "Nonane fraction", a mixture of isomeric C_9 and C_{10} alkanes, in pentane. Left chromatogram: split injection on to a 12 m × 0.32 mm I.D. glass capillary column coated with OV-73 of 0.3-μm film thickness. Centre chromatogram: 0.5 μl on-column injection on to the same separation column equipped with a wide-bore, 10 m × 0.50 mm I.D. pre-column (butt connector); it is a very similar chromatogram except that the solvent peak is broadened owing to longitudinal diffusion in the retention gap (r.g.) (having an internal volume exceeding that of the separation column by a factor more than two). The 100-μl injection resulted in improved separation of the early peaks compared with the 0.5-μl injection owing to the solvent trapping process: the solutes were released near the exit of the pre-column at the end of the solvent evaporation period (see Fig. 2). Isothermal runs at 40°C with the exception that the 100-μl volume was introduced at ambient temperature (returning to 40°C after withdrawal of the syringe).

retention times, primarily of the early eluted peaks. The retention time of the major peak in Fig. 9 is at least doubled if the sample volume is increased from 0.5 to 100 μl. As in many other applications in capillary GC, it is strongly recommended to use relative retention times.

5.2. Preparation of retention gap capillaries

The preparation of capillary columns was a mysterious task for many years and this outdated impression sometimes still leads to the opinion that the preparation of columns should be left to commercial producers. The availability of commercial columns is indispensable (and the columns available today are indeed excellent compared with the often very poorly made glass capillaries on the market a few years ago). However, laboratories with a high consumption of columns should reconsider preparing columns themselves because of the arguments put forward by Grob[36] and also because it is highly economical and allows one to have many more columns than could be purchased.

The use of long retention gaps is another strong argument favouring the treatment of capillaries in the laboratory. The price of commercial fused-silica retention gap tubing inevitably is high. Fused-silica retention gaps are convenient if frequent removal of a contaminated inlet is necessary, as glass retention gaps necessitate straightening of the inlet whenever a piece of the pre-column is removed. However, if longer retention gaps are used for injection of large sample volumes, this practice is no longer possible as fused silica becomes an unreasonably expensive form of capillary tubing. Glass capillary retention gaps are of almost the same quality and, if prepared by the user, are available in almost unlimited numbers. Deactivation can be carried out with long pieces and the actual work involved in the preparation of a pre-column 100-m length is about 2 h.

Deactivation methods for uncoated pre-columns are identical with those used for the preparation of coated columns[35]. It is therefore an obvious step to coat part of the deactivated tube to produce a tailor-made column for injection of large sample volumes. It is unlikely that columns with built-in retention gaps for large injections will be available from stock for a wide range of interesting applications because there are far too many parameters to be adjusted: the type of stationary phase, the film thickness of the stationary phase, deactivation by trimethylsilylation or phenyldimethylsilylation, the length of the retention gap and the separation column and possibly their inner diameters.

6. SUMMARY

The technique of injecting liquid samples of volumes up to more than 100 μl is described. It relies on the reconcentration of the solute bands by well known mechanisms: the solvent effects for volatile sample components and the retention gap technique for higher boiling solutes. The involved technical parameters are discussed and recommendations given concerning selection of the retention gap, separation column, injection speed and chromatographic conditions.

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CHREV. 196

RECENT DEVELOPMENTS IN ISOTACHOPHORESIS

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CONTENTS

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1. INTRODUCTION

Recently (2nd–6th September, 1984), the 4th International Symposium on Isotachophoresis, ITP-84[1], was successfully held in Hradec Králové, Czechoslovakia, and included basic and advanced courses[2]. The symposium illustrated that capillary isotachophoresis has successfully matured both as an analytical separation method and as a micropreparative method. Isotachophoresis is now a fairly advanced analytical method with a theory permitting the establishment of the possibilities of both its separation capabilities and its qualitative and quantitative aspects. In addition, advanced instrumentation allowing the realization of theoretical predictions is now available. From the viewpoint of information theory, isotachophoresis seems[3] to be comparable to other advanced separation methods, *e.g.*, liquid chromatography, as an identification method.

The number of published practical applications of analytical isotachophoresis continues to grow and we may presume that this method (together with ion chromatography) will soon dominate in the analysis of ionogenic substances in solutions. This conclusion is supported by a number of reviews that have appeared since 1981; the contents of these reviews can be characterized as follows:

(1) Brief descriptions of the basic principles of isotachophoresis[4–17] with appropriate examples of possible analytical applications[18–20], aimed, in a number of instances, at a certain field, *e.g.*, pharmacy[21–23], analysis of beverages[24], inorganic chemistry[25] or biochemistry[26].

(2) Reviews of applications of analytical isotachophoresis in a certain field, *e.g.*, the food industry[27] or the chemical industry[28]; analyses of compounds of a certain type[29], *e.g.*, inorganic compounds[30,31], amino acids, peptides and proteins[32–34] or nucleotides[35].

(3) Methodological reviews devoted, *e.g.*, to qualitative aspects[36], to the selection of electrolyte systems[37], to electrolyte systems for low-[38] and high-molecular-weight[39] compounds, to the isotachophoresis of complexes[40–43] or cations[44,45] or to classification of isotachophoresis from the viewpoint of moving boundary methods[46–48].

(4) Reviews of the physico-chemical principles of isotachophoresis[49–52].

(5) Reviews of instrumentation[53–56].

Unfortunately, the current literature on isotachophoresis is confused as the fundamental book on the subject[57] is 9 years old, there was a considerable delay in the publication of papers presented at the 3rd International Symposium on Isotachophoresis in Goslar[58], complete, critical reviews covering the recent isotachophoretic literature are lacking (the most recent reviews date back to 1981[59,60]; see also ref. 19) and, in 1980, the regular publication of a chronological literature reference list[61] was stopped.

The aim of this review is to summarize critically and as completely as possible original papers and reviews from the period 1981–84, particularly the last three international symposia on isotachophoresis (ITP-80, Eindhoven, The Netherlands; ITP-82, Goslar, F.R.G.; and ITP-84, Hradec Králové, Czechoslovakia), and to characterize the present state of analytical isotachophoresis.

2. THEORY

2.1. Mathematical description of isotachophoresis

The basic description of isotachophoretic systems starts from general equations for the electrophoretic process, which include the following:

(i) transport processes in the presence of homogeneous chemical reactions in solutions of electrolytes (usually in a one-dimensional form, where time and migration path are independent variables);

(ii) the relationship between electric current and ion migration;

(iii) Ohm's law for electrolyte systems;

(iv) electroneutrality in solution;

(v) definition of the equilibrium constants;

(vi) definition of analytical concentrations of substances present in the form of various sub-species in the solution;

(vii) protolytic equilibria of the solvent (up to now always water).

The analytical solution of such a system of equations is very difficult even for a simple case and an explicit solution giving the dependence of the concentrations of various substances on time and migration path is apparently not obtainable. The present approach is to formulate and solve mathematical descriptions of simplified physico-chemical models, corresponding to calculations with one of the following aims:

(i) Calculation of the composition of equilibrium isotachophoretic zones and respective effective mobilities for qualitative and quantitative analysis. Here, diffusion is neglected and steady-state zones are assumed. Transport processes are described by algebraic equations and a mathematical description of the whole system is represented by a system of algebraic equations. The required equations (for the case of weak polybasic acids and bases) and the iterative procedure (the so-called RFQ method) were described earlier[57]. The applicability of this method was extended to systems in which complex-forming equilibria[62] are present, including the case[63] where several ligands are coordinated to a metal. This method has recently been used for the calculation of the equilibrium zone characteristics in the construction of zone existence diagrams, which are important tools for the evaluation of zone stability and separability (see section 3.2).

(ii) Modelling of the separation dynamics. Transport processes are described here by partial differential equations and the system of equations is solved by computation. This enables the processes in an electrophoretic system to be modelled with respect to space and time. The physico-chemical model is considerably simplified and the corresponding simplified transport equations contain only a few terms.

The dynamics of the isotachophoretic process in a simple case of uni–univalent electrolytes, where transport equations included only the electromigration term, was studied in this manner[64,65]. The separation of a two-component sample, including various cases of non-ideal injection and the effects of an impurity present in the leading electrolyte or in the terminator, was described.

A more general procedure[66,67] also takes into consideration the influence of diffusion on the dynamics of the electrophoretic process. Relatively low current densities were used and the influence of diffusion on the character of the migrating

boundaries is well illustrated by the simulation results. Three fundamental boundary types appearing in the isotachophoresis of protolytes were analysed[68] by means of the above method: the classical sigmoid shape of the boundary between the two zones; the boundary with a decrease in the electric field intensity (appearing in the case of inversion of effective mobilities); and the boundary between a zone and the zone of a free weak acid (H^+ termination; see section 3.1). It can be concluded from the results that diffusion can, in some instances, disturb the isotachophoretic effect or to prevail over it, despite the fact that the basic model predicts stable zones.

Thormann and Mosher[69] dealt with the theoretical and numerical analysis of the shape and the width of the boundary between weak electrolytes. With the assumption of sufficiently weak electrolytes and equality of ionic mobilities, u, they obtained for the boundary width (characterized by the change in the molar fraction of one component from p to $1-p$) the relationship

$$W = (\rho' + 2) \ln\left(\frac{1-p}{p}\right)\left[\frac{FRTc_1 (u + u_X)}{I\rho'}\right] \tag{1}$$

[$\rho' = (K_1 - K_2)/K_2$, K_1 and K_2 are the dissociation constants, X = counter ion, T = absolute temperature, R = gas constant, F = Faraday's constant], from which it follows that the boundary sharpness improves with the difference in dissociation constants and with increasing current, I, whereas the increase in the concentration c_1 reduces the boundary sharpness. It is of interest that the theory here predicts an asymmetric shape of the boundary for weak electrolytes, which is also supported by computer-simulated model examples.

A very general approach has recently been presented[70] in which the system incorporating also the kinetics of chemical processes has been solved numerically. The simulation of model cases was limited, however, to the $Na^+ \rightarrow H^+$ (acetic acid) boundary, where, with regard to the high rate constants of acid–base reactions, no quantitative statement about the real width of the mentioned boundary could be made. Simulation with substantially lower rate constants indicated that the boundary width increases with the decreasing recombination rate constant.

(iii) Analytical solution for a certain simplified model. A general mathematical theory of electrophoresis, very widely conceived, and which has been presented only recently[71,72] should be mentioned here. This theory, however, when applied to isotachophoresis[72,73], is considerably simplified, including neglect of diffusion. The mathematical solution is performed only for the case of migration of strong or weak acids with H^+ as the counter ion. This leads to a series of Riemann invariants, the first one being constant in any position of the separation tube. This led Babskii and co-workers to the formulation of the concept of "electrolytic memory". The expression of the first invariant in the form

$$R_1 = \sum_i \frac{K_i c_i (u_H + u_i)}{K_i u_i} = \sum_{i,H} \frac{c_i}{r_i} \tag{2}$$

($r_i = u_i/u_H$) leads to its identity with the classical form[57] of the Kohlrausch regulating function, and "electrolytic memory" can therefore be understood only as the classical principle of the concentration adjustment formulated in another way.

2.2. Application of isotachophoresis to the evaluation of physico-chemical data

Primary information that can be obtained from isotachophoretic measurements and that serves for physico-chemical interpretations is the step height provided by a conductometric or potential gradient detector. This value reflects the specific conductivity and/or effective mobility of the compound in its zone. In general, it is a function of (i) the physico-chemical constants of separated compounds, and hence of ionic mobilities and equilibrium constants of the chemical equilibria involved, and (ii) the working conditions, especially the leading electrolyte composition, which determines the concentration and pH in the zone for given physico-chemical constants.

Obviously, for the evaluation of ionic mobilities and equilibrium constants it is necessary to know the effective mobility and the pH of the zone. The latter is, however, difficult to obtain by experiment. This problem was overcome by Kiso and co-workers[74–81], who developed a method based on iterative calculation starting from the dependence of the pH of the zone on the equilibrium constants and on computer simulation of the isotachophoretic steady state. To achieve the maximum accuracy of the results, ionic mobilities are measured first under conditions of total dissociation, then the complete simulation is performed with the values of the studied constants changing parametrically, until a good fit is obtained with the experimental dependence of the effective mobility on the parameters of the leading electrolyte. Kiso and co-workers applied the above method to the determination of the dissociation constants[74–76] of some weak acids and the stability constants of complexes of a number of cations with some organic hydroxy acids[77–80]. The ionic mobilities, dissociation constants, effective mobilities, effective charges, pH values of zones and slopes of calibration graphs were tabulated[81] for 287 anions in 31 electrolyte systems.

To calculate limiting ionic mobilities from experimental isotachophoretic data, a method was proposed[82] that is based on a computer solution of the system of equations describing the steady state and also including corrections for temperature and ionic mobility. A similar model was used by other workers[83], who evaluated the dissociation constants of weak acids from the dependence of experimental effective mobilities on the pH of the leading electrolyte.

A method[84] based on the measurement of the electric potential gradient was described for the determination of relative ionic mobilities. The arrangement used eliminates some interferences and leads to reproducible and precise results; the values are related to constant ionic strength in the zone.

For qualitative purposes, the measurement of the so-called "relative apparent mobility" by isotachophoretic experiments carried out at a constant voltage across the column with the use of the measured sample as the terminator was described[85–87].

The relative apparent mobility is obtained as the reciprocal of the time of passage of the boundary through the detector. The practical use of this parameter is, however, very restricted as it is not directly proportional to the electrophoretic mobility.

Determination of the Kohlrausch regulating function was described by Hjertén *et al.*[88], who used for its calculation the values of the mobilities and concentrations determined experimentally. For a fully dissociated bivalent anion (5-sulphosalicylate) they found good coincidence for the leading and the sample zones. For cationic analysis of the copper and cobalt zones with acetate as the counter ion they obtained different values, which was explained by the formation of complexes.

The possibility of determining complex formation constants of metals with neutral ligands was described by Stover[89], who reported the separation of alkali metals and ammonium with the use of 18-crown-6-ether neutral ligand in the leading electrolyte.

Fujishita *et al.*[90] described a method for the determination of relative ionic weight (M), which is based on an empirical correlation of experimental data. They showed that the currently used equation[91] containing $M^{-1/2}$ can be substituted, to a good approximation, by the expression $h_r = a + b\,M/|Z|$ where h_r is the relative step height and $|Z|$ the effective charge.

3. FUNDAMENTAL ANALYTICAL ASPECTS

In order to perform successful isotachophoretic analyses, it is necessary that a suitable electrolyte system, in which the sample substances separate, is available. Further, qualitative data permitting the identification of steps in the record and quantitative data permitting conversion of step lengths to analysed quantities should also be available.

3.1. Selection of electrolyte systems

The selection of an electrolyte system for every given analytical problem is aimed not only at a complete separation but also at the possibility of quantitative evaluation within the minimum possible time[92]. The following procedure was proposed for the selection of electrolyte systems[93]:

(i) a preliminary system is chosen;

(ii) whether all the substances in question, when analysed, form stable zones in this system is investigated; and

(iii) whether all of these substances can be separated from one another is investigated.

If the system according to (ii) or (iii) is not satisfactory, another system is taken and the procedure is repeated, starting from (i).

When all the zones are stable and all the substances are separable, it is necessary for the suitability of the electrolyte system to be tested by additional criteria:

(iv) by investigating whether the given electrolyte system is also convenient in the instrumentation used from the viewpoint of the amounts and ratios of the sample components; and

(v) when the procedure is developed for routine analysis, it is necessary to optimize it also from the viewpoint of the analysis time. It is possible, in principle, that the optimization procedure will again lead to the starting point (i).

3.2. Stability of zones

The stability of zones has two independent, but not unrelated, aspects, which are specified as follows.

(i) Correctness of isotachophoretic migration

From this viewpoint the zone is stable as long as it migrates between correctly

selected leading and terminating electrolytes where its front and rear boundaries are permanently sharp. The general condition of the sharp zone boundary is closely associated with the migration order and was formulated[94] for the boundary between the front zone X and the rear zone Y as follows:

$$\bar{u}_{X,X} > \bar{u}_{Y,X} \tag{3a}$$

$$\bar{u}_{X,Y} > \bar{u}_{Y,Y} \tag{3b}$$

where $\bar{u}_{X,Y}$ is the effective mobility of substance X in zone Y. Its validity suggests that substance X possesses a higher effective mobility than substance Y in both zones X and Y.

Provided that the zone fulfills the above conditions in the sense of migration between the leading and the terminating zones, then the system of electrolytes is considered to be correct[44].

From this viewpoint, the ions of the solvent play a significant role, as they can penetrate through the whole electrolyte system and disturb correct migration substantially. An aqueous medium, where both H^+ and OH^- have absolutely the highest ionic mobilities, is particularly concerned. The migration behaviour of H^+ in acidic cationic systems was described[95–97] and conditions were defined under which the system is correct with respect to H^+ and H^+ can serve as the terminator. Further, the concept of the effective mobility of H^+, $\bar{u}_{H,H}$, was introduced, with the aid of which correct migration can be described by condition 3b for Y = H. It holds in general that the effective mobility of H^+ increases with a decrease in the leading electrolyte concentration and in the pK_a value of the acid of the counter ion and with an increase in the content of the free acid in the leading electrolyte. The concept of the effective mobility of OH^- in anionic systems was formulated[93] in a similar manner.

The zone existence diagram was proposed[94] to define the range of substances that provide stable zones in a given system. Calculated effective mobilities of substances in their zones are plotted against the pH of these zones. With the aid of the parametric network in this diagram, the determination of equilibrium parameters of the zones of substances of known ionic mobilities and dissociation constants is simple. Hence the diagram permits a simple determination of zone stabilities of given substances, as the margin contours in the diagram define the range of existence of correct zones and thus the point corresponding to a sample substance must lie inside this area.

(ii) Analytical stability of zones

From this viewpoint the stable zone can be characterized as a zone that contains, independent of time, a constant amount of the analyte. This means that outside the sample zone proper no sample is present. Hence the zone of an analyte neither is enriched with this analyte from the leading or the terminating electrolyte nor decreases owing to migration of the analyte into the leading or terminating zone. At a constant composition of the leading electrolyte, the volume of the zone and its steady-state characteristics (concentration, pH) are also constant; in a tube of constant cross-section both the front and rear zone boundaries migrate at the same velocity.

The analytical stability of the zone is a natural property given by the chemical nature of substances, especially by the chemical stability of the separated substance in the subsequent zone. Analytically unstable zones can appear in practice particularly when the selectivity between the separated substances is affected by complex formation. According to the mechanism that ensures stability or causes the instability of a zone, zones can be classified into certain types[41,98], as follows.

(a) In simple stable zones, complex-forming reactions do not proceed and it is therefore migration of either uncomplexed metal cations or kinetically inert complexes.

(b) Equilibrium stable zones are characterized by the presence of fast complex-forming equilibria. The separated substance migrates as a mixture of various ionic sub-species that are chemically unstable outside the zone proper and are interconverted by fast reactions on the boundary.

(c) Tailing unstable zones are characterized by a slow reaction rate at the rear zone boundary that is comparable to the migration velocity. The existence of tailing zones was shown[98,99] for the cationic migration of lanthanum with the use of cyclohexanediaminetetraacetic acid as the counter ion. It follows from theoretical analysis that the amount of the substance escaping from the zone and subsequently contained in the tail is proportional to the intensity of the electric field in the next zone.

(d) Bleeding unstable zones are characterized by the fact that a certain component of the substance can leave the zone via the rear boundary and is chemically stable in the next zone. Bleeding zones were investigated[100,101] by studying the example of anionic migration of EDTA and NTA complexes. The theoretical model shows that the zone instability is proportional to the conditional complex stability constant (and hence to pH).

3.3. Separability of compounds

In the 1970s, the separability of substances was commonly investigated by a procedure based on the measurement of effective mobilities (or proportional detection signals) of separated substances in several systems and on finding a system where the differences in the effective mobilities obtained are maximal. This system was then considered as the separation optimum. In practice, this already classical procedure has often been combined with graphical processing, *e.g.*, of the $\bar{u} = f(pH_L)$ type. Recently, the possibility has been shown of obtaining the required dependences of effective mobilities by computer simulation[102,103] instead of by elaborate measurements. This procedure was used to find the separation conditions for some organic acids with the use of Ca^{2+} as a complexing counter ion and to find the conditions for the determination of trifluoroacetic acid in urine.

This procedure is obviously based on experimental determination and/or calculation of the mobilities of individual compounds in their zones (*i.e.*, parameters of the $\bar{u}_{i,i}$ type). As shown earlier[92,94], such a procedure is not generally correct and, in principle, it may fail. The reason is that the separability is affected by the effective mobilities of compounds in the mixed zone. Unfortunately, these values are not accessible by direct measurement. This difficulty can be overcome by a procedure[93,104] that converts the problem of separability into the problem of the determination of

migration order. This procedure, in principle, eliminates the necessity for knowing parameters of the mixed zone, as it starts from the evaluation of the equilibrium state. Data of two types are the basis here: (i) $\bar{u}_{i,i}$ and pH_i, corresponding to the evaluation of the steady state; and (ii) $\bar{u}_{i,j}$ determined by calculation from a known pH_j. If the compounds are separable, then their migration order can be determined unambiguously on the basis of conditions for their self-sharpening boundary (conditions 3a and 3b).

To evaluate the separability of a group of substances X from one given substance Y, the zone existence diagram[93,94,104] can be utilized, in which the sequence contours, corresponding to equalities in conditions 3a and 3b, are drawn through the point corresponding to the substance Y. The whole diagram is then divided into regions containing points of compounds migrating in the X → Y and/or Y → X zone order and, moreover, a third region where both conditions 3a and 3b do not apply simultaneously and the zone order cannot be determined unambiguously. Here substances X and Y are not separable and create a stable mixed zone. As has been shown[94], the composition of such a mixed zone must be constant, which indicates that a sample will give, in principle, a zone of the pure substance that is in excess, migrating in front of the stable mixed zone. Although the risk of the confusion of such a pair of zones with a totally separated pair is high, calibration with a standard sample of variable composition usually permits a correct evaluation.

Another approach to the question of separability, including also quantitative aspects, was described by Gebauer and Boček[92]. They started from a model of the separation process of a binary mixture. The quantitative description of separability is based on the concept of selectivity expressed in terms of the relative difference in effective mobilities in the mixed zone. This quantity is accessible directly from a simple experiment and expresses the maximum separable volume of the mixed zone relative to the volume of the leading electrolyte. The concept of separation speed expresses the volume velocity of separation and makes it possible to take the analysis time into account. The two quantities mentioned above permit a simple and rapid optimization procedure, particularly for mixtures, where the problem can be simplified to the separation of a binary mixture.

3.4. Modifications of the isotachophoretic system

Recently, some papers have appeared that describe the theory and/or experiments for systems different from the basic "classical" structure of isotachophoretic systems.

The first modification represents a simplification in which, instead of a common counter ion, only a solvent ion (here H^+) migrates in the opposite direction. These systems were described earlier[105] ("buffer-free" isotachophoresis[106]). A more detailed description of such systems, starting from the application of the general moving boundary theory, has recently been presented[107,108]. The results obtained for equilibrium zone parameters extend existing simple theoretical models for basic systems and thus represent a more valuable contribution for practical applications than a complex mathematical elaboration[71] of the same system that provides comparable results.

The system in which a mixture of several compounds is used as the counter

ion is another possibility. A detailed description of such systems using counter ions consisting of up to six components has been published[109,110]. These systems provide a better correlation between the pH of the leading electrolyte and the effective mobilities of the separated compounds and hence permit a simple selection of the separation conditions by pH changes. Another case of the use of a mixed counter ion comes from the field of isotachophoresis of inorganic cations[97], where one counter ion provides complexing effects and the second counter ion provides sufficient buffering of the system, which the complex-forming counter ion of a strong acid is not able to ensure.

Further modifications of electrolyte systems were shown by the description[111,112] of "combined" systems (see also ref. 113), where the leading electrolyte contains a certain amount of the terminator or the terminator contains a certain amount of the leading compound, or both the possibilities are combined. In these systems, the number of compounds that migrate isotachophoretically decreases. This is considered to be the advantage of the system in the sense of an increase in the selectivity.

Although the systems mentioned above are sometimes designated as "non-isotachophoretic", they belong to the general concept of isotachophoresis. Even these systems permit isotachophoretic migration of stable isotachophoretic zones in the sense of the present understanding (see section 3.2).

3.5. Quantitative aspects

Some authors have recently treated quantitative analysis from the viewpoint of its simplification. The basic equation relating the zone length (in units of charge, Q, or time, t) with the amount of compound i, n_i:

$$Q_i = t_i I = n_i F/T_i \tag{4}$$

suggests that the calibration factor is given virtually only by the reciprocal transference number, T_i, of compound i in its zone.

With a suitable choice of the leading electrolyte, the influence of the variations in its composition on the accuracy of the determination of the transference number (and hence also of the calibration factor) was shown[114] to be negligible. The so-called tolerance diagrams, showing the influence of variations in pH and concentration of the leading electrolyte on the accuracy of anionic analyses, indicate that the tolerance range increases rapidly with increasing pH above 5.

Hirokawa and Kiso[115] used computer simulation to analyse the factors that affect the bias of the values of T_i in eqn. 4 caused by neglecting the influence of H^+ and OH^- ions. They also concluded that the simplification is justified (and the bias is in the range of a few percents) as long as the pH of the leading electrolyte is in the range 5–9. With a knowledge of respective ionic mobilities, the calculated calibration constants can then be used instead of the experimental calibration graph.

Eqn. 4 was also discussed by Svoboda *et al.*[116] in connection with the possibility of using isotachophoresis as an absolute analytical method. In the view of Svoboda *et al.*, prerequisites are precise experimental values of the effective mobilities or the concentrations of substances in their zones.

An important factor, directly related to quantitative aspects, is the width of the zone boundary, which influences the detection limit of the analysis. Electroosmotic flow is one of the quantities that can influence the boundary width substantially. The possibility of affecting the electroosmotic flow (and hence the boundary sharpness) by the addition of surface-active agents to the leading electrolyte was studied[117] both theoretically and experimentally. The zeta potentials of the separation capillary walls were measured in commonly used solutions containing various non-ionogenic or cationic detergents. The boundary widths in a model mixture separated in these systems were also determined. The conclusion was that the disturbing effects can be eliminated best by means of a higher concentration and a lower pH of the leading electrolyte containing an additive admixture.

For evaluation of very short zones using UV detection, the zone area (in terms of integrated zone absorbance) was suggested[118] as a parameter suitable for quantitation.

4. INSTRUMENTATION

4.1. Introduction

The instrumentation aspects of isotachophoresis are well developed. Basic instrumentation for analytical isotachophoresis resulted from developments in the 1970s and a detailed description can be found in a comprehensive book[57] and a review[60]. The heart of the instrumentation is an isotachophoretic column, which is composed of a separation capillary, injection system, electrode chambers and detection cell. The column is connected to a stabilized power supply. The detector response to the isotachophoretic zones passing through the detection cell is processed in the detection device and the resulting signal is registered by a recorder as an isotachopherogram. There are two types of design of the main separation unit, *viz.*, the isotachophoretic column. The first is of the module type[57], *i.e.*, the components such as the injection system, electrolyte chambers and detection cell are independent modules and can be assembled as desired. The advantages of the modular equipment are its versatility and the possibilities of replacing various units and of adjusting the equipment to the demands imposed by a given analytical problem. In the second type[119,120], the isotachophoretic column is formed by a compact block and the separation capillary is created as a flat groove in this block. The compact equipment offers the advantages that both the separation capillary and the detection cell are thermostated with the same efficiency (the cell is made up from a part of the capillary without any connections or any changes in the inner diameter) and, moreover, a series of detection contacts can be placed along the separation path. The instrumentation used at present utilizes the advantages of both of the above possibilities. Various functional elements of the basic instrumentation have been developed fairly diversely since the end of the 1970s, and their present state can be discussed separately.

Micropreparative systems are also discussed separately; either their construction is a modification of the basic analytical equipment in continuous preparation or these systems differ from the basic analytical arrangement substantially in continuous preparation with hydrodynamic flow perpendicular to the direction of electromigration.

4.2. Injection system

An accurate and reproducible injection of a known and/or constant volume of the sample between of the leading and the terminating solutions is a fundamental requirement for the performance of quantitative analyses. By using the experience gained with chromatography, several satisfactory constructions are now available for sample injection, microsyringes and sampling valves made of electrically insulating material (*e.g.*, PTFE) being used to introduce the samples. Therefore, descriptions of new types of injection systems now appear only rarely; mostly sampling valves of simple construction[121] are involved or sampling systems are used the construction of which is motivated by special demands, such as sampling of extremely large sample volumes (up to 1 ml) (see section 4.3)[122].

In general, present-day sampling systems make it possible to inject volumes up to 30 μl and meet the demand of a sharply focused sample zone between the leading and the terminating solutions.

4.3. Separation capillary

The development of separation capillaries from the basic instrumentation to the present state has made considerable progress as a result of demands for an increase in the separation capacity for the purposes of analytical separations of mixtures of components with considerably different concentrations (*e.g.*, body fluids). The separation capacity can be increased by extending the length of the separation capillary, but the analysis time and the maximum voltage required also increase. An elegant solution to this problem was the use of a concentration cascade[123] based on the principle of two different concentration levels of the leading electrolyte, where the separation capacity is increased in a chemical manner. The separation capillary is divided into two virtually independent units, which can be considered as two connected columns.

From the instrumental point of view, the column coupling system[124] frequently used today[54,55] has led to significant progress. Essentially, it consists of the pre-separation unit with a capillary of larger diameter (*e.g.*, 0.8 mm) equipped with the tell-tale detector and bifurcation block, behind which an analytical capillary of small inner diameter (*e.g.*, 0.2 mm) is connected. At the beginning of the analysis, the driving current passes through the pre-separation capillary only. At the end of this capillary, within a short distance before the bifurcation block, the tell-tale detector is located, which enables one to follow the sample migration and the level of the separation obtained. At a suitable moment, the driving current is switched so that it passes through the analytical capillary and thus introduces the required sample zones into it. This is analogous to the so-called "heart cutting" technique, a procedure used in chromatography to separate a group of microcomponents from macrocomponents. Microcomponents introduced into the analytical capillary are then further analysed separately. The use of the column coupling system also makes it possible to use different leading electrolytes in the pre-separation and analytical capillaries and thereby to affect the subsequent separation. The column coupling system is now being used with advanced home-made[57] and commercial[125], or commercial equipment can be adapted to this purpose[126]. The column coupling system enables one to

separate mixtures containing components in ratios up to 1:1000 without increasing demands on the power supply voltage and without prolonging the analysis time in comparison with a simple system.

A mechanical manner of increasing the separation capacity is volume coupling[127], where the separation capillary is divided into two parts: a replaceable part of the capillary of an optionally larger diameter and thus also with a greater separation capacity and a permanent part with the detector. The parts are assembled with the aid of suitable centring rings without flexible sealings. This design provides simple adaptability of the equipment to the demand of a given analysis without extra demands on the voltage applied across the column.

Direct analysis of trace components in biological materials is, however, still beyond the possibilities of current isotachophoretic instrumentation. For this purpose, a column has recently been developed with which large sample volumes (up to 1 ml) can be analysed[121]. It permits the determination of microcomponents at concentrations up to 10^5 times higher than those of macrocomponents. The sample is introduced by a large-volume sampling valve and the pre-separation run is carried out in a channel with a larger cross-section (*e.g.*, 1 × 20 mm). Isotachophoretic zones are stabilized in this part of the column with the aid of a granulated hydrophilic gel. Major components of the sample are cut out from the separation channel in a similar manner to the procedure with the column coupling capillary system. Microcomponents are then introduced via a tapered channel into an analytical capillary (0.2 mm I.D.), where the final separation and detection are effected.

4.4. Detection

Detection is of key importance to practical applications of separation methods, and therefore also to isotachophoresis. For this reason, the detectors for isotachophoresis have been of central research interest since the beginning of the renaissance of isotachophoresis as an instrumental analytical method. The first universal on-line detector, which played a very significant role in the development of isotachophoresis, was the thermocouple detector[57], which senses the temperature of the migrating zones. Owing to its low resolving power (*i.e.*, its ability to detect zones shorter than about 5 mm) in the early 1970s. the thermocouple was replaced with universal contact detectors, which sense the electric resistance or potential gradient in zones.

The detection cell of these detectors is incorporated in the separation capillary and sensing platinum contacts are placed directly in its walls. The measured electric voltage or resistance is amplified by an electronic device and registered by the recorder as an isotachopherogram. The input part of the detection electronics is insulated galvanically from the output, *e.g.*, by means of a transformer[57] or an optical coupler. The design of the detection cells used allows zones shorter than 0.1 mm to be detected, which ensures the detection of zone volumes of the order of nanolitres in commonly used capillaries. A number of papers were devoted to the construction of suitable detection cells and to the processing electronics during the 1970s. Subsequently, descriptions of new designs appeared only rarely[128,129] and their contribution was the improvement or simplification of the fabrication technology of the detection cell.

Remarkable progress in the development of contact detections in recent years

was made by multi-channel detection[119,130,131]. Here, equidistant electrodes creating the sensing array are placed along the whole separation capillary. The electrodes are formed by vapour deposition of SnO_2 layers on a glass support plate, which serves as the bottom part of the separation capillary of the compact-type isotachopherograph. In the course of the analysis the voltage across the neighbouring contacts is scanned periodically with the aid of carbon contacts moved by a stepping motor. This zone-scanning system has been used mainly for automation of isotachophoretic analyses and for the investigation of separation dynamics (see section 4.6).

The disadvantage of contact detectors is the polarization of the sensing electrodes, which has an adverse effect on the utility of the signal magnitude for qualitative analysis (the step height is distorted). To solve this problem, a contactless high-frequency conductivity detector[132] was proposed in the 1970s. The measuring electrodes of this detector are placed on the outer walls of the separation capillary and the zone conductivity is measured through the capillary wall with the aid of a high-frequency voltage (1 MHz). Although this detection procedure is not yet used in practical applications, recent work[133] showed that an improved design of the electronic section and especially thermostatting of the whole system provide promising results. The equipment described here has considerable sensitivity (detection of zones 0.3 mm in length), which, however, depends of the electrolyte concentration. A concentration of 10^{-3} *M* Cl^- was found to be optimal for the leading electrolyte.

Detection based on differences in the refractive index of various zones was one of the first methods used in electrophoresis. In capillary isotachophoresis, the commercial instrumentation described most recently[134] is equipped with refractometric detection. This enables one to perform isotachophoretic analyses in glass capillaries of 0.1 mm diameter in which the zone migration is compensated with a counter flow of the leading electrolyte at a high concentration (of the order of moles per litre). Refractometric detection is performed in such a manner that the capillary is irradiated along its total length with a narrow light beam and the diffraction image is projected on a screen. The disadvantage of this system is the necessity to work with high electrolyte concentrations, which results in slow analyses (hours or tens of hours).

The development of a selective UV-absorption detector[57] for this technique was an outstanding contribution to the development of isotachophoresis in the 1970s. The UV detector is now a common component of commercial apparatus. In the ideal case, the zones of substances absorbing light at a selected wavelength (254 or 280 nm) are registered as rectangular pulses on passage through the detection cell. The UV detector was also proposed for universal detection[135,136] by using a counter ion the absorption of which is dependent on concentration and pH.

A commercial photometric detector for high-performance liquid chromatography (HPLC) was modified for the UV detection of isotachophoretic zones[137]. It permits the easy selection of wavelengths in the range 200–400 nm.

Simultaneous detection at two different wavelengths represented significant progress in the use of the UV detector for capillary isotachophoresis. Sometimes, when detecting zones of compounds that have similar molar absorptivities at a given constant wavelength, the resulting steps in the detection record can hardly be distinguished. In this instance, simultaneous measurement of UV absorption at two different wavelengths[138] enables one to obtain two mutually different qualitative data

on the same zone, which significantly aids in the identification of the compound in the given zone. The detector is designed in such a way that two independent and mutually perpendicular light beams of suitable wavelengths pass through a given point of the capillary — the detection cell. The changes in the intensities of the light beams are then detected and registered simultaneously.

A very simple UV detector has recently been developed[139] for use in commercial apparatus[125]. A suitable wavelength of the UV light of a contactless mercury lamp is selected with the aid of an interference filter and the light passes through a slit (0.2 × 0.3 mm) placed directly on the separation capillary. For detection a phototransistor is employed, in front of which is placed a wavelength shifter that converts UV light to the wavelength required by the phototransistor.

Great attention has recently been devoted to the development of new selective detectors for capillary isotachophoresis, which would facilitate the identification of compounds in the detected zones. Sensing of absorption spectra in isotachophoretic zones[140] is one of the possibilities. A common spectrophotometer is used, the measuring cuvette being replaced with a suitable window through which the separation capillary passes. On passage of the zone through the window, the analysis is interrupted and the absorption spectrum, which can then be compared with the spectrum of the pure compound, is recorded. To record the spectrum, it is necessary for the investigated zone to fill reliably the whole detection cell (the required zone length was 0.3 mm with the equipment described).

Fluorimetric detection is a highly selective method. In isotachophoresis, the equipment designed initially for the dual-wavelength UV detection was employed for fluorimetric zone detection[141]. Zones of fluorescing compounds or of compounds quenching counter-ion fluorescence were detected. Although the material of the separation capillary used forming the detection cell walls (PTFE) absorbs 96% of the incident light and, subsequently, also a portion of the passed or emitted light, good results were obtained with this arrangement for the reliable selective detection of a whole series of compounds, *e.g.*, B vitamins, quinine.

Radiometric detection of compounds labelled with radioactive isotopes[142] is a specific detection method. Its principle is the detection of the radiation emitted from the labelled compound zone passing the window of a Geiger–Müller (GM) tube. The material used for the separation capillary (PTFE) permits the detection of β-radiation, the energy of which is 0.7 MeV and higher (*e.g.*, ^{24}Na, ^{32}P, ^{36}Cl, ^{40}K, ^{42}K, ^{89}Sr). In experiments described[142], a separation capillary of I.D. 0.3 mm and a wall thickness of 0.13 mm was pressed into a slit of width 2 mm shielded with lead and situated directly on the inlet window of the GM tube. Although the optimum arrangement has probably not yet been achieved, the sensitivity obtained is considerable. For instance, in the case of $^{32}PO_4^{3-}$, the zone, which could not be distinguished in the conductivity record, was detected reliably with a radiometric detector.

Electrochemical detection, owing to its high sensitivity and specificity, is widely utilized in modern liquid chromatography. Its direct use in isotachophoresis is hindered by the presence of the driving electric field. Although this detection procedure has already been tested in isotachophoresis[143], where the electrochemical detector was attached to the separation capillary via an elution block in which the separated zones were flushed continuously into the detection cell, it seems likely that, after establishing a suitable geometrical arrangement and electric separation, iso-

tachophoresis will gain another sensitive specific detector. A two electrode arrangement (Pt, glassy carbon) was used and the detection limit found for easily oxidizable compounds (ascorbic acid) was about 1 pmole.

4.5. Electrode chambers, electrodes and power supply

Since the end of the 1970s, electrode chambers and the means of their connection to the separation capillary, electrodes and stabilized current supply have not changed significantly and detailed descriptions can be found in a book[57] and a review[60].

The chamber, filled with the leading electrolyte, is connected to the separation capillary via a semipermeable membrane (Cellophane). The terminator chamber is connected via a multi-way switching valve, which is open in the course of the analysis. Recent modifications have concerned only the material of the chambers, *e.g.*, simple equipment[144] made of fluorinated polymers [PTFE, poly(chlorotrifluoroethylene)] was developed for isotachophoresis in non-aqueous media.

A modification of the electrode chamber construction was described[145] in connection with anionic analyses at high pH. Here, interference by carbonates is a serious problem and can be solved[145] by using a closed system where the solutions in the electrode chambers are not accessible to atmospheric carbon dioxide.

The basic parameters of the sources of stabilized d.c. currents have not changed since the end of the 1970s[57]. A source with a maximum voltage of up to 30 kV, capable of supplying 10–500 μA of stabilized current, can be used for common applications.

4.6. Automation and data processing

Considerable attention has recently been devoted to simplification of the isotachophoretic analyser operation with the aid of control units that permit programmed control of the analysis and its record. Such control units are particularly suitable for work with column coupling systems[53,125,146]. They make it possible to programme the entire analysis in which the zone system obtained by a preliminary sample separation can be cut into four segments, two of which are further analysed and the other two are excluded from the subsequent analysis. A control unit[147,148], which permits flow programming and recorder control in the course of the analysis, was also described for the LKB commercial analyser[149].

Several procedures aimed at simplifying the quantitative evaluation of analyses have been proposed. The coulometric system[150] is based on an unambiguous relationship between the mass transfer by the electric current and the electric charge passed. Here, the analysis record resembles a common time record, but the time axis is replaced with the electric charge passed. The analysis record is independent of the separation equipment used and the separation current. The advantage of the principle is a high accuracy and uniformity of the output information, suitable for automation.

Another procedure for processing measured records is based on electronic evaluation of the step lengths by a modified chromatographic integrator (Shimadzu) or on an on-line microcomputer[148,151]. To permit the use of common chromatographic integrators for the quantitative evaluation of the isotachopherogram, a procedure

was developed for the conversion of the isotachopherogram into a record similar to a chromatogram[152,153]. In this instance, the time dependence of the signal, which is equal to dt/dh, where t is time and h the magnitude of the conductivity detector signal, is recorded. The conversion is performed by a microcomputer, which processes the signal of an A/D converter connected to the detector. Quantitative evaluation is then performed with a common chromatographic integrator.

An important contribution to qualitative assessment of the analysis record is based on computer processing of the signals from a dual-wavelength UV detector[138]. The processing is based on the calculation of the ratio of absorbances at different applied wavelengths in various zones. This ratio has a characteristic constant value for a certain group of related compounds, and then one can reliably interpret the analysis record. To investigate the course of the separation process and to evaluate automatically analyses immediately after the steady state has been reached, multichannel detection[130,154] is employed, where a detection array of sensing electrodes is in contact with the electrolyte along the separation path. The potential gradient distribution along the separation path is scanned by means of a moving contact at short time intervals, and is processed by a microcomputer. The processing is based on rapid periodical evaluation of the lengths of separate zones, (*e.g.*, every second). Constancy of zone lengths in several subsequent evaluations indicates that the steady state has been reached. The analysis is then evaluated and the results are printed out.

4.7. Micropreparative systems

Current capillary isotachophoretic analysers can be used for micropreparative purposes in a discontinuous arrangement only. Once the separation has been performed, the analysis is discontinued and the analysed compound zone is isolated by a suitable method. Either direct cutting of the capillary section containing the compound under analysis[155]or isolation with the aid of a microsyringe[156–158] can be used for this purpose. A fractionation valve[159], which is placed at the end of the separation capillary, was developed for the same purpose. When the zone in question passes through this valve, the driving current is switched off and the compound is flushed out for subsequent treatment. With a suitably selected amount of sample the yield is 90–100%. With the aid of those micropreparative techniques, isotachophoresis was coupled with mass spectrometry[160,161] and liquid chromatography[155].

The Tachofrac[162] is a device that permits the trapping of separated zones on a strip of cellulose acetate. This device is supplied with the LKB 2127 Tachophor commercial analyser.

Continuous free-flow isotachophoresis[163–165] is used for micropreparative purposes in an entirely different way. This technique, developed especially for micropreparative purposes, uses a laminary flow of thin (*ca.* 0.5 mm) layers of the liquid leading and terminating electrolytes for zone stabilization. The sample is pumped continuously between these two flows, electric current is applied and hence isotachophoresis proceeds perpendicularly to the electrolyte flows. At a suitably selected flow-rate and electric current, individual zones of the substances separated can be collected by means of a fraction collector at the outlet of the system. Up to several grams of pure chemicals can be prepared daily by this means. A commercially available apparatus[166] makes it possible to perform continuous isotachophoresis[167,168] in addition to *e.g.*, zone electrophoresis or isoelectric focusing.

The apparatus for isotachophoretic electrodesorption, developed mainly for the characterization and determination of biologically active compounds[169], was modified also for the micropreparative electrodesorption of monoclonal antibodies (for milligram amounts)[170]. Here, the adsorption element with an affinity sorbent is employed in which the protein in question is adsorbed. Then, this element is inserted into the isotachophoretic separation capillary and, in the subsequent isotachophoretic analysis, the determined protein is released by electrodesorption and, after the separation, is isolated with a microsyringe.

4.8. Commercial instrumentation

At present there are commercially available three analytical capillary isotachophoretic analysers and one instrument for continuous flow isotachophoresis.

The LKB Tachophor 2127[149], still available, was the first commercially available isotachophoretic analyser. It is equipped with a PTFE separation capillary, 0.5 mm in diameter, in lengths of 230–800 mm. The capillary is thermostated within the range 3–29°C. The sample is injected with the aid of a microsyringe via a septum. The power supply, with a maximum voltage of 30 kV, provides a stabilized d.c. current up to 500 μA. Either UV (254, 280, 340 and 365 nm) or conductivity detection is used. The Tachofrac fraction collector[162] is an optional device making it possible to trap separated zones on a cellulose acetate strip and subject them to additional, *e.g.*, immunological analysis. To simplify the operation, the instrument is equipped with a control unit. Replaceable elements of resistant TPX plastic are supplied for work with non-aqueous solvents.

Shimadzu (Japan) introduced the second commercially available apparatus for capillary isotachophoresis. The IP-1B apparatus[171] is supplied with a PTFE capillary, 0.5 mm in diameter, 200–1000 mm in length, with the possibility of thermostating at 5–20°C.

The later IP-2A apparatus[172] is equipped with capillaries of diameter 1 and 0.5 mm, with optional lengths. By this means (volume coupling) the separation capacity can be optimized for a given problem. The power supply, with a maximum voltage of 30 kV, provides up to 500 μA of stabilized d.c. current. The sample is injected with a microsyringe via a septum. The materials of construction used (PTFE, glass) permit the use of non-aqueous solvents. UV and potential gradient detection are used. In addition to the basic unit, equipment for establishing a counter flow and a device for electronic measurement of step lengths in the isotachopherogram are also available.

An analyser[125] equipped with a column coupling system is manufactured in Czechoslovakia. The pre-separation capillary, 0.8 mm in diameter, and the attached analytical capillary, 0.3 mm in diameter, are each 200 mm long. The sample is introduced either with an injection valve 25 μl in volume or with a microsyringe via a septum. The use of Perspex as the material of construction for electrode chambers restricts the working range to aqueous solutions. The power supply, with a maximum voltage of 16 kV, provides a stabilized d.c. current of up to 500 μA. Conductivity detection is used with the detection cells at the ends of both capillaries. The instrument is equipped with an automation unit controlling the column-coupling system and analysis timing.

Although the apparatus for continuous free-flow electrophoresis, the Elphor VaP 21[166], is not an analytical one, it can be classified as such in view of the separation efficiency obtained and its operation in a free solution. It makes it possible to perform isotachophoretic separations in a flat rectangular channel of optional dimensions, 50–200 × 250 × 0.3–1 mm, between two glass plates of good thermal conductivity. The sample is introduced continuously into the system at a flow-rate of 0.3–100 ml/h. A 90-piece fraction collector is placed at the outlet. The driving current power supply operates within the ranges 750 V/1330 mA to 3000 V/330 mA. The cooling system enables one to work in the temperature range 4–25°C and its cooling power allows the dissipation of up to 1200 W of electric power in the separation system. The apparatus is provided with a scanning UV detector (Elphor Scan) and for automatic control and evaluation of the separation an Apple II microcomputer with software on a floppy disc is available.

5. APPLICATIONS OF ISOTACHOPHORESIS

In the past, many reviews have been devoted to practical applications of isotachophoresis. The last review[60] covers the literature up to the end of 1980. This section is aimed at reviewing applications published since 1981 and earlier applications that were not included in the last review[60]. The section is divided up according to the origin of the samples, which enabled us to draw together the papers dealing with similar analytical problems, such as the presence of identical or similar major components or comparable analytical concentrations. In the field of clinical applications, the material is further classified according to the character of the analysed substances.

5.1. Analysis of body fluids

5.1.1. Inorganic anions

The heavy metals Ca, Fe, Zn, Pb, Cu and Al were analysed by isotachophoresis in deproteinated serum[173]. Prior to the analysis, the metals were concentrated by chromatography on Chelex. A selective complex-forming counter ion of α-hydroxyisobutyric acid was used to achieve the separation.

Sulphate was analysed in body fluids of urological patients[174]. Their urine was analysed after acidification with 0.1 *M* HCl and their plasma after ultrafiltration. The recovery of exogenic sulphate in plasma was 92–104% and in urine 87–103%.

5.1.2. Organic acids

Pyruvate, lactate, acetoacetate and β-hydroxybutyrate, *i.e.*, acids that play the most significant role in the acid–base balance, were determined in serum within 25 min by direct analysis[175,176]. A good correlation between isotachophoresis and enzymatic tests was found for the determination of lactate in plasma for both normal and elevated values[177]. Lactic acidosis, ketoacidosis and their mixed forms were thus easily distinguished. When the clinical state of the patient became critical, some additional acids appeared in the record. Both the patient's state and his chance of surviving could be evaluated reliably from the general profile of the isotachopherogram.

Citrate, which forms a complex with calcium ions and thus affects the formation of urinary calculi, was determined directly in urine[178]. To dissolve the citrate complex prior to the analyses, urine had to be kept at room temperature for at least 2.5 h. The minimum detectable amount was 0.2–0.4 nmole. The results correlated well with the enzymatic test with citrate lyase. The specificity of isotachophoretic analysis was confirmed by the disappearance of the citrate zone when analysing urine incubated with citrate lyase. Simultaneous determination of oxalate and citrate in 24-h urine was also described[179]. After adding $ZnCl_2$ to urine, a phosphate zone was also observed in the isotachopherogram and, in the urine of a patient suffering from oxalosis, glycolate was also found. Citrate was also determined in human semen plasma[180]; physiological values ranged from 32 to 44 nmol/l.

Anions in urinary calculi were analysed by isotachophoresis in 14 min[181]. The calculus was ground and, after its dissolution in 1 *M* HCl, oxalate and phosphate were determined at pH 3.68 and, after dissolution in 1 *M* NaOH, urate, xanthine and cystine were determined at pH 7.7.

Methylmalonic acid levels were investigated in the urine of rats[182] and of patients with methylmalonic aciduria[183]. Regarding the detection limit of 0.5 μg and the physiological concentration range, methylmalonic acid was not found in the urine of healthy individuals.

Ascorbic acid was determined in leucocytes and in the urine of healthy persons[184]. The ascorbic acid zone was identified in the isotachopherogram by the prolongation of the zone after the addition of ascorbate and by the disappearance of the zone when the sample was incubated with ascorbate oxidase. Concentrations of 5–35 mg/l of ascorbic acid and 18–46 μg of ascorbic acid per 10^8 cells were found in undiluted urine by analysis in 30–35 min and, after ultrasonic treatment, in leucocytes.

The use of 95% methanol as the solvent permitted the determination of taurochendeoxycholic, taurocholic, glycocholic and glycochendeoxycholic acids in human bile[185].

A series of papers on the determination of oxalate in urine was supplemented with additional work[186–189]. The two most frequently used leading electrolytes (HCl–NaCl and HCl–β-alanine)[190] were compared. HCl–NaCl gave a higher reproducibility and a better recovery of oxalate.

Valproate, which is used for the treatment of primary epilepsy, was determined by direct isotachophoretic analysis of serum with a reproducibility of better than 2%[191].

Analytical isotachophoresis was also used[192,193] for the determination of acetate in the blood of patients during haemodialysis and the results were comparable to those obtained by an enzymatic method ($r = 0.95$), the reproducibility being 3.9%. The maximum acetate levels were found 180 min after the start of haemodialysis.

The content of trifluoroacetic acid, a metabolite of halothane, an inhalation anaesthetic, in the blood and urine of anaesthetized patients was monitored by isotachophoresis[103,194,195]. The presence of trifluoroacetate in urine could be detected by isotachophoresis eleven days after the operation.

Thiodiacetic acid, a major metabolite of carcinogenic vinyl chloride, was determined in the urine of persons exposed to vinyl chloride vapour[196,197]. The reproducibility of the analysis was about 3%, the minimum detectable concentration was $6 \cdot 10^{-6}$ mol/l and the analysis time was 45 min.

Formate was determined in serum and haemolysed blood after methanol intoxication with a reproducibility 3%[198]. The maximum concentration of formate in serum was 17 mmol/l.

The metabolites of ethylene glycol, *i.e.*, glycolate, glyoxalate and oxalate, were determined in the blood of intoxicated persons[199]. Whereas negligible amounts of glyoxalate and oxalate were found, the level of glycolic acid reached 17–29 mmol/l.

5.1.3. Amino acids and peptides

γ-Aminobutyric acid was determined in rat brain by cationic isotachophoresis[200]. Rat brain was homogenized, γ-aminobutyric acid was concentrated by ion-exchange chromatography and the eluate was then analysed for γ-aminobutyric acid with a detection limit of 1 nmol.

Cystathionine and its derivatives[201,202] were determined by anionic isotachophoresis in different rat tissues and in the urine of patients with cystathioninuria. The minimum detectable amount was about 1 nmol and the recovery was 92–95%.

Carnitine, which is important for activated fatty acid transfer and whose insufficiency in the organism manifests itself clinically, was determined in serum by cationic isotachophoresis[203]. The serum was incubated in alkaline medium, carnitine was trapped on an ion-exchange resin and 20–30 μl of the sample were analysed within 10–15 min with a detection limit of 0.2 nmol. The reproducibility was about 2.7% and the recovery was 108–116%. The method enables one to determine free carnitine and carnitine bearing short acyl groups. Levels of 42–62 μmol/l were found in the serum of blood donors.

Cysteine derivatives have been determined in human urine[204] and taurine in various rat tissues[205,206].

Isotachophoresis has been used several times for the determination of unidentified peptides in urine or the ultrafiltrate of serum from uraemic patients (or after fractionation of these samples by gel chromatography, gradient elution chromatography etc.)[207–212]. In the UV detector record characteristic zones were observed, the peptidic character of which was confirmed by amino acid analysis.

Tripeptide glutathione and its dimer glutathione disulphide were determined by isotachophoresis in homogenates from various rat tissues[213,214]. The purity of the isotachophoretic zones was checked by incubation of the sample with N-ethylmaleimide, dithiothreitol and glutathioreductase.

Iminopeptides were determined by isotachophoresis in the urine of patients with prolidase deficiency, which manifests itself clinically as iminopeptiduria[215]. Alkalinized urine was subjected to chromatography on Chelex, the fractions were evaporated to dryness and again chromatographed on Diaion. By applying this procedure to samples of urine from healthy persons, all peptides were removed. In the urine of patients with iminopeptiduria, five characteristic zones, which contained twelve dipeptides with terminal proline, were found by isotachophoresis.

Cytostatic methotrexate was determined in plasma by isotachophoresis[216,217]. Prior to the analysis, methotrexate in plasma was precipitated with silver ions and the precipitate was washed, dissolved in Tris buffer and analysed by anionic isotachophoresis. The minimum detectable concentration of methotrexate in plasma was 2.5 μg/l when the column coupling system was used.

5.1.4. Purines, pyrimidines and organic bases

Uric acid, an increased level of which in blood is a symptom of gout, was determined by isotachophoresis with a reproducibility of better than 2%[218]. The purity of the uric acid zone was illustrated by the disappearance of the zone after incubation of the sample with uricase. The results correlated well with those of an enzymatic determination ($r = 0.98$).

Everaerts and co-workers[219–223] determined purines and pyrimidines in urine, serum and in the ultrafiltrate of serum from healthy persons, hyperuraemic patients and patients with Lesch–Nyhan syndrome. Orotic, uric and hippuric acids, xanthine, hypoxanthine, allopurinol, guanosine and adenine were found in the isotachopherogram. In analysis of urine, steps due to orotidine and pseudoxanthine also appeared. When medical treatment with allopurinol was applied, steps due to oxopurinol and allopurinol also appeared[220]. In order to distinguish the zones in the UV record more clearly, non-UV-absorbing spacers were used. The purity of some zones (hypoxanthine, xanthine, allopurinol, uric acid) was checked by analysing samples incubated with enzymes that react with these compounds prior to the analysis[219,221].

Hypoxanthine, as a marker of tissue hypoxia, was determined in serum by isotachophoresis after deproteinization and ion-exchange chromatography[224]. An average hypoxanthine concentration of 0.91 μmol/l was found in the blood of 18 healthy blood donors; in the umbilical blood of newborns the hypoxanthine range was 3–75 μmol/l. The reproducibility was about 5% and the minimum determinable concentration was 10 nmol/l.

The composition of RNA bases from rat livers was determined by isotachophoresis after extraction and hydrolysis with KOH[225]. Isotachophoresis was used to study the influence of 5-fluoroorotate on nucleotide labelling with [^{3}H]orotate[226].

Theophylline, which is used to treat asthma, was determined in ultrafiltered serum[227]. For quantitation the absorbance of the spike between serine and bicine spacers was measured. The detection limit was 1 mg/l with an analysis time of 12 min. Theophylline bound to some proteins was also studied.

Cytostatic adriamycin and its main metabolite adriamycinol were determined in plasma after extraction with butanol[228]. Isotachophoresis did not separate these two cytostatics from one another.

The local anaesthetic trimecaine and both its des-ethylated metabolites were determined by isotachophoresis in plasma after deproteinization and extraction with chloroform[229]. The minimum determinable concentration was 50 μg/l and the reproducibility was 5%. The newer local anaesthetics heptacaine and its 2-alkoxy derivatives were determined in an analogous manner.

Quinine was determined in the extract of urine extracted with isopropanol–dichloromethane[230]. It was found that 2–15% of administered quinine was excreted by urine; the detection limit was 69 μg per 48 h.

Isotachophoresis was also used to determine spermine in semen[231].

5.1.5. Nucleotides and nucleosides

Nucleotide levels in muscles were determined in order to study their metabolism in Duchenne muscular dystrophy[232]. Suppressed levels of ATP, ADP and AMP were found in the isotachopherogram when muscle extract from patients with the above disease was analysed.

Isotachophoresis was also used to study the levels of ATP, diphosphoglycerate, phosphate and lactate in erythrocytes in stored blood[233,234]. The reproducibility of the analyses was better than 3% and enabled one to monitor concentration changes of these compounds during storage.

The contents of ATP, ADP, AMP, c-AMP, IMP, NADH, NAD^+, creatine phosphate, glucose-6-phosphate and pyruvate[235] were determined in heart and bone muscles of the dog, pig, frog and hamster. The values found were in agreement with literature data obtained by other methods.

Various rat tissues were analysed for ATP and ADP, using non-absorbing spacers[236,237]. The reproducibility of the determination of ATP was 2.2% and the recovery was 100–101%. Levels of UDP glucuronate in rat liver after narcosis with diethyl ether, halothane, enflurane, isoflurane and ketamine with diazepam were suppressed, as shown by an isotachophoretic study[238,239]. Isotachophoresis was used to study some nucleotides in human lymphocytes and in the culture of human fibroblasts[240]. Malignant hyperthermia was studied on a pig model. Levels of purine nucleotides, phosphocreatine, lactate and phosphate, as possible markers of malignant hyperthermia susceptibility[241], were investigated. Some further papers[242–245] also reported the determination of nucleotides, especially ATP.

Cytostatic 5-fluorouracil and its analogue 5-deoxy-5-fluorouridine were determined in plasma and serum by isotachophoresis, using a UV detector after deproteinization and ion-exchange chromatography[246]. Five minutes after their intravenous application (15 mg/kg body weight) the maximum concentration in plasma (120–260 μmol/l) was reached. The reproducibility of the analysis was 4–7%. A cytostatic arabinofuranosylcytosinetriphosphate was analysed in leukaemic cells after incubation and repeated washing[247]. The minimum detectable amount was 15 pmol with the use of the UV detection.

5.1.6. Proteins

In the past, protein analyses mainly involved the analysis of cerebrospinal fluid, particularly in cases of multiple sclerosis[248–254]. Isotachophoresis permitted the determination of the permeability of the blood–cerebrospinal fluid barrier for various IgG fractions and for albumin and also permitted a quantitative study of the synthesis of immunoglobulins G inside this barrier. Proteins were determined in cerebrospinal fluid in cases of neurinoma[255], meningitis[256], meningoencephalitis[248–250], acute leukaemia[257] and subarachnoid haemorrhage[250,258]. In the presence of high salt contents, dialysis of the sample is recommended prior to starting the analysis[259].

A number of studies have dealt with the isotachophoresis of serum proteins. To distinguish and characterize the zones better, mixtures of defined substances (amino acids and Good buffers) are frequently used as spacers[248–250,255,260–262]. Small, sharp peaks, the appearance of which is poorly reproducible, often appear in isotachopherograms of serum proteins. The existence of such peaks was explained as the presence of proteins that could have been denatured in the course of the isotachophoretic separation[263].

Isotachophoresis was used to determine immunoglobulins G in the serum of patients suffering from some infectious and non-infectious diseases. Significant differences were found in the composition of serum proteins of patients with liver cirrhosis and paraproteinaemia[255].

Serum lipoproteins pre-stained with Sudan Black B were determined by capillary isotachophoresis with photometric detection at 570 nm[264]. As the preparation proceeded in a free solution, chylomicrons did not remain at the start as in gel electrophoresis but migrated between low-density and very-low-density lipoproteins. High-density lipoproteins were successfully determined using UV detection[265,266].

Thrombin was determined in human blood by isotachophoresis within less than 20 min[267,268]. The purity of thrombin in the isotachophoretic zone was verified by incubation of the sample with heparin prior to the analysis; the thrombin zone disappeared from the isotachopherogram.

In order to determine proteins in body fluids, capillary isotachophoresis was combined with electrophoresis in agarose[269], with isotachophoresis in polyacrylamide gel[270] and with immunoelectrophoresis[271]. Further, isotachophoresis was applied to the determination of proteins in bovine serum, cerebrospinal fluid and bovine aqueous humour[272] and to the determination of crystallines in eye lens[273,274].

5.2. Drugs and their production

Arabonic acid, an intermediate in riboflavine production, was determined in samples of glucose oxidized in aqueous alkaline solutions with a reproducibility of 3.5%[275]. In addition to arabonic acid, oxaloacetic, formic, glycolic, lactic, gluconic and 3-hydroxypropionic acids were identified. The content of arabonic acid in mother liquors was about 0.1 mol/l and in crystalloids it was higher than 89%. Isotachophoresis was used for monitoring the enzymatic cleavage of deacetoxycephalosporin G, in which phenylacetic and 7-aminodeacetoxycephalosporanic acids[276] are produced; the latter is an intermediate in the preparation of new antibiotics of the cephalosporin type. The course of the enzymatic reaction could easily be studied by determining any of the three components of the reaction mixture.

Mucolytic S-carboxymethylcysteine was determined in syrups and capsules with a reproducibility of 0.7% and a detection limit of 10^{-10} mol[21]. In preparations kept for 6 weeks at 50°C, cysteinesulphinic acid was found as the main degradation product.

Sulphites, which are frequently added to drugs as antioxidants, and sulphates, produced by their oxidation, were determined by isotachophoresis, among other methods, in infusion solutions of amino acids[277]. The minimum detectable amounts were 0.2 mmol/l for sulphites and 0.1 mmol/l for sulphates.

The synthetic peptides alanylglutamine and tyrosinyllysyltyrosine, which are suitable for parenteral administration of glutamine and tyrosine because of their solubility in water and their thermal stability, were determined by isotachophoresis in less than 20 min[278–280].

Ascorbic and nicotinic acids, thiamine, pyridoxol, pyridoxal, pyrodoxamine and nicotinamide were determined in vitamin preparations by isotachophoresis[281]. The recovery was 93–101% and the reproducibility was better than 2%.

Isotachophoresis was also used to determine the following drugs: quinine[230], insulin[282], inorganic ions in crude drug preparations[283], valproate[284], codeine and phenyltoloxamine (reproducibility 1.0%, minimum detectable concentration 0.5 ppm)[285], the aminoglycoside antibiotics tobramycin, spectinomycin, clindamycin and lincomycin (minimum detectable amount 1.6 nmol, reproducibility 2%)[286], the

local anaesthetics procaine, lidocaine and tetracaine (reproducibility about 1.7%, detection limit 1.8 nmol)[287], ephedrine, norephedrine, norpseudoephedrine, phenylephedrine, carbinoxamine, methoxymethylmorphine and yohimbine (reproducibility 3–5%)[288], the peptide drugs saralasin, gonadorelin and protirelin (reproducibility about 2%)[289], ephedrine (reproducibility 1.0%), pyridine-4-aldoxime (minimum detectable amount 10 pmol)[290] and phosphonoacetate[291]. Paper isotachophoresis[292,293] with an on-line conductivity detector was used successfully for cationic and anionic determinations of various drugs (quinine, ephedrine, codeine and aminophenazone).

5.3. Food production

In this field, isotachophoresis has been used especially for determinations of organic acids, preservatives and flavours.

Isotachophoresis has proved to be an excellent analytical method for food preservatives, *e.g.*, sorbic, benzoic and propionic acids and *p*-benzoic acid esters (reproducibility better than 2%)[294–297], thiabendazole and diphenylol (reproducibility about 2%)[298], and food additives, *e.g.*, glutamate, GMP, IMP[299], mono-, di- and triphosphates, citrate, glutamic and ascorbic acids, glucono-δ-lactone in meat products (reproducibility about 1%)[300], glutamate in soup (reproducibility better than 2%)[301], cyclamate and saccharin sweeteners in dietetic drinks, tins and some other foodstuffs (reproducibility 0.4–2%)[302] and quinine in drinks (detection limit 5 mg/l)[230].

Great attention was devoted to determinations of acids in wines[303–309]. Acetic, succinic, gluconic, lactic, malic, malonic, phosphoric and sulphuric acids were determined in wines by using chlorate as the internal standard, and the reproducibility was about 2%[303].

The following acids were also determined in foods: citric acid in citrus fruit juices[310], succinic acid in fish extract[311], ascorbic and dehydroascorbic acids in citrus fruit concentrate (reproducibility 1–2%)[312], 2-pyrrolidone-5-carboxylic acid in tomatoes and tomato products[313], lactic, acetic, citric and phosphoric acids in sauerkraut brine (reproducibility about 1%, minimum determinable amount 3–5 ng)[314] and phosphoric, formic, citric, malic, glycolic, lactic and acetic acids in coffee[315].

Tetrodotoxin, which is the main toxin in poisoning by puffer fish, was determined by isotachophoresis in the fish extract with a limit of determination of 0.25 μg[316].

The histamine content in fish and fish products[317,318] was determined by isotachophoresis within 30 min. Isotachophoretic analysis of extracts from mackerel, tuna and herring (reproducibility about 1%, recovery 99%) revealed a rapid increase in histamine concentration after storage of the fish for 3–4 days at 4°C.

Isotachophoresis also proved to be very useful in the following food analyses; EDTA determination in mayonnaise and margarine (reproducibility 3–6%, minimum detectable concentration 10 ppm)[319], nucleotide determination in muscles of frozen carps[320], sinalbin and sinigrin determination in mustard seeds (reproducibility 2%, detection limit 2.4 nmol)[321], determination of inorganic acids in Miso[322], determination of cyanides in apricot kernel and plums and in products made from these fruits[323], determination of theanine, aspartic and glutamic acids in various species of green tea[324], and determination of amino acids in sweet pepper fruits[325].

5.4. Environmental analysis

The herbicide Asulam was determined in soil after extraction, centrifugation, filtration and repeated concentration[326]. The detection limit was 0.5 ng. If preparative isotachophoresis was performed prior to the analysis, 0.02 ppm of Asulam could be detected and 0.1–0.2 ppm determined.

Cationic triazine and quaternary herbicides were determined by isotachophoresis in water and soil[327]. For the determination of quaternary herbicides (chlormequat, paraquat, diquat) the soil was boiled with 50% sulphuric acid, the herbicides were extracted into dichloromethane, the organic layer was evaporated to dryness and an aqueous solution of residue was analysed. The analysis gave a 70–80% recovery and the sensitivity of the method was *ca.* 10 ppb. Triazine herbicides (atrazine, simazine and prometryne) were derivatized to quaternary ammonium salts prior to analysis. The recovery was 95–98% and the limit of determination was 10 ppb.

Phenol was determined in industrial waste waters by direct analysis with a detection limit of 0.1 ppm[328]. A 50-μl volume of the sample was required for the analysis and the analysis time was 20 min. Phenol concentrations of 76 ppm were found in waste waters and 33 ppm in purified waste waters.

Butylamine, which is used in numerous industrial applications, was determined in air[329]. The air was bubbled through 0.1 *M* HCl, the solution was evaporated in a stream of nitrogen and the residue was dissolved in water and analysed. The detection limit of the method was 1 nmol and the recovery was 99.5–118%. The time of the isotachophoretic analysis was 10 min.

Triethylamine and 2-dimethylaminoethanol were determined in air samples from polyuretane foam production[330]. The air was bubbled through 20 mmol/l HCl and the trapped amines were concentrated by adsorption on silica gel. The recoveries were 97–117 and 75–95% for triethylamine and dimethylaminoethanol, respectively.

Chloride, sulphate, nitrate, nitrite, fluoride and phosphate were determined in river water by isotachophoresis[331]. The reproducibility of the determinations of sulphate, nitrate and chloride was better than 2%, and that of nitrite, fluoride and phosphate was better than 5%. The detection limits were 60 ppm for nitrite and 30 ppm for phosphate and fluoride. The analysis time was 25 min.

Isotachophoresis was also used to determine sulphate, phosphate and organic acids in sewage[332], heavy metals (Fe, Cu, Ni, Cd, Co, Zn and Pb) in river water[205] and chloride, sulphate, nitrate, potassium, sodium, calcium and magnesium in surface water[333].

5.5. Chemical industry

In the analysis of industrial or, more often, potential industrial samples, isotachophoresis was used most frequently for the determination of phosphoric acid derivatives such as condensed phosphates[334–340], aminoalkylphosphonates[341] and acid alkyl phosphonates[342–344].

In connection with the growing use of biological technology for the preparation of new compounds, isotachophoresis was used to analyse lysine in fermentation broth[345], lower fatty acids[346] in cultures of microorganisms and organic acid products[347–349] from alkane fermentation. Some papers were devoted to the analysis of

acidic products from the degradation of saccharides[350–355] and methoxybenzene derivatives in degradation products from cellulose hydrothermolysis[356].

The analysis of waste water from metal coatings for Cu complexes[357], ballpoint pen ink for chloride and sulphate[358], cosmetic preparations for chondroitin sulphate[359], permanent waving lotion for thioglycolic and dithioglycolic acids and cysteine[360] and industrial samples for Fe^{2+} and Fe^{3+} (ref. 361), the determination of chlorocarboxylic acid produced by phenol chlorination[362], sulphur oxo acids in sodium sulphide[363], inorganic ions in crude phosphoric acid[364], the veterinary drug amprolium in feedstuffs[365], sulphur oxo acids[366], the explosive 2-(5-cyanotetraazolato)pentaminecobalt(III)[367] and halogenated cobaltocarboranes, used as extraction agents in nuclear chemistry[368], are additional industrial applications of isotachophoresis.

5.6. Miscellaneous

Isotachophoresis was used in enzymology to measure prolidase activity in the serum of iminoaciduric patients[369] and to study the enzymatic transfer of sulphate[370], and to study glucuronidation[371], the metabolism of ascorbic acid[372] and methionine[373], nucleotide-dependent enzymatic processes[374] and reactions of glutathione-S-reductase[375] and adenylate kinase[376].

In protein chemistry, isotachophoresis served to study substitution of dog serum albumin[377], to investigate the bound of 8-aniline-naphthalenesulphonic acid to serum albumin[378], to determine enzyme–immunoglobulin conjugates used in enzyme immunoassay[379], to determine proteins[380], to control the synthesis of the C-terminal pentapeptide bombinine[381,382], to determine virus membrane glycoproteins[383], to study the influence of lyophilization on the protein composition in reference sera[384], to investigate formate dehydrogenase inactivation[385,386], and to determine fetuin in nasal secretion[387] and nuclear proteins in regenerating rat livers[388].

In peptide chemistry, isotachophoresis was applied to the determination of low-molecular-weight compounds in peptide preparations[389–391], the reduced and oxidized forms of glutathione[392] and lysylalanine[393] and to the investigation of the deiodination of iodotyrosine during peptide hydrolysis[394].

In the chemistry of nucleotides, isotachophoresis was applied to the determination of nucleotides produced by microorganisms after high-voltage and ultrasonic treatment[395], to the determination of adenine nucleotides[396,397] and to the control of nucleotide synthesis[398].

Isotachophoresis further served to analyse venom from spiders and snakes[399,400] and stinging insects[401,402], plant alkaloids[403] and allergens[404,405], royal jelly for 10-hydroxy-Δ^2-decenic acid[406], incubated human saliva for putrescine, cadaverine and agmatine[407,408], the composition of the polar lobe of the first cleavage stage of *Nassarius reticulatus* embryos[409], products from the reaction of ammona and acetone[410], humic acid[411], dithiocarbamates[412], alginates[413], hyaluronates[414], cyclohexylamine[415], hydroxybiphenyl conjugates[416], 5-aminolevulic acid and its derivatives porfobilinogen and 4,5-dioxovaleric acid[417], formaldehyde and formic acid[418], acids produced by *Candida albicans*[419] and sialic acid[420].

Successful separations of model mixtures suggest possibilities of using isotachophoresis in practice. Cationic analyses in the past 4 years include separations

of alkali metals and ammonium in a methanolic medium[421], using associates with 18-crown-6-ether[89,422] of varying stability, separations of alkaline earth metals with cyclohexanediaminetetraacetic acid[423] and α-hydroxybutyrate[79] counter ions, separations of K, Na, Ca and Mg with sulphate[120] and the N-oxide of nitrilotris(methylphosphonic) acid[333] counter ions, the complete separation of lanthanides with α-hydroxyisobutyrate as a complex-forming counter ion[424] and the separation of a mixture of Ba, Na, Ca, Mg, Mn, Cd, Co, Zn, Ni, Pb and Cu[173].

Anionic isotachophoretic separations of inorganic compounds include separations of iodide, bromide and chloride and $[Fe(CN)_6]^{3-}$, $[Fe(CN)_6]^{4-}$ and perchlorate with the addition of α-cyclodextrin to the leading electrolyte[422], the separation of chloride, bromide and iodide in a methanolic medium[421] and the investigation of condensation of chromate into bichromate[134] and the formation of various polyvanadates[425].

Separations of coordination compounds include those of *cis* and *trans* isomers produced by the pyrolysis of the green complex of *trans*-$[CoCl(en)_2]Cl$ in which Cl was replaced with water[426], nine cationic nitrosylnitrato complexes of ruthenium with three pairs of *cis-trans* isomers produced by acidic hydrolysis of $[RuNO(NO_3)_3(H_2O)_2]$[426–428] and chelates of Fe(II), Co(II), Cu(II) and Ni(II) with 1,10-phenanthroline in acetonitrile medium[429].

Fatty acids[430], nucleotides with Mg^{2+} counter ion[431], polyamines with citrate counter ion[432] and organic acids[433] were separated among organic model mixtures.

Finally, we can also mention some papers[434–438] that involve isotachophoresis to a small extent and do not fit the above classifications.

6. SUMMARY

This paper is summarizing the contributions to the analytical capillary isotachophoresis published during the period 1981–1984. It characterizes the present state of the method and covers theory, fundamental analytical aspects, instrumentation and applications.

Special attention was payed to the fundamental analytical aspects, and a detailed discussion is given of the selection of electrolyte systems, stability of zones and separability of substances.

The present commercial instrumentation is also briefly described.

7. ADDENDUM

To cover more recent progress in isotachophoresis as represented by the last International Symposium on Isotachophoresis, ITP-84, as completely as possible, the preparation of the manuscript was based on the book of the symposium abstracts[1] and on the actual presentations in the lectures and poster sessions. Hence this text includes a number of papers that did not appear in the symposium volume (*J. Chromatogr.*, Vol. 320, No. 1). On the other hand, owing to some differences between the actual symposium presentations and the final proceedings, which appeared just when this manuscript was submitted for publication, a few papers from this volume must be referred to here. They concern the use of the concentration cascade technique on the commercial LKB instrument[439], desorption isotachophoresis[440], UV detection

at 206 nm[441], the determination of mobilities and pK values[442], dynamics of isotachophoretic separations[443] and the determination of sodium in serum[444].

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