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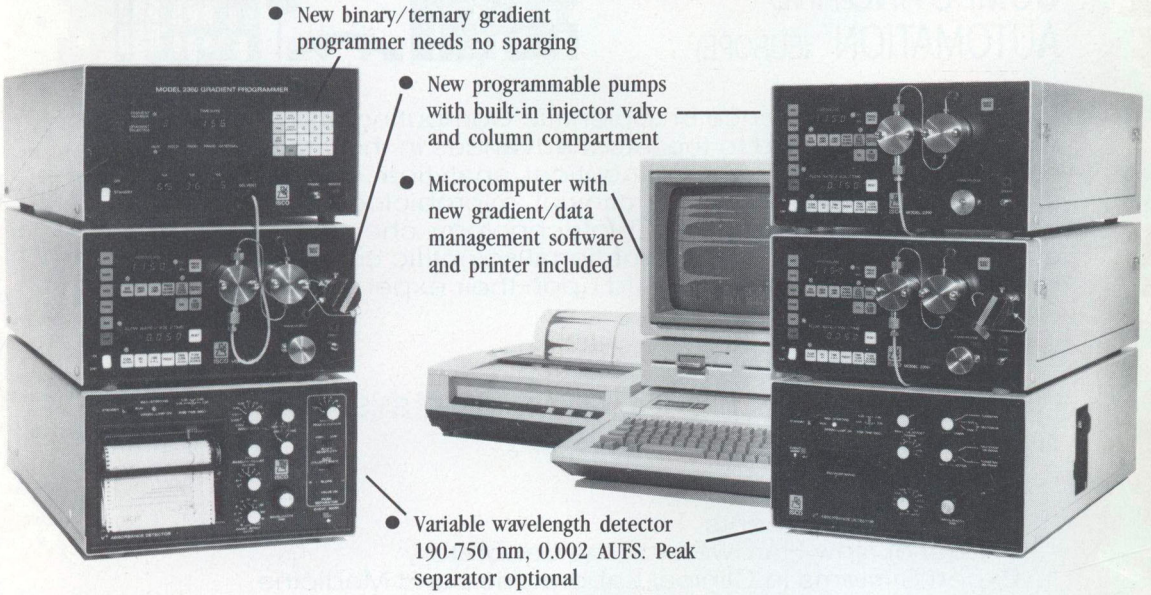

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OPTIMIZATION OF TEMPERATURE PROGRAMMING IN GAS CHROMATOGRAPHY WITH RESPECT TO SEPARATION TIME

I. TEMPERATURE PROGRAMME OPTIMIZATION FUNDAMENTALS

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SUMMARY

The ranges of separability of neighbouring component pairs in a given mixture, separated isothermally on a given chromatographic column, are defined. These ranges are calculated by approximation functions fitted to the measured values of the retention times and peak widths during isothermal analyses. The sequence of the most difficult to separate component pairs is determined within the temperature separability ranges of the component pairs of the mixture. This sequence determines the strategy for calculation of the optimum temperature programme, and every step of this sequence determines the substrategy. The purpose of the strategy is to find the optimum temperature trajectory (programme) and the purpose of the substrategy is to find the optimum subtrajectory, *i.e.*, a part of the optimum trajectory. The determination of the strategy and the corresponding substrategies is presented for mixtures of components that do not change their mutual position during isothermal separations within the whole temperature range.

INTRODUCTION

Temperature programming in gas chromatography (GC) has been studied since this technique was used for the separation of mixtures with widely different boiling points of the individual components. Various workers have dealt with this problem from different viewpoints. Harris and Habgood[1] presented a relationship for the calculation of the retention time during linear temperature programming and investigated the influence of the temperature increment on the resolution of two neighbouring peaks. De Wet and Pretorius[2] studied the effects of temperature on the plate height. Giddings[3] studied the influence of temperature programming on the

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time of analysis and Giddings[4,5], Scott[6] and others studied the effect of temperature on the column efficiency and the separability of mixtures. Studying the temperature influence, these workers usually based their theories on the knowledge of the composition of the column packing and of the individual components of the mixture.

In order to optimize the temperature programme for a given separation problem, Bártů[7] and Bártů and Wičar[8] proposed a procedure in which the rate of migration and broadening of a chromatographic zone during a temperature programme, $T_P(t)$, is predicted. With respect to heat transfer, the oven–column system is regarded as a first-order static system characterized by a time constant H_c. In this model, the actual column temperature $T(t)$ differs from the measured oven temperature $T_P(t)$.

Although already published before[7,8], the set of basic equations (1–15) is summarized here for the sake of readability.

In a multi-step temperature programme, the column temperature within the kth programme step, $T_k(t)$, is

$$T_k(t) = T_{S,k} + (T_{N,k} - T_{S,k})(1 - e^{-t/H_c}) + D_k[t - H_c(1 - e^{-t/H_c})] \quad (1)$$

where D_k is the rate of increase or decrease of oven temperature in the kth step, and $T_{S,k}$ and $T_{N,k}$ are the column and oven temperatures at the beginning of the kth step, respectively. For the oven temperature in the kth step, the following equation holds:

$$T_{P,k}(t) = T_{N,k} + D_k t \quad (2)$$

Eqns. 1 and 2 are recursive; the column and the oven temperatures at the end of the kth step obviously represent the initial temperatures for the $(k + 1)$th step:

$$T_{S,k+1} = T_k(t_k); \qquad T_{N,k+1} = T_{P,k}(t_k) \quad (3)$$

where t_k is time at the end of the kth step.

At the end of the kth step, the position of the nth component zone is determined by the sum of distances passed by the zone in the column during all previous programme steps 1, 2, ..., k:

$$L_c = L_{1,n} + L_{2,n} + \ldots + L_{x,n} = \sum_{k=1}^{x} L_{k,n} \quad (4)$$

where L_c is the column length and $L_{k,n}$ is the distance travelled by the nth component zone in the kth step. By definition, $L_{x,n}$ is the distance travelled by the nth component zone within the xth step when the zone leaves the column.

In a dimensionless form, eqn. 4 reads

$$1 = l_{1,n} + l_{2,n} + \ldots + l_{x,n} = \sum_{k=1}^{x} l_{k,n} \quad (5)$$

where $l_{k,n} = L_{k,n}/L_c$. The distance $l_{k,n}$ are determined by

$$l_{k,n} = \int_0^{t_k} \{1/t_{A,n}[T_k(t)]\}\, dt \tag{6}$$

where t_k is the duration of the kth step and

$$t_{A,n}(T) = A_{t,n} \exp(B_{t,n}/T) + C_{t,n} \tag{7}$$

is the approximate dependence of the retention time of the nth component on temperature, obtained by interpolation of a set of isothermal retention data[7].

Substitution of $l_{k,n}$ from eqn. 6 into eqn. 5 yields

$$1 = \sum_{k=1}^{x} \int_0^{t_{k,n}} \{1/t_{A,n}[T_k(t)]\}\, dt = \int_0^{t_{AP,n}} \{1/t_{A,n}[T(t)]\}\, dt \tag{8}$$

where $t_{AP,n}$ is the calculated value of the retention time of the nth component for a given temperature programme.

The retention time of the nth component is given by the sum of the upper integration limits in eqn. 8:

$$t_{AP,n} = t_{1,n} + t_{2,n} + \ldots + t_{x,n} = \sum_{k=1}^{x} t_{k,n} \tag{9}$$

where $t_{k,n}$ is the retention time increment of the nth component in the kth programme step.

In the course of a temperature programme, the width of the nth component zone at the column outlet, $S_{AP,n}$, could be regarded as composed of increments generated in the individual program steps, $S_{k,n}$:

$$S_{AP,n}(t_{AP,n}) = S_{1,n} + S_{2,n} + \ldots + S_{x,n} = \sum_{k=1}^{x} S_{k,n} \tag{10}$$

The zone width increment generated within the kth programme step is given by[7,8]

$$S_{k,n} = s_{A,n}[T_k(t_{k,n})] \frac{\sqrt{L_c L_{k,n}(t_{k,n})}}{t_{A,n}[T_k(t_{k,n})]} - s_{A,n}[T_k(0)] \frac{\sqrt{L_c L_{k,n}(0)}}{t_{A,n}[T_k(0)]} \tag{11}$$

where

$$s_{A,n}(T) = A_{s,n}\, e^{B_{s,n}/T} + C_{s,n} \tag{12}$$

is the approximate dependence of the width at half-height of the nth component peak

on temperature, obtained by interpolation of experimental isothermal data. $L^0_{k,n}(0)$ and $L^0_{k,n}(t_{k,n})$ are the distance travelled by the nth component zone in the column at the beginning and at the end of the kth programme step, respectively.

Eqn. 11 can be expressed in its integral form:

$$S_{k,n} = \int_0^{t_{k,n}} \frac{\mathrm{d}}{\mathrm{d}t} \left\{ s_{A,n}[T_k(t)] \frac{\sqrt{L_c L^0_{k,n}(t)}}{t_{A,n}[T_k(t)]} \right\} \mathrm{d}t \tag{13}$$

where

$$L_{k,n}(t) = L_{1,n} + L_{2,n} + \ldots + L_{k-1,n} + \int_0^t \frac{L_c}{t_{A,n}[T_k(\tau)]} \mathrm{d}\tau \tag{14}$$

is the distance passed by the nth component zone in the column up to a given time t.

At the column outlet, the zone width $S_{AP,n}$ and the peak width $s_{AP,n}$ are mutually dependent according to the relationship[7,8]

$$s_{AP,n} = S_{AP,n}(t_{AP,n}) \frac{t_{A,n}[T_x(t_{x,n})]}{L_c} \tag{15}$$

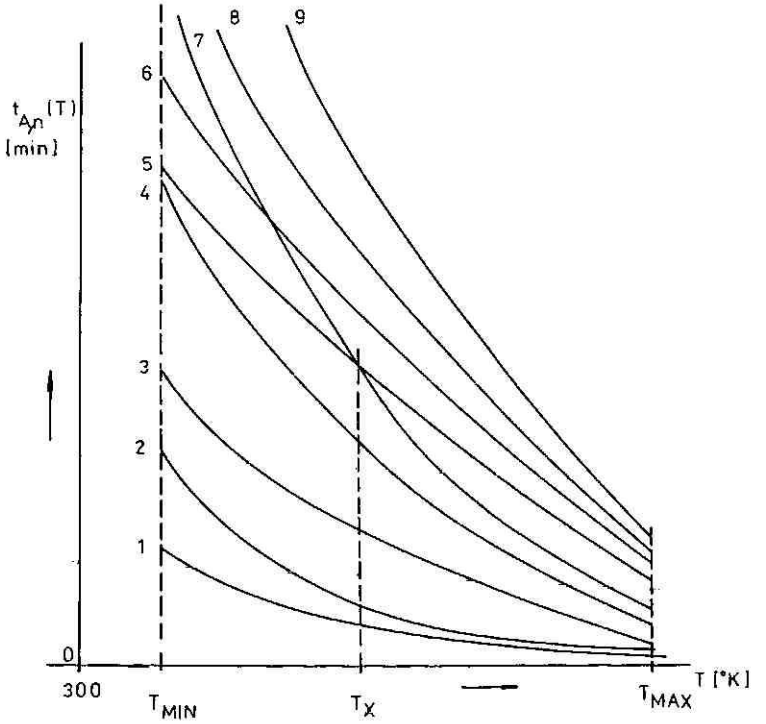

Fig. 1. An example of approximation functions of a nine-component mixture.

In the process of optimization of the temperature programme, the substantial volume of calculations is represented by the evaluation of $t_{AP,n}$ and $s_{AP,n}$ and is obviously proportional to the number of components in the mixture to be separated.

DISCUSSION

Temperature separability range

The following considerations, which lead to a significant reduction in the computation volume, are based on the analysis of the properties of the approximation functions $t_{A,n}(T)$. An example of a set of approximation functions, corresponding to a hypothetical nine-component mixture, is presented in Fig. 1. T_{MIN} and T_{MAX} are physical temperature limits determined by both the apparatus and the column. For each pair of neighbouring components m, n there are three possible arrangements of the corresponding approximation functions $t_{A,m}$ and $t_{A,n}$ (*cf.*, function numbers in Fig. 1):

A. (functions 1, 2):

$$t_{A,m}(T_{MIN}) - t_{A,n}(T_{MIN}) \geqslant t_{A,m}(T_{MAX}) - t_{A,n}(T_{MAX})$$

B. (functions 4, 5):

$$t_{A,m}(T_{MIN}) - t_{A,n}(T_{MIN}) < t_{A,m}(T_{MAX}) - t_{A,n}(T_{MAX})$$

C. (functions 5, 7):

$$t_{A,m}(T_{MIN}) > t_{A,n}(T_{MIN}), \quad t_{A,m}(T_{MAX}) < t_{A,n}(T_{MAX})$$

In the last instance the approximation functions intersect. Consequently, there must be at least one temperature T_x in the $\langle T_{MIN}, T_{MAX} \rangle$ interval at which $t_{A,m}(T_x) = t_{A,n}(T_x)$ and both peaks corresponding to the m, n component pair coincide. An isothermal separation of such a pair of components at $T < T_x$ results in a retention order which is reversed at $T > T_x$ (Fig. 2).

Let us define the temperature separability range for a given pair of components m, n:

$$T_H[m, n] \geqslant T \geqslant T_L[m, n] \tag{16}$$

For every T within the range given by eqn. 16 the compound pair is separated whenever the resolution is sufficient, or

$$R_{m,n} = \frac{t_{A,m}(T) - t_{A,n}(T)}{1.7\,[s_{A,m}(T) + s_{A,n}(T)]} \geqslant 1 \tag{17}$$

To determine the temperature separability range for a given mixture, first the temperatures T_x for all component pairs within the temperature limits T_{MIN}, T_{MAX} are sought, using the approximation functions $t_{A,n}(T)$. The T_x temperatures are arranged into a square matrix $T_x[N, N]$, where N is the total number of components. By convention, the matrix subscripts are determined by the isothermal retention time values at the lowest temperature T_{MIN}:

$$t_{A,1}(T_{MIN}) < t_{A,2}(T_{MIN}) < \ldots < t_{A,N}(T_{MIN}) \tag{18}$$

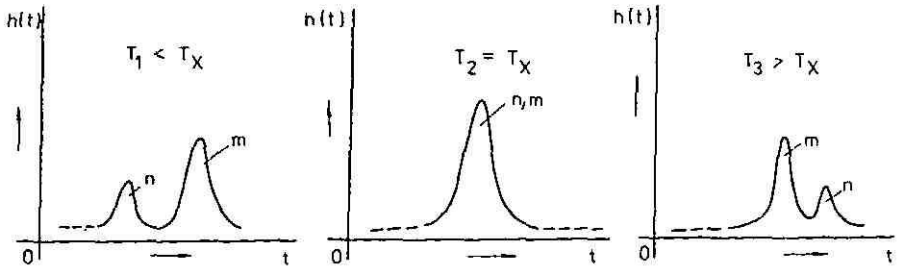

Fig. 2. Partial isothermal chromatograms with components changing their mutual positions at various temperatures.

If the mixture does not contain any component pair with intersecting approximation functions (mixtures of "type I"), then $T_x[N, N] = 0$; otherwise the non-zero elements are located above the principal diagonal:

$$T_x[i, j] \; i > j \qquad i = 1, 2, \ldots, N - 1; \qquad j = 2, 3, \ldots, N \tag{19}$$

In an isothermal separation, the resolution of a component pair i, j changes with the separation temperature. It is assumed that merely one maximum resolution value $R_M[i, j]$ exists, at a temperature $T_M[i, j]$ within the T_{MIN}–T_{MAX} range, whenever the approximation functions $t_{A,i}(T)$, $t_{A,j}(T)$ do not intersect in the same temperature range.

Two maximum resolution values are expected, if $T_x[i, j] \neq 0$ ("type II" mixtures); one, $R_M[i, j]$, at a temperature $T_M[i, j]$ in the T_{MIN}–$T_x[i, j]$ range, the other, $R_M[j, i]$, at $T_M[j, i]$ in the $T_x[i, j]$–T_{MAX} range.

The temperature dependence of the zone width is at least one order of magnitude smaller than that of the retention time. We may therefore expect that the maximum resolution value:

$$R_{m,n} = \frac{t_{A,m}(T) - t_{A,n}(T)}{1.7\,[S_{A,m}(t_{A,m}/L_c) + S_{A,n}(t_{A,n}/L_c)]} \tag{20}$$

in a given temperature range is mainly due to the maximum value of the numerator in eqn. 20. As the analytical determination of the maximum value of $R_{m,n}$ is not possible, iteration methods have to be applied. The temperature dependence of resolution is presented schematically in Fig. 3. The corresponding approximation functions intersect in (b) and (c) and do not in (a).

The maximum resolution values R_M, obtained by iteration, and the corresponding temperatures T_M are arranged into square matrices $R_M[N, N]$ and $T_M[N, N]$. In both matrices, the principal diagonals contain the resolution values and temperatures of neighbouring components, provided the corresponding approximation functions do not intersect. (The $R_M[m, n]$, $T_M[n, n]$ elements correspond to the n, $n + 1$ component pair.) In the case of intersection, two resolution values and related temperatures correspond to each component pair. For the components n, m, $T_M[n, m]$ and $R_M[n, m]$ are in the $T_X[n, m]$–T_{MAX} range and $T_M[m, n]$ and $R_M[m, n]$ are in the T_{MIN}–$T_X[n, m]$ range. The resolution of more remote components, *i.e.*, of those

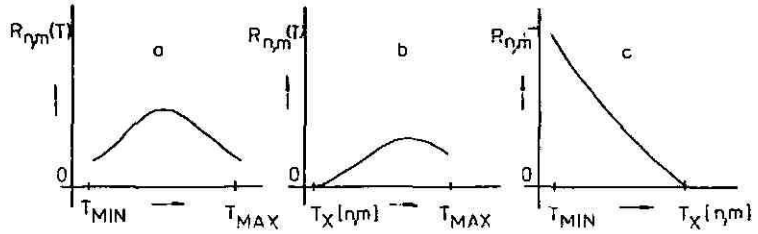

Fig. 3. Examples of the dependence of the resolution on temperature for various component pairs.

bearing the subscripts n, $n + i$ with $i \geq 2$, is not calculated, provided the corresponding approximation functions do not intersect.

In the next step, both matrixes R_M and T_M are used to determine the lower and the upper temperature separability limits according to eqns. 16 and 17. The separability limits T_L, T_H, together with the corresponding resolution values R_L, R_H, are again arranged into square matrices $T_L[N, N]$, $T_H[N, N]$, $R_L[N, N]$, and $R_H[N, N]$. The individual elements are determined by iteration. The temperatures in the principal diagonals of both T_L and T_H belong to

$$\begin{aligned} &T_L[n, n] \in \langle T_{MIN}, T_M[n, n]\rangle \\ &T_H[n, n] \in \langle T_M[n, n], T_{MAX}\rangle \\ &R_L[n, n] \geq 1, \quad R_H[n, n] \geq 1 \end{aligned} \tag{21}$$

provided $T_M[n, n] \neq 0$ (see Fig. 4a).

For components with intersecting approximation functions (Fig. 4b), the temperatures $T_L[N, N]$, $T_H[N, N]$ are

$$\begin{aligned} &T_L[n, m] \in \langle T_x[n, m], T_M[n, m]\rangle \\ &T_H[n, m] \in \langle T_M[n, m], T_{MAX}\rangle \\ &T_L[m, n] \in \langle T_{MIN}, T_M[m, n]\rangle \\ &T_H[m, n] \in \langle T_M[m, n], T_x[n, m]\rangle \\ &R_L[n, m] \geq 1, \quad R_H[n, m] \geq 1 \\ &R_L[m, n] \geq 1, \quad R_H[m, n] \geq 1 \end{aligned} \tag{22}$$

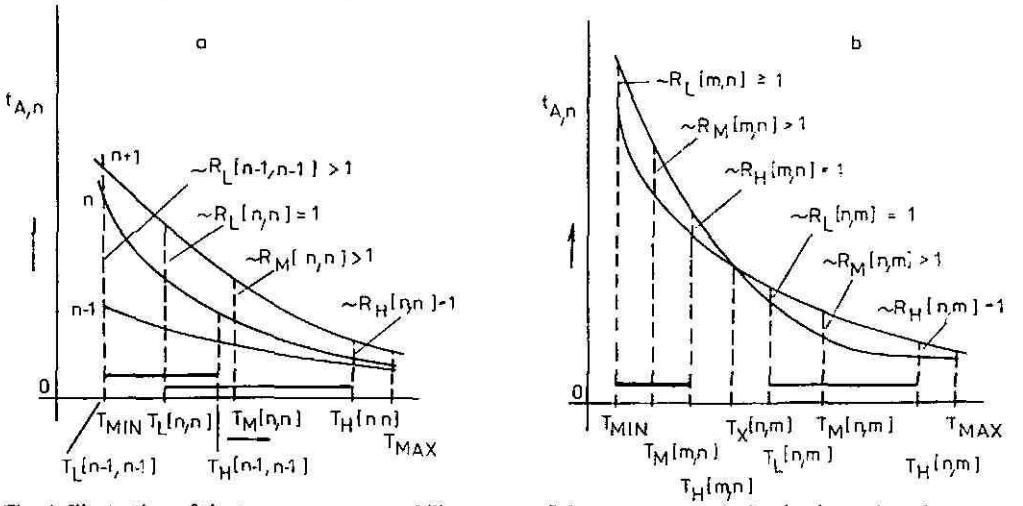

Fig. 4. Illustration of the temperature separability ranges of the components during isothermal analyses.

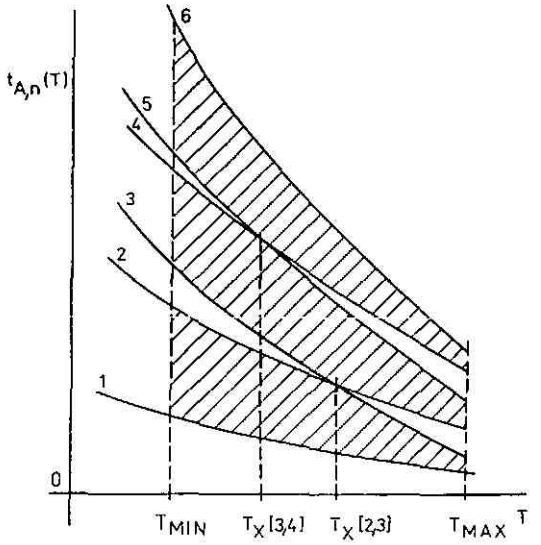

Fig. 5. Illustration of the reduced ranges for calculation of temperature separability ranges.

If any of the elements of R_H is less than one (even the maximum resolution does not satisfy), we put

$$\begin{aligned} T_H[n, m] &= T_L[n, m] = T_M[n, m] \\ R_H[n, m] &= R_L[n, m] = R_M[n, m] \end{aligned} \tag{23}$$

The matrices T_x, T_M, T_H, T_L, R_M, R_H and R_L summarize the knowledge on the mutual positions of the approximation functions in a concise form.

If the approximation functions corresponding to a component pair n, $n + 1$ do not intersect, but at least one of them is crossed by the approximation function of another more distant component (Fig. 5), the separability range of the particular pair n, $n + 1$ is to be corrected. In this instance the R_H, T_H, R_L, T_L matrices are calculated in reduced intervals, as shown by the hatched areas in Fig. 5 for $n = 1$, 3, 5.

Strategies and substrategies for temperature program optimization

The aim of the optimization is to develop a procedure for computation of the oven temperature programme $T_p(t)$ such that for the resolution of any component pair of the separated mixture there holds

$$\begin{aligned} R_{n,m} &\geqslant 1 \qquad n = 1, 2, \ldots, N - 1 \\ &\qquad\quad m = 2, 3, \ldots, N \end{aligned} \tag{24}$$

and, simultaneously, the retention time of the component that leaves the column last is minimal:

$$t_{AP,E} = \min \tag{25}$$

For a mixture of type I (the most common case in GC)

$$t_{A,1}(T) < t_{A,2}(T) < \ldots < t_{A,N}(T), \qquad T \in \langle T_{MIN}, T_{MAX} \rangle \tag{26}$$

and the resolution matrices $R_L[N, N]$, $R_H[N, N]$ together with the temperature separability matrices $T_L[N, N]$, $T_H[N, N]$ contain non-zero elements exclusively in the principal diagonal. Optimization conditions 24 and 25 can here be modified to

$$R_{n,n+1} \geqslant 1; \qquad t_{AP,N} = \min \qquad n = 1, 2, \ldots, N - 1 \tag{27}$$

The separability ranges for a nine-component mixture are presented in Fig. 6; the dashed numbers refer to T_L. The diagonal elements of the T_H matrice can be arranged to form an ascending sequence:

$$T_H[n_1, n_1] < T_H[n_2, n_2] < \ldots < T_H[n_k, n_k] \tag{28}$$

where $n_1, n_2, \ldots, n_k$ are the neighbouring pairs of components $n = 1, 2, \ldots, N - 1$, sorted with respect to the upper temperature separability limit, T_H. The first term of the sequence determines the maximum temperature at which all the components of the mixture could be separated with $R \geqslant 1$ by isothermal separation. The isothermal separation of the mixture at the temperature determined by the second term results in the separation of all components at $R \geqslant 1$, except the first pair.

Generally, the ith term of the sequence determines the temperature at which all components except the first $i - 1$ pair are separated at $R \geqslant 1$ by isothermal separation.

For the mixture in Fig. 6 the sequence

$$T_H[3, 3] < T_H[1, 1] < T_H[6, 6] < T_H[5, 5] \tag{29}$$

is obtained, or in abbreviated form

$$3, 1, 6, 5, 8, 2, 4, 7 \tag{29a}$$

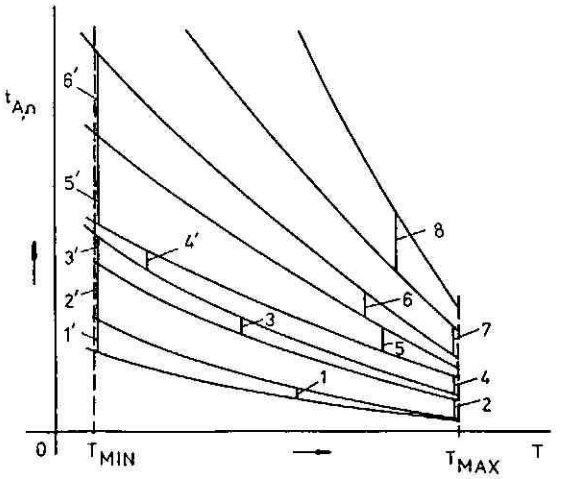

Fig. 6. Approximation functions of a nine-component mixture with marked temperature separability ranges.

Sequence 29 or 29a could serve for the design of an idealized temperature programme $T_P^*(t)$. Such a programme guarantees the separation of all components at $R \geqslant 1$ in a time $t_{AP,9} < t_{A,9}(T_H[3, 3])$, *i.e.*, in a time shorter than necessary for isothermal separation at $R \geqslant 1$. The idealized temperature programme starts, in accordance with sequence 29, with an isothermal step at $T_H[3, 3]$; the duration of this step is determined by the retention time of the fourth component, $t_{A,4}(T_H[3, 3])$. The temperature of the next programme step again follows from sequence 29. The second term $T_H[1, 1]$, however, is of no importance as the first pair of components will have left the column long before the elution of the fourth component is completed at the end of the first programme step. The next term, $T_H[6, 6]$, and the retention time of the seventh component, $t_{AP,7}$, therefore determine both the temperature and the duration of the second programme step. For $t_{AP,7}$ there holds

$$t_{A,7}(T_H[6, 6]) < t_{AP,7} < t_{A,7}(T_H[3, 3]) \tag{30}$$

The fourth term of sequence 29 is meaningless as both the fifth and sixth components will leave the column before the seventh component elutes. Therefore, $T_H[8, 8]$ is the temperature of the last programme step and the programme ends at $t_{AP,9}$:

$$t_{A,9}(T_H[8, 8] < t_{AP,9} < t_{A,9}(T_H[3, 3]) \tag{31}$$

The ideal temperature programme for the separation of the mixture from Fig. 6 is depicted in Fig. 7.

Generally, to design an ideal temperature programme, a new sequence is formed:

$$T_H[n_i, n_i] < T_H[n_j, n_j] < \ldots < T_H[n_1, n_1] \tag{32}$$

from eqn. 28 merely by excluding some of its terms to guarantee that

$$n_i < n_j < \ldots < n_1; \qquad n_{k+1} - n_k > 1 \tag{33}$$

The last inequality reflects the fact that, at the moment of elution of the kth component, the resolution of the k, $k + 1$ pair, $R_{k,k+1}$ is irrelevant. If $R_{k,k+1} < 1$ there is no possibility of influencing it; if $R_{k,k+1} > 1$ one has to focus on the resolution of the next component pair, $R_{k+1,k+2}$.

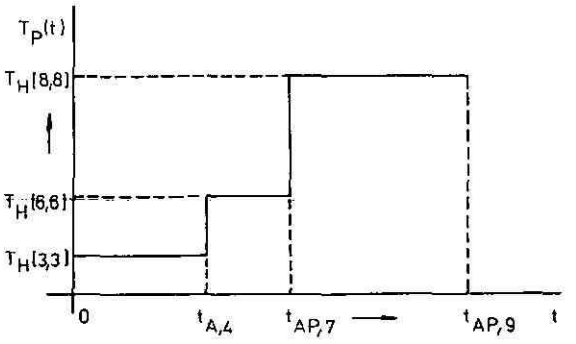

Fig. 7. Idealized temperature programme for the separation of the nine-component mixture from Fig. 6.

Sequence 29 now becomes

$$T_H[3, 3],\ T_H[6, 6],\ T_H[8, 8] \tag{34}$$

or in a concise form

$$3, 6, 8 \tag{34a}$$

The component pairs making up sequence 34 are called the most difficult to separate component pairs in the given mixture. The above-derived idealized temperature programme, $T_P^*(t)$, is of course unrealistic as it requires instant temperature changes of the column and, more important, does not satisfy optimization condition 26; it has served merely to illustrate the fundamental concept of the most difficult to separate component pair of a mixture.

With respect to the algorithm of the temperature programme optimization, sequence 32 and each of its members determine the strategy and substrategy, respectively, of the optimization process. The goal of the optimization strategy is to design the optimal temperature programme for a given separation. Consequently, the aim of each substrategy is to find the optimal subtrajectory $T_{P,k}(t)$, *i.e.*, one segment of the optimal temperature program $T_P(t)$.

To fulfil condition 26 there are merely instrumental limitations regarding the shape of the optimal segment $T_{P,k}(t)$. For the sake of simplicity, each segment $T_{P,k}(t)$ is assembled from two linear program sections, $T_{P,1,k}(t)$ and $T_{P,2,k}(t)$, one of which is isothermal and the other is represented by a linear temperature increase or decrease.

For a mixture containing N_s most difficult to separate component pairs (*i.e.*, requiring N_s substrategies), the maximum number of programme sections is $2\ N_s$. A completely separated N-component mixture consists of $N - 1$ component pairs. In accordance with the sequence 28:

$$T_H[n_1, n_1]\ T_H[n_2, n_2] \ldots T_H[n_{N-1}, n_{N-1}] \tag{35}$$

and the maximum number of the corresponding substrategies is determined by the component pairs 1, 3, ..., $N - 1$. The maximum number of substrategies for even and odd N is $N/2$ and $(N + 1)/2$, so the maximum number of linear programme sections is N or $N + 1$, respectively.

Fig. 8 presents two limiting acceptable subtrajectories $T_{P,1,k}(t)$ and $T_{P,2,k}(t)$ in the kth substrategy. In the following considerations, the subscript k in eqns. 1–10 is split into subscripts i, k, where $i = 1, 2$.

Solution of individual substrategies

The design of the first substrategy differs from all successive ones as $T_{N,1,1} = T_{S,1,1}$ and $T_{N,1,1}$ is an independent variable. In all other successive programme segments, the initial oven and column temperatures are different and are given by eqn. 3. Consequently, in the first substrategy we look for those values of the independent variables

$$T_{N,1,1},\ D_{1,1},\ D_{2,1},\ t_{1,1},\ t_{2,1} \tag{36}$$

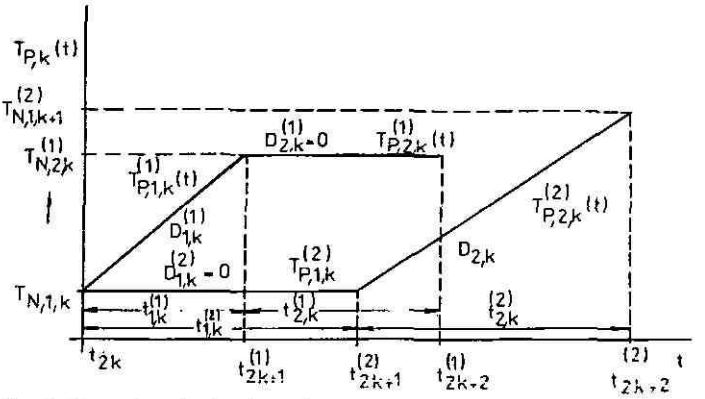

Fig. 8. Examples of subtrajectories.

that lead to the minimum of the criterion function, *i.e.*, that minimize the retention time of the second component of the first most difficult to separate component pair:

$$t_{AP,n_1+1} = t_{1,1} + t_{2,1} = \min \tag{37}$$

The criterion function 37 is defined by the sum of the upper integration limits in eqn. 8 and is subject to the following constraints:

$$\begin{aligned} &D_{\mathrm{MIN}} < D_{i,1} < D_{\mathrm{MAX}} \\ &T_{\mathrm{MIN}} < T_{P,i,1} < T_{\mathrm{MAX}} \\ &T_L[n_1, n_1] < T_{N,1,1} < T_H[n_1, n_1] \\ &0 < t_{2,1} < t_{A,n_1+1}(T_H[n_1, n_1]) \\ &R_{n_1,n_1+1} - 1 \geqslant 0 \\ &t_{1,1} + t_{2,1} < t_{\mathrm{MAX}} \end{aligned} \tag{38}$$

where t_{MAX} is an arbitrarily chosen time limit for a given separation. With regard to the next substrategy, it is desirable for the oven temperature at the end of the first substrategy, $T_{N,1,2}$, to approach the upper separability range of the next most difficult to separate component pair as closely as possible:

$$T_{N,1,2} = T_{P,2,1}(t_{2,1}) \rightarrow T_H[n_2, n_2] \tag{39}$$

In the second substrategy, the closer the subtrajectory approaches the optimum, the higher is the temperature at the end of the first substrategy. This is especially important in cases where $T_H[n, n]$ does not differ much from the maximum temperature T_{MAX}, and, consequently, the retention time cannot be decreased by further temperature increase. Condition 39 can be regarded as an extension of the constraints 38.

To solve the minimization problem 37, t_{AP,n_1}, s_{AP,n_1} and s_{AP,n_1+1} have to be calculated (see the resolution value R_{n_1,n_1+1} in constraints 38.

For components denoted by $n < n_1$, the values of

$$t_{AP,n}, s_{AP,n}, R_{n,n+1}, n = 1, 2, \ldots, n_1 - 1 \tag{40}$$

may be calculated according to eqns. 5–15. For the remaining components ($n > n_1$

+ 1), merely increments generated within the first substrategy are calculated:

$$\begin{aligned} {}^{1}I_{AP,n} &= I_{1,1,n} + I_{2,1,n} \\ {}^{1}t_{AP,n} &= t_{AP,n_1+1} \\ {}^{1}S_{AP,n} &= S_{1,1,n} + S_{2,1,n},\ n = n_1 + 2, n_1 + 3, \ldots, N \end{aligned} \tag{41}$$

where the left superscripts relate to the end of a given substrategy.

Generally, in the kth substrategy optimum values of

$$D_{1,k},\ D_{2,k},\ t_{1,k},\ t_{2,k} \tag{42}$$

are sought for by minimizing the criterion function

$$t_{AP,n_k+1} = t_{AP,n_{k-1}+1} + t_{1,k} + t_{2,k} = \min \tag{43}$$

and are subject to the following constraints:

$$\begin{aligned} &D_{\mathrm{MIN}} < D_{i,k} < D_{\mathrm{MAX}} \\ &T_{\mathrm{MIN}} < T_{P,i,k}(t) < T_{\mathrm{MAX}} \\ &t_{1,k} + t_{2,k} < t_{\mathrm{MAX}} \\ &R_{n_k,n_k+1} - 1 \geqslant 0 \\ &T_H[n_k, n_k] < T_{N,1,k+1} = T_{P,2,k}(t_{2,k}) \leqslant T_H[n_{k+j}, n_{k+j}] \\ &j = 1, 2, \ldots, N_s - k, \qquad i = 1, 2 \end{aligned} \tag{44}$$

In the last inequality, the subscript j normally equals unity. There are some instances, however, where the resolution R_{n_k,n_k+1} substantially exceeds unity and we may therefore continue increasing the temperature up to the next T_H value (which is an equivalent of $j = 2, 3, \ldots$).

After the values of the independent variables 42 have been determined, the values

$$I = \sum_{k=1}^{x} {}^{k}I_{AP,n};\quad t_{AP,n} = \sum_{k=1}^{x} {}^{k}t_{AP,n};\quad S_{AP,n} = \sum_{k=1}^{x} {}^{k}S_{AP,n} \tag{45}$$

for $n = n_{k-1} + 2, n_{k-1} + 3, \ldots, n_{k-1}$, and resolutions

$$R_{n,n+1} \text{ for } n = n_{k-1} + 1, n_{k-1} + 2, \ldots, n_k - 1 \tag{46}$$

are calculated.

The increments for the components $n = n_k + 2, n_k + 3, \ldots, N$ in the kth substrategy are expressed by

$$\begin{aligned} {}^{k}t_{AP,n} &= t_{1,k} + t_{2,k} \\ {}^{k}S_{AP,n} &= S_{1,k,n} + S_{2,k,n} \\ {}^{k}I_{AP,n} &= I_{1,k,n} + I_{2,k,n} \end{aligned} \tag{47}$$

In the last substrategy, the last constraint 44 becomes

$$T_{P,i,k}(t) < T_{MAX} \tag{48}$$

Generally, within the kth strategy the most difficult to separate components migrate in the column for a given time interval at $T > T_H[n_k, n_k]$ (where $R_{n,n+1} < 1$) and at $T < T_H[n_k, n_k]$ (where $R_{n,n+1} > 1$) for the remainder of the time.

CONCLUSIONS

This paper has introduced basic terms for the determination of the optimum temperature programme in the gas chromatographic separation of an arbitrary mixture on an arbitrary column under a constant carrier gas flow-rate. The optimization task, *i.e.*, the procedure of derivatization of substrategies and the introduction of conditions for the solution of substrategies is based on the approximation functions $t_{A,n}(T)$, $s_{A,n}(T)$ obtained during experimental isothermal analyses of the mixture.

LIST OF SYMBOLS

$A_{s,n}$ constant of the approximation function $s_{A,n}(T)$;
$A_{t,n}$ constant of the approximation function $t_{A,n}(T)$;
$B_{s,n}$ constant of the approximation function $s_{A,n}(T)$;
$B_{t,n}$ constant of the approximation function $t_{A,n}(T)$;
$C_{s,n}$ constant of the approximation function $s_{A,n}(T)$;
$C_{t,n}$ constant of the approximation function $t_{A,n}(T)$;
$D_{i,k}$ rate of the oven temperature increase or decrease in the kth substrategy and in the ith section;
D_k rate of the oven temperature increase or decrease in the kth programme step;
D_{MAX} maximum rate of the $D_{i,k}$ or D_k;
D_{MIN} minimum rate of the $D_{i,k}$ or D_k;
H_c time constant of the column in the oven;
$h(t)$ chromatogram;
L_c column length;
$L_{k,n}$ distance travelled by the nth component zone in the kth programme step;
$L_{k,n}(t)$ distance travelled by the nth component zone until time t in the kth programme step;
$L_{k,n}(0)$ distance travelled by the nth component zone until the beginning of the kth substrategy;
$L_{x,n}$ distance travelled by the nth component zone within the xth step when the zone leaves the column;
${}^{k}l_{AP,n}$ dimensionless distance travelled by the nth component zone within the kth substrategy;
${}^{k}l_{AP,n}(0)$ dimensionless distance travelled by the nth component zone until the beginning of the kth substrategy;
$l_{i,k,n}$ dimensionless distance travelled by the nth component zone within the kth substrategy and ith section;

$l_{k,n}$ dimensionless distance travelled by the nth component zone within the kth programme step;

$l_n(t)$ dimensionless distance travelled by the th component zone from the beginning until time t;

N total number of components in a given mixture;

N_S total number of substrategies in the strategy;

n_k, $n_k + 1$ most difficult to separate pair in the kth substrategy;

$R_H[n, m]$ resolution of the component pair n, m at the upper limit of the temperature separability range $T_H[n, m]$;

$R_H[n, n]$ resolution of the component pair n, $n + 1$ with non-intersecting approximation function $t_{A,n}(T)$, $t_{A,n+1}(T)$;

$R_L[n, m]$ resolution of the component pair n, m at the lower limit of the temperature separability range $T_L[n, m]$;

$R_L[n, n]$ resolution of the component pair n, $n + 1$ with non-intersecting approximation functions $t_{A,n}(T)$, $t_{A,n+1}(T)$;

$R_M[n, m]$ maximum resolution of the component pair n, m at the temperature $T_M[n, m]$ within the temperature range $\langle T_{MIN}, T_{MAX}\rangle$;

$R_M[n, n]$ maximum resolution of the component pair n, $n + 1$ with non-intersecting approximation functions $t_{A,n}(T)$, $t_{A,n+1}(T)$;

$R_{n,m}$ resolution of the component pair n, m at a given temperature;

$S_{AP,n}$ calculated zone width of the nth component at the column outlet;

${}^kS_{AP,n}$ calculated increment of the zone width of the nth component within the kth substrategy;

${}^kS_{AP,n}(0)$ calculated increment of the zone width of the nth compoonent until the beginning of the kth substrategy;

$S_{i,k,n}$ calculated increment of the zone width of the nth component within the kth substrategy and the ith section;

$S_{k,n}$ calculated increment of the zone width of the nth component within the kth programme step;

$S_{x,n}$ calculated increment of the zone width of the nth component within the xth step when the zone leaves the column;

$s_{A,n}(T)$ approximation function of the width at half-height of the nth component peak on temperature;

$s_{AP,n}$ calculated peak width of the nth component at the column outlet;

T temperature;

$T(t)$ actual temperature programme in the column;

$T_H[n, m]$ highest temperature at which the components n, m are separated with $R_{n,m} \geqslant 1$;

$T_H[n, n]$ highest temperature at which the components n, $n + 1$ with non-intersecting functions $t_{A,n}(T)$, $t_{A,n+1}(T)$ are separated with $R_{n,n+1} \geqslant 1$;

$T_{i,k}(t)$ actual temperature in the column within the kth substrategy and the ith section;

$T_k(t)$ actual temperature in the column within the kth programme step;

$T_L[n, m]$ lowest temperature at which the components n, m are separated with $R_{n,m} \geqslant 1$;

$T_L[n, n]$ lowest temperature at which the components n, $n + 1$ with non-intersecting function $t_{A,n}(T)$, $t_{A,n+1}(T)$ are separated with $R_{n,n+1} \geqslant 1$;

$T_M[n, m]$ temperature at which the components n, m are separated with the maximum resolution $R_{n,m}$;

$T_M[n, n]$ temperature at which the components n, $n + 1$ with non-intersecting function $t_{A,n}(T)$, $t_{A,n+1}(T)$ are separated with the maximum resolution $R_{n,n+1}$;

T_{MAX} maximum allowed temperature in the oven;

T_{MIN} minimum allowed temperature in the oven;

$T_{N,k}$ temperature in the oven at the beginning of the kth programme step;

$T_{N,i,k}$ temperature in the oven at the beginning of the ith section in the kth substrategy;

$T_P^*(t)$ idealized temperature programme;

$T_P(t)$ temperature programme in the column;

$T_{P,k}(t)$ kth step in the temperature programme $T_P(t)$;

$T_{P,i,k}(t)$ temperature programme in the column within the kth substrategy and the ith section;

$T_{S,k}$ temperature in the column at the beginning of the kth program step;

$T_{S,i,k}$ temperature in the column at the beginning of the ith section in the kth substrategy;

$T_X[n, m]$ temperature at which the functions $t_{A,n}(T)$ and $t_{A,m}(T)$ intersect;

$T_x(t_{x,n})$ temperature at which the nth component leaves the column outlet;

t time;

$t_{A,n}(T)$ approximation function of the retention time of the nth component on temperature;

$t_{AP,n}$ calculated retention time of the nth component at the column outlet;

${}^k t_{AP,n}$ calculated retention time of the nth component within the kth substrategy;

t_k duration of the kth programme step;

$t_{i,k}$ computed duration of the temperature programme $T_{P,i,k}(t)$;

t_{MAX} maximum allowed time of analyses;

$t_{x,n}$ computed duration of the xth temperature programme step when the nth component leaves the column outlet.

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OPTIMIZATION OF TEMPERATURE PROGRAMMING IN GAS CHROMATOGRAPHY WITH RESPECT TO SEPARATION TIME

II*. OPTIMIZATION OF THE INDIVIDUAL TEMPERATURE PROGRAMME SUBSTRATEGIES

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SUMMARY

The temperature programme optimization substrategies for a mixture with non-intersecting retention time approximation functions are described. For mixtures of compounds whose elution functions intersect, the determination of strategies for possible solutions and of the corresponding substrategies for temperature programme optimization is derived. Heuristic methods for the minimization of the retention times of the most difficult to separate component pairs are presented. Further, the calculation of retention times and peak widths for optimization purposes is discussed.

INTRODUCTION

In Part I[1], the problem of optimizing the temperature programme for a given separation problem on a given column was converted into a minimization problem of retention times of the most difficult to separate component pairs. It was shown that these component pairs determine the total number of substrategies or the total number of partial optimization problems. The analysis was limited to mixtures that do not contain components with intersecting retention time approximation functions while simultaneously obeying the inequality

$$\max_n\{T_L[n, n]\} < \min_n\{T_H[n, n]\} \qquad n\in\langle 1, 2, \ldots, N-1\rangle \tag{1}$$

* For Part I, see ref. 1. Symbols used here are defined in Part I.

(*i.e.*, mixtures of type I). These mixtures represent the majority of instances in practice.

The optimization in this instance is equivalent to the minimization of a single strategy, resulting in a single optimal temperature programme. If condition 1 is not met, at least one of the substrategies is split into two independent substrategies. In this event there is not necessarily a single optimum solution (a single optimum temperature programme).

For mixtures with intersecting approximation functions (mixtures of type II) several strategies can be derived, none of them necessarily fulfilling all the constraints. In this instance no single optimum temperature programme exists.

The optimization of each substrategy is equivalent to the minimization of the retention time of the second component of that component pair which determines the given substrategy. At the same time, several constraints[1] have to be fulfilled. For this purpose, the standard NAG library minimization programs[2,3], based on minimization of adjacent Lagrange functions by gradient methods, were originally utilized. In the course of the optimization experiments, the disadvantages of these methods became evident (inefficient consumption of both computing time and computer memory). Therefore, a heuristic minimization algorithm has been developed.

DISCUSSION

Decomposition of substrategies

Fig. 1 illustrates the approximation functions of a type I mixture. The components n_z and $n_z + 1$ belong to the kth substrategy, and therefore

$$T_L[n_z, n_z] > T_H[n_k, n_k] \tag{2}$$

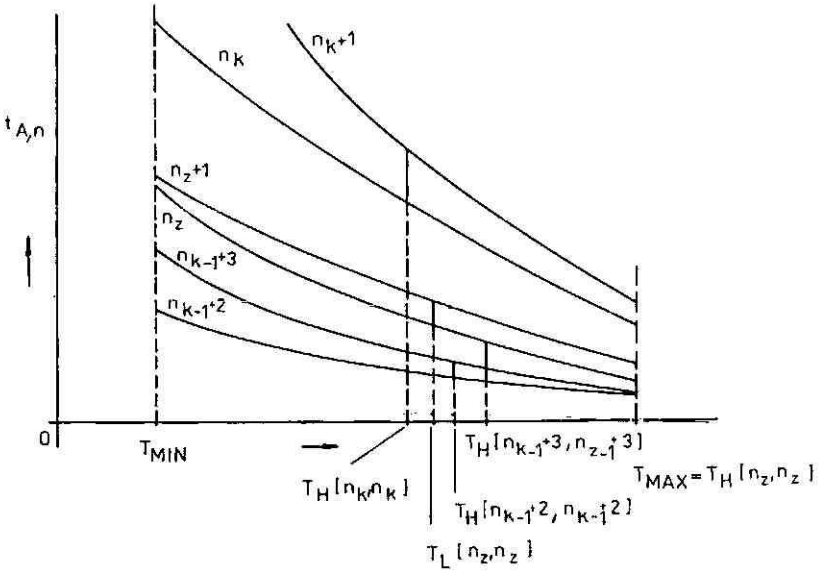

Fig. 1. Subtrajectory for the component pair n_z, $n_z + 1$.

For all the other components $n \in \langle n_{k-1} + 2, n_k - 1 \rangle$ there holds:

$$T_L[n_z, n_z] < T_H[n, n] \tag{3}$$

Consequently, the original kth substrategy decomposes into two independent substrategies k_1 and k_2. The k_1th substrategy relates to component pair n_z, $n_z + 1$ and the k_2th substrategy to component pair n_k, $n_k + 1$. For the $k - 1$th substrategy, the last constraint (*cf.*, Part I, eqn. 44) is

$$T_{N,1,k_1} = T_{P,k-1}(t_{2,k-1}) > T_L[n_z, n_z] \tag{4}$$

In the k_2th substrategy, we seek a temperature programme $T_{P,k_2}(t)$ involving one segment with a linear temperature decrease[1]. Generally, with substrategies including components of the n_z, $n_z + 1$ type, there is not necessarily an optimal temperature programme as all the possible programmes lead to $R_{n_z, n_z+1} < 1$ or $R_{n_k, n_k+1} < 1$. In this instance two independent separations are necessary.

If for the n_z, $n_z + 1$ component pair in the kth substrategy the inequality

$$T_L[n_z, n_z] < T_H[n, n] \qquad n = n_{k-1} + 2, \ldots, n_k + 1 \tag{5}$$

is valid and, simultaneously

$$T_H[n_{k-1} + 2, n_{k-1} + 2] > T_H[n_{k-1} + 3, n_{k-1} + 3] > \ldots > T_L[n_z, n_z] \tag{6}$$

(where $n_k = n_z + 2$) holds, the kth substrategy decomposes into $[n_z - (n_{k-1} + 2)]/2 + 2$ individual substrategies, provided that $n_z - (n_{k-1} + 2)$ is even; otherwise the number of substrategies is $n_z - (n_{k-1} + 2) + 1]/2 + 2$.

Determination of substrategies for mixtures of type II

The formulation of this problem is analogous to that for mixtures with non-intersecting approximation curves (mixtures of type I). For mixtures of type I there is only one strategy, *i.e.*, a single sequence of substrategies leading to a single optimum temperature programme. In an extreme case, if some of the substrategies decompose, no single optimum temperature programme must necessarily exist. For type II mixtures, there are a number of possible strategies depending on the number of intersecting pairs. Some of these (possibly all) strategies will not lead to a satisfactory solution and other ones might, but only one of the solvable strategies leads to the shortest retention time of the last component. This strategy has the optimum trajectory.

Mixtures of type II are characterized[1] by non-zero elements $T_L[n,m]$ and $T_L[m,n]$ in the matrices $T_L[N, N]$ and $T_H[N, N]$. Fig. 2 illustrates a mixture of type II containing two pairs of components with intersecting approximation functions (components 2, 3 and 5, 6). The vertical solid lines between the curves mark the upper temperature separability limit of the particular pair of components, and the dot-and-dash line marks the lower limit. In the matrices $T_H[N, N]$ and $T_L[N, N]$ there are, in addition to the diagonal elements, also elements with subscripts 2, 3; 3, 2; 5, 6; and 6, 5. Let the subrow belonging to the nth element be denoted as a group of

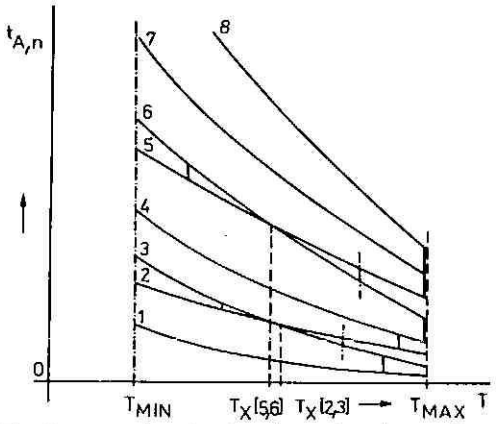

Fig. 2. Approximation functions of a mixture of type II.

elements with subscripts n, n; $n, n+1;\ldots;$ n, N and the subcolumn as a group of elements with subscripts n, n; $n+1, n;\ldots;$ N, n. For determining the individual strategies, the elements of the principal diagonal of the matrix $T_H[N, N]$ are ordered according to their value. If the elements are out of the diagonal in some row (and thus in some column), first of all the lowest temperature value in the subrow (or in the subcolumn) is sought for and used as an element of the strategy, provided that

$$\max_m\{T_L[n, m]\} < \min_m\{T_H[n, m]\} \qquad m = n, n+1,\ldots, N \tag{7}$$

or

$$\max_m\{T_L[m, n]\} < \min_m\{T_H[m, n]\} \qquad m = n, n+1,\ldots, N \tag{8}$$

The conditions 7 and 8 signify that the components with intersecting approximation functions are separated with $R_{n,m} > 1$ at the lowest temperature of the subrow (or the subvcolumn). On the other hand, this temperature value cannot be used for determining the substrategies, because there will always be the possibility of insufficient separation ($R_{n,m} < 1$) of at least for one component pair.

Hence every row in the matrix $T_H[N, N]$ with non-zero elements out of the principal diagonal doubles every strategy step if conditions 7 and 8 are fulfilled. In the case illustrated in Fig. 2, four strategies are theoretically possible, given by the substrategies for the most difficult to separate pairs: I, 6–5; II, 3–2, 5–6; III, 2–3, 6–5, 7; and IV, 2–3, 5–6, 7 (6–5 refers to the pair of components 6 and 5, and 7 to the pair of components 7 and 8). Generally, if the approximation functions of the mixture intersect n times, then maximally 2^n strategies exist.

If none of these strategies is satisfactory (*i.e.*, in every strategy then is at least one pair with resolution $R_{n,m} < 1$), more analyses must be executed with partial temperature programmes.

Two situations may influence the design of individual substrategies. In the mixture being analysed, there might be component pairs or groups that are not of

interest; hence their resolution can be arbitrary. If such a pair happens to be the most difficult to separate pair, the corresponding substrategy is either changed or deleted. The other situation is exemplified by mixtures that contain components that cannot be separated at all ($R_M[n, m] < 1$). Any temperature programme other than $T_P(t) = T_M[n, m]$ leads to a further resolution loss with pairs of this type.

Heuristic method of optimization

The retention time of a component $t_{AP, n_1 + 1}$ is minimized in the first substrategy while looking for the optimal temperature incrtements $D_{1, 1}$; $D_{2, 1}$, and for the corresponding time intervals $t_{1, 1}$; $t_{2, 1}$. Moreover, the independent variable $T_{N, 1, 1}$, *i.e.*, the initial temperature at the beginning of the temperature programme, is optimized.

The heuristic method for the minimization of the retention times of the most difficult to separate components is based on the following considerations, as explained for a mixture of type I. The most difficult to separate components in the kth substrategy migrate, until the components corresponding to the previous substrategy (n_{k-1}, $n_{k-1} + 1$) are eluted, at a mean velocity lower than the characteristic velocity (the mean velocity of the components during isothermal analysis at a temperature T_H, at which the components are separated with $R_{n, n+1} = 1$)[2]. Hence, their resolution in the column is greater than 1 until this moment. After the elution of the components n_{k-1}, $n_{k-1} + 1$, a subtrajectory $t_{1, k}$, $D_{1, k}$, $t_{2, k}$, $D_{2, k}$ is calculated, so that the pair n_k, n_{k+1} is eluted in the shortest time ($t_{1, k} + t_{2, k} =$ min). As the resolution value for the components n_k, $n_k + 1$ at the beginning of the kth substrategy in the column is greater than 1, the substrajectory at which the column temperature $T(t) > T_H[n_k, n_k]$ can be sought in the kth substrategy. At these temperatures, in isothermal separations, the resolution would be insufficient: $R_{n_k, n_k + 1} < 1$. However, in the subtrajectory considered at this temperature, the

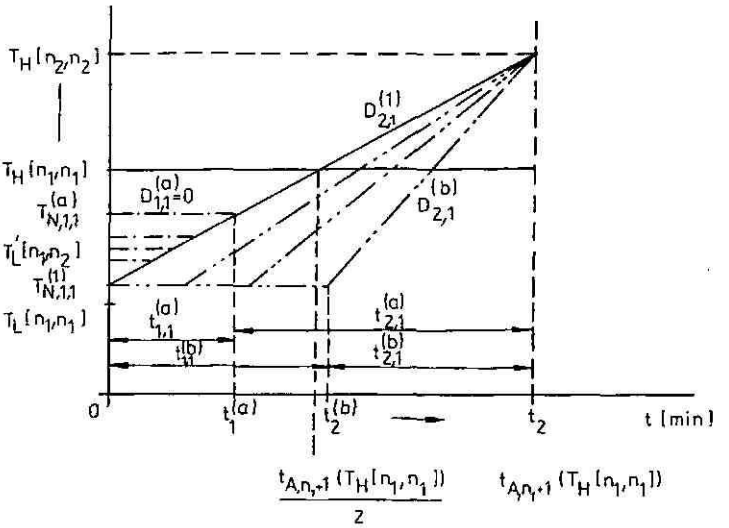

Fig. 3. Optimization of subtrajectories in the 1st substrategy.

resolution of the peak pair decreases simultaneously with the component retention time increments.

By iteration, $t_{AP,\,n_k+1}$ can be minimized. The values of $t_{1,\,k}^{(i)}$, $D_{1,\,k}^{(i)}$, $t_{2,\,k}^{(i)}$ and $D_{2,\,k}^{(i)}$ in the ith iteration step are determined according to the magnitude of $R_{n_k,\,n_k+1}^{(i-1)}$, the boundary conditions being fulfilled[1]. For $R_{n_k,\,n_k+1}^{(i-1)} > 1$, the programmed temperature can be increased in the iteration step $i+1$ and, consequently, the value of $t_{AP,\,n_k+1}$ can be decreased. In contrast, a temperature decrease leads to an increase in $t_{AP,\,n_k+1}$ for $R_{n_k,\,n_k+1}^{(i-1)} < 1$.

Consider first the 1st substrategy where a course of the temperature programme $T_{P,\,1}(t)$, consisting of two linear sections, is searched for so that $R_{n,\,n+1} = 1$. Simultaneously, the temperature at the end of the first subtrajectory should be as close as possible to the temperature of $T_H[n_2, n_2]$. The shortest retention time of the component $n_1 + 1$ on isothermal separation, $t_{A,\,n_1+1}(T_H[n_1, n_1])$, is known from the temperature separability ranges of the components. The temperature programme will be partially below the value of $T_H[n_1, n_1]$, and partially above this value. The retention time will be $t_{AP,\,n_1+1} \approx t_{A,\,n_1+1}(T_H[n, n])$. Fig. 3 illustrates the processes leading to such temperature programmes. The first estimation of the trajectory $D_{1,\,1}^{(1)}$, $t_{2,\,1}^{(1)}$, $D_{2,\,1}^{(1)}$, $t_{2,\,1}^{(1)}$ and $T_{N,\,1,\,1}^{(1)}$ is determined as follows. The values of the temperature increments will be chosen as

$$\begin{aligned} D_{1,\,1}^{(1)} &= 0 \\ D_{2,\,1}^{(1)} &= 2(T_H[n_2, n_2] - T_H[n_1, n_1])/t_{A,\,n_1+1}(T_H[n_1, n_1]) \end{aligned} \tag{9}$$

and the initial temperature

$$T_{N,\,1,\,1}^{(1)} = T_H[n_2, n_2] - D_{2,\,1}^{(1)}\, t_{A,\,n_1+1}(T_H[n_1, n_1]) \tag{10}$$

If $T_{N,\,1,\,1}^{(1)} < T_L[n_1, n_1]$, which is indicated by $T_L[n_1, n_1]$ in Fig. 3, then $t_1^{(1)} \neq 0$, but holds that

$$t_1^{(1)} = \{T_L'[n_1, n_1] - (T_H[n_2, n_2] - D_{2,\,1}^{(1)}\, t_{A,\,n_1+1}(T_H[n_1, n_1]))\}/D_{2,\,1}^{(1)} \tag{11}$$

and expression 10 reduces to

$$T_{N,\,1,\,1}^{(1)} = T_L[n_1, n_1] \tag{12}$$

For a trajectory determined in this way $t_{AP,\,n_1}^{(1)}$, $t_{AP,\,n+1}^{(1)}$, $s_{AP,\,n_1}^{(1)}$, $s_{AP,\,n_1+1}^{(1)}$ and $R_{n_1,\,n_1+1}^{(1)}$ are calculated. According to the magnitude of the value of $R_{n_1,\,n_1+1}^{(1)}$ iterations are started. For $R_{n_1,\,n_1+1}^{(1)} > 1$, the value of $T_{N,\,1,\,1} \in \langle T_{N,\,1,\,1}^{(1)}, T_H[n_1, n_1]\rangle$ is increased until the value of $T_{N,\,1,\,1}^{(a)}$ for which $R_{n_1,\,n_1+1}^{(a4)} = 1$ is found. The subtrajectory is then determined by the values of $T_{N,\,1,\,1}^{(a)}$, $t_{1,\,1}^{(a)}$, $D_{1,\,1}^{(a)} = D_{1,\,1}^{(1)}$, $t_{2,\,1}^{(a)}$, $D_{2,\,1}^{(a)} = D_{2,\,1}^{(1)}$. If $R_{n_1,\,n_1+1}^{(1)} < 1$, $T_{N,\,1,\,1}^{(1)}$ remains constant and $t_{1,\,1}$ is changed in the course of the iterative calculations. Consequently, the magnitude of $D_{2,1}$ is adapted until the values of $T_{N,\,1,\,1}^{(1)}$, $t_{1,\,1}^{(b)}$, $t_{2,\,1}^{(b)}$, $D_{1,\,1}^{(1)}$, $D_{2,\,1}^{(b)}$ for which $R_{n_1,\,n_1+1} = 1$ are found. The value of $D_{2,\,1}$ is restricted by the condition $D_{2,\,1} \leqslant D_{\mathrm{MAX}}$; if the solution leads to greater $D_{2,1}$

values then $D^{(b)}_{2,1} = D_{MAX}$. By subsequent calculations a subtrajectory is derived with a value for $T_{N,1,2} < T_H[n_2, n_2]$. Then it holds for the initial temperature:

$$T^{(1)}_{N,1,1} = T_H[n_1, n_1] - D_{MAX}\, t_{A,n_1+1}(T_H[1, n_1])/2 \tag{13}$$

For $T^{(1)}_{N,1,1} < T_L[n_1, n_1]$ eqn. 11 is modified to

$$t^{(1)}_{1,1} = \{T_L[n_1, n_1] - (T_H[n_1, n_1] - D_{MAX}\, t_{A,n_1+1}(T_H[n_1, n_1]))\}/D_{MAX} \tag{14}$$

According to the magnitude of the resolution $R^{(1)}_{n_1,n_1+1}$, the subtrajectory is calculated in the same way as in the previous case.

Calculation of kth substrategy

The temperature $T_{N,1,k}$ at the beginning of the kth substrategy is known. Fig. 4 shows the procedure for looking up the kth subtrajectory. For the enitial temperature programme in the kth subtrajectory we chose

$$\begin{aligned} D_{1,k} &= D_{MAX} \\ t^{(1)}_{1,k} &= (T_H[n_{k+1}, n_{k+1}] - T_{N,1,k})/D_{1,k} \\ D^{(1)}_{2,k} &= 0 \end{aligned} \tag{15}$$

and the value of $t^{(1)}_{2,k}$ is implicitly given by the calculated value of t_{AP,n_k+1}[*i.e.*, $t^{(1)}_{2,k} = t_{AP,n_k+1} - t^{(1)}_{2k+1}$]. If $T_{N,1,k} > T_H[n_{k+1}, n_{k+1}]$ holds, the calculation of $t^{(1)}_{1,k}$ involves the nearest higher temperature which fulfils the condition $T_{N,1,k} < T_H[n_{k+j}, n_{k+j}]$. Next, the values of t_{AP,n_k}, t_{AP,n_k+1}, s_{AP,n_k} s_{AP,n_k+1} and R_{n_k,n_k+1} are calculated. If $R^{(1)}_{n_k,n_k+1} > 1$, the other temperature programme of the same shape with the values of $t^{(2)}_{1,k} > t^{(1)}_{1,k}$ is chosen. By

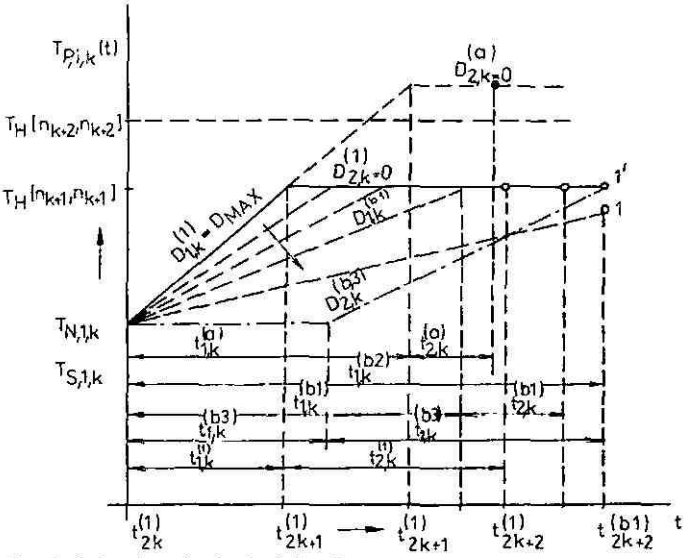

Fig. 4. Subtrajectories in the kth substrategy.

iteration, the value of $t_{1,k}^{(a)}$ for which $R_{n_k,\,n_k+1}^{(a)} = 1$ is calculated. If $R_{n_k,\,n_k+1}^{(1)} < 1$, the temperature programme in the iteration steps is set according to eqn. 15 and, at the same time, the value of $D_{1,k}^{(i)}$ is decreased [thus increasing the value of $t_{1,k}^{(i)}$]. By approximating $R_{n_k,\,n_k+1}^{(b1)} = 1$ at the temperature $T_{N,2,k} = T_H[n_k, n_k]$, the calculations are stopped. For $T_{N,2,k} < T_H[m_k, n_k]$ (the end point of such subtrajectory is marked by the number 1 in Fig. 4) a subtrajectory is searched for, leading to the same retention times and ending at the temperature $T_{N,2,k}$ (point 1 in Fig. 4). The optimum subtrajectory is defined by

$$\begin{aligned} &D_{1,k}^{(1)} = 0 \\ &t_{1,k}^{(i)} + t_{2,k}^{(i)} = t_{1,k}^{(b2)} \\ &0 < D_{2,k}^{(i)} < D_{\mathrm{MAX}} \end{aligned} \tag{16}$$

At the same time, the value of $D_{2,k}^{(b3)}$ and the corresponding time intervals are determined:

$$\begin{aligned} t_{n,k}^{(b3)} &= (T_H[n_{k+1}, n_{k+1}] - T_{N,1,k}^{(i)})/D_{2,k}^{(b3)} \\ t_{1,k}^{(b3)} &= t_{1,k}^{(b2)} - t_{2,k}^{(b3)} \end{aligned} \tag{17}$$

and

$$R_{n_k,\,n_k+1}^{(b3)} \approx 1; \qquad T_{N,1,k+1} \approx T_H[n_{k+1}, n_{k+1}]$$

The heuristic procedure described is applied for the calculation of the optimum trajectory of type I mixtures. The same procedure is used in the case of split subtrajectories and for type II mixtures. The programme always looks for the first local minimum $t_{AP,\,n_k+1}$, fulfilling the given conditions for the prescribed shape of linear temperature sections.

Calculation of retention times and peak widths

While optimizing the temperature programme, the retention time increments, the zone widths and the position of components that are still in the column are calculated in individual substrategies. The distance passed by a zone in the kth substrategy is given by[1]

$${}^{k}l_{AP,\,n} = l_{1,k,n} + l_{2,k,n} = \int_0^{t_{1,k}} 1/t_{A,\,n}[T_{1,k}(t)]\mathrm{d}t + \int_0^{t_{2,k}} 1/t_{A,\,n}[T_{2,k}(t)]\mathrm{d}t \tag{18}$$

In practice, two cases can occur: either $t_{1,k}$ and $t_{2,k}$ are known, or one and possibly both upper limits of the integral in eqn. 18 are unknown. In the latter instance, these times are calculated from

$$1 = \sum_{i=1}^{k-1} {}^{i}l_{AP,\,n} + {}^{k}l_{AP,\,n} \tag{19}$$

The upper limits of the integrals 18 are known for the components $n \in \langle n_{k+2}, N \rangle$ in the kth substrategy. Hence, $t_{1,k}$ and $t_{2,k}$ are implicitly determined by the retention time of the slower component from the pair determining the kth substrategy[1]. The upper limit of at least one integral in eqn. 18 is unknown for the components $n \in (n_{k-1} + 2, n_k + 1)$. The peak widths are always calculated for the known values of the upper limits[1].

The calculation of the function values from eqn. 18 can be divided into two groups: calculation of a definite integral of an analytically given function: and calculation of the integral to its upper limit. The calculation of a definite integral is one of the most com,mon problems in numerical mathematics. Popular solution methods are those of Newton-Cotes, Tschebyshew and Gaus[4]. The implementation of these methods on computers requires discrete numbers of points of the integrated functions. The number of points can be adapted to the integration range according to the slope of the integrated function[5].

The calculation oif eqn. 18 and the zone width increment, eqn. 13[1], can be transformed into the solution oif a set of two linear differential equations. The first equation in the kth substrategy is

$$\frac{\mathrm{d}^k l_{AP,n}}{\mathrm{d}t} = 1/t_{A,n}[T_k(t)] \tag{20}$$

with the initial condition

$${}^k l_{AP,n}(0) = \sum_{i=1}^{k-1} {}^i l_{AP,n} \tag{21}$$

and the calculation is terminated if

$$l_{AP,n}({}^k t_{AP,n}) = 1 \tag{22}$$

The second equation is

$$\frac{\mathrm{d}^k S_{AP,n}}{\mathrm{d}t} = \left[s_{A,n}[T_k(t)] \frac{L_c}{t_{A,n}[T(t)]} \sqrt{l_n(t)} \right] \tag{23}$$

with the initial condition

$${}^k S_{AP,n}(0) = \sum_{i=1}^{k-1} {}^i S_{AP,n} \tag{24}$$

In the first substrategy it is supposed that ${}^1 l_{AP,n}(0) = l_{MIN}$, which does not influence the solution significantly but removes the effect of singularity in eqn. 23 for $t = 0$. The differentials can be solved by any numerical method utilized for the solution of linear differential equation systems, *e.g.*, by Runge-Kutta's, Adam's or Gear's methods[6].

CONCLUSION

The arrangement of substrategies in single strategies for the optimization of temperature programmes for type II mixtures has been described. An heuristic method for the minimization of the retention times of difficult to separate pairs of components, determining individual substrategies, has been presented in detail. This method is advantageous with respect to demands on computer memory and convergency speed compared with commercial programs for minimization (*cf.*, the libraries supplied with medium- or large-sized computers).

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DETERMINATION OF PIPERAZINE IN WORKING ATMOSPHERE AND IN HUMAN URINE USING DERIVATIZATION AND CAPILLARY GAS CHROMATOGRAPHY WITH NITROGEN- AND MASS-SELECTIVE DETECTION

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SUMMARY

A reliable routine method is presented for the determination of piperazine down to the sub-ppm level in aqueous solutions and in urine. The method includes a two-phase derivatization procedure with ethyl- or isobutyl chloroformate as the reagent, followed by a capillary gas chromatographic determination using nitrogen- or mass selective detection. The addition of ammonia ensured a quantitative recovery. Detection limits for piperazine in urine were *ca.* 20 ng/ml using nitrogen-selective and *ca.* 1 ng/ml with mass-selective detection. The calibration plots were linear in the investigated range, 100–10 000 ng/ml with nitrogen-selective and 30–3000 ng/ml with mass-selective detection. The precision was *ca.* 6% at a concentration of 300 ng/ml. Acid anhydrides were investigated as alternative reagents in the two-phase derivatization procedure, and heptafluorobutyric acid anhydride in aqueous solutions gave approximatively 100% recovery. However, in urine the recoveries of the investigated acid anhydride derivatives were unsatisfactory.

INTRODUCTION

Piperazine is a substance with many applications, *e.g.*, as a constituent of drugs, as an hardener in the manufacture of polymers and as an antioxidant. It and its derivatives are produced in large quantities, leading to considerable risks of work place contamination.

During the last 10–15 years, evidence of the potential health hazards of amines has been presented[1–4]. Hagmar *et al.*[5] have shown that piperazine in the air can induce asthma. Furthermore, some of the piperazine inhaled is transported with saliva to the stomach, where it partly reacts with nitrite forming the presumptively carcinogenic N-mononitrosopiperazine[6]. As a consequence, knowledge of the exposure levels in air and their relationship to human uptake and secretion is important. The large

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number of samples which need to be analysed in this case, both of the air and of body fluids such as urine, makes the development of simple routine methods highly desirable.

Older colorimetric methods as used by Rogers[7] and modified by Hanna and Tang[8] are not sufficiently specific and sensitive for analysis of piperazine at low concentrations in urine. A more recent method presented by Fletcher *et al.*[9] is based on acylation by acetic acid anhydride and subsequent analysis by gas chromatography (GC) on packed columns. However, the working range, 50–500 μg/ml of piperazine in urine, is much higher than that applicable for biological monitoring of occupational exposure. Furthermore, the use of acetic acid anhydride is questionable, since as suggested in their paper, acylated piperazine may also be formed by biotransformation.

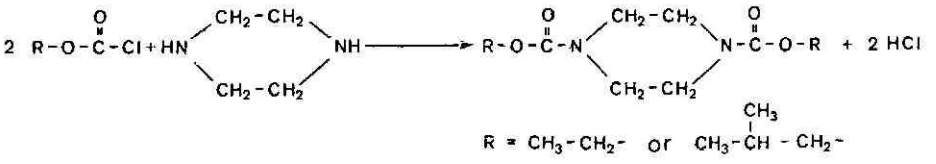
Methods used up to now in our laboratories for air samples (see ref. 5) do not have the desired simplicity for large numbers of samples. Furthermore, the precision of about 30% is not always sufficient. More recent methods for determination of free amines, including piperazine, in aqueous salt solutions developed at our laboratories[10,11] give a precision of about 2% and would seem applicable also to urine samples. However, with a matrix of urine there are certain drawbacks. Repeated injections result in changes of the chromatographic system, manifested as broadened peaks and prolonged retention times, necessitating frequent calibrations.

For routine measurements there is great value in a stable chromatographic system with no memory effects. Thus a work-up procedure such as extraction is in this case more or less necessary. If a derivatization step is also included, which usually gives a substance of lower basicity and polarity, it would be possible to use highly efficient capillary columns for the GC determinations. For piperazine, which is very soluble in water, a direct extraction to an organic solvent is not relevant. In such cases a two-phase derivatization which simultaneously combines extraction and derivatization is very attractive. Such procedures for amines in aqueous solutions have been developed with chloroformates[12] and acid anhydrides[13] and acid chlorides[14] as reagents.

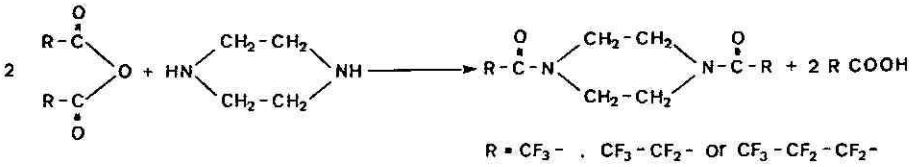
Air sampling of amines is generally performed in acidified aqueous solutions. For highly water-soluble amines such as piperazine this procedure gives 100% sampling efficiency. The determination of piperazine in both air and urine is accordingly performed from a water-based matrix. The possibility of using a similar work-up procedure for both air and urine samples has been taken into account in our work towards routine methods for piperazine determination.

In this paper we describe procedures for the routine determination of piperazine in aqueous solutions and in urine. They are based on two-phase derivatization followed by capillary GC using nitrogen-selective or mass-selective detectors. The derivatization reactions are illustrated below.

Carbamate formation:



Amide formation:



EXPERIMENTAL

Equipment

A Varian Model 3700 gas chromatograph was equipped with a Varian thermionic specific detector and a Grob-type on-column injection system with water-cooling designed and manufactured in our laboratories. Typical settings for the detector were: gas flow-rates, 6 ml/min of hydrogen and 200 ml/min of air; bead heating current, 5.3 scale divisions; bias voltage, 10 V; detector temperature, 270°C. Make-up gas for the detector (6 ml/min of nitrogen) was added to a carrier gas of helium. The carrier gas was dried over molecular sieve 5A and deoxygenated using an "Indicating Oxytrap" (Chrompack, Middelburg, The Netherlands). Chromatograms were recorded on Servogor Model 310 recorders and a Hewlett-Packard Model 3390 A integrator was used for peak evaluation. A Shimadzu GCMS-QP1000 EI/CI quadrupole gas chromatograph–mass spectrometer with a Shimadzu autosampler (AOC-9) was used for identification and quantitative routine analysis. The autosampler was used in connection with a Shimadzu splitless injection system (SPL-69). An Heidolph 2001 Rotavapor, rotating evaporator (Heidolph Elektro, Kelheim, F.R.G.) connected to an aspiration pump was used for evaporation. A Model 3E-1 centrifuge (Sigma, Harz, F.R.G.) was used for separation of phases.

Columns

A Duran 50 borosilicate glass capillary column (15 m × 0.32 mm I.D.) coated with PS-255 as stationary phase (film thickness 0.75 μm), produced in our laboratories[13], was used in the Varian GC system. Two other columns supplied by Chrompack were used both in the Varian and in the Shimadzu GC systems. These columns, Chrompack Aryl 17 CB (15 m × 0.32 mm I.D., film thickness 0.15 μm) and Chrompack CP-Sil 8CB (25 m × 0.32 mm I.D., film thickness 1.1 μm), are both chemically bonded.

Chemicals

Chemicals used were piperazine, piperazine hydrochloride, ethyl chloroformate, isobutyl chloroformate and toluene from Janssen (Beerse, Belgium), trifluoroacetic acid anhydride (TFAA), pentafluoropropionic acid anhydride (PFPA) and heptafluorobutyric acid anhydride (HFBA) from Pierce (Rockford, IL, U.S.A.) and di-*n*-butylamine from Fluka (Buchs, Switzerland).

Preparation of standard solutions of piperazine

Standard solutions were prepared by spiking alkaline aqueous solutions or urine with piperazine. These solutions were used for the investigations of recovery

and the linearity of peak height *versus* added amount of piperazine after performing the whole analysis procedure. Peak heights were compared to those of standards of the derivatives prepared as outlined below.

Synthesis of piperazine derivatives

Carbamate standards. Piperazine (10 g) was added to a mixture of 200 ml of 5 *M* sodium hydroxide and 200 ml of toluene, and about 5 ml of pyridine. The chloroformate reagent (40 ml) was added dropwise during 5 min with constant stirring. The toluene layer was separated and washed several times with distilled water. After evaporation of the toluene solvent in a rotating evaporator, the residue was dissolved in toluene–isooctane (1:1) and recrystallized at about −15°C.

Amide standards. Each amide standard was prepared by adding the acid anhydride reagent to a solution of piperazine in toluene. The completeness of the reaction was monitored by GC with flame ionization detection (FID). The solution was shaken with a 1 *M* phosphate buffer at pH 10 to remove the excess of reagent and acid formed. The toluene solution was separated and further diluted in toluene to the appropiate concentration of the amide derivative. Standard solutions prepared in this way are stable for at least 3 weeks at room temperature at concentrations corresponding to about 100 ng/ml of piperazine.

Two-phase derivatization and sample work-up procedure

With chloroformates as the derivatization reagents. A 1-ml aqueous sample was added to a mixture of 2 ml of 1 *M* phosphate buffer at pH 10, 2 ml toluene and 1 ml of 2.5% (w/w) aqueous ammonia. The mixture was shaken and 20 μl of chloroformate reagent were added. The mixture was shaken for 1 min and centrifuged at 4000 rpm for 40 min. A 1-ml aliquot of the toluene layer was evaporated for about 15 min. For the ethyl chloroformate derivative, the temperature was kept at 25°C and for the isobutyl chloroformate derivative, at 40°C. A 1-ml volume of toluene was added to the dry residue. The toluene solution contained an internal standard comprising the isobutyl chloroformate derivative of di-*n*-butylamine at the appropriate concentration. The toluene layer was analysed by GC. The same procedure was used for urine samples.

With acid anhydrides as the derivatization reagents. A 2-ml aqueous sample was added to a mixture of 2 ml toluene and 2 ml of 1 *M* phosphate buffer at pH 10. The mixture was shaken, 40 μl acid anhydride were added and the mixture was immediately shaken vigorously for 1 min. An aliquot of the toluene layer was analysed by GC.

RESULTS AND DISCUSSION

Standards

The identity of the piperazine derivatives was confirmed by gas chromatography–mass spectrometry (GC–MS). The purity of the derivatives was checked by GC using FID and was higher than 99%. For the chloroformate derivatives, the preferred products for routine analysis, the purity was further examined by elemental analysis. The experimental values for carbon, hydrogen and nitrogen differed by less than 0.2% from the calculated ones. The remaining amount of chloroformate reagent

was controlled by determining the chlorine content, which was found to be less than the detection limit or certainly less than 0.1%.

Derivatization reactions

Chloroformates as reagents. Parameters for the derivatization of different amines have been extensively studied in both single- and two-phase systems by Hartwig and co-workers[12,15,16]. In general, derivatizaton in a two-phase system with an appropriate pH in the aqueous phase will be faster than in a single-phase system. The choice of pH dependes on the basicity of the amine considered. For the reaction to occur at a reasonable rate, a considerable population of the nitrogen atoms must be unprotonated. For a volatile amine such as methylamine, the time required for quantitative reaction in a two-phase system at pH 10.3 was found to be between 20 and 280 min, depending on the reagent concentration[12]. For piperazine, with a pK_a value of 9.83 for the first step, we have found the reaction rate with isobutyl chloroformate in a two-phase system to be at least five times higher at pH 10.5 than at pH 7. At pH 10.5 the reaction is quantitative in less than 5 min. In a single-phase system with toluene as solvent the time for quantitative reaction of piperazine is in the order of 20–30 min. For very strong bases such as hexamethylenediamine (pK_a 11.86), no reaction occurs at pH 7 whereas the reaction is quantitative in about 10 min using a pH 12 phosphate buffer. The dependence of the reaction rate on pH may be used selectively to determine aromatic amines in the presence of aliphatic ones.

A basic compound soluble in an organic phase may be added to improve the neutralization of the hydrochloric acid formed and increase the reaction rate. We found such an addition to be necessary in order to get a quantitative recovery. Pyridine may be used but ammonia is even better, promoting 100% recovery for piperazine derivatives of both isobutyl chloroformate and ethyl chloroformate.

Acid anhydrides as reagents. Acid anhydrides have been used as derivatizing agents for amines in single-phase systems with good results[17–19]. Two-phase derivatization of some amines with PFPA has been demonstrated by Skarping *et al.*[13]. One example here is the reaction between 2,4- or 2,6-toluenediamine and PFPA, where quantitative recovery was obtained and the time required for complete reaction was less than 1 min. The derivatives were stable in toluene solution for several weeks. The usefulness of PFPA and some other perfluoro fatty acid anhydrides for the derivatization of piperazine is discussed below in the recovery experiments.

Choice of reagent. When determining piperazine in aqueous solutions, both chloroformates and acid anhydrides may be used. In samples, where disturbing peaks appear, a change from one type of reagent to the other may solve the separation problem. The work-up procedure using anhydrides is simpler, since it does not involve an evaporation step. Both types of derivatives exhibit excellent chromatographic behaviour.

In urine samples, only chloroformates can be used as reagents since acid anhydrides such as HFBA do not give quantitative recovery. This may to a large extent depend on the excessive foaming occurring when these reagents are shaken with urine. The loss using HFBA for the derivatization is in the order of 10–30% at piperazine concentrations of 1000 μg/ml and the precision of the analysis will accordingly be rather poor. The choice between the two chloroformate reagents is rather free; they both give fragment ions with high selectivity. The detection limits

are about equal with both nitrogen-selective and mass-selective detectors although they are somewhat better for the first eluting ethyl chloroformate derivative.

Work-up procedure

For chloroformate derivatives. The evaporation step to dryness described in the Experimental may serve several purposes, such as enrichment, removal of reagent excess or possibility to choose an optimum solvent for the subsequent GC or LC analysis. The most important of these, when using Varian's nitrogen-selective detector, is the removal of excess of chloroformate, since the injection of such a reagent influences the detector sensitivity. The response is at first rapidly increased, then decreases exponentially with time to a normal response in about half an hour. Whether other nitrogen-selective detectors give the same type of problem is not clear. Hartwig and co-workers[12] used an Hewlett-Packard thermionic nitrogen-sensitive detector but they did not mention this problem. The main reason for using the evaporation step also before mass-selective detection is the increase in capillary column lifetime. However, the possibility to enrich the component of interest may be important in future work on the analysis of N-mononitrosopiperazine at very low concentrations.

Other procedures for removal of the excess of reagent are available. Hartvig and co-workers[12] reacted the chloroformate with N,N-dimethylethylenediamine and extracted the derivative formed with an acidic phase. The addition of strong alkali to the aqueous phase, thereby catalysing the reaction of chloroformate to the corresponding alcohol, may be another alternative. Instead of removing the excess of reagent, one could use a standardized procedure for the chromatographic determinations with strict time control. We prefer the first alternative in order to avoid memory effects in the chromatographic system.

For acid anhydride derivatives. No additional work-up is needed after derivatization if the GC analysis is performed shortly thereafter. Otherwise the organic layer ought to be separated from the aqueous phase.

Mass spectra

Mass spectra of isobutyl- and ethyl chloroformate derivatives of piperazine were obtained for the purposes of identification and for chosing suitable fragment ions for quantitative analysis. Fig. 1 shows spectra of the derivatives obtained in the electron impact (EI) mode. Several characteristic fragments appear suitable for mass fragmentography, *e.g.*, for the ethyl chloroformate derivative the molecular ion (M) at *m/e* 230, the M − 15 fragment with loss of CH_3, the M − 29 fragment with loss of C_2H_5 and the M − 45 fragment with loss of C_2H_5O, and for the isobutyl chloroformate derivative the molecular ion (M) at *m/e* 286, the M 15 fragment with loss of CH_3, the M − 73 fragment with loss of C_4H_9O and finally the M − 101 fragment with loss of $C_4H_9CO_2$.

Capillary GC with nitrogen-selective detection

Derivatization from aqueous solutions. After derivatization according to the procedures described, 1-ml portions of each organic solution containing the derivative were mixed together giving equimolar amounts of each component. A 2-μl volume of this solution was injected onto two different capillary columns, giving the

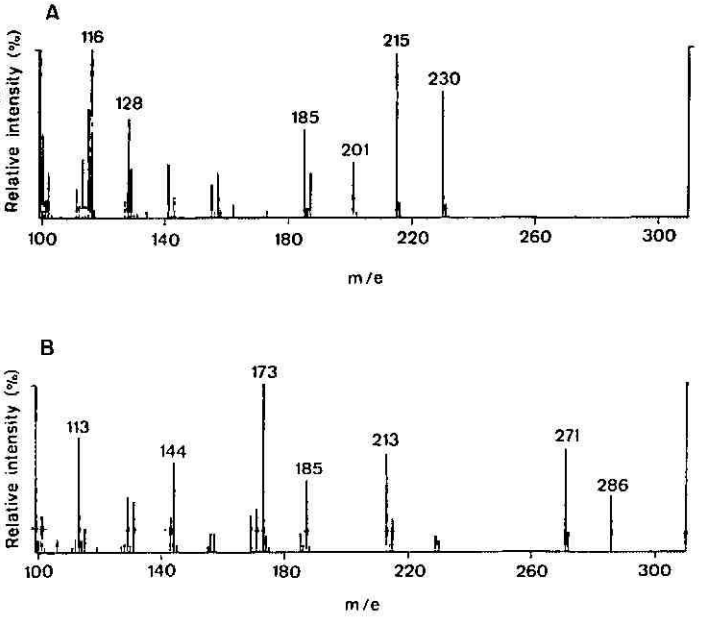


Fig. 1. Mass spectra of piperazine derivatives with ethyl chloroformate (A) and isobutyl chloroformate (B) obtained by electron impact ionization and positive ion monitoring.

chromatograms shown in Fig. 2. The chromatographic behaviour of all derivatives is excellent. Thus, in a situation where disturbing peaks may appear, the reliability of quantitative and qualitative analysis may be increased by using more than one derivative for the analysis or by changing from one column to the other.

Derivatization from urine. Fig. 3 shows chromatograms of isobutyl- and ethyl chloroformate derivatives of piperazine together with corresponding blank traces. Interfering peaks have been found, by analysing more than 50 different blank urine samples, to correspond to less than 20 pg/μl of piperazine, using either of these two reagents. The use of a more polar column such as Aryl 17 CB gave a somewhat better resolution of the ethyl chloroformate derivative of piperazine from possible interfering peaks. Accordingly, the accuracy and precision are somewhat improved and the detection limit somewhat lower.

Capillary GC with mass-selective detection

This technique has mainly been used for the two investigated chloroformate derivatives of piperazine. Fig. 4 shows some peaks of fragment ions of the derivatives at a piperazine concentration of 30 pg/μl with an injection volume of 1 μl. Several peaks are suitable for a quantitative analysis. In our routine analysis we have chosen the molecular ion (M) at *m/e* 286 for the analysis of the isobutyl chloroformate derivatives and that at *m/e* 230 for the ethyl chloroformate derivative.

Quantitative analysis

Chromatographic linearity. A linear variation of the peak height with injected

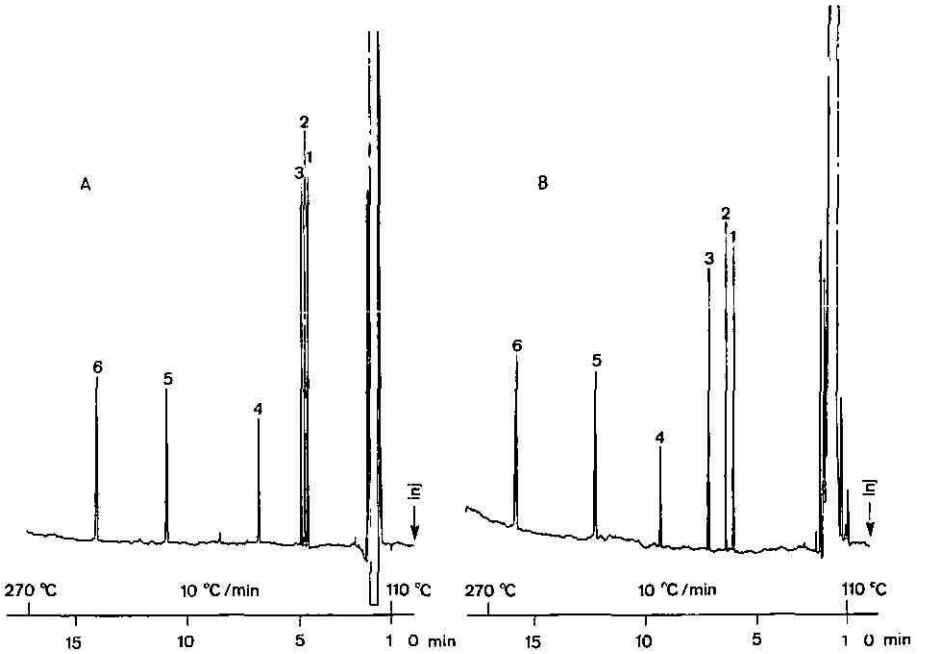


Fig. 2. Chromatograms of piperazine derivatives with nitrogen-selective detection: TFAA (1), PFPA (2), HFBA (3), ethyl chloroformate (5) and isobutyl chloroformate (6) at concentrations of 70 pg/μl with respect to piperazine. Internal standard: the isobutyl chloroformate derivative of di-*n*-butylamine (4) at a concentration of 130 pg/μl. Columns: (A) fused silica (15 m × 0.32 mm I.D.), bonded stationary phase Aryl 17 CB, film thickness 0.15 μm; (B) fused silica (25 m × 0.32 mm I.D.), bonded stationary phase CP-Sil 8CB, film thickness 1.1 μm. Injection: 2 μl on-column. Temperature programming as shown. Carrier gas (helium) at 0.7 kg/cm² (A) and 1.0 kg/cm² (B). Detector: bead heating current, 5.3 scale divisions; bias voltage, −10 V, temperature, 270°C. Flow-rates: hydrogen, 6 ml/min, air, 200 ml/min, make-up gas (nitrogen), 6 ml/min. Attenuation: $4 \cdot 10^{-12}$ A.f.s.

amount was found for all five piperazine derivatives both with nitrogen-selective detection and on-column injection and with mass-selective detection and splitless injection (see Fig. 5). The concentration range was 30–5000 pg/μl with respect to piperazine and 2 μl were injected using nitrogen-selective detection and 1 μl using mass selective detection.

Recovery. The recovery was studied at two concentrations, 300 and 3000 μg/l, by spiking aqueous solutions and urine with piperazine and performing the derivatization procedures as described in the Experimental. Peak heights were compared to those of standards by using nitrogen-selective detection.

For amide derivatives, which give unsatisfactory recovery and precision from urine samples, only aqueous solutions were considered. Of the reagents investigated (TFAA, PFPA, HFBA) only HFBA gives approximatively 100% conversion. This was confirmed by analysing the toluene layer containing the derivative by GC and comparing with standards. Corresponding figures were *ca.* 35% for TFAA and *ca.* 50% for PFPA. The recoveries could be increased to about 70% by two replicate additions of reagent. A change of the experimental conditions may further increase

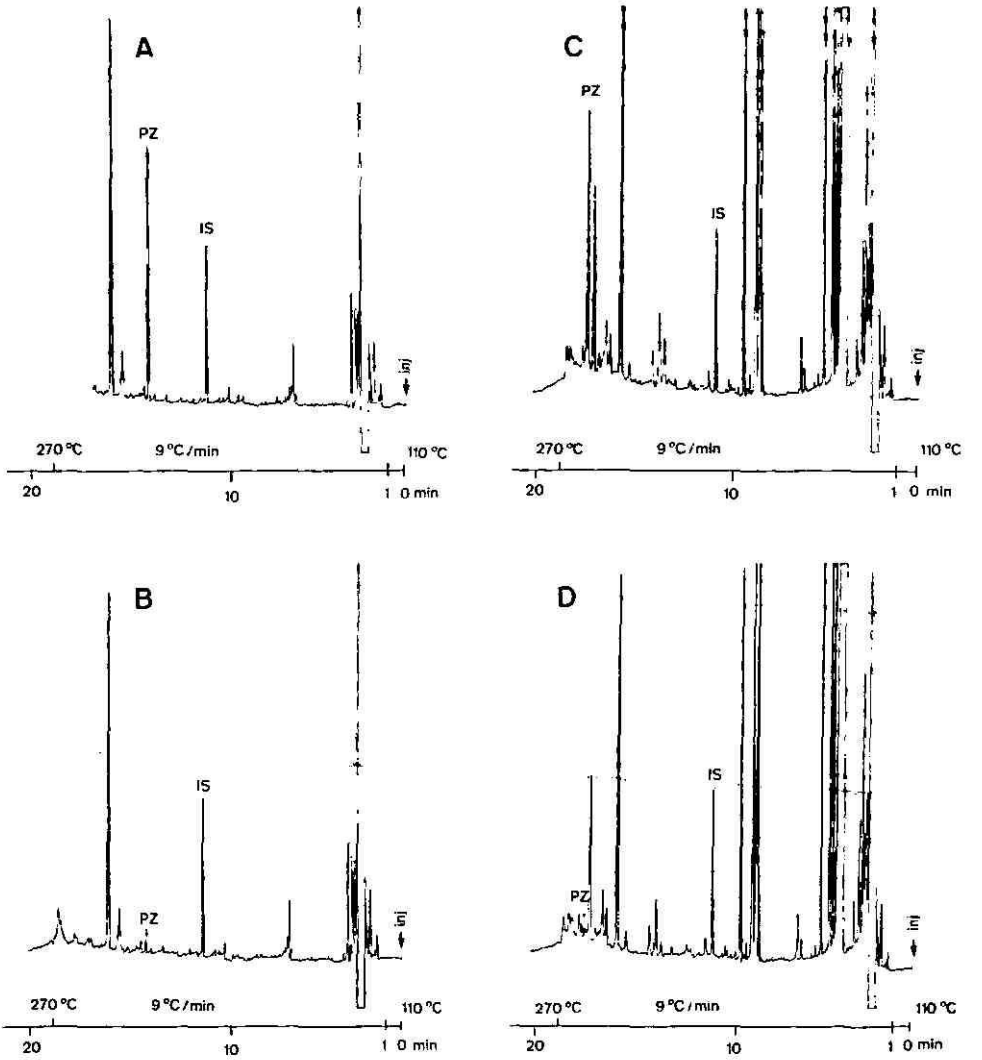


Fig. 3. Chromatograms of piperazine (PZ) at 500 pg/μl in urine treated with ethyl chloroformate (A) and isobutyl chloroformate (C) using nitrogen-selective detection. Internal standard (IS): the isobutyl chloroformate derivative of di-*n*-butylamine at concentrations of 200 pg/μl. Corresponding blank traces were for ethyl chloroformate in urine (B) and for isobutyl chloroformate in urine (D). Column: fused silica (25 m × 0.32 mm I.D.), bonded stationary phase CP-Sil 8CB, film thickness 1.1 μm. Carrier gas (helium) at 0.7 kg/cm^2. Temperature programming as shown. Attenuation: $8 \cdot 10^{-12}$ A.f.s. Other conditions as in Fig. 2.

the recovery. As quantitative recovery is obtained in toluene solution, the losses in this case may to a considerable extent depend on a competing hydrolysis reaction.

With ethyl- or isobutyl chloroformate as reagent, the recoveries were close to 100%, using ammonia as catalyst (0.4% of the total volume of the reaction mixture as described above). Figures obtained at 300 pg/μl were 101 ± 4% for ethyl chlo-

roformate and 98 ± 3% for isobutyl chloroformate at a 95% degree of confidence, based on twelve measurements. The amounts of ammonia and chloroformate reagents added are not critical. Variation of the concentration of ammonia between 0.33 and 1.65% and of the chloroformate reagents between 5 and 50 μl did not significantly change the recovery. A variation of pH between 8 and 10.5 in the phosphate-buffered urine or aqueous solution also did not change the recovery (with 0.33% ammonia and 20 μl chloroformate reagent).

Attempts were made to use pyridine, 0.33–1%, instead of ammonia as the catalyst. An 100% recovery was obtained for the ethyl chloroformate derivative, but in this case the urine blank exhibited a lot more peaks which may disturb the analysis. For the isobutyl chloroformate derivative only about 90% recovery was obtained. In this case the recovery was about the same as without any catalyst which was 91 ± 2% at a 95% degree of confidence based on fifteen measurements. For ethyl chloroformate without catalyst the recovery was only 75–80% with a relative standard deviation of 20–25% based on ten measurements. The lower recoveries without

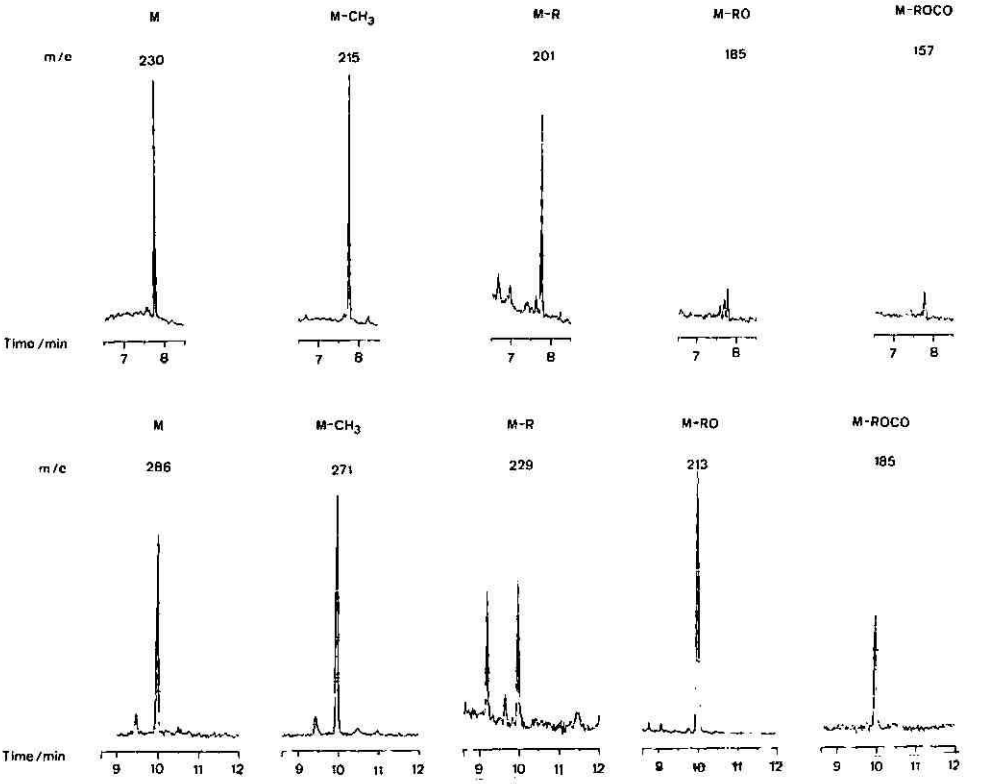


Fig. 4. Multiple ion detection of piperazine as chloroformate derivatives from urine corresponding to 30 pg/μl of piperazine. Topline: molecular ion (230) and fragments of ethyl chloroformate derivative. Bottomline: molecular ion (286) and fragments of isobutyl chloroformate derivative. Column: fused silica (15 m × 0.32 mm I.D.), bonded stationary phase Aryl 17 CB, film thickness 0.15 μm. Inlet pressure of helium: 0.1 kg/cm². Injection: 1 μl, splitless. Temperature programming: isothermal at 110°C (1 min) then raised at 10°C/min to a final temperature of 250°C. For the structure of the fragments, see the formulae given in text.

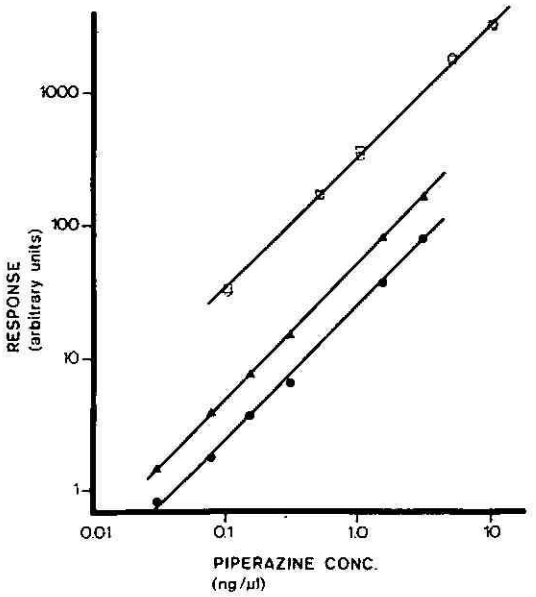


Fig. 5. Calibration curves for piperazine in urine as the corresponding chloroformate derivatives using nitrogen-selective or mass-selective detection: ethyl chloroformate derivative with nitrogen-selective (□) and with mass-selective detection (▲); isobutyl chloroformate derivative with nitrogen-selective (○) and with mass-selective detection (●). Peak height measurement was used with nitrogen-selective detection and peak area counts were used for mass fragmentography. The ratios between the values for the piperazine derivative and for the internal standard (the isobutyl chloroformate derivative of di-*n*-butylamine) are plotted. Chromatographic conditions as in Figs. 3 and 4.

catalyst are essentially due to losses during the derivatization step and not in the subsequent work-up procedures. This was confirmed by spiking the sample solution with piperazine derivatives of the two chloroformate reagents and performing the evaporation and work-up procedure. Calculated values of the recoveries were just 1–2% below 100%. These differences are of course not statistically significant, but nevertheless, small adsorption effects may well be present, leading to minor losses also during the evaporation step.

Calibration curves. Fig. 5 shows calibration curves in urine for piperazine derivatives of ethyl- and isobutyl chloroformate with nitrogen-selective or mass-selective detection. Different amounts of piperazine were added to aqueous solutions or to urine and the entire derivatization and work-up procedure was performed. For each piperazine concentration, three determinations were made with a duplicate injection into the gas chromatograph. Each point on the curve represents the average value. All plots are virtually linear in the concentration range investigated, *i.e.*, 100–10000 pg/μl with respect to piperazine using nitrogen-selective detection and 30–3000 pg/μl using mass selective detection.

In aqueous solution in the corresponding concentration range, as expected, linear plots were obtained for all amide and chloroformate derivatives investigated, using both detectors. The chromatograms and the linear calibration plots show that any adsorption effect is of no practical importance.

Detection limit. Using nitrogen-selective detection the responses for all deriv-

atives are essentially the same. In aqueous solutions, where the chromatograms appear to be free from interfering peaks, the detection limits are less than 3 pg piperazine/μl. Values for HFBA presented in ref. 19 are about the same. In urine with the use of ethyl- or isobutyl chloroformate, small interfering peaks in the range of 0–15 pg/μl may appear, giving a detection limit in the order of 20 pg piperazine/μl.

Detection limits using mass-selective detection for the derivatives considered above were calculated as three times the noise level for peaks at a derivative concentration corresponding to 30 pg piperazine/μl urine (same concentrations as in Fig. 4). The molecular ion fragment peak of the ethyl chloroformate derivative gives a detection limit of 1.5 pg piperazine/μl and the corresponding value for the molecular ion of the isobutyl chloroformate derivative is 2 pg/μl. Some other fragment ions with good selectivity give about the same results. For the ethyl chloroformate derivative these are the ions at *m/e* 230, 215 and 201 where the most sensitive ion fragment at *m/e* 215 gives a detection limit of 1 pg/μl. Suitable ions for the isobutyl chloroformate derivative are those at *m/e* 286, 271, 213 and 185, where the most sensitive fragment ion at *m/e* 213 gives a detection limit of 1 pg/μl. Since no interfering peaks appear in this case, neither in aqueous solutions nor in urine, and the derivatives show no or very little adsorption in the chromatographic system, the detection limit depends only on the performance of the detector.

Accuracy and precision. When analysing a substance by GC after a work-up procedure and especially when this substance is initially present in a complicated matrix such as urine, the addition of an internal standard in the sample before the analysis is strongly recommended. Repeated injections may lead to changes in the separation process, *e.g.*, because of increasing adsorption on the column, or to changes in detector sensitivity with time. Changes in the injected amount with time, especially using splitless injection, may also occur. Such changes can usually be discovered and corrected for by using a combination of internal and external standards. The quotients of the peak heights and of the retention times can be used to evaluate the stability of the chromatographic system over a period of time. An increasing reliability in the identification thus results which improves the accuracy, especially

TABLE I

PRECISION IN THE GC ANALYSIS OF PIPERAZINE AS ITS ETHYL CHLOROFORMATE DERIVATIVE

Injected volume: 1 μl. Internal standard: the isobutyl chloroformate derivative of di-*n*-butylamine. An autoinjector was used for splitless injection and on-column injections were performed manually.

*Detection mode**	*Injection technique*	*Injected amount (ng)*	*Relative standard deviation (%)*		*No. of observations*
			Extern. std.	*Intern. std.*	
MF	Splitless	3	5.0	1.3	35
MF	Splitless	0.3	12.5	2.7	43
MF	Splitless	0.07	4.2	4.7	14
TSD	On-column	5	3.7	4.1	12
TSD	On-column	0.5	3.3	4.1	14
TSD	On-column	0.05	4.6	3.0	12

* MF = Mass fragmentography; measurement of the peak area of the molecular ion at *m/e* 230. TSD = Varian nitrogen-selective detector, with peak height measurement.

when the resolution between the substance peak of interest and peaks emanating from the matrix is not too good.

If the analyses are performed over a short period of time, during which the chromatographic system may be considered unchanged, one usually has to pay a price of lower precision when using an internal standard, compared to the use of only an external standard. In Table I, the precision has been calculated using an internal or an external standard. The value of an internal standard in cases where the chromatographic system changes with time is demonstrated. Considering the values at 0.3 ng/μl, with mass selective detection one finds that the use of an internal standard has greatly improved the precision (from 13 to 3%). As outlined above, the combination of an internal and an external standard may be used to ascertain reliable results even with small changes in the chromatographic system for a period of time. This implies relatively frequent injections of standards, but with the use of an autoinjector the sample throughput is still high. Normally we use a standard injection each fifth injection.

As internal standard we have used the isobutyl chloroformate derivative of di-*n*-butylamine and as external standard the analyte. The internal standard chosen behaves similarly to the piperazine derivatives in the chromatographic system and also with respect to the two detectors used for the analysis. A still better standard with respect to changes in the sensitivity of the mass selective detector would obviously be a deuterium-labelled piperazine derivative. However, the inadequate resolution of the unlabelled and labelled derivatives on the capillary column prevents its use with nitrogen-selective detection, which would necessitate different internal standards depending on the choice of detection mode.

The overall precision of the system, work-up procedure and GC analysis, is about 6% at a piperazine concentration of 300 pg/μl in urine, as shown in the recovery experiment. The contributions from the two operations are about equal as calculated from the results in Table I.

Choice of approach for the analysis

As shown above, several derivatives may be used for the analysis of piperazine in aqueous samples or in urine. The concentration of piperazine in aqueous samples after air sampling is usually high enough to permit a free choice between these derivatives. However, since the chloroformate reagents can also be used for urine samples, we have chosen chloroformates for routine analysis. Normally we prefer the ethyl chloroformate derivative due to its shorter elution time. The choice between detection modes, nitrogen-selective or mass-selective detection, is rather arbitrary. Somewhat higher precision is generally obtained using nitrogen-selective detection, but the detection limit is somewhat in favour of the mass-selective approach. However, the piperazine concentration in actual samples is usually so high that this difference in detection limit is of little or no importance. Splitless injection with an autosampler is preferred since it gives a higher throughput than manual on-column injection, about 45 samples per 24 h in the former case, compared to about 14 samples during an 8 h working day in the latter. The thermal stability of the piperazine derivatives formed is sufficient to permit splitless injection. This may not always be true, *e.g.*, chloroformate derivatives of N-mononitrosopiperazine show some degradation in the injection port, when using splitless but not with on-column injection.

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CHEMICAL CHANGES OF ORGANIC COMPOUNDS IN CHLORINATED WATER

XII*. GAS CHROMATOGRAPHIC–MASS SPECTROMETRIC STUDIES OF THE REACTIONS OF METHYLNAPHTHALENES WITH HYPOCHLORITE IN DILUTE AQUEOUS SOLUTION

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SUMMARY

The products of aqueous chlorination reactions of naphthalene, 1- and 2-methylnaphthalenes, 1,2-, 1,3-, 1,4-, 1,5-, 1,8-, 2,3- and 2,6-dimethylnaphthalenes and 2,3,5-trimethylnaphthalene with hypochlorite have been determined by gas chromatography–mass spectrometry. They included chloro-substituted, oxygenated (quinones) and hydroxylated (phenols) compounds which were readily formed at room temperature. The extent of the reactions was shown to depend on the pH, the initial concentrations of both compounds and the number of methyl substituents and their positions. Monochlorinated compounds were identified in the chlorinated water under those conditions utilized for water treatment.

INTRODUCTION

The disinfection of water and waste water with chlorine is a well established procedure. However, the increasing presence and variety of aquatic pollutants raises the question of the chemical fate of these contaminants when subjected to aqueous chlorination[1]. Even the numerous studies of phenol, which established the intermediate formation of mono-, di- and trichlorophenols[2–9], leave the question of the end-products of this reaction unresolved[10].

Several polynuclear aromatic hydrocarbons (PAHs) in aqueous solution are degraded by addition of chlorine[11,12], chlorine dioxide[13–16] or sodium hypochlorite[17–21]. These reactions may occur at the site of chlorine addition as well as throughout a water distribution system[22]. PAHs have been suggested as the precursors of at least a portion of the mutagens produced in some chlorination processes[22]. The levels

* For Part XI, see ref. 33.

0021-9673/86/$03.50

of these ubiquitous PAH compounds may be increased by the presence of coal-tar coatings inside pipes and water storage tanks[23,24].

In order to provide further insight into the possible rôle of organic compounds in the formation of chlorine-substituted compounds and of chlorine-induced mutagens, this laboratory has continued the study of the aqueous chlorination chemistry of organic compounds[7,9,10,25–33]. The present report describes a detailed study of the product distributions of several methylated naphthalenes which were chosen for study because of their previous identification in unpurified water[34] and drinking water[35–37].

EXPERIMENTAL

Materials

Naphthalene, methylnaphthalenes and ethylnaphthalenes were obtained from Tokyo Chemical Industry Co. (Tokyo, Japan), Nakarai Chemical Ltd. (Kyoto, Japan) and Wako Pure Chemical Industry Co. (Osaka, Japan). 1- and 2-chloronaphthalenes, 1,2- and 1,4-dichloronaphthalenes, 4-chlorophthalic acid and other compounds, which are expected to be formed during chlorination of naphthalenes with aqueous chlorine, were commercially available reagents. Hypochlorite solution was prepared by diluting sodium hypochlorite solution (*ca.* 10% available Cl, Nakarai Chemicals Ltd.) with 1 *M* disodium hydrogenphosphate–potassium dihydrogen phosphate buffer solution, pH 7. The hypochlorite concentrations were determined by iodometric titration.

Treatment of aqueous naphthalene solutions with hypochlorite and extraction of reaction mixture

A mixture of 200 ml of hypochlorite solution and each naphthalene compound dissolved in 1 ml of methanol was shaken in a separatory funnel at ambient temperature, in order to prevent leakage of the compound from the reaction system. After the desired reaction time, the residual chlorine was removed by addition of an equivalent volume of sodium thiosulphate solution. The reaction mixture was then acidified to pH 2 with 0.1 *M* hydrochloric acid before extraction with three 40-ml volumes of diethyl ether. The solvents were dried over anhydrous sodium sulphate and 2 ml of methanol were added to prevent evaporation of the reaction products during concentration under vacuum at 40°C to suitable volumes for gas chromatographic (GC) and GC–mass spectrometric (GC–MS) analyses.

Product resolution and characterization

A Shimadzu GC-6A gas chromatograph equipped with a flame ionization detector and 2 m × 3 mm I.D. glass column packed with 2% OV-1 on Uniport HP (60–80 mesh) was programmed from 60 to 240°C at 5°C/min. The nitrogen carrier gas flow-rate was 50 ml/min. A Shimadzu Model Chromatopac-1A data system was used to determine the retention times and peak areas on the chromatograms.

An Hitachi M-80 mass spectrometer–gas chromatograph equipped with an Hitachi M-003 data-processing system was used for the qualitative analyses of samples under the following conditions: ion source, temperature 250°C; trap current, 70 μA; electron energy, 70 eV. A glass column (2 m × 3 mm I.D.) packed with 2%

OV-1 on Uniport HP (60–80 mesh) was used for the GC separation of the diethyl ether extracts. The oven temperature of the gas chromatograph was programmed from 120 (sometimes 60 or 80) to 240°C at 5°C/min. The products were identified by comparison of their retention times and mass spectra with those of authentic compounds.

RESULTS AND DISCUSSION

In a preliminary examination of the reaction of individual methylnaphthalenes with hypochlorite in dilute aqueous solution, the decrease in the concentration of the active chlorine during contact with each of these compounds was followed by iodometric titration. It was observed that a much slower decrease occurred at low concentration, less than 1 mg/l, of methylnaphthalenes, in comparison with aqueous phenolic solutions[7,26,27,29]. In the present work, therefore, each methylnaphthalene (10^{-3} *M*) was allowed to react with 10^{-2} *M* hypochlorite and the products identified.

A small amount of methanol (less than 1%) was used in many reactions to provide sufficient quantities of methylnaphthalene products for analysis and to minimize the formation of methylnaphthalene crystals. The use of such small amounts of this solvent did not appear to alter the product distribution based on chromatographic comparison with reactions in the absence of methanol. In contrast, reaction solutions containing crystalline methylnaphthalene tended to yield only chloro products rather than both the chloro and oxygenated products observed in homogeneous solutions.

GC–MS analysis of chlorination products of naphthalene

A typical GC–MS (total ion current) trace of a diethyl ether extract of neutral naphthalene solution (3.9 mmol/l) after treatment with hypochlorite (20 equiv. of chlorine per mol of compound) for 24 h is shown in Fig. 1. At least ten reaction products can be seen on the chromatogram. Some of the peaks were identified on the basis of their retention times and mass spectra as compared with those of authentic compounds. Compounds corresponding to other peaks were determined from the mass spectrum of each peak (Fig. 2).

Fig. 2B and D show the mass spectra of the peaks in scans 77 and 127, respectively, which are the major GC–MS peaks in the diethyl ether extract from chlorine-treated naphthalene solution. The molecular ion (M^+) of the peak of scan 77 occurs at *m/z* 162, having one chlorine atom, and a fragment ion occurs at *m/z* 127, which arises by the loss of one chlorine atom from the molecular ion. The compound corresponding to the peak of scan 127 gave a molecular ion at *m/z* 196, having two chlorine atoms, and two fragment ions at *m/z* 161 ($M^+ - Cl$) and at *m/z* 126 ($M^+ - 2\ Cl$). The mass spectra and retention times of these compounds were in agreement with those of 1-chloronaphthalene and 1,4-dichloronaphthalene, respectively.

The mass spectrum of the compound (Fig. 2E) corresponding to the peak of scan 196 in Fig. 1 gave a molecular ion at *m/z* 262, having three chlorine atoms, and fragment ions at *m/z* 227 ($M^+ - Cl$), *m/z* 199 ($M^+ - COCl$), *m/z* 164 ($M^+ - Cl - COCl$) and *m/z* 136 ($M^+ - 2\ COCl$). This suggests a formula $C_{10}H_5Cl_3O_2$. From the nature of the reaction of naphthalene with hypochlorite in water and the mass

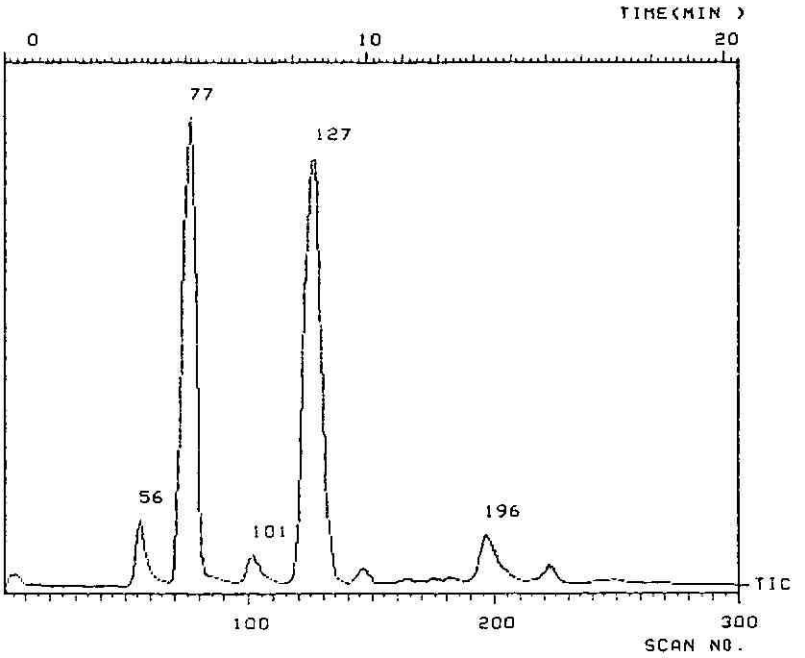


Fig. 1. Mass chromatogram (total ion current) of a diethyl ether extract of a naphthalene solution (3.9 mmol/l) after treatment with hypochlorite (20 equiv. of chlorine per mol of compound) at room temperature for 24 h. Mass spectrum for each peak as in Fig. 2. Compounds as in Table I. The GC column temperature was raised from 80 to 240°C at 5°C/min. For other GC–MS conditions, see Experimental.

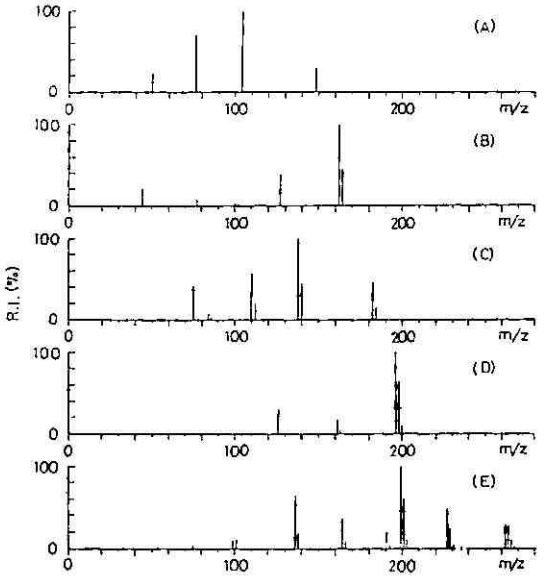


Fig. 2. Mass spectra of peaks of scan No. 56 (A), 77 (B), 101 (C), 127 (D) and 196 (E) in Fig. 1.

spectrum, this compound is considered to be a trichlorodihydroxynaphthalene. However, its exact nature could not be determined because authentic compounds were not available.

Fig. 2A shows the mass spectrum of the compound corresponding to the peak of scan 56 in Fig. 1. The molecular ion (M^+) is at *m/z* 148 and the most abundant fragment ion is as *m/z* 104, which arises by loss of carbon dioxide from the molecular ion. Other fragment ions occur at *m/z* 76 ($M^+ - CO_2 - CO$) and *m/z* 50 ($M^+ - CO_2 - CO - C_2H_2$). The retention time and mass fragmentation pattern of this compound are in agreement with those of anhydrous phthalic acid.

The compound corresponding to the peak of scan 101 in Fig. 1 gave a molecular ion at *m/z* 182, having one chlorine atom, and three fragment ions at *m/z* 138 ($M^+ - CO_2$), *m/z* 110 ($M^+ - CO_2 - CO$) and *m/z* 75 ($M^+ - CO_2 - CO - Cl$). This was interpreted as being due to an anhydrous phthalic acid with one chlorine atom. Since the GC retention time did not agree with that of anhydrous 4-chlorophthalic acid, the corresponding compound is probably the 3-chloro derivative. This result, in addition to the above finding, leads to the conclusion that chlorination of aqueous naphthalene solution with hypochlorite produces not only chloronaphthalenes and chlorohydroxynaphthalenes, but also chlorophthalic acids by oxidative cleavage of the aromatic ring.

A summary of chlorination products identified or determined from their GC retention times and mass spectra is presented in Table I.

The occurrence of mono-, di- and trichloronaphthalenes in chlorine-treated naphthalene solution has been confirmed by Smith *et al.*[19] by GC analysis of a benzene and XAD-2 extract from the reaction solution. However, no oxidative ring-cleavage compounds (chlorophthalic acids) were reported, because such highly polar compounds could not be isolated from the aqueous solution by the benzene or XAD-2 extraction procedures. Other oxidizing reagents, *i.e.*, potasium permanganate, peracetic acid and hydrogen peroxide, have been reported to oxidize naphthalene under rather drastic conditions to form phthalic acid[38], *o*-carboxycinnamic acid[39] and 1- and 2-naphthols[40], respectively. Oxidation by chlorine dioxide is known to produce mono- and dichloronaphthalenes and phthalic acid[14].

GC–MS analysis of chlorination products of methylnaphthalene

Typical GC–MS (total ion current) traces of diethyl ether extracts from individual methylnaphthalene solutions after treatment with an excess of hypochlorite at room temperature for 2 or 16 h are shown in Figs. 3–6. Some of the peaks were identified on the basis of the retention times and mass spectra compared with those of authentic compounds. Compounds corresponding to other peaks were determined from the mass spectrum of each peak in the same manner as described for the naphthalene products.

Figs. 3–6 show that the treatment of methylnaphthalenes with hypochlorite in buffered solutions of pH 7 produces mono- and dichloro-substituted compounds as the predominant products, minor products being chlorinated polyhydroxy compounds (phenols) or quinones. (MS data for each compound appearing on the chromatograms in Figs. 3–6 are available from the authors). Unfortunately, the nature of the main peak of the diethyl ether extract from chlorine-treated 1,2-dimethylnaphthalene solution could not be determined because this compound gave a complex mass spectrum.

TABLE 1

PRODUCTS OF REACTION OF NAPHTHALENE WITH CHLORINE IN NEUTRAL WATER

Reaction conditions and GC–MS conditions as in Fig. 1.

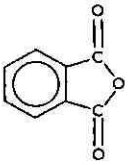
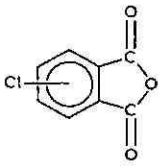
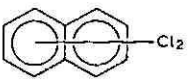
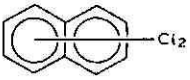
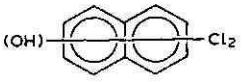
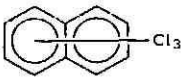
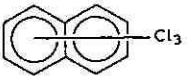
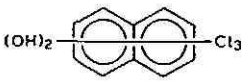
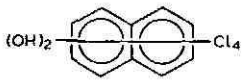
Proposed structure	*Scan No.*	*Molecular ion (m/z)*	*Amount**	*m/z (relative intensity)*
	56	148	Minor	148(30), 104(100), 76(65), 50(21)
	77	162	Major	164(35), 162(100), 127(35), 77(5), 44(21)
	101	182	Minor	184(14), 182(44), 140(30), 138(100), 112(19), 110(6), 75(40)
	127	196	Major	200(10), 198(64), 196(100), 163(5), 161(17), 126(28)
	146	196	Minor	200(11), 198(64), 196(100), 163(7), 161(33), 126(29), 75(3)
$C_{10}H_8Cl_2O$	163	214	Trace	218(13), 216(58), 214(100), 142(13), 139(33), 123(73), 103(24), 76(29)
	172	212	Trace	216(8), 214(63), 212(100), 113(17)
	175	230	Trace	234(30), 232(90), 230(100), 195(20), 181(25), 162(8), 160(34), 133(88), 105(9)
	180	230	Trace	234(36), 232(91), 230(100), 195(91)

TABLE I (*continued*)

Proposed structure	*Scan No.*	*Molecular ion (m/z)*	*Amount**	*m/z (relative intensity)*
	196	262	Minor	266(9), 264(28), 262(28), 229(25), 227(38), 203(9), 201(64), 199(100), 164(33), 136(54)
	222	296	Minor	302(1), 300(10), 298(25), 296(15), 265(12), 263(34), 261(36), 237(29), 235(93), 233(100), 199(31), 197(43), 163(50), 135(25), 127(12)

* Derived semiquantitatively from the GC–MS peak areas, relative to the area of starting material. Major, over 5%; minor, less than 5 to 0.5%; trace, near detection limit (0.05%).

Treatment of 1-methylnaphthalene with hypochlorite in water has been reported to produce over twenty chlorinated compounds including chloro derivatives, quinones, phenols and carboxylic acid[12]. Hypochlorite treatment of C_2-naphthalenes present in the aromatic fraction of diesel fuel was shown to give monochloro-C_2-naphthalenes[18]. Mono- and dichloro-substituted compounds and phthalic acid have also been identified by Taymaz *et al.*[14] when 1- and 2-methylnaphthalenes were treated with chlorine dioxide in aqueous solutions.

Effect of the experimental conditions on the reactions of methylnaphthalenes with hypochlorite in aqueous solution

GC analysis of the diethyl ether extracts indicated that a much slower decrease in the amount of the original compound in water occurs even though at longer reaction times (Fig. 7) and at higher molar ratios of hypochlorous acid to compound (Fig. 8), in comparison with those reactions observed for aqueous solutions of phenolic compounds[7,27,29]. Monochloronaphthalene was present at higher concentrations in water when naphthalene was treated with hypochlorite for a long time and at high molar ratios of hypochlorous acid to compound. This was followed by the formation of a small amount of dichloro-substituted compounds, as is seen in Figs. 7 and 8. The fact that the monochloro-substituted compound is mainly formed can be explained by the deactivating effect of chlorine substituents. The amounts of ether-extractable and chromatographiable compounds decreased with increasing contact time and increasing molar ratio of hypochlorous acid to compound. This indicates the formation of highly water-soluble and non-volatile compounds during the secondary reactions in chlorine-treated naphthalene solution.

In contrast to the naphthalene–hypochlorite reaction, it was found in the methylnaphthalene–hypochlorite reactions that a rapid decrease in the amount of the original compounds occurs with formation of monochloro-substituted compounds (Table II). This tendency was, however, strongly dependent upon the number of methyl substituents and their positions in the naphthalene ring. 2-Methyl-, 1,2- and 1,3-dimethyl- and 2,3,5-trimethylnaphthalenes readily reacted with hypochlorous acid in water. A moderate reactivity was found for the 1-methyl-, 2-ethyl- and 1,8-

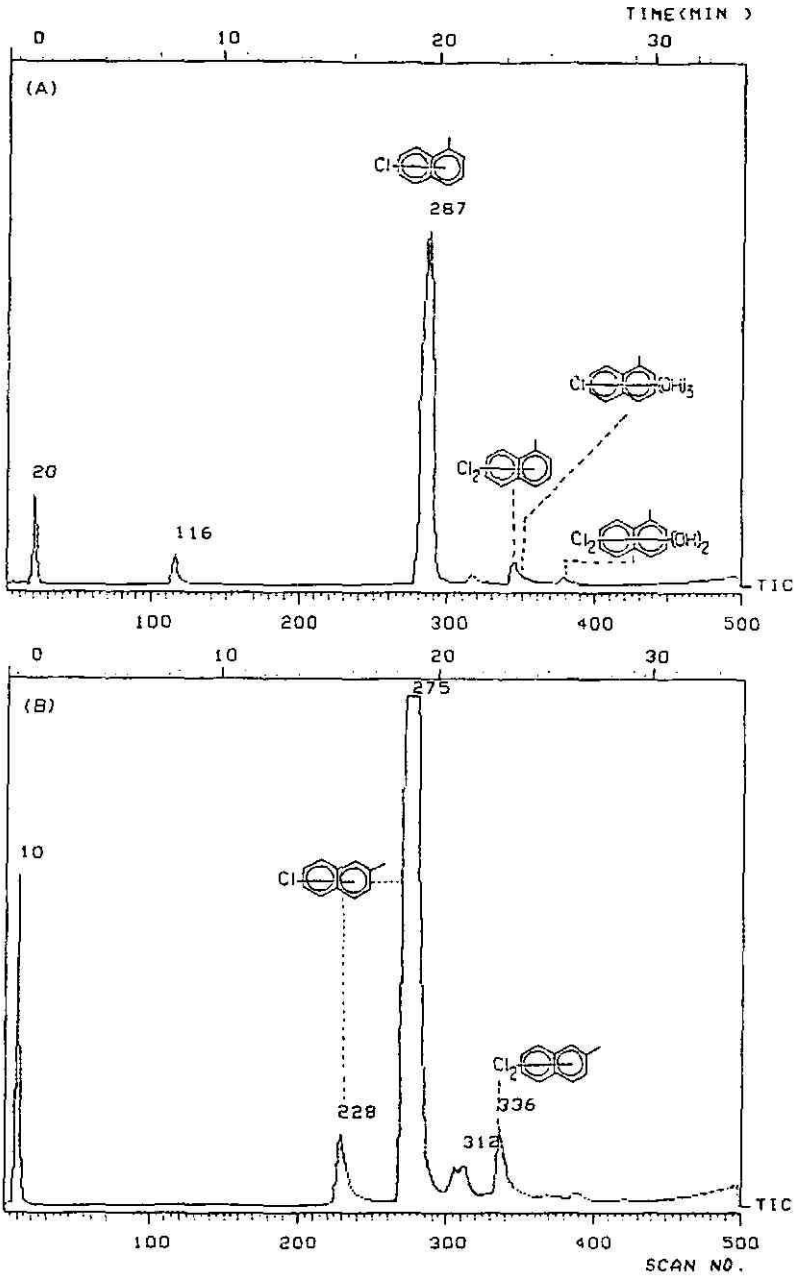


Fig. 3. Mass chromatograms (total ion current, TIC) of diethyl ether extracts of 1- (A) and 2-methylnaphthalene (B) solutions (3.5 mmol/l) after treatment with hypochlorite (20 equiv. of chlorine per mol of compound) at room temperature for 16 h. The GC column temperature was programmed from 60 (10-min hold) to 240°C at 5°C/min. For other GC–MS conditions, see Experimental.

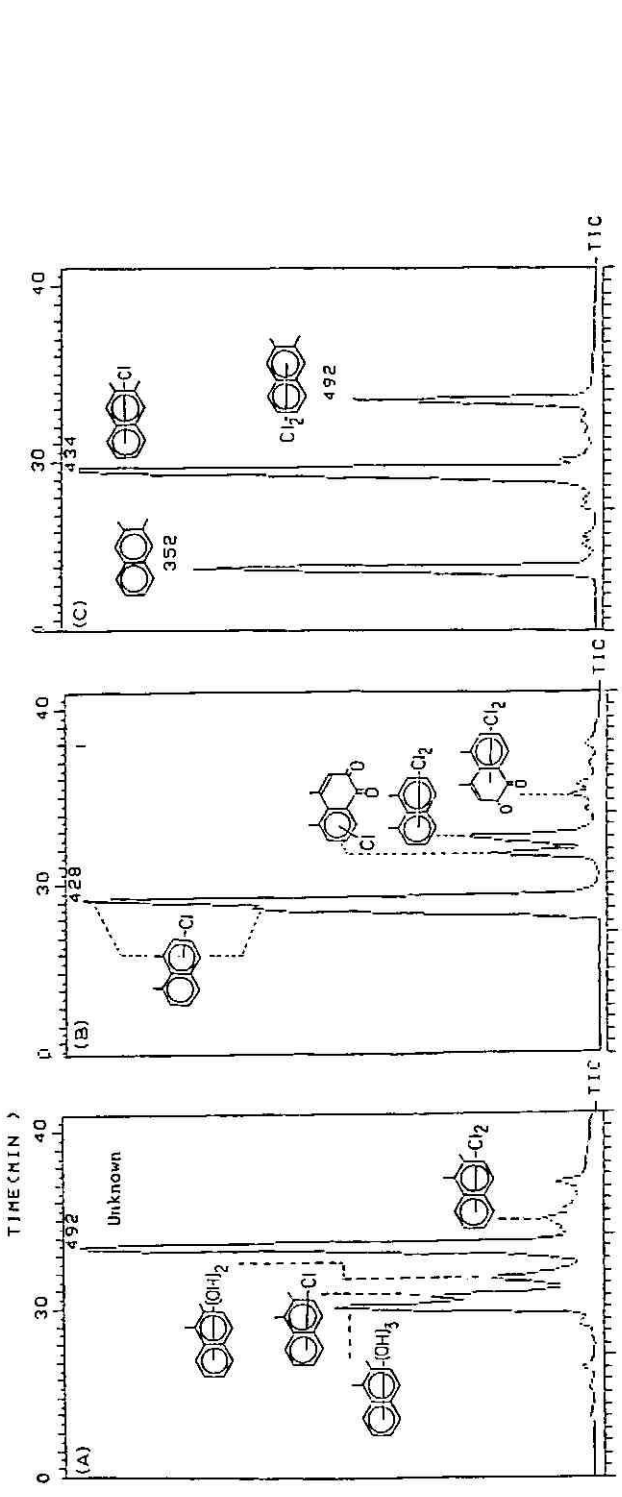


Fig. 4. Mass chromatograms (total ion current) of diethyl ether extracts of 1,2- (A), 1,8- (B) and 2,3-dimethylnaphthalene (C) solutions (3.2 mmol/l) after treatment with hypochlorite (20 equiv. of chlorine per mol of compound) at room temperature for 2 h. Other details as in Fig. 3.

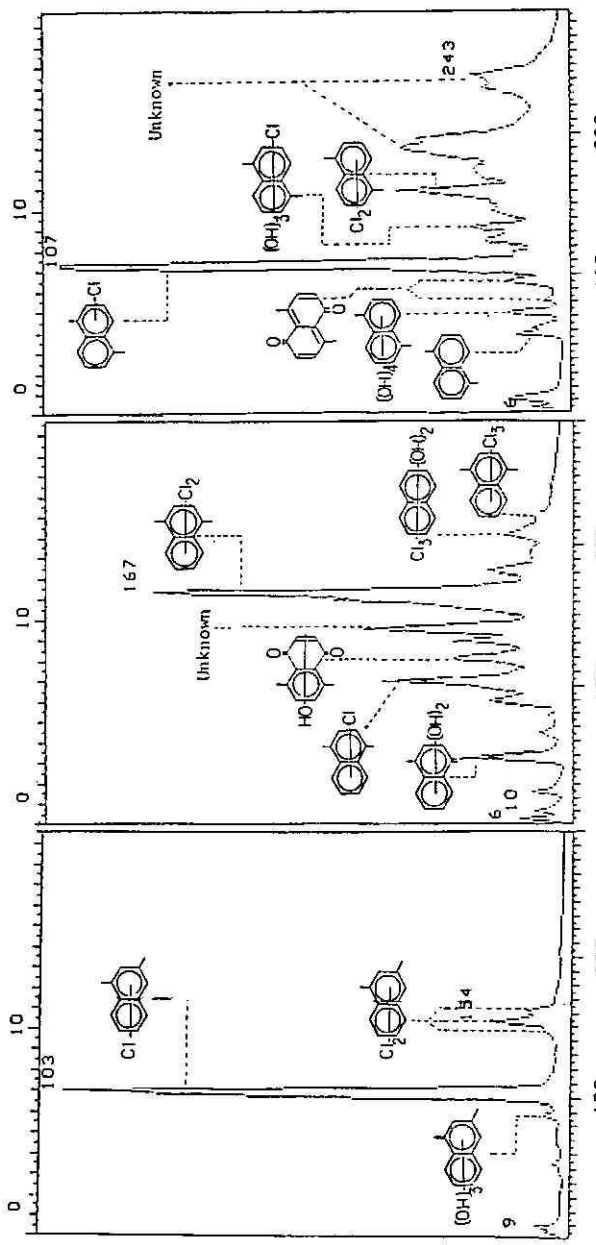


Fig. 5. Mass chromatograms (total ion current) of diethyl ether extracts of 1,3- (A), 1,4- (B) and 1,5-dimethylnaphthalene (C) solutions (3.2 mmol/l) after treatment with hypochlorite (20 equiv. of chlorine per mol of compound) at room temperature for 2 h. The GC column temperature was programmed from 120 to 240°C at 5°C/min. For other GC–MS conditions, see Experimental.

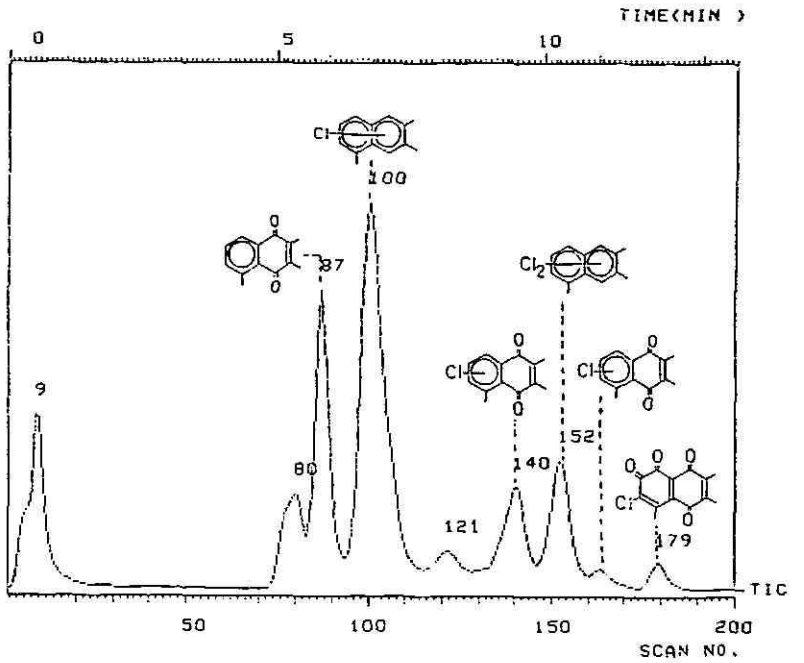


Fig. 6. Mass chromatogram (total ion current) of a diethyl ether extract of 2,3,5-trimethylnaphthalene solution (0.294 mmol/l) after treatment with hypochlorite (20 equiv. of chlorine per mol of compound) at room temperature for 2 h. The GC column temperature was programmed from 140 to 240°C at 5°C/min. For other GC–MS conditions, see Experimental.

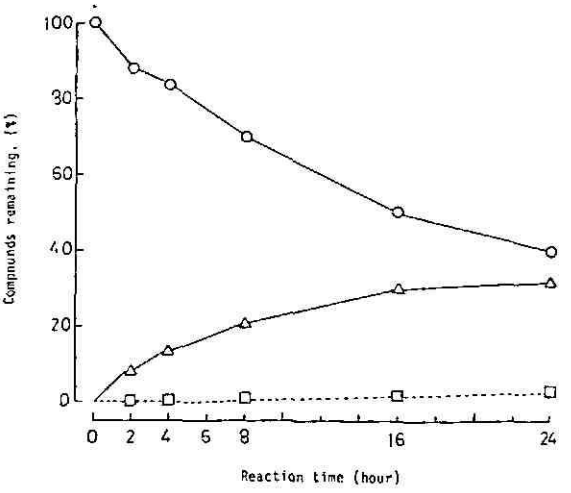


Fig. 7. Time course of the reaction of naphthalene (0.39 mmol/l) with an excess of hypochlorite at room temperature. The yields are derived from GC peak areas, relative to the area of starting material. O—O, Starting material; △—△, monochloronaphthalenes; □—□, dichloronaphthalenes. Each point represents the average of three experiments.

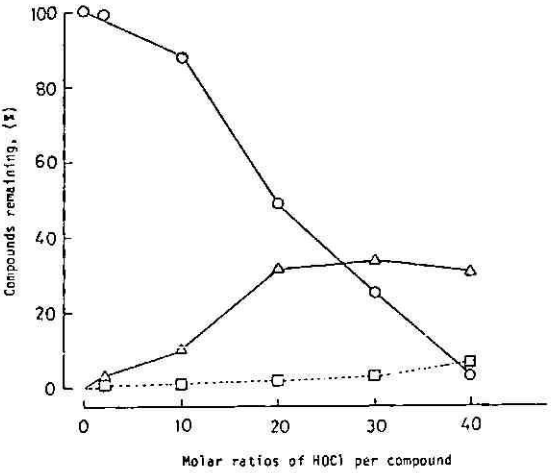


Fig. 8. Residual amounts of reaction products in aqueous naphthalene solutions (0.39 mmol/l) after treatment with hypochlorite (various equivalents of chlorine per mol of compound) at room temperature for 16 h. Other details as in Fig. 7.

and 2,3-dimethylnaphthalenes, and low reactivity for the 1-ethyl-, 1,4-, 1,5- and 2,6-dimethylnaphthalenes.

The distribution of the chlorinated products can be explained in terms of the electrophilic substitution by chlorine. Methyl substituents are activating and *o-/p*-directing; if they are *m*-substituted, *e.g.*, 1,3-dimethyl- and 2,3,5-trimethylnaphthalene, the activating effect is enhanced, whereas if they are *p*-substituted, *e.g.*, 1,4-, 1,5- and 2,6-dimethylnaphthalenes, the effect is weakened.

Fig. 9 shows the results of GC determinations of diethyl ether extracts from naphthalene solutions after treatment with an excess of hypochlorite at various pH values for 16 h. Dichlorinated compounds were detected at high concentrations in acidic solution, but large amounts of monochloro-substituted compounds were obtained under neutral conditions. These chlorinated compounds were hardly detected when naphthalene was treated with an excess of hypochlorite at pH 10. This can be explained in terms of the concentrations of undissociated hypochlorous acid in the chlorinated water. Therefore, the effect of pH on the reaction of naphthalene with hypochlorite in water is correlated with the titration curve of hypochlorous acid as shown in Fig. 9.

A summary of the GC determinations of the diethyl ether extracts from the other methylnaphthalene solutions after treatment with an excess of hypochlorite at various pH values for 1 h is shown in Table III. The effect of pH described above was also observed in these reactions.

In order to confirm whether similar reactions take place under conditions present in water treatment, different initial concentrations of naphthalene were treated with an excess of hypochlorite at pH 5, 7 and 9 for 16 h. The results of GC determinations of the diethyl ether extracts from the chlorinated naphthalene solutions are shown in Fig. 10. It was observed that the reactions of naphthalene with hypochlorite in water proceed more rapidly with increasing initial concentrations of

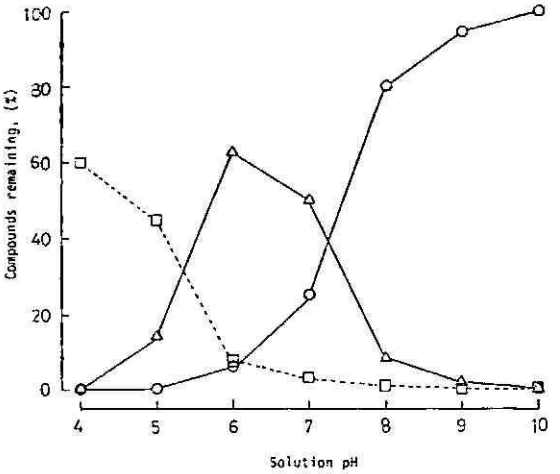


Fig. 9. Residual amounts of reaction products in aqueous naphthalene solutions (0.39 mmol/l) after treatment with an excess of hypochlorite at various pH values and room temperature for 16 h. Other details as in Fig. 7.

both compounds and with decreasing pH. However, no chlorinated compounds were detectable when 5 ppm of naphthalene were treated with hypochlorite under alkaline conditions. In contrast, the monochlorinated compound was detected in higher concentrations after treatment of even lower concentrations of naphthalene with an excess of hypochlorite under neutral and acidic conditions.

It was worth stressing the relationship between the results reported here and the customary chemical treatment of waste water. Industrial wastes are frequently treated with heavy doses of chlorine to destroy odours, to disinfect the waste water and to improve sedimentation and filtration behaviour. As already mentioned, naph-

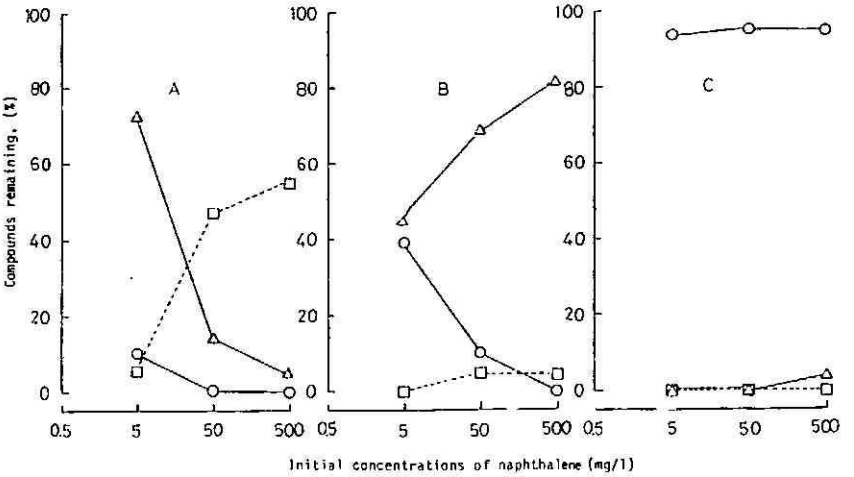


Fig. 10. Effect of the initial concentrations of compounds on the residual amounts of reaction products of aqueous naphthalene solutions after treatment with an excess of hypochlorite (20 equiv. of chlorine per mol of compound) at pH 5 (A), 7 (B) and 9 (C) for 16 h. Other details as in Fig. 7.

TABLE II

CHLORINATION PRODUCTS OF METHYLNAPHTHALENES IN WATER AFTER TREATMENT WITH AN EXCESS OF HYPOCHLORITE AT 20°C FOR 1 h

Each compound (5 mg) dissolved in 1 ml of methanol was shaken with 100 ml of water (pH 7) containing 25 mg of chlorine. After adjusting to pH 2, the reaction mixture was extracted with diethyl ether (3 × 20 ml) for GC analysis.

Compound	*Residual amounts of products (%)**				
	Original	*Cl_1 deriv.*	*Cl_2 deriv.*	*Other*	*Total*
Naphthalene	79.3	1.5	N.D.**	N.D.	80.80 ± 3.73***
1-Methylnaphthalene	38.5	31.8	N.D.	N.D.	70.30 ± 2.29
2-Methylnaphthalene	12.2	60.3	N.D.	N.D.	72.50 ± 2.29
1-Ethylnaphthalene	70.8	11.1	N.D.	N.D.	81.90 ± 5.12
2-Ethylnaphthalene	44.5	45.4	N.D.	N.D.	89.90 ± 1.98
1,2-Dimethylnaphthalene	7.6	61.2	N.D.	18.5	87.30 ± 2.04
1,3-Dimethylnaphthalene	N.D.	68.0	N.D.	2.1	70.10 ± 2.59
1,4-Dimethylnaphthalene	52.0	25.3	N.D.	N.D.	77.30 ± 5.80
1,5-Dimethylnaphthalene	70.9	5.0	N.D.	N.D.	75.90 ± 1.82
1,8-Dimethylnaphthalene	36.6	35.3	N.D.	N.D.	71.90 ± 3.85
2,3-Dimethylnaphthalene	28.5	40.1	1.1	N.D.	68.70 ± 5.01
2,6-Dimethylnaphthalene	55.1	15.5	N.D.	N.D.	70.60 ± 3.67
2,3,5-Trimethylnaphthalene	5.0	53.0	N.D.	11.4	69.40 ± 5.24

* Yields derived from GC peak areas, relative to the area of the starting material.
** Not detected by GC analysis.
*** Average and standard deviation for three samples.

TABLE III

CHLORINATION PRODUCTS OF METHYLNAPHTHALENES IN WATER AFTER TREATMENT WITH AN EXCESS OF HYPOCHLORITE AT ROOM TEMPERATURE FOR 1 h, AS A FUNCTION OF pH

Procedure and details as in Table II.

Compound	*pH*	*Residual amount of product (%)*				
		Original	*Cl_1-deriv.*	*Cl_2-deriv.*	*Other*	*Total*
Naphthalene	5	65.0	19.0	Trace	N.D.	84.00 ± 1.50
	7	88.0	2.0	N.D.	N.D.	90.0 ± 3.00
	9	79.6	N.D.	N.D.	N.D.	79.60 ± 0.96
2-Methylnaphthalene	5	4.4	90.5	N.D.	N.D.	94.90 ± 3.31
	7	32.3	60.0	N.D.	N.D.	92.30 ± 4.35
	9	94.8	3.7	N.D.	N.D.	99.40 ± 1.91
1,4-Dimethylnaphthalene	5	28.3	65.5	N.D.	N.D.	93.80 ± 1.42
	7	78.6	17.7	N.D.	N.D.	96.30 ± 2.31
	9	100	N.D.	N.D.	N.D.	100 ± 5.00
2,3-Dimethylnaphthalene	5	14.5	59.9	3.3	N.D.	77.70 ± 1.21
	7	66.1	34.5	0.4	N.D.	91.00 ± 1.00
	9	72.2	13.4	N.D.	N.D.	85.70 ± 2.04
2,3,5-Trimethylnaphthalene	5	N.D.	66.9	5.0	8.6	80.50 ± 2.23
	7	36.5	48.3	Trace	2.2	87.00 ± 1.05
	9	77.0	2.0	N.D.	N.D.	79.00 ± 2.17

thalene and methylnaphthalenes are common components in such waste water[36], unpurified water[34], and coal-tar coatings of water-supply systems[23,24] and this has led to the suggestion that chlorinated naphthalenes could be generated during the chlorine treatment of such waters and in the water-supply system. This suggestion was clearly supported by an earlier report[37] in which several chlorinated naphthalenes and other polynuclear aromatic hydrocarbons were found to be present in drinking water.

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GAS CHROMATOGRAPHIC DETERMINATION OF TETRAMETHYLSUCCINONITRILE IN POLY(VINYL CHLORIDE) PRODUCTS IN CONTACT WITH FOOD

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SUMMARY

A method is described for the determination of tetramethylsuccinonitrile (TMSN) in poly(vinyl chloride) (PVC) products in contact with food. TMSN was extracted from PVC with dichloromethane, steam distilled, extracted from the distillate with dichloromethane, then determined with a gas chromatograph equipped with a nitrogen–phosphorus detector and a 6-ft. glass column packed with Chromosorb W coated with 5% Thermon 1000 plus 0.5% phosphoric acid. Recoveries between 98.3 and 100.6% were obtained in the range of 0.5–12.5 ppm of TMSN by the use of nitrobenzene as an internal standard. The detection limit was 0.05 ppm of TMSN in 1 g of PVC products. The highest residue to TMSN found in PVC products in contact with food was 13.9 ppm.

INTRODUCTION

Tetramethylsuccinonitrile (TMSN, tetramethylbutanedinitrile) is the major decomposition product of 2,2'-azobisisobutyronitrile[1,2] which is used as a forming agent in the production of plastics or as a catalyst for the polymerization of vinyl compounds such as vinyl chloride[3,4]. It is known to have acute toxicity; LD_{50} is 18 mg intraperitoneal (i.p.) per kg body weght in mice[5], and the lethal function in hamsters is considered to be the central neurotoxicity[6].

Nevertheless, few analytical studies of residual TMSN in plastics have been reported. Mandik and Culkova[7] reported an analytical method for TMSN in polystyrene using gas chromatography (GC) and flame ionization detection (FID). The sample was dissolved in carbon disulphide and the detection limit was 5 ppm. This method, however, could not be applied to poly(vinyl chloride) (PVC) products because of its low sensitivity and the presence of many interfering peaks near the retention time of TMSN.

The present paper describes a clean-up method for extracts obtained from PVC and a sensitive GC method for the determination of TMSN. The method was used to determine residual TMSN in PVC products in contact with food.

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EXPERIMENTAL

Apparatus

An Hewlett-Packard Model 57110A gas chromatograph, equipped with a nitrogen–phosphorus detector, was used with a Shimadzu Chromatopac C-R1A integrator. The coiled glass column (6 ft. × 2 mm I.D.) was packed with Chromosorb W AW DMCS (80–100 mesh) coated with 5% Thermon 1000 plus 0.5% phosphoric acid.

The steam distillation apparatus with ground-glass joints was almost the same type as that described in ref. 8. The distillation flask had a volume of 100 ml. Biotron BT10 20 3500 was used to homogenize the samples.

An high-performance liquid chromatograph Model L-2000 (Yanagimoto) equipped with an ODS-A column (250 mm × 4.6 mm I.D.) and a gas chromatograph–mass spectrometer Model JMS-DX300 (Jeol) equipped with a Carbowax 20M column (10 m × 0.530 mm I.D.) were used for clean-up and confirmation of TMSN, respectively.

Reagents

All solvents and reagents were reagent grade. TMSN (98% pure) was obtained from Tokyo Chemical Industry (Tokyo, Japan).

Samples

Food packages and a wrapping film made of PVC were obtained from markets in Tokyo.

Standard and internal standard solutions

TMSN and nitrobenzene (99.5% pure) (each 100 mg) were weighed into separate 100-ml volumetric flasks, dissolved with methanol and made up to 100 ml. Both solutions (1000 μg of TMSN or nitrobenzene per ml) were used as stock solutions, and were stable for at least 1 month in a refrigerator. These solutions were diluted in methanol to definite concentrations before use. The diluted solutions of TMSN were used as standard solutions and those of nitrobenzene were used as internal standard solutions. For the recovery test, the stock solution of TMSN was diluted in dichloromethane to definite concentrations.

Preparation of test solutions

Samples were cut into small pieces. A 1-g amount of the pieces was put into a 50-ml centrifuge-tube and 20 ml of dichloromethane were added. After 10 min, the mixture was homogenized with the homogenizer for 30 s and then left to stand for 10 min. Methanol (20 ml) was added to the homogenate and the mixture was centrifuged at 2000 rpm (700 *g*) for 5 min. The supernatant was transferred to a round-bottom flask. The pellet in the centrifuge-tube was suspended with 20 ml of dichloromethane and the suspension was left to stand for 10 min. Methanol (20 ml) was added to the suspension and the mixture was centrifuged for 5 min. The supernatant was added to the former extract. The solvent was evaporated to 5–7 ml under reduced pressure at 30–35°C. The concentrate was transferred to a distillation flask with 2 ml of dichloromethane, and 10 ml of water and 3 g of sodium chloride were added. The

mixture was steam distilled till 70 ml of distillate including organic solvents were obtained. A definite amount of nitrobenzene, usually 1 ml of the 25 ppm solution, was added to the distillate as the internal standard. The mixture was shaken with 50 ml of dichloromethane. The extraction was repeated two more times with 30 ml of dichloromethane. The solvent layers were pooled, dried with 2 g of anhydrous sodium sulphate and concentrated to 5–7 ml under reduced pressure at 30–35°C. The extract was further concentrated to a definite volume, usually 1 ml, under a stream of nitrogen gas. This solution was used for GC. In the recovery test, a definite amount of TMSN in dichloromethane was added after the sample had been weighed and placed into a centrifuge-tube.

Determination

TMSN was determined by GC under the following conditions: nitrogen carrier gas, 45 ml/min; hydrogen, 3 ml/min; air 100 ml/min; injection port, 150°C; column, 125°C; detector, 250°C; injection volume, 2 μl. TMSN and nitrobenzene appeared at retention times of 3.5 and 4.5 min, respectively. All results were calculated as the mean ± S.D. of three experiments.

Confirmation

To confirm TMSN, the solution for GC was obtained by the method described with 10 g of sample, and was concentrated to 0.2 ml. The concentrate was subjected to high-performance liquid chromatography (HPLC), eluting with 50% methanol at the flow-rate of 0.78 ml/min. The fraction eluted between 5.5 and 6.5 ml which corresponded to the retention volume of TMSN was shaken with 10 ml of dichloromethane. The dichloromethane layer was dried with a small amount of anhydrous sodium sulphate and concentrated to 0.1 ml under a stream of nitrogen gas. A 5-μl portion of the concentrate was injected for gas chromatography–mass spectrometry. The conditions were as follows: ionization voltage, 70 eV; carrier gas (helium), 15 ml/min; column temperture, 70°C for 4 min, then raised to 130°C at 4°C/min.

RESULTS AND DISCUSSION

Using 5% Thermon 1000 plus 0.5% phosphoric acid as the liquid phase in GC, sharp peaks of TMSN and nitrobenzene were obtained. A similar chromatogram was observed with 5% polyethylene glycol 20M, but on Amipack 124, 3% OV-17, 5% polyethylene glycol plus 1% potassium hydroxide, Chromosorb 101 or 5% Thermon 1000 the peaks were not as sharp. A linear response on the column packed with 5% Thermon 1000 plus 0.5% phosphoric acid was observed between 0.1 and 5 ppm of TMSN: $y = 0.2598x - 0.0003$, $r = 0.9999$, where y = peak area of TMSN relative to that of 25 ppm of nitrobenzene as an internal standard, x = ppm of TMSN and r = correlation coefficient. The detection limit of TMSN was 0.05 ppm in GC.

The method recommended[4] for the determination of TMSN in work-place air is GC with FID. Biber and Fales[9] developed an analytical method for TMSN in heptane using capillary column GC; the detection limit was 0.05 ppm using nitrogen–phosphorus detection (NPD), although application to plastic products was not reported. Mandik and Culkova[7] used GC–FID for the determination of TMSN

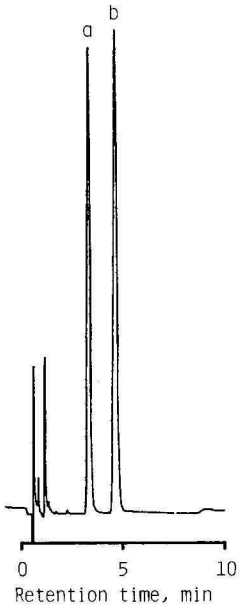

Fig. 1. A typical gas chromatogram of the extract obtained from a PVC product. Peaks: a = tetramethylsuccinonitrile; b = nitrobenzene (internal standard).

in impact-resistant polystyrene, and reported that the detection limit was 5 ppm. These two methods are direct injection methods, *i.e.*, the heptane solution of TMSN or carbon disulphide solution of PVC was used for GC, and were not subjected to extraction or any other pretreatment for purification.

With PVC products, TMSN in dichloromethane extracts could not be determined by direct injection because of the presence of many interfering substances on

TABLE I

CONTENTS OF TETRAMETHYLSUCCINONITRILE IN PVC PRODUCTS IN CONTACT WITH FOOD, AND RECOVERY TESTS

Sample	*TMSN content** (*μg/g*)	*TMSN spiked* (*μg*)	*Found* (*μg*)	*Recovery** (*%*)
Case 1	1.27 ± 0.05	1.0	2.27 ± 0.05	100.0 ± 4.7
2	9.79 ± 0.92			
3**	13.94 ± 0.07	12.5	26.27 ± 0.39	98.6 ± 3.2
Film 1	n.d.***			
Cup 1	0.06 ± 0.05	0.5	0.55 ± 0.02	98.0 ± 3.5
2	7.25 ± 0.05	5.0	12.28 ± 0.19	100.6 ± 3.9

* Mean ± S.D. ($n=3$).
** Confirmed by GC–MS.
*** Less than 0.05 ppm.

the chromatograms, despite the use of NPD. Most of these substances were removed by steam distillation of the dichloromethane extract, and no peak was observed at the retention time of the internal standard. A typical gas chromatogram is shown in Fig. 1.

Recoveries of TMSN by the proposed method were between 98.3 and 100.6% (Table I). Recoveries were reduced by dryness of the extracts when the solvent of dichloromethane extracts obtained from PVC and from steam distillate was evaporated; then the concentration of the extracts was stopped at about 5 ml. The amounts of TMSN spiked in the recovery tests were roughly the same as the TMSN content in the samples, except for sample Cup 1 where the content of TMSN was almost the same as the detection limit. The recoveries and standard deviations were not affected by the amount of TMSN.

The residual concentrations of TMSN in food packages made of PVC are shown in Table I. The concentrations of TMSN ranged from n.d. (less than 0.05 ppm) to 13.9 ppm. In general, the concentrations were lower than those in polystyrene, between n.d. (less than 5 ppm) and 85 ppm[7].

TMSN in PVC product Case 3 was confirmed by the coincidence of the GC and HPLC retention times with that of authentic TMSN and by the mass spectrum.

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EFFECTS OF MODIFIER AND MOLECULAR STRUCTURE OF SOME COUMARINS ON RETENTION IN REVERSED-PHASE HIGH-PERFORMANCE THIN-LAYER AND COLUMN CHROMATOGRAPHY*

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SUMMARY

Coumarins, furocoumarins and pyranocoumarins (29 compounds) were investigated in silanized silica (HPTLC RP-2 and RP-18 and HPLC RP-18)–water organic modifier (methanol, acetonitrile, dioxane, tetrahydrofuran and methanol containing acetic acid) systems. The physico-chemical properties of these compounds were characterized by their hydrophobicity parameters (R_{Mw}), determined by extrapolation of the linear relationships for retention data in binary solvent systems [$R_M = f(\varphi)$] to pure water. The effect of individual substituents on retention was quantified by using the group contribution parameter ΔR_M or $\Delta \log k'$.

INTRODUCTION

Coumarins are interesting biologically active compounds[1–4] and methods for their determination include thin-layer (TLC)[5,6] and column high-performance liquid chromatography (HPLC)[7–14].

In reversed-phase systems with non-polar chemically bonded stationary phases, the retention of solutes is mainly controlled by adjusting the type and concentration of the organic modifier in an aqueous–organic eluent[15–18]. Solvation effects play an important role and the relationship between capacity factor ($\log k'$) and the modifier content depends strongly on the molecular structure of the solute[19,20].

The effect of the qualitative and quantitative eluent composition in retention is an important problem owing to the use of mixed solvents to control the eluent strength and selectivity[21–24]. The linear relationship between the logarithm of the capacity factor and the composition of the eluent can be described by the simple equation[25–28]

$$\log k'_{w,\,mod} = \log k'_w - b\varphi_{mod}$$

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where φ_{mod} is the volume fraction of the modifier (0.01 × vol.-%), b is a constant ($b = \log k'_w - \log k'_{mod}$), $\log k'_w$ (lipophilicity index) is the logarithm of the capacity factor for 100% water as eluent and the subscripts w and mod denote components of the eluent (water and organic modifier, respectively). The usefulness of chromatographic R_{Mw} and $\log k'_w$ values as an expression of the lipophilic character of molecules is generally recognized[29–33].

The investigated series of coumarins are increasingly used as taxonomic markers in the chemotaxonomic identification of plant species and as models for a group of drugs undergoing chemical modification *in vivo*, which justifies the systematic study of their retention behaviour in thin-layer and column chromatography[34,35]. In this regard, the aim of this work was to examine how the characteristics of the sorbent, the type of modifier used and the structure of the solutes influence their retention.

Quantitative correlations between R_M (HPTLC) and $\log k'$ (HPLC) values were obtained; the slope of these relationships permits the practical application of HPTLC data to the optimization of HPLC systems, including preparative separations[36–38].

EXPERIMENTAL

TLC was performed on 10 × 10 cm pre-coated HPTLC plates of RP-18 F_{254} or RP-2 F_{254} (E. Merck, Darmstadt, F.R.G.); 1-μl samples of the solutes (1.0 mg cm^{-3} in methanol) were spotted 1 cm from the edge and eluted with appropriate eluents in saturated tanks over a distance of 8.5 cm. The spots were localized under UV light at 254 nm.

Column HPLC was carried out using a liquid chromatograph (produced at the Institute of Physical Chemistry of the Polish Academy of Sciences, Warsaw, Poland) with a 200-ml syringe pump, a 5-μl sample injection valve and a UV detector (254 nm). A stainless-steel column (250 × 4 mm I.D.) was packed with 10 μm Li-Chrosorb RP-18 (E. Merck). The flow-rate was 1.2 cm^3 min^{-1}. The eluents were degassed in an ultrasonic bath. The temperature was kept at 21 ± 2°C. The column dead volume was determined using an aqueous solution of sodium nitrate as the non-retained compound.

The coefficients in the regression equations were calculated from the experimental data by the multiple regression analysis using the least-squares method.

Materials

The coumarins used (Table I) were obtained from the Department of Chemistry, Royal Danish School of Pharmacy, Copenhagen, Denmark. Analytical-reagent grade organic solvents were purchased from E. Merck.

RESULTS AND DISCUSSION

The HPTLC results are shown as plots of R_M *versus* percentage of methanol (Figs. 1 and 2), as R_M *versus* R_M relationships obtained using different systems (Figs. 3–5) as a plot of $\log k'$ *versus* R_M (Fig. 6) and also as parameters of regression equations describing the linear relationships between experimental R_M values and the percentage of concentration of organic modifier in the eluent. The results in Figs. 1

and 2 indicate that in most instances linear relationships were obtained for almost all of the investigated concentration range. The lines sometimes cross (change in the sequence of R_M values) or spread fan-wise. The increase in the selectivity at higher water contents in the eluent is caused by an increasing contribution of hydrophobic interactions, especially for less polar compounds. In reversed-phase systems, in which solvation effects are very important, the retention depends on the molecular structure of the solute; the introduction of small polar or non-polar groups into a large sample molecule influences the retention significantly (Figs. 1 and 2). Compounds with polar hydroxy groups in the molecule, such as umbelliferone, esculetin, scopoletin, dihydrooroselol and *cis*- and *trans*-khellactone, have the weakest retention if an aqueous–organic eluent is used. The methylation of hydroxy groups markedly increased the retention of, *e.g.*, osthol, O-prenylumbelliferone and ostruthin (the spots were always near the start). Similar retentions were obtained for furocoumarins: isoimperatorin, peucenidin, libabotin, athamantin, archangelicin and columbianadin. Of the pyranocoumarins, the greastest hydrophobicity and the longest retention times were shown by visnadin and disenecioyl *cis*-khellactone. In order to elute these non–polar compounds, which possess large hydrophobic fragments in the molecule, the eluent strength must be increased, which can be achieved by increasing the content of organic modifier. At low concentration of the modifier these compounds cannot

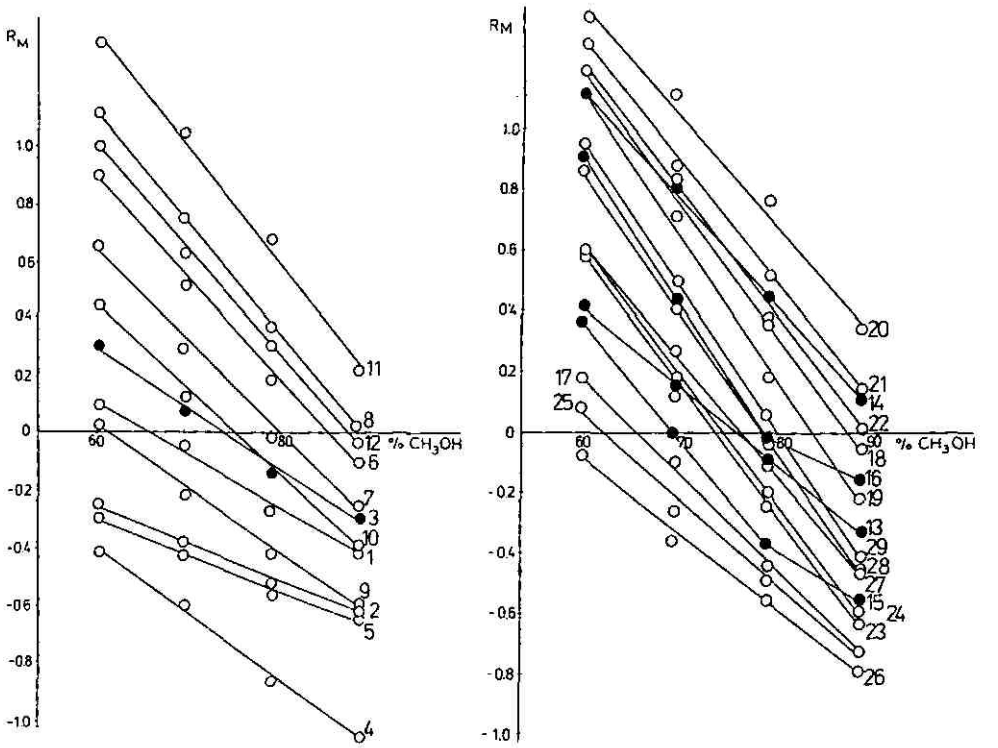
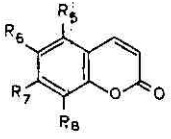
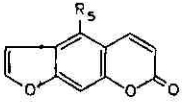


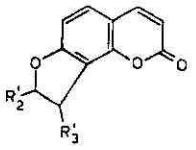
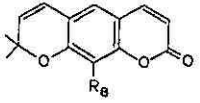
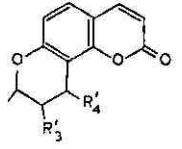
Fig. 1. R_M values plotted against the concentration of methanol in the eluent. Active solid: octadecylsilica. Notation of solutes as in Table I.

Fig. 2. As Fig. 1 for further solutes.

TABLE I

COMPOUNDS INVESTIGATED

Type	*Structure*	*No.*	*Substituents*	*Name*
Simple coumarins		1	None	Coumarin
		2	$R_7 = OH$	Umbelliferone
		3	$R_7 = OCH_3$	Herniarin
		4	$R_6 = R_7 = OH$	Esculetin
		5	$R_6 = OCH_3$, $R_7 = OH$	Scopoletin
		6	$R_6 = CH_2CH=C(CH_3)_2$, $R_7 = OH$	Demethylsuberosin
		7	$R_7 = OH$, $R_8 = CH_2CH=C(CH_3)_2$	Osthenol
		8	$R_7 = OCH_3$, $R_8 = CH_2CH=C(CH_3)_2$	Osthol
		9	$R_7 = OCH_3$, $R_8 = CH_2CHC(CH_3)_2$ (OH OH)	Merazin hydrate
		10	$R_7 = OCH_3$, $R_8 = CH_2CH-C(CH_3)_2$ (O)	Merazin
		11	$R_7 = OCH_2CH=C(CH_3)_2$	O-prenylumbelliferone
		12	$R_6 = OCH_2CH=C(CH_3)CH_2CH_2CH=C(CH_3)_2$, $R_7 = OH$	Ostruthin
Furano-coumarins		13	None	Psoralen
		14	$R_5 = OCH_2CH=C(CH_3)_2$	Isoimperatorin
		15	$R_5 = OCH_2CH-C(CH_3)_2$ (OH OH)	Oxypeucedanin hydrate
		16	$R_5 = OCH_2CH-C(CH_3)_2$ (O)	Oxypeucedanin

Dihydro-furano-coumarins		17	R'_2 = $C(OH)(CH_3)_2$	Dihydrooroselol
		18	R'_2 = $C[OCOCH_2CH(CH_3)_2](CH_3)_2$, R'_3 = $OCOCH_3$	Peucenidin
		19	R'_2 = $C[OCOC(CH_3)=CHCH_3](CH_3)_2$, R'_3 = $OCOCH_3$	Libanotin
		20	R'_2 = $C[OCOCH_2CH(CH_3)_2](CH_3)_2$, R'_3 = $OCOCH_2CH(CH_3)_2$	Athamantin
		21	R'_2 = $C[OCOC(CH_3)=CHCH_3](CH_3)_2$, R'_3 = $OCOC(CH_3)=CHCH_3$	Archangelicin
		22	R'_2 = $C[OCOC(CH_3)=CHCH_3](CH_3)_2$	Columbianadin
Pyrano-coumarins		23	None	Xanthyletin
		24	R_8 = OCH_3	Luvangetin
Dihydro-pyrano-coumarins		25	R'_3 = R'_4 = OH	*cis*-Khellactone
		26	R'_3 = R'_4 = OH	*trans*-Khellactone
		27	R'_3 = $OCOCH=C(CH_3)_2$, R'_4 = $OCOCH_3$	Samidin
		28	R'_3 = $OCOCH(CH_3)CH_2CH_3$, R'_4 = $OCOCH_3$	Visnadin
		29	R'_3 = R'_4 = $OCOCH=C(CH_3)_2$	Disenecioyl *cis*-khellactone

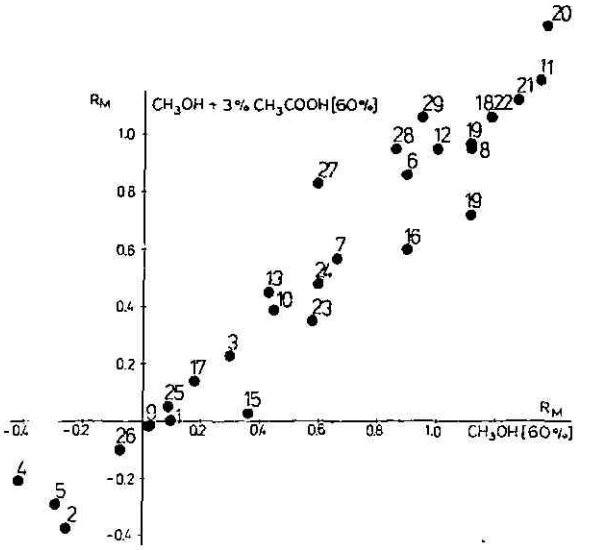


Fig. 3. R_M *vs.* R_M relationships. Active solid: octadecylsilica. Notation of solutes as in Table 1. Eluent compositions indicated on the axes.

be separated. In these instances the range of measurable data of coumarins whose retentions (log k') show a linear dependence on the concentration of the modifier can be extended by extrapolation of experimental $R_M = f(\varphi)$ relationships to a pure water medium.

It should be pointed out that extrapolation over a wide concentration range

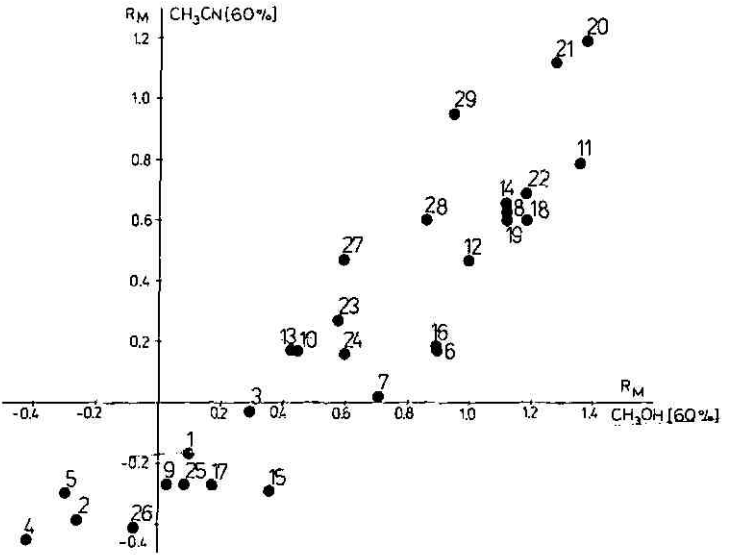


Fig. 4. As Fig. 3.

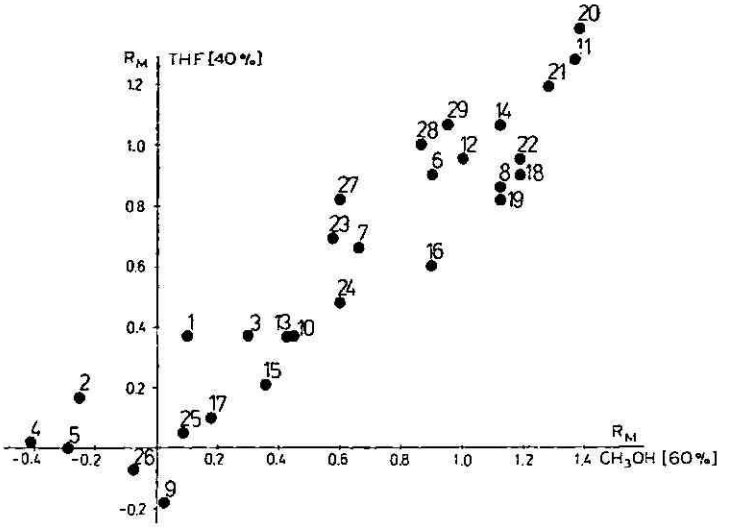


Fig. 5. As Fig. 3.

(φ_{mod} = 50–0%), assuming linear relationships over the whole concentration range, may give unreliable results. The structure of the stationary "brush" phase changes for water-rich eluents, which is also reflected by increased zone spreading[39]. Therefore, extrapolated (idealized) log k'_w or R_{Mw} values, although more or less different from the real parameters obtained for the system RP sorbent–100% water[40], may provide better characteristics of the lipophilicity of the solutes for the given water–modifier systems. However, the extrapolated R_{Mw} values may differ for the individual modifiers.

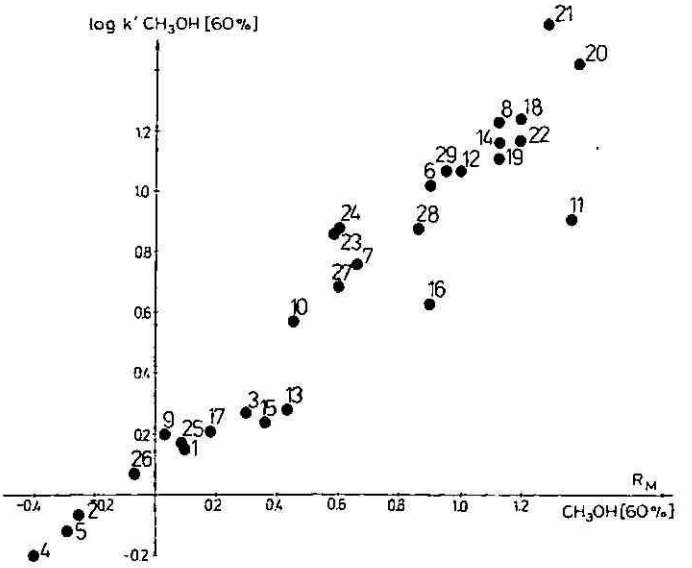


Fig. 6. Log k' *vs.* R_M relationships.

The theoretical capacity factors R_{Mw} thus estimated are summarized in Table II as the parameter a in the equation

$$R_M = a - b\varphi \quad (1)$$

Theoretically, the R_{Mw} value of an individual solute should be content for a given stationary phase, independent of the organic modifier used in an aqueous–organic phase; however, for the investigated coumarins the R_{Mw} values derived from different solvent systems depend on the nature of the organic modifier and also on the composition range used for determining the experimental data. The extrapolated R_{Mw} values (a) obtained using the methanol–water system were correlated with R_{Mw} values

TABLE II

PARAMETERS a (INTERCEPT) b (SLOPE) AND r (CORRELATION COEFFICIENT) OF THE LINEAR RELATIONSHIPS IN EQN. 1

*Compound**	*System I (RP-18; methanol concentration range 60–30%)*			*System II (RP-18; methanol + 3% acetic acid, concentration range 50–90%)*			*System III (RP-18; tetrahydrofuran concentration range 40–70%)*		
	a	*b*	*r*	*a*	*b*	*r*	*a*	*b*	*r*
1	1.17	1.77	0.992	1.74	2.82	0.994	1.40	2.56	0.999
2	0.56	1.35	0.997	1.38	2.87	0.998	1.02	2.09	0.998
3	1.49	2.00	0.996	2.19	3.23	0.998	1.40	2.56	0.999
4	0.92	2.21	0.998	0.65	1.45	0.999	0.88	2.17	0.999
5	0.41	1.18	0.986	0.99	2.16	0.998	0.90	2.25	0.999
6	2.88	3.34	0.997	3.18	3.85	0.996	2.48	3.98	0.997
7	2.38	2.92	0.995	2.60	3.45	0.998	2.02	3.34	0.997
8	2.76	2.84	0.967	2.71	2.96	0.999	2.35	3.80	0.996
9	1.27	2.09	0.998	1.72	2.92	0.999	0.64	2.03	0.999
10	2.01	2.66	0.995	2.45	3.41	0.998	1.40	2.50	0.999
11	3.69	3.82	0.994	2.84	2.70	0.998	3.26	4.91	0.997
12	2.80	3.22	0.997	2.71	2.96	0.999	2.51	4.02	0.996
13	1.95	2.55	0.999	2.28	3.05	0.999	1.04	1.76	0.998
14	3.19	3.43	0.999	2.02	2.15	0.997	2.63	4.01	0.997
15	2.18	3.10	0.999	2.12	3.47	0.996	1.05	2.14	0.997
16	3.68	4.65	0.999	3.39	4.68	0.998	1.77	2.94	0.997
17	1.94	2.98	0.998	1.88	2.91	0.996	0.79	1.69	0.994
18	3.73	4.02	0.999	3.55	4.19	0.998	2.41	3.82	0.997
19	3.85	4.53	0.997	2.82	3.25	0.972	2.16	3.38	0.999
20	3.47	3.40	0.999	3.65	3.76	0.998	3.32	4.88	0.999
21	3.53	3.77	0.999	3.01	3.14	0.999	2.93	4.40	0.998
22	3.66	4.07	0.996	3.88	4.68	0.996	2.11	3.70	0.999
23	2.94	3.98	0.998	2.96	4.31	0.997	1.84	2.93	0.997
24	2.96	3.96	0.998	2.29	2.96	0.995	1.42	2.36	0.999
25	1.66	2.64	0.979	1.83	2.93	0.998	0.71	1.64	0.997
26	1.31	2.34	0.995	1.50	2.65	0.997	0.53	1.49	0.993
27	2.73	3.54	0.999	2.92	3.49	0.996	2.05	3.17	0.996
28	3.48	4.39	0.999	3.01	3.45	0.998	2.44	3.66	0.997
29	3.69	4.58	0.999	3.07	3.35	0.998	2.56	3.80	0.998

* See Table I.

for other investigated systems (solvent systems denoted by Roman numerals as in Table II):

$$R_{Mw}\,(\mathrm{I}) = 1.17\,R_{Mw}\,(\mathrm{II}) - 0.39 \quad (n = 29,\ r = 0.884) \tag{2}$$

$$R_{Mw}\,(\mathrm{I}) = 1.03\,R_{Mw}\,(\mathrm{III}) + 0.66 \quad (n = 29,\ r = 0.838) \tag{3}$$

$$R_{Mw}\,(\mathrm{I}) = 1.06\,R_{Mw}\,(\mathrm{V}) + 0.15 \quad (n = 29,\ r = 0.856) \tag{4}$$

The moderate correlation coefficients obtained for systems with methanol containing acetic acid (II), tetrahydrofuran (III) or dioxane (V) as modifier showed that the molecular properties of the solute in both of the compared systems contribute

System IV (RP-18; acetonitrile concentration range 50–90%)			*System V (RP-18; dioxane concentration range 40–80%)*			*System VI (RP-2; methanol concentration range 40–80%)*			*System VII (RP-18 methanol 60%), log k*
a	*b*	*r*	*a*	*b*	*r*	*a*	*b*	*r*	
1.56	2.82	0.997	1.18	2.48	0.997	0.87	1.55	0.992	0.15
1.16	2.64	0.998	1.31	3.15	0.991	1.25	2.47	0.996	−0.07
1.74	2.93	0.999	2.19	4.02	0.998	1.94	3.14	0.996	0.27
1.12	2.64	0.999	1.00	3.18	0.998	1.15	2.77	0.999	−0.20
1.21	2.35	0.998	0.67	2.08	0.998	1.22	2.31	0.999	−0.12
2.24	3.38	0.996	3.00	4.57	0.999	2.79	3.84	0.997	1.02
2.15	3.50	0.998	2.61	4.37	0.998	2.59	3.79	0.997	0.76
2.51	3.12	0.998	3.14	4.43	0.996	1.37	2.25	0.986	1.23
1.55	2.98	0.997	1.14	2.88	0.998	1.90	3.32	0.996	0.20
2.06	3.12	0.998	2.42	4.17	0.996	2.37	3.64	0.996	0.57
2.85	3.38	0.997	2.95	3.66	0.999	1.65	2.74	0.997	0.91
2.33	3.06	0.998	2.39	3.27	0.998	1.21	2.31	0.997	1.07
1.90	2.09	0.999	1.15	2.03	0.998	1.93	2.90	0.999	0.28
2.33	2.80	1.000	2.45	3.91	0.991	1.75	2.70	0.994	1.16
0.87	1.97	0.993	1.04	2.18	0.996	1.81	2.88	0.999	0.24
1.59	2.38	0.995	2.11	3.28	0.989	1.64	2.73	0.987	0.63
1.39	2.81	0.998	1.58	2.85	0.998	1.74	2.74	0.998	0.21
2.29	2.82	0.997	2.88	4.11	0.999	3.42	4.39	0.997	1.24
2.43	3.04	0.999	2.87	4.14	0.999	3.63	4.54	0.996	1.11
3.25	3.43	0.992	3.55	4.30	0.999	3.70	4.43	0.997	1.42
3.28	3.63	0.999	3.35	4.40	0.998	3.78	4.61	0.996	1.55
2.52	3.06	0.999	3.00	4.17	0.997	3.61	4.78	0.998	1.17
1.52	2.13	0.993	2.45	3.94	0.987	1.90	2.68	0.998	0.86
1.62	2.51	0.990	2.17	3.35	0.999	3.42	4.39	0.997	0.88
1.39	2.81	0.998	1.34	2.56	0.998	1.58	2.56	0.998	0.17
0.94	2.33	0.985	1.04	2.22	0.998	1.25	2.22	0.996	0.07
2.16	2.84	0.997	2.70	3.72	0.997	3.40	4.26	0.998	0.69
2.26	2.80	0.998	3.10	4.13	0.977	2.99	3.72	0.999	0.88
2.95	3.37	0.998	3.00	3.96	0.998	3.64	4.47	0.998	1.07

TABLE III

LOG k' (HPLC), R_M (HPTLC), ΔLOG k' AND ΔR_M VALUES OF COUMARINS

Selectivities relative to coumarin (1) for solutes 2–13 and 23, to psoralen (3) for solutes 14–22 and to xanthyletin (23) for solutes 24–29.

*Compound**	*HPLC RP-18, 60% methanol*		*HPTLC RP-18, 60% of modifier in the eluent*												*From System 1 in Table II,* ΔR_{M_w}
			Methanol		*Methanol + 3% acetic acid*		*Acetonitrile*		*Dioxane*		*Tetrahydro-furan*		*RP-2, methanol*		
	log k'	*Δlog k'*	R_M	ΔR_M	R_M	ΔR_M	R_M	ΔR_M	R_M	ΔR_M	R_M	ΔR_M	R_M	ΔR_M	
1	0.15	0.00	0.10	0.00	0.00	0.00	−0.17	0.00	−0.31	0.00	−0.14	0.00	−0.07	0.00	0.00
2	−0.07	−0.22	−0.25	−0.35	−0.37	−0.37	−0.39	−0.22	−0.60	−0.29	−0.23	−0.09	−0.19	−0.12	−0.61
3	0.27	0.12	0.30	0.20	0.23	0.23	−0.03	0.14	−0.19	0.12	−0.14	0.00	0.10	0.17	0.32
4	−0.20	−0.35	−0.41	−0.51	−0.21	−0.21	−0.45	−0.28	−0.90	−0.59	−0.43	−0.29	−0.50	−0.43	−0.25
5	−0.12	−0.27	−0.29	−0.39	−0.29	−0.29	−0.30	−0.13	−0.57	−0.26	−0.45	−0.31	−0.17	−0.10	−0.76
6	1.02	0.87	0.90	0.80	0.86	0.86	0.17	0.34	0.23	0.54	0.05	0.19	0.48	0.55	1.71
7	0.76	0.61	0.66	0.56	0.57	0.57	0.02	0.19	−0.05	0.26	0.02	0.16	0.29	0.36	1.21
8	1.23	1.08	1.12	1.02	0.95	0.95	0.63	0.80	0.43	0.74	0.02	0.16	−0.05	0.02	1.59
9	0.20	0.05	0.03	−0.07	−0.02	−0.02	−0.27	−0.10	−0.60	−0.29	−0.57	−0.43	−0.05	0.02	0.10
10	0.57	0.42	0.45	0.35	0.39	0.39	0.17	0.34	−0.10	0.21	−0.14	0.00	0.19	0.26	0.84
11	0.91	0.76	1.36	1.26	1.19	1.19	0.79	0.96	0.73	1.04	0.27	0.41	0.05	0.12	2.52
12	1.07	0.92	1.00	0.90	0.95	0.95	0.47	0.64	0.43	0.74	0.09	0.23	−0.18	−0.11	1.63
13	0.28	0.13	0.43	0.33	0.45	0.45	0.17	0.34	−0.07	0.24	0.00	−0.14	0.27	0.34	0.00
14	1.16	0.88	1.12	0.69	0.72	0.27	0.65	0.48	0.41	0.48	0.19	−0.19	0.12	0.15	1.24
15	0.24	−0.04	0.36	−0.07	0.03	−0.42	−0.29	−0.46	−0.31	−0.24	−0.25	−0.25	0.09	−0.18	0.23
16	0.63	0.35	0.90	0.47	0.60	0.15	0.18	0.01	0.02	0.09	0.02	0.02	−0.02	−0.29	1.75
17	0.21	−0.07	0.18	−0.25	0.14	−0.31	−0.27	−0.44	−0.10	−0.03	−0.25	−0.25	0.09	−0.18	−0.01
18	1.24	0.96	1.19	0.76	1.06	0.61	0.60	0.43	0.43	0.50	0.07	0.07	0.83	0.56	1.78
19	1.11	0.83	1.12	0.69	0.96	0.51	0.60	0.43	0.37	0.44	−0.12	−0.12	0.95	0.68	1.90
20	1.42	1.14	1.38	0.95	1.38	0.93	1.19	1.02	0.79	0.86	0.37	0.37	1.06	0.79	1.52
21	1.55	1.27	1.28	0.85	1.12	0.67	1.12	0.95	0.72	0.79	0.25	0.25	1.06	0.79	1.58
22	1.17	0.89	1.19	0.76	1.06	0.61	0.69	0.52	0.48	0.55	0.18	0.18	0.72	0.45	1.71
23	0.86	0.71	0.58	0.48	0.35	0.35	0.27	0.44	0.09	0.40	0.05	0.19	0.27	0.34	0.00
24	0.88	0.02	0.60	0.02	0.48	0.13	0.16	−0.11	0.18	0.09	0.00	0.05	−0.29	−0.56	0.02
25	0.17	−0.69	−0.09	−0.49	0.05	−0.30	−0.27	−0.54	−0.17	−0.26	−0.29	−0.34	0.05	0.22	−1.23
26	0.07	−0.79	−0.07	−0.65	−0.10	−0.45	−0.41	−0.68	−0.31	−0.40	−0.39	−0.44	−0.09	−0.36	−1.63
27	0.69	−0.17	0.60	0.02	0.83	0.48	0.47	0.20	0.50	0.41	0.14	0.09	0.75	0.48	−0.21
28	0.88	0.02	0.86	0.28	0.95	0.60	0.60	0.33	0.79	0.70	0.21	0.16	0.79	0.52	0.54
29	1.07	0.21	0.95	0.37	1.06	0.71	0.95	0.68	0.66	0.57	0.25	0.20	1.00	0.73	0.75

* See Table I.

differently to the retention for various modifier systems.

A significantly worse correlation was obtained for the RP-2 sorbent as the stationary phase:

$$R_{Mw}\,(\mathrm{I}) = 1.95\,R_{Mw}\,(\mathrm{VI}) + 0.81 \qquad (n = 29,\ r = 0.695) \tag{5}$$

indicating that the characteristics of the support also influence the retention.

The best correlation coefficient was obtained for the relationship R_M *vs.* log k' values at 60% of methanol in the eluent in both of the compared techniques:

$$R_M = 1.01 \log k' - 0.06 \qquad (n = 29,\ r = 0.955) \tag{6}$$

the slope being close to unity and the intercept nearly zero. The mechanism of the separation processes in the two systems was the same and the R_M values easily determined in the HPTLC system can be used in the optimization of HPLC systems.

The HPLC selectivity parameter $\alpha = k_2/k_1$ corresponding to $\Delta R_M = \log k'_2/k'_1$ shows the effect of individual substituents on the retention and the selectivity of the developing systems.

Table III summarizes the log k', Δlog k', R_M and ΔR_M values obtained for 60% organic modifier in the eluent. The ΔR_M values are differentiated for the various investigated modifiers owing to the differences in the polarity of the substituents and in the interaction with the eluent and the stationary phase.

With 60% of methanol as modifier (Table III), $\Delta R_M(\mathrm{OH}) = -0.35$ (compounds 1 and 2). A methoxy group in the molecule increases the hydrophobic properties, $\Delta R_M(\mathrm{OCH_3}) = 0.20$ (compounds 1 and 3). Prenylation of umbelliferone (2) to O-prenylumbelliferone (11) causes a large increase in retention: ΔR_M (O-prenyl) = 1.61. ΔR_M (geranyloxy) = 1.25 for compounds 2 and 12. Also, the addition of a condensed furan or pyran ring to the basic coumarin structure increases the retention; ΔR_M for compounds 1 and 13 is 0.33 and for compounds 1 and 23 it is 0.48.

Comparison of dihydroxyfuranocoumarins possessing large radicals shows that all the investigated compounds give high retention times, except dihydrooroselol (17) with a hydrophilic isopropanol group in the molecule. Methanol–water and methanol-containing acetic acid are the most selective eluents with respect to substituent effects on retention. For other investigated modifiers the ΔR_M values are significantly lower. The lowest ΔR_M values were obtained for 60% of tetrahydrofuran as eluent (see Table III).

The selectivity of the systems used was also illustrated by R_M *vs.* R_M correlations taking 60% of methanol as the reference modifier (Figs. 3–5). The compounds with R_M values lying on the same vertical line cannot be resolved when methanol is used and the compounds with R_M values lying on a horizontal line cannot be separated in the second correlated system. As shown in Fig. 3, the addition of 3% acetic acid to the methanol–water eluent significantly improved the separation of compounds 8, 14 and 19 and of 6 and 16, which cannot be separated with 60% methanol, but the selectivity for compounds 8, 12, 19 and 20 deteriorates. The pair of positional [demethylsuberosin (6) and osthenol (7)] and *cis-trans* isomers [*cis*-khellactone (25) and *trans*-khellactone (26)] were well separated in the systems used.

The results indicate that for the investigated modifiers the eluent strength

changes more or less depending on the molecular structure of the solutes[41]. For coumarins, tetrahydrofuran and dioxane were found to be the solvents with greatest eluent strength, but were less selective.

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METHYLATED CYCLODEXTRIN-BONDED STATIONARY PHASES FOR LIQUID CHROMATOGRAPHY

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SUMMARY

Ethylenediamine-monosubstituted per-O-methyl-α- and -β-cyclodextrin were coupled to succinamidopropyl silica. The unreacted, terminal carboxyl groups of the resulting methylated cyclodextrin stationary phases do not interact significantly with several disubstituted benzene derivatives having various functional groups. However, the secondary amino –NH– group in the spacer arm linking the cyclodextrin units to silica gel interacts strongly with solutes having small pK_a values, such as aminobenzoic and nitrobenzoic acids. Methylated α-cyclodextrin stationary phases with non-nitrogen-containing spacer arms were prepared by reaction of bare silica gel with two organotrichlorosilanes incorporating methylated α-cyclodextrin. These new stationary phases do not exhibit the strong interaction with the benzoic acids.

INTRODUCTION

Recently, cyclodextrins (CDs) have been immobilized on silica gel beads via several different spacer arms[1–4], and the unmodified CD stationary phases obtained have been shown to be useful in the liquid chromatographic separation of structural isomers[1–3], enantiomers[4,7–10] and diastereomers[5].

We previously compared the retention behaviour of disubstituted benzene derivatives on several β-CD stationary phases obtained by reaction of diethylenetriamine-monosubstituted β-CD with succinamidopropyl, glutaraldehyde-activated and epoxy-substituted silica derivatives[3]. It was found that the unmodified β-CD stationary phase prepared from succinamidopropyl silica (Su-Silica) is superior to the others, because the unreacted, terminal carboxyl groups of Su-Silica do not interact significantly with solutes having various functional groups. This spacer arm, however, contains both amido and secondary amino –NH– groups and it is probable that the –NH– groups affect the solute retention in some cases. On the unmodified CD stationary phases obtained by coupling ethylenediamine-monosubstituted CDs to Su-Silica, we had to use mixtures of methanol and phosphate buffer instead of methanol and water in order to elute amino-, chloro-, methyl- and nitrobenzoic acids, and their retention was strongly dependent upon the ionic strength of the buffer[11,12]. Com-

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pared with these solutes which are readily ionizable to anionic forms, the retention of cresol, nitroaniline and dinitrobenzene was scarcely affected by a change in ionic strength. On the other hand, the benzoic acids could readily be eluted with water or methanol–water after acylation of these unmodified CD stationary phases with acid anhydrides. This phenomenon strongly suggests the influence of the –NH– groups in the spacer arm on the retention of the acids. Feitsma *et al.*[10] and Fujimura *et al.*[6] recently referred to the anomalous retention behaviour of aromatic carboxylic acids on their unmodified β-CD stationary phases prepared by treating amino-bonded silicas with monotosylated β-CD. It was proposed that the secondary amino groups in their spacer arms and the unreacted, terminal amino moieties participate in the retention.

Acylation of the unmodified CD stationary phases effected remarkable changes in solute retention, as mentioned above. In this treatment, it is possible to acylate both the –OH groups of the CD units and the –NH– groups in the spacer arm, *i.e.*, $-(CH_2)_3NHCO(CH_2)_2CONH(CH_2)_2NH-CD$. Therefore, in order to investigate the effect of the spacer arm on the retention, we used the methylated CD stationary phases reported previously[13]. These stationary phases, having $-OCH_3$ instead of –OH groups in their CD units, were acetylated with acetic anhydride in pyridine, together with the stationary phases without CD units. The retention behaviour of several solutes was studied on these stationary phases before and after acetylation. Further, an attempt was made to obtain methylated CD stationary phases having the same spacer arm described above, but by a simplified procedure. We also tried to prepare methylated α-CD stationary phases with non-nitrogen-containing spacer arms.

EXPERIMENTAL

Materials and chromatography

Silica gel (super micro bead B-5, mean particle diameter 5.9 μm and specific surface area 411 m^2/g) and α-CD were gifts from Fuji-Davison (Kasugai, Japan) and Wako (Osaka, Japan), respectively. All other reagents of analytical reagent grade were obtained from Wako or Tokyo Kasei (Tokyo, Japan).

All chromatographic studies were carried out with an LC-6A pump (Shimadzu, Kyoto, Japan), a special dampner (Gasukuro Kogyo, Tokyo, Japan), a KHP-UI-130A injector (Kyowa Seimitsu, Tokyo, Japan) and a 440 UV detector operating at 254 nm (Waters, Milford, MA, U.S.A.).

Each stationary phase was packed into a stainless-steel column (10 cm × 0.4 cm I.D.) by a balanced density slurry method. The flow-rate of the eluent (water, methanol–water or methanol–0.15 *M* phosphate buffer) was 1.0 ml/min. The concentration of the sample solutes was 0.2 m*M*, and a volume of less than 20 μl was injected.

Preparation of methylated CD stationary phases

Careful treatment of α-CD (10 g) or β-CD (7.5 g) with trityl chloride (10 g) in pyridine gave mono(6-O-trityl)-α-CD or -β-CD as a main product, respectively. Ethylenediamine-monosubstituted per-O-methyl-α-CD (Me-α-en) or -β-CD (Me-β-en) was prepared by methylation of all the remaining hydroxyl groups of each mono-

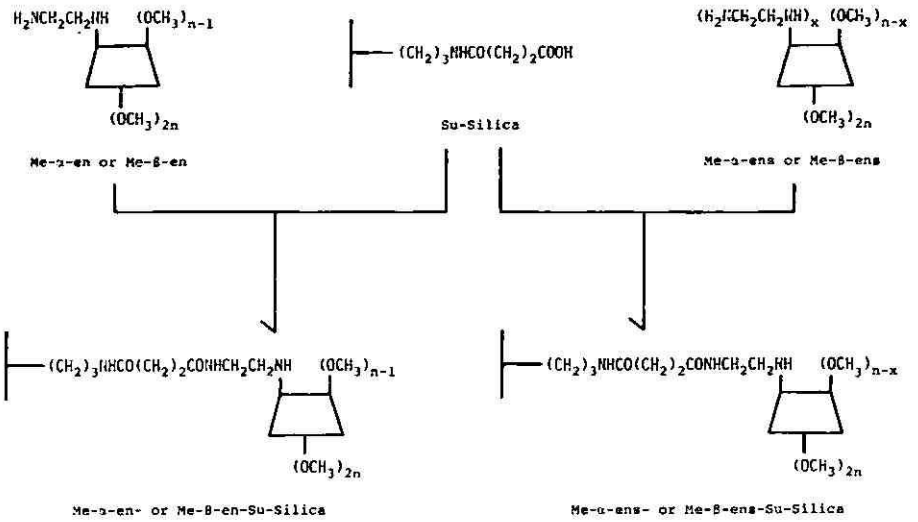

Fig. 1. Immobilization of ethylenediamine-substituted per-O-methyl-CDs on Su-Silica. α-CD; $n = 6$; β-CD; $n = 7$; $x = 1$–3 for Me-α-ens and Me-β-ens.

trityl-CD, removal of the trityl group and tosylation of the free hydroxyl group produced, followed by displacement of the sulphonate with ethylenediamine.

Me-α-en or Me-β-en was coupled to Su-Silica as described previously (Fig. 1), and the stationary phase obtained is denoted by Me-α-en-Su-Silica or Me-β-en-Su-Silica, respectively[13].

A mixture of mono-, di- and tri-(6-O-trityl)-α- or -β-CD was obtained by reaction of trityl chloride (10 g) with α-CD (4.5 g) or β-CD (5 g) for 10 h at room temperature in a smaller amount of pyridine compared with the above-mentioned monotritylation. By treating each mixture in much the same way as described above, ethylenediamine-substituted per-O-methyl-α-CD (Me-α-ens) or -β-CD (Me-β-ens) was derived and gave the corresponding methylated CD stationary phase (Me-α-ens-Su-Silica or Me-β-ens-Su-Silica, respectively).

The amounts of ethylenediamine-substituted per-O-methyl-CDs immobilized were evaluated by elemental analysis: 46.5 μmol/g for Me-α-en-Su-Silica, 58.2 μmol/g for Me-β-en-Su-Silica, 48.5 μmol/g for Me-α-ens-Su-Silica and 35.2 μmol/g for Me-β-ens-Su-Silica.

Preparation of stationary phases without CD units

N-Ethylethylenediamine or *n*-propylamine was immobilized instead of the CD derivatives on Su-Silica; the resulting stationary phases are denoted by Et-en-Su-Silica or Pr-Su-Silica, respectively. The amount of N-ethylethylenediamine immobilized was 199 μmol/g and that of n-propylamine, 341 μmol/g.

Acetylation of stationary phases

Each stationary phase (1.5 g) was suspended in pyridine (20 ml) and acetic anhydride (6 ml) was added. After stirring for 6 h at 45°C, the acetylated stationary phase was filtered off, thoroughly washed successively with methanol, water and

methanol and dried *in vacuo*. The acetylated stationary phases are denoted by prefixing the names of the parent stationary phases by Ac-.

RESULTS AND DISCUSSION

Effect of unreacted carboxyl groups on retention

The amount of carboxyl groups on Su-Silica utilized in this work is 993 μmol/g, and the proportion of the carboxyl groups used for the immobilization of ethylenediamine-monosubstituted CD (Me-α-en or Me-β-en) is only about 1/20. It is therefore assumed that the unreacted carboxyl groups will affect the retention of solutes on the methylated CD stationary phases. Table I gives the retention times of the *ortho*, *meta* and *para* isomers of eight disubstituted benzene derivatives having various functional groups on Su-Silica in methanol–water (20:80). These solutes are apparently rapidly eluted, and the contribution of the unreacted carboxyl groups on Su-Silica to the retention is considered to be small.

Effect of spacer arm on retention

Tables II and III give the retention times of the eight solutes with methanol–water (20:80) on the methylated CD stationary phases before and after acetylation. Comparison of these results with those in Table I indicates that the coupling of Me-α-en and Me-β-en to Su-Silica has brought about increased retention of the solutes. This apparently suggests a positive contribution of the methylated CD units to the retention. In spite of a smaller amount of the methylated CD units in Me-α-en-Su-Silica, it retained the solutes more strongly than did Me-β-en-Su-Silica. This fact implies that the disubstituted benzene derivatives can fit the methylated α-CD cavity better than the methylated β-CD one. On Me-α-en-Su-Silica, *p*-iodoaniline could not be eluted within 30 min with methanol–water (20:80) and gave a retention time of 22.15 min with methanol–water (30:70). Being retained much more strongly, *p*-nitrophenol was eluted in 10.95 min with methanol–water (40:60). Moreover, neither aminobenzoic nor nitrobenzoic acid could be eluted within 60 min with any eluent of methanol–water (0:100–50:50), on Me-α-en-Su-Silica and Me-β-en-Su-Silica.

However, the treatment of these stationary phases with acetic anhydride in

TABLE I

RETENTION TIMES (min) OF DISUBSTITUTED BENZENE DERIVATIVES ON Su-SILICA

Eluent: methanol–water (20:80). t_0 = 1.10 min.

Solute	*o-*	*m-*	*p-*
Cresol	1.80	1.90	1.90
Iodoaniline	2.45	2.55	2.55
Toluidine	1.90	2.20	2.80
Nitroaniline	2.30	1.80	2.05
Nitrophenol	1.95	1.95	2.00
Dinitrobenzene	2.40	1.75	1.65
Aminobenzoic acid	1.10	1.45	1.50
Nitrobenzoic acid	1.60	1.70	1.50

TABLE II

RETENTION TIMES (min) ON METHYLATED α-CYCLODEXTRIN STATIONARY PHASES BEFORE AND AFTER ACETYLATION

Eluent: methanol–water (20:80).

Solute	*Me-α-en-Su-Silica**			*Ac-Me-α-en-Su-Silica***		
	o-	*m-*	*p-*	*o-*	*m-*	*p-*
Cresol	3.05	3.65	4.55	4.25	5.05	5.85
Iodoaniline	7.50	20.15	–***	9.85	18.05	29.75
Toluidine	2.35	2.45	2.60	4.25	5.80	8.70
Nitroaniline	7.60	5.65	22.90	8.05	5.85	14.45
Nitrophenol	8.15	10.30	–***	6.10	9.05	11.15
Dinitrobenzene	4.80	2.95	2.60	7.15	4.30	3.95
Aminobenzoic acid	–***	–***	–***	2.55	1.60	2.75
Nitrobenzoic acid	–***	–***	–***	1.40	1.90	2.05

* t_0 = 1.00 min.
** t_0 = 1.20 min.
*** Not eluted.

pyridine resulted in ready elution of the benzoic acids, as shown in Tables II and III. This remarkable change in the retention is also true for the unmodified CD stationary phases obtained by coupling ethylenediamine-monosubstituted CDs to Su-Silica, as described in the Introduction. In the case of the unmodified CD stationary phases, the –OH groups of the CD moieties are also acetylated. Therefore, in order to explain convincingly the effect of the spacer arm on the retention, we acetylated the methylated CD stationary phases whose CD moieties were not modified. As is seen in Fig.

TABLE III

RETENTION TIMES (min) ON METHYLATED β-CYCLODEXTRIN STATIONARY PHASES BEFORE AND AFTER ACETYLATION

Eluent: methanol–water (20:80).

Solute	*Me-β-en-Su-Silica**			*Ac-Me-β-en-Su-Silica***		
	o-	*m-*	*p-*	*o-*	*m-*	*p-*
Cresol	3.85	4.00	4.15	5.45	5.65	5.65
Iodoaniline	5.45	10.05	9.70	8.75	13.65	13.00
Toluidine	2.25	2.35	2.25	4.00	5.05	6.60
Nitroaniline	5.05	4.70	7.20	7.80	6.55	9.25
Nitrophenol	5.90	6.40	11.55	5.80	8.05	9.05
Dinitrobenzene	5.70	2.65	2.60	9.50	4.25	4.15
Aminobenzoic acid	–***	–***	–***	2.55	1.65	1.60
Nitrobenzoic acid	–***	–***	–***	1.35	1.75	1.65

* t_0 = 1.00 min.
** t_0 = 1.10 min.
*** Not eluted.

1, the spacer arm contains three –NH– groups, *i.e.*, two amido and one secondary amino groups. Acetylation with acetic anhydride is readily performed for primary and secondary amino groups but not for amido, –NH–, ones. Consequently, the remarkable change in the retention of the acids is ascribed to acetylation of the secondary amino –NH– group.

If this inference is reasonable, a similar remarkable change in the retention is expected for a stationary phase containing a secondary amino group but not CD units. N-Ethylethylenediamine was coupled to Su-Silica to produce Et-en-Su-Silica [–$(CH_2)_3NHCO(CH_2)_2NHC_2H_5$], and the retention behaviours of the solutes both on Et-en-Su-Silica and its acetylated stationary phase (Ac-Et-en-Su-Silica) were studied (Table IV). The expected large decrease in the retention after acetylation is apparent for aminobenzoic and nitrobenzoic acids, and for *o*- and *p*-nitrophenol. These solutes have smaller pK_a values, compared with the others. Rough calculations show that the percentage of the anionic form at pH 6.8, for instance, is 98.8% for *p*-aminobenzoic acid (pK_a = 4.89), 99.96% for *p*-nitrobenzoic acid (pK_a = 3.43) and 28.9% for *p*-nitrophenol (pK_a = 7.19). On the other hand, only 2.4% of *m*-nitrophenol (pK_a = 8.4) is in the anionic form at the same pH, and the retention of *m*-nitrophenol is not significantly influenced by acetylation of Et-en-Su-Silica. These results strongly suggest a strong interaction of the anionic forms of the solutes with the secondary amino group in the spacer arm. A similar large decrease in the retention of aminobenzoic and nitrobenzoic acids or of *o*- and *p*-nitrophenol was also observed by using methanol–phosphate buffer instead of methanol–water as eluents. This fact is most likely caused by the masking the secondary amino group, thus removing or reducing the strong interaction mentioned above.

It is of interest to examine the retention behaviour of aminobenzoic and nitrobenzoic acids both on Pr-Su-Silica and the stationary phase obtained by treating it with acetic anhydride, because these two stationary phase contain amido –NH– but no amino –NH–. No appreciable change in the retention was observed after the

TABLE IV

RETENTION TIMES (min) ON Et-en-Su-SILICA AND Ac-Et-en-Su-SILICA

Eluent: methanol–water (20:80).

Solute	*Et-en-Su-Silica**			*Ac-Et-en-Su-Silica***		
	o-	*m-*	*p-*	*o-*	*m-*	*p-*
Cresol	2.95	3.05	3.10	4.75	4.90	4.95
Iodoaniline	4.00	4.20	4.10	7.65	8.85	8.50
Toluidine	2.05	2.10	2.00	3.15	3.20	3.15
Nitroaniline	4.05	2.65	3.35	6.80	4.30	5.45
Nitrophenol	22.95	5.25	27.50	5.90	6.05	6.10
Dinitrobenzene	4.35	2.45	2.25	8.15	4.25	3.90
Aminobenzoic acid	–***	–***	–***	6.20	4.50	4.35
Nitrobenzoic acid	–***	–***	–***	6.25	11.05	10.95

* t_0 = 0.85 min.

** t_0 = 1.15 min.

*** Not eluted.

treatment with acetic anhydride. This suggests that the amido –NH– has not been acetylated. N-Propylacetamide (1 g) was treated with acetic anhydride (20 ml) in pyridine (30 ml) for 6 h at 45°C. The isolated compound gave an identical infrared spectrum to that of the starting N-propylacetamide. No acetylated product could be obtained. This model reaction also indicates that the amido –NH– in the spacer arm is not acetylated.

Methylated CD stationary phases derived from mixtures of tritylated CDs

α- or β-CD was treated with an excess of trityl chloride in pyridine to give a mixture of mono-, di- and tri-(6-O-trityl)-α- or -β-CD. Methylation of all the remaining hydroxyl groups of the derivatives in each mixture and then removal of the trityl groups produced a mixture of the corresponding hydroxy-per-O-methyl derivatives. Hitherto, the methylated CD stationary phases had been prepared by reaction of Su-Silica with ethylenediamine-monosubstituted per-O-methyl-CDs derived from monohydroxy-per-O-methyl-CDs. Their isolation from the mixtures by column chromatography is quite time-consuming and troublesome. If the mixtures can be used for preparing methylated CD stationary phases without the column isolation step, it is of great advantage. Therefore, the mixture of the hydroxy-per-O-methyl derivatives of α- or β-CD was treated as described in the Experimental. The resulting mixture of ethylenediamine-substituted per-O-methyl-α-CD (Me-α-ens) or -β-CD (Me-β-ens) was coupled to Su-Silica. The methylated CD stationary phases obtined (Me-α-ens-Su-Silica and Me-β-ens-Su-Silica) were compared with those from ethyl-

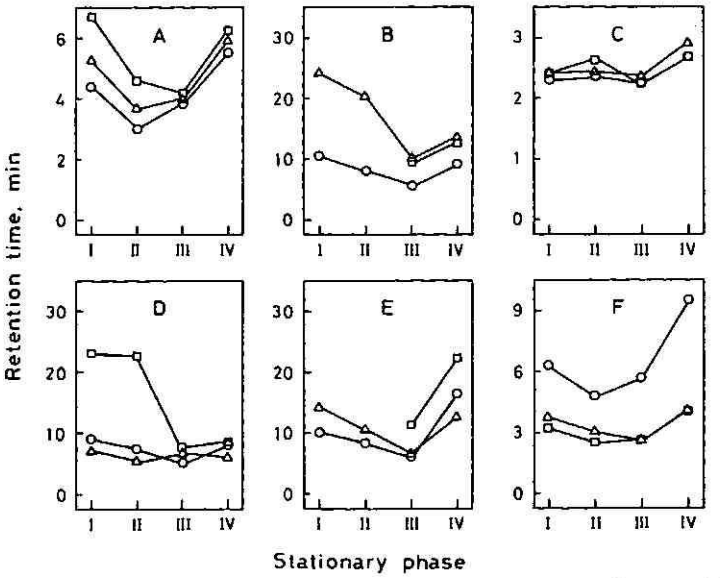

Fig. 2. Retention times of disubstituted benzene isomers, *ortho* (○), *meta* (△) and *para* (□), on Me-α-ens-Su-Silica (I), Me-α-en-Su-Silica (II), Me-β-en-Su-Silica (III) and Me-β-ens-Su-Silica (IV). Eluents: methanol–water (30:70) for I; methanol–water (20:80) for II–IV. Solutes: (A) cresol; (B) iodoaniline; (C) toluidine; (D) nitroaniline; (E) nitrophenol; (F) dinitrobenzene. *p*-Iodoaniline and *p*-nitrophenol were not eluted from I and II within 30 min.

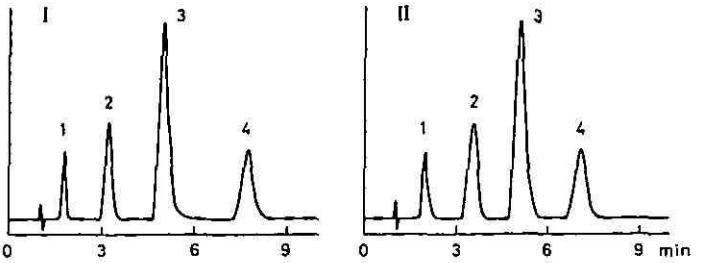

Fig. 3. Separation of antiepileptic drugs on Me-β-en-Su-Silica in methanol–water (20:80) (I) and on Me-β-ens-Su-Silica in methanol-water (30:70) (II). Solutes: 1 = primidone; 2 = phenobarbital; 3 = carbamazepine; 4 = phenytoin.

enediamine-monosubstituted per-O-methyl-CDs (Me-α-en-Su-Silica and Me-β-en-Su-Silica).

Fig. 2 shows the retention times of six disubstituted benzene derivatives on these four methylated CD stationary phases. It is apparent that the use of Me-α-ens or Me-β-ens results in no decrease in the selectivity. The solutes are retained on Me-α-ens-Su-Silica more strongly than on Me-α-en-Su-Silica. The same statement is true for the methylated β-CD stationary phases. This may be due to the extra ethylenediamine moieties in Me-α-ens or Me-β-ens. Further work is needed to explain this stronger retention.

Fig. 3 also gives an example showing no decrease in the selectivity in the separation of antiepileptic drugs.

It is shown that the use of Me-α-ens or Me-β-ens gives the methylated CD stationary phase much more readily without decrease in the selectivity, compared with the use of Me-α-en or Me-β-en.

Attempt to prepare methylated α-CD stationary phases with non-nitrogen-containing spacer arms

On our CD stationary phases, CDs are bonded to silica gel via a nitrogen-containing spacer arm. Consequently, a strong interaction with the spacer arm is observed for solutes having small pK_a values, as described above. In order to enhance the specificity of CD units, CD stationary phases with non-nitrogen-containing spacer arms are expected to be favourable. Armstrong and DeMond[4] first published results obtained with unmodified CD stationary phases having a non-nitrogen-con-

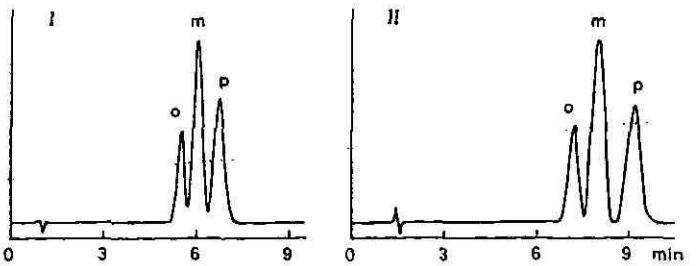

Fig. 4. Separation of cresol isomers on Me-α-pe-Silica (I) and on Me-α-pr-Silica (II) in methanol–water (30:70). *o*, *m* and *p* denote *ortho*, *meta* and *para* isomers, respectively.

taining spacer arm. On these stationary phases, CDs are coupled to a silica gel derivative with an active terminal group[14]. The existence of the unreacted, terminal group is unavoidable in this case, which is similar to our stationary phases as already mentioned.

We have, therefore, started to prepare CD stationary phases by reaction of bare silica gel with organohalogenosilanes incorporating CDs. Two trichlorosilanes, *i.e.*, mono[6-O-(5-trichlorosilylpentyl)]-per-O-methyl-α-CD and mono{6-O-[2-(3-trichlorosilylpropoxy)ethyl]}-per-O-methyl-a-CD, were synthesized and coupled to silica gel. The resulting stationary phases are denoted by Me-α-pe-Silica and Me-α-pr-Silica, respectively. The preliminary results for these stationary phases will be described briefly.

Fig. 4 shows typical liquid chromatograms for a mixture of *o*-, *m*- and *p*-cresol on these methylated α-CD stationary phases with non-nitrogen-containing spacer arms. The three isomers are well separated on both stationary phases and are eluted in the order *o*- < *m*- < *p*-. This elution order is consistent with the generally accepted order of stability for inclusion complex formation. On these stationary phases, as expected, aminobenzoic and nitrobenzoic acids can readily be eluted with eluents which do not contain phosphate buffer, which suggests no or little interaction between the solutes and the non-nitrogen-containing spacer arms. On the other hand, cresol, iodoaniline and nitroaniline were retained on Me-α-pe-Silica and Me-α-pr-Silica more strongly than on Me-α-en-Su-Silica. This may be ascribed to differences in the hydrophobicity of the spacer arms. Further work is now in progress, and the results together with the preparation procedures will be reported elsewhere.

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SIMULTANEOUS DETERMINATION OF BIOGENIC AMINES AND MORPHINE IN DISCRETE RAT BRAIN REGIONS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

A simple and sensitive method has been developed for the simultaneous determination of norepinephrine, epinephrine, dopamine, 5-hydroxytryptamine, 5-hydroxyindoleacetic acid, and morphine in discrete rat brain regions by reversed-phase high-performance liquid chromatography with electrochemical detection. Perchloric acid extracts of the tissue were directly injected into the chromatographic system. Each of these compounds gave a linear response over the range of 20–160 ng/ml cerebellar homogenate (0.4–3.2 ng on column). Recoveries of these compounds, added to the homogenates, were complete when compared with standards dissolved in perchloric acid. The average between-run coefficients of variation for all these compounds were lower than 7.4% over the range of 20–160 ng/ml, and the within-run coefficients of variation at 20 ng/ml were lower than 8.7%. The present method has been applied to a study of the effects of intraperitoneal administration of morphine on biogenic amines in several discrete rat brain regions.

INTRODUCTION

There has been an exponential increase in the number of literature accounts of the use of high-performance liquid chromatography (HPLC) for neurochemical analysis of endogenous biogenic amines in biological samples[1–20]. A recent study of the pharmacokinetics and pharmacodynamics of morphine, necessitating the simultaneous measurement of biogenic amines and morphine in whole brain, also employed HPLC[21]. The availability of a method for the simultaneous determination of biogenic amines and morphine would prove useful in studies addressing the relation between morphine-induced changes in biogenic amine transmission and the behavio-

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ral effects of the drug. Changes in steady-state levels or turnover rates of biogenic amines in various brain regions after morphine administration could be interpreted in the light of the knowledge of the relative levels and rates of elimination of morphine in those regions.

Unfortunately, the method of Ishikawa *et al.*[21] who used HPLC with organic solvent extraction as a means of purification, not only gave poor chromatographic separation but also resulted in low recoveries for both biogenic amines and morphine. We describe here a modification of the method of Kim *et al.*[15], originally used for measuring biogenic amines in various brain regions, which allows a sensitive and specific simultaneous determination of biogenic amines and morphine by the use of HPLC with electrochemical detection (ED).

The present method has been applied to a study of the effects of intraperitoneal administration of morphine on the biogenic amine levels in several discrete regions of the rat brain. The levels of morphine in blood were also measured by HPLC at various time intervals after a single i.p. injection of graded doses of morphine.

EXPERIMENTAL

Materials

Norepinephrine bitartrate (NE) (No. A-9512), epinephrine (E) (No. E-4375), dopamine hydrochloride (DA) (No. H-8502), 5-hydroxytryptamine creatinine sulfate complex (5-HT) (No. H-7752), 5-hydroxyindoleacetic acid (5-HIAA) (No. H-8876) and 3,4-dihydroxybenzylamine hydrobromide (DHBA) (D-7012) were purchased from Sigma (St. Louis, MO, U.S.A.), citric acid (B-10081), disodium hydrogen orthophosphate (No. 10249) and morphine sulfate (Lot No. 88675) were from British Drug Houses (Toronto, Canada), disodium ethylenediaminetetraacetic acid (EDTA) (S-311) and 70% perchloric acid (PCA) (ACS Reagent, A-229) were from Fisher (Fair Lawn, NJ, U.S.A.), sodium octyl sulfate (No. 10577) was from Eastman Kodak (Rochester, NY, U.S.A.) and methanol (HPLC Grade) was from Caledon Labs. (Georgetown, Canada). Water was deionized and glass distilled.

HPLC apparatus

HPLC determinations were performed with a Beckman (Irvine, CA, U.S.A.) Model 330 isocratic liquid chromatograph and a Model 110A Pump, a Bioanalytical Systems (West Lafayette, IN, U.S.A.) Model LC-4B amperometric detector and a Hewlett-Packard (Palo Alto, CA, U.S.A.) 3390A recording integrator. A glassy carbon working electrode was set at 0.8 V *vs.* an Ag/AgCl reference electrode. Sensitivity of the detector was maintained from 0.2 to 1.0 nA f.s., depending on the concentration of the substance to be measured. Separation was performed on a 250 mm × 4.6 mm I.D. Altex Ultrasphere ODS column (C_{18} reversed-phase, particle size 5 μm) preceded by a guard column (Guard-Pak, C_{18}, Waters Assoc., Milford, MA, U.S.A.) with isocratic elution.

Mobile phase

The mobile phase (pH 4.2) contained 50 m*M* citric acid, 100 m*M* disodium hydrogen orthophosphate, 0.38 m*M* sodium octyl sulfate and 0.5 m*M* EDTA, to which 15% (v/v) methanol was added. The mobile phase was filtered through a

0.45-μm filter (Millipore, Bedford, MA, U.S.A.) and then degassed under vacuum before use. A flow-rate of 1.2 ml/min (2500 p.s.i.) at ambient temperature was employed.

Preparation of standard solutions

Standard solutions of NE, E, DA, 5-HT, 5-HIAA, DHBA, and morphine (each 0.1 mg/ml expressed as the free base) were prepared in 0.1 *M* PCA, which had been previously filtered and degassed. These solutions were freshly prepared every two weeks; the morphine solution was stored at +4°C, and the other solutions at −70°C.

Animal procedures

Experiment I. Adult, male Sprague Dawley rats (Charles River, Montreal, Canada), weighing 250–290 g were housed individually in an environmentally controlled room at 21–23°C and 40% relative humidity, with lighting on from 7:00–19:00 h, for one week before use. They received food and water *ad libitum*, except that food was removed 24 h prior to sacrifice. On the day of experiment, the rats were injected intraperitoneally with 1 ml morphine (20 mg/kg) in saline or saline (per 100 g body weight). The rats were sacrificed by decapitation at 30, 75, and 120 min after the injection and the brains were removed rapidly. Each brain was dissected on an ice-chilled glass plate, and the striatum, hypothalamus, hippocampus, and midbrain-thalamus were separated, as described by Glowinski and Iversen[22]. The tissue was frozen on dry ice, and stored at −70°C until assayed. In order to determine whether the present technique is useful for determining morphine levels resulting from lower doses, rats were injected intraperitoneally with 5 mg/kg morphine and the midbrain-thalamus was dissected out and processed as described above. The cerebellum was saved to make a pooled cerebellum homogenate for use in the preparation of standard curves, as described below. An equal number of brains from each treatment (morphine or saline) and time group was processed each day in order to minimize variability of results.

Experiment II. Adult, male Sprague Dawley rats, weighing 245–260 g were kept under constant environmental conditions, as described above. Each rat was injected intraperitoneally with either 5, 10, or 20 mg of morphine (in 1.0 ml saline/100 g body weight). Blood samples (100 μl), collected from the tail vein at 15, 30, 60, 90, 120, and 180 min after injection, were carried through the entire procedure of Kim and Kats[24] for morphine determinations, using HPLC–ED.

Preparation of tissue samples for biogenic amines and morphine

Frozen tissue wet weights [mean ± standard error of the mean (S.E.M.)] were 65 ± 2, 69 ± 1, 107 ± 2, and 205 ± 5 mg for striatum, hypothalamus, hippocampus, and midbrain-thalamus, respectively. Individual samples were placed in polypropylene tubes, set in an ice-water bath, and were homogenized in 1.0 ml of 0.1 *M* PCA, containing 40 ng of DHBA as an internal standard, for 45 s by the use of a Polytron homogenizer (Brinkman Instrument, NY, U.S.A.). Homogenates were centrifuged at +4°C for 20 min at 35 550 *g*. The supernatants were separated and either immediately analyzed or stored at −70°C for assay later, within two weeks.

Supernatants were injected into the HPLC system, in a volume of 20 μl, by means of a 50-μl Hamilton syringe.

Standard curve

Known amounts (20–160 ng) of NE, E, DA, 5-HT, 5-HIAA and morphine in 1.0 ml aliquots of the pooled cerebellar homogenate (100 mg of tissue per ml of 0.1 *M* PCA) were taken through the entire procedure, DHBA (40 ng) being added to each of these samples as an internal standard. An identical set of samples of these compounds was made up in 1.0 ml of 0.1 *M* PCA. The measured levels of endogenous NE, E, DA, 5-HT, and 5-HIAA, contained in aliquots of the same homogenate without these added exogenous compounds, were subtracted from those of the corresponding standard samples. To construct the standard curve, the NE/DHBA, E/DHBA, DA/DHBA, 5-HT/DHBA, 5-HIAA/DHBA, and morphine/DHBA response ratios, corrected for the endogenous responses, were plotted against the amounts of each of these compounds, injected into the HPLC system. Actual amounts of these compounds, injected into the HPLC system, were 0.4–3.2 ng, while the amount of DHBA was 0.8 ng.

Statistical analysis

Data were analysed using one-way analysis of variance and Duncan's multiple range tests[23].

RESULTS AND DISCUSSION

Representative chromatograms of NE, E, DA, 5-HT, 5-HIAA, and morphine are shown in 0.1 *M* PCA (Fig. 1A) and in midbrain-thalamus homogenates, obtained from saline-treated (Fig. 1B) and morphine-treated (Fig. 1C) rats. No interfering endogenous compounds were apparent. Biogenic amines in frozen tissue samples were found to be completely stable when stored at −70°C for up to three months.

Limits of detection (*i.e.*, to give a recorder peak height of at least 0.1 a.u.f.s.) are summarized in Table I. The linearity of the concentration response relation for each of these compounds was established over the range of 20–160 ng in 1.0 ml of cerebellar homogenate (0.4–3.2 ng on column). Linear regression analysis from standard curves in cerebellar homogenates indicated that the correlation coefficients for all these compounds were higher than 0.9943. Recoveries of each of these compounds (20–160 ng) added to the homogenates were calculated by comparison of peak areas with results obtained with the corresponding samples in 0.1 *M* PCA, as well as with that of internal standard DHBA. The recoveries were essentially complete (Table I). The average between-run and within-run coefficients of variation for all these compounds were lower than 7.4 and 8.7%, respectively (Table I).

The ion-pairing reagent, 0.38 m*M* sodium octyl sulfate, permitted a large improvement in resolution. A recent report describes the principles and methodology of HPLC with ED and ion-pairing reagents[31]. We have found that a lower concentration, *i.e.* 0.14 m*M*, resulted in shortening the retention times for all compounds, but the resolution of 5-HT and morphine was poor. A higher (> 20%) methanol concentration in the mobile phase also resulted in a poor resolution of both these compounds. In previous studies of HPLC methods for catecholamines and indoles[15],

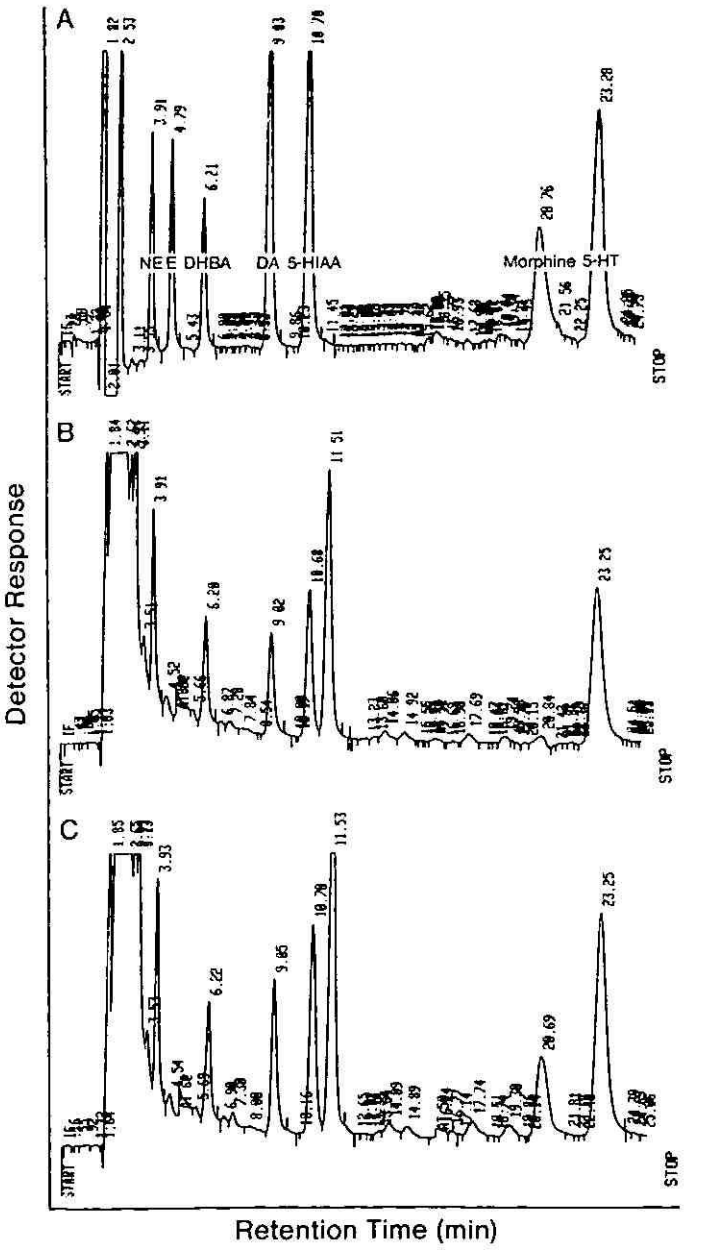

Fig. 1. (A) Reversed-phase HPLC separation of NE, E, DA, 5-HT, 5-HIAA and morphine standards (each 0.8 ng), and their internal standard DHBA (0.8 ng) in 0.1 *M* PCA). (B) Representative chromatogram demonstrating the HPLC separation and detection of NE, DA, 5-HT, and 5-HIAA from a portion of a single midbrain-thalamus obtained with a saline-treated rat. (C) Representative chromatogram demonstrating the HPLC separation and detection of NE, DA, 5-HT, 5-HIAA, and morphine from a portion of a single midbrain-thalamus obtained with a morphine-treated rat (20 mg/kg) at 30 min prior to sacrifice.

TABLE I

RECOVERY, PRECISION, AND LIMIT OF DETECTION OF THE HPLC DETERMINATION OF NE, E, DA, 5-HT, 5-HIAA AND MORPHINE IN CEREBELLAR HOMOGENATES

n = 5. C.V. = coefficient of variation.

Compound	*Recovery (%)*,** (mean ± S.D.)*	*Between-run C.V. (%)****	*Within-run C.V. (%)§*	*Detection limit (ng)*
NE	98 ± 7	5.6	7.7	0.1
E	94 ± 4	4.3	5.6	0.1
DA	100 ± 9	5.1	4.8	0.05
5-HT	110 ± 9	7.4	8.7	0.1
5-HIAA	106 ± 3	6.4	6.6	0.05
Morphine	108 ± 5	4.6	8.4	0.2

* Relative to the value for 0.1 M PCA solution.
** Over the range of 20–160 ng/ml homogenate.
*** Determinations over the range of 20–160 ng/ml homogenate.
§ Determinations at 20 ng/ml homogenate.

we had improved resolution at pH 4.2–4.5, which is a higher pH than is generally used. In the present work, varying the pH value of the mobile phase within the range of pH 4.2–5.0 did not critically influence the retention times for all compounds, and produced no differences in detector responses.

Different oxidation potentials (0.72–0.85 V) were also employed in the present study to obtain effective resolution. At a potential of 0.72 V, the detector response for biogenic amines was not decreased, but that for morphine was significantly decreased, and broadening of the peak was observed. At potentials of 0.8–0.85 V, the detector response for biogenic amines remained the same, but higher resolution for morphine was obtained. Under the experimental and mobile phase conditions described here, all compounds were readily oxidized at 0.8 V with sufficiently high cificity of analysis to detect biogenic amines and morphine simultaneously. The retention and resolution of biogenic amines and morphine were constant over 1000 runs on the same column. Maintenance of column efficiency was accomplished by (a) washing the column with 800–1000 ml of methanol–water (2:3) after 100–120 analyses and (b) replacing the guard column after every 250 analyses.

The concentrations of morphine in the hypothalamus of rats, sacrificed at 30 and 75 min after intraperitoneal administration, were significantly higher than those found in the midbrain-thalamus and striatum ($p < 0.001$ and $p < 0.025$, respectively), and the concentration of morphine in the hippocampus at 75 min was significantly higher than that of midbrain-thalamus or striatum ($p < 0.025$ and $p < 0.05$, respectively) (Fig. 2). The lowest morphine concentration measured in brain after a 20 mg/kg dose was 150 ng/g, in the 120 min sample of midbrain-thalamus (Fig. 2). This corresponds to an absolute sample size of about 0.6 ng on the column. The concentration of morphine in the midbrain-thalamus of rats sacrificed at 30 min after intraperitoneal administration of 5 mg/kg morphine was found to be 88 ± 5 ng (mean ± S.E.M., n = 5). Since the lower limit of detection is about 0.2 ng, it should be possible to measure tissue morphine values after doses in the 5–10 mg/kg range, *i.e.* after low analgesic rather than cataleptic doses. The peak levels of morphine

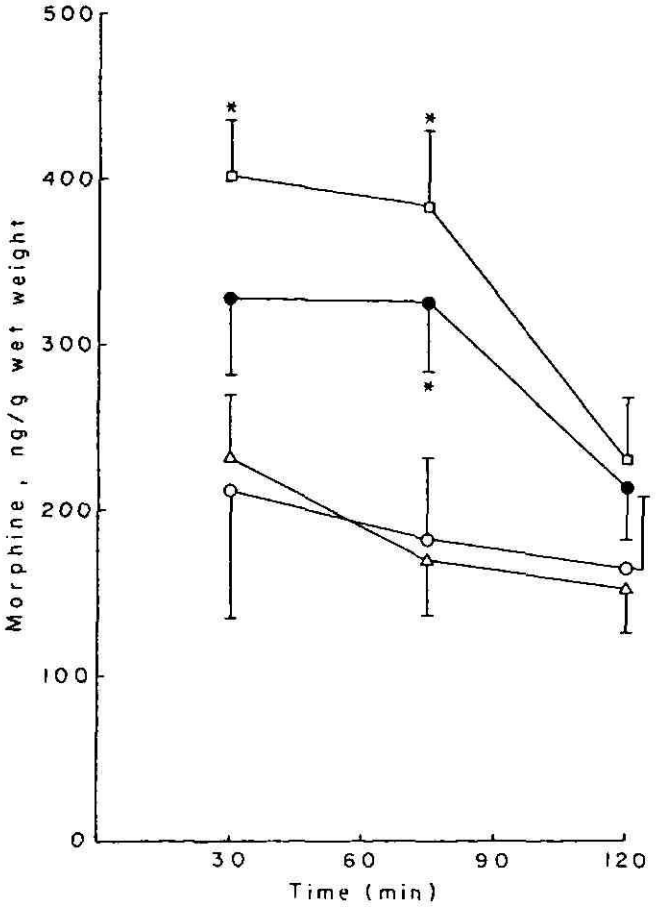

Fig. 2. Morphine concentrations in various brain regions of the rat at different time intervals after intraperitoneal injection of 20 mg/kg morphine: (□) hypothalamus, (●) hippocampus, (△) midbrain-thalamus, and (○) striatum. The points and vertical bars represent mean ± S.E.M. from four or five rats. An asterisk (*) means significantly different from midbrain-thalamus and striatum at p values 0.001, 0.025, or 0.05.

occurred at 30 min in all brain regions studied. The maximum blood level of morphine was also found to occur at 30 min after intraperitoneal administration of 5–20 mg/kg morphine (Fig. 3). Clearance of morphine from the blood appeared to follow an exponential time course, with an apparent biological half life of about 70 min, regardless of the dose given.

The baseline levels of biogenic amines found in the various brain regions in saline control rats are shown in Table II. The values found in hypothalamus, hippocampus, and midbrain-thalamus are in good agreement with those reported in the literature, but NE and DA levels in the striatum are 50–100% higher than most published values. The reason for the difference from other published values does not appear to lie in the HPLC technique, since it applies only to the striatum. Since our tissue weights for striatum were considerably lower than those reported by other

TABLE II

EFFECTS OF INTRAPERITONEAL MORPHINE ADMINISTRATION ON BIOGENIC AMINES IN VARIOUS BRAIN REGIONS OF THE RAT

A single intraperitoneal dose of either saline or morphine (20 mg/kg) was given at 30, 75, or 120 min prior to sacrifice. Since no statistical differences were found in the levels of biogenic amines between saline-treated rats at 30, 75, and 120 min prior to sacrifice, the data of the three groups were combined.

Experimental group	*Time of dose sacrifice (min)*	*Number of rats*	*Amount found (ng/g wet weight) (mean ± S.E.M.)*			
			NE	*DA*	*5-HT*	*5-HIAA*
Striatum						
Saline control		15	597 ± 39	15823 ± 514	728 ± 37	287 ± 15
Morphine	30	5	626 ± 61	16136 ± 1556	913 ± 108§	401 ± 70**
	75	5	697 ± 82	16720 ± 1200	728 ± 121	413 ± 63***
	120	5	599 ± 49	16667 ± 790	798 ± 46	383 ± 20§
Hypothalamus						
Saline control		15	3002 ± 123	462 ± 25	1023 ± 62	186 ± 19
Morphine	30	5	2487 ± 210*	413 ± 38	614 ± 51§	214 ± 18
	75	5	2396 ± 70***	465 ± 24	1016 ± 86	249 ± 28
	120	5	3134 ± 256	515 ± 36	950 ± 88	240 ± 35
Hippocampus						
Saline control		15	786 ± 36	43 ± 1	394 ± 22	103 ± 5
Morphine	30	5	814 ± 50	43 ± 4	422 ± 35	138 ± 12§
	75	5	897 ± 41*	46 ± 2	262 ± 11***	100 ± 11
	120	5	829 ± 38	45 ± 2	358 ± 26	151 ± 5§
Midbrain-thalamus						
Saline control		20	1184 ± 22	256 ± 8	1176 ± 33	210 ± 7
Morphine	30	10	1381 ± 54§	311 ± 12§	1385 ± 65**	284 ± 19§
	75	10	1181 ± 74	277 ± 26	1096 ± 32	236 ± 14
	120	10	1295 ± 33**	306 ± 10§	1423 ± 52§	341 ± 24§

* $p < 0.05$ compared to saline control.
** $p < 0.01$ compared to saline control.
*** $p < 0.025$ compared to saline control.
§ $p < 0.005$ compared to saline control.

investigators, it is conceivable that their larger samples contained substantial amounts of extra-striatal tissue with low catecholamine content, which diluted the NE and DA in the striatal tissue proper.

The effects of intraperitoneal administration of morphine on regional levels of biogenic amines are summarized in Table II. A significant increase in 5-HT at 30 min and a more sustained increase in 5-HIAA levels were found in the striatum, while NE and DA content in this region remained unchanged. In the hypothalamus, morphine administration resulted in a significant decrease in 5-HT and NE levels, with a return towards basal levels by 75–120 min. While NE and 5-HIAA levels were found to be increased in the hippocampus, a transient decrease in 5-HT concentration was noticeable 75 min after morphine administration. In the midbrain-thalamus both catecholaminergic and indoleaminergic transmissions were affected by morphine administration, *i.e.* NE, DA, 5-HT, and 5-HIAA levels were significantly increased at

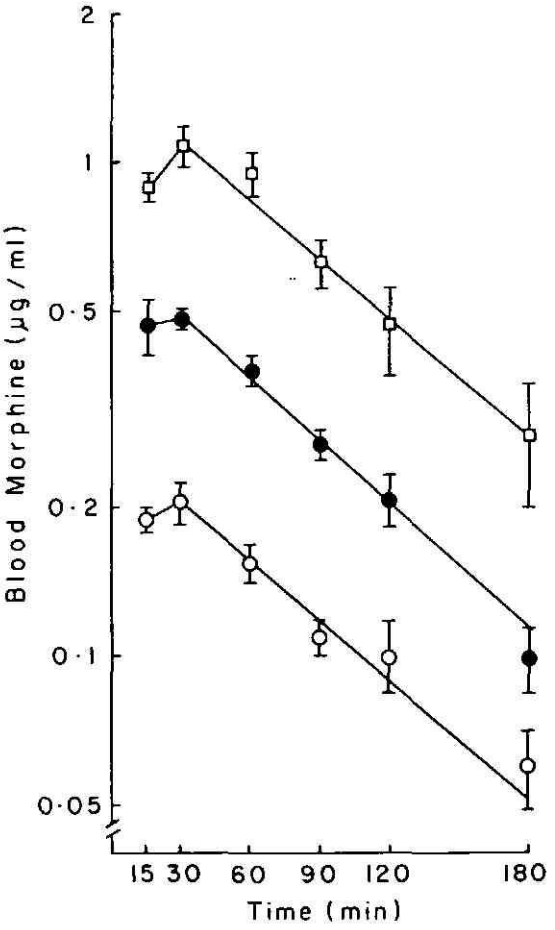

Fig. 3. Blood concentrations of morphine at various time intervals after intraperitoneal injection of 5 (○), 10 (●) or 20 (□) mg/kg of morphine. The points and vertical bars represent mean ± S.E.M. from five rats.

30 and 120 min, but there was a transient return to or below baseline levels at 75 min.

It is apparent that morphine influences the activities of catecholamines and indoles in different ways in various discrete brain regions. Our observation that acute morphine administration caused a decrease of NE level in the hypothalamus is consistent with findings by other investigators[25,26] and suggests that morphine acts either to stimulate NE release or to decrease its synthesis in this brain region. Johnson *et al.*[27] also reported that morphine administration produced slight, transient decreases in NE levels in most brain areas, and increased rates of NE biosynthesis in the hypothalamus. This combination of findings is more consistent with stimulation of NE release. On the other hand, other investigators have reported no changes in NE or DA levels in the hypothalamus[28] after acute morphine injection, or even a trend towards increased hypothalamic NE level[29]. However, we were unable to confirm a report of decreased NE level in the hippocampus[26]. Striatal DA was reported to be depleted and DA turnover rates increased in areas of striatum, hypothalamus, and midbrain[27]; in contrast, we found increased levels in the midbrain-thalamus, and no change in the other regions. Although steady-state concentration of 5-HT was not greatly affected by morphine administration, the level of 5-HIAA in the striatum was reported to be elevated[30]. Our findings are essentially in agreement with this report. The contradictory findings of morphine-induced enhancement or inhibition of biogenic amine release may indicate variability related to individual or strain differences, doses or morphine, routes of administration, or different environmental situations.

In the present study, the supernatant of the homogenates was injected directly into the column, eliminating the purification step of organic extraction. This eliminated a possible source of error, a loss in sensitivity and unsatisfactory recovery. The major advantages of the present method are simplicity of sample processing (elution time of 24 min for all these compounds), high sensitivity, and reproducibility sufficient to permit differential measurements of all of the substances in question in tiny samples from different regions of a single rat brain.

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PURIFICATION AND SOME PROPERTIES OF THREE SERINE CARBOXYPEPTIDASES FROM *ASPERGILLUS NIGER*

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SUMMARY

Three enzymes exhibiting peptidyl-L-amino acid hydrolase and esterase activities have been purified by immobilized metal-ion affinity chromatography and ion-exchange chromatography. The three enzymes were entirely free of the acid protease activity that normally exists along with them in the crude culture filtrates of *Aspergillus niger*. Although all three *exo*-peptidases possessed nearly identical molecular weights (*ca.* 140 000), isoelectric points (*ca.* 5.0) and other properties, their affinities for the two substrates tested, carbobenzoxy-L-Glu-L-Tyr and benzoyl L-arginine ethyl ester, differed. All three peptidases were inhibited by phenylmethanesulphonyl fluoride, indicating that they are serine carboxypeptidases. They were also inhibited by tosyl phenylalanine chloromethyl ketone, suggesting the presence of a histidyl residue in their active sites. The differences in the number of accessible histidyl residues on the enzyme surfaces could explain the differences in their retentions on Cu^{2+}–iminodiacetate–Sepharose 6B.

INTRODUCTION

Many carboxypeptidases have already been isolated from plants[1–6] and microorganisms, mostly fungi[7–16], and Zuber and Matile[17] have termed them acid carboxypeptidases owing to their maximal activity in the acidic region of the pH scale. However, Hayashi and Bai[18] preferred to call them serine carboxypeptidases owing to the presence of a reactive serine residue in their active sites as evidenced by their inhibition in the presence of phenylmethanesulphonyl fluoride (PMSF) and diisopropyl phosphofluoridate (DFP).

Many *Aspergilli* are prolific producers of serine carboxypeptidases. In fact, these enzymes are secreted into the culture medium as proteolytic complexes and very often both *endo*- and *exo*-peptidase activities are present. Such cases have been reported by Ichishima[19] and Panneerselvam and Dhar[15,20] for *Aspergillus saitoi* and *Aspergillus fumigatus*, respectively. The initial aim of our work was to find a method that facilitates the separation of *endo*- and *exo*-peptidase activities from the crude extracts of *Aspergillus niger* culture media. The use of immobilized metal-ion affinity

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chromatography (IMAC) on Cu^{2+}–iminodiacetate (IDA)–Sepharose 6B, not only helped us to achieve this[21] but also to isolate and study three iso-carboxypeptidases, referred to as carboxypeptidases I, II and III, by using special elution conditions.

The enzymes were characterized using classical techniques such as sodium dodecyl sulphate polyacrylamide gel ectrophoresis (SDS-PAGE) after a final ion-exchange chromatographic step.

EXPERIMENTAL

Chemicals

The crude culture filtrate of *Aspergillus niger* was a generous gift from Drs. Chow Ching Cheng and J. M. Lebeault of the Division des Procédés Biotechnologiques of the Université de Technologie de Compiègne.

The carboxypeptidase substrates carbobenzoxy-L-Glu-L-Tyr (CGT) and benzoyl L-arginine ethyl ester (BAEE), iminodiacetic acid (IDA; disodium salt), phenylmethanesulphonyl fluoride (PMSF) and tosylphenylalanine chloromethyl ketone (TPCK) were obtained from Sigma (St. Louis, MO, U.S.A.), casein from Merck (Darmstadt, F.R.G.), ethylenediaminetetraacetate (EDTA; sodium salt) and $CuSO_4 \cdot 5H_2O$ from Prolabo (Paris, France) and Sephadex G-50 and Sepharose 6B from Pharmacia (Uppsala, Sweden).

Methods

Determination of protein concentration. The routine determination of protein concentration was carried out by measuring the absorption at 280 nm using a Jobin Yvon Model JY201 spectrophotometer.

Carboxypeptidase assay. The peptidyl-L-amino acid hydrolase activity of the extract was assayed using 10 m*M* CGT by high-performance liquid chromatography (HPLC) as described[22]. One unit of peptidyl-L-amino acid hydrolase activity is defined as the amount of enzyme required to release 1 μM of L-tyrosine from the substrate at 30°C and pH 2.7.

The esterase activity of the enzyme was assayed spectrophotometrically using the method described by Schwert and Takaneka[23] by measuring the increase in absorbance at 260 nm accompanying the hydrolysis of 10 m*M* BAEE in 50 m*M* acetate buffer (pH 5.0). One unit of enzyme activity is the amount of enzyme needed to hydrolyse 1 μM of BAEE at 30°C and pH 5.0.

Acid protease assay. The acid protease activity in each step of the purification was determined using 2% casein as described[21]. One unit of enzyme activity is the amount of enzyme required to liberate 1 μg of L-tyrosine per minute at 39°C and pH 2.7. However, it should be noted that the action of the carboxypeptidases on the peptides released from the casein by the endopeptidase action of the acid protease is not taken into account. For both carboxypeptidase and acid protease, the specific activity is defined as the number of units of activity per unit absorbance of the enzymes at 280 nm.

Preparation of affinity sorbents. The affinity sorbent, Cu^{2+}–IDA–Sepharose 6B, was prepared according to ref. 24. Iminodiacetate, the metal-chelating agent, was fixed after epoxy activation of Sepharose 6B. The IDA–Sepharose 6B obtained was equilibrated and prepared with 50 m*M* $CuSO_4$ as described elsewhere[21].

Purification procedures. Purification was effected as in ref. 21, with certain modifications.

Ammonium sulphate precipitation. The culture medium was filtered and subjected to ammonium sulphate precipitation using 90% saturation.

Desalting and affinity chromatography. Unlike in ref. 21, a single step was employed to simultaneously desalt and separate *endo-* and *exo*-peptidase activities using a Sephadex G-50 column (45 × 3.2 cm I.D.) coupled to a Cu^{2+}–IDA–Sepharose 6B column (20 × 2.5 cm I.D.) in tandem. In a typical experiment, 30 ml of the ammonium sulphate extract containing 1740 absorbance units (280 nm) were injected into the Sephadex G-50 column and then washed with 50 m*M* acetate starting buffer (pH 5.3). The flow-rate was maintained at 112 ml/h (13.9 cm/h for the desalting column and 22.8 cm/h for the affinity column) and 10-ml fractions were collected. The first peak (excluded) that appears at the outlet of the desalting column passed directly into the Cu^{2+} column. The Sephadex column was then disconnected and both columns were washed separately with the starting buffer. When the flow-through peak from the Cu^{2+}–IDA–Sepharose 6B column was complete, the starting buffer was replaced with the first elution buffer, 5 m*M* Gly–HCl (pH 3.0). After the appearance of the first eluted peak a second elution buffer, 100 m*M* Gly–HCl (pH 3.0), was used to continue the elution.

Ion-exchange chromatography. The three active peaks obtained after IMAC were subjected to anion-exchange chromatography on DE-52 cellulose (Whatman) for further purification. The gel was conditioned according to the manufacturer's instructions. The elution conditions are described in the legends to the figures.

The active fractions were pooled, concentrated and used for the characterization. .

Partial characterization of the carboxypeptidases

Electrophoretic studies. The homogeneity and the molecular weights of the three enzymes were determined by SDS-PAGE (7%), conventional PAGE as described[25] and gradient gel electrophoresis using Pharmacia PAA 4/30 gradient gels. In all instances, thyroglobulin (MW 669000), ferritin (440000), catalase (232000), lactate dehydrogenase (140000) and bovine serum albumin (67000) from the Pharmacia electrophoresis calibration kit were used as standards. The bands were revealed using Coomassie Brilliant Blue as described[25].

Isoelectric focusing was carried out in the pH range 3.5–9.5 using Ampholine Pagplate (LKB, Bromma, Sweden). Standards were from the Pharmacia calibration kit, ranging from p*I* 3.5 to 9.3. The bands were revealed by silver staining according to Merril *et al.*[26].

Kinetic and other studies. The Michaelis constants (K_m) of the enzymes for the two substrates, BAEE and CGT, were determined using Lineweaver–Burk double reciprocal plots.

The effect of inhibitors on the enzymes was studied using 2 m*M* and 100 μM solutions of PMSF and TPCK, respectively. Other characteristics such as pH and temperature optima for activity, the stability of the enzymes at different pHs and temperatures were also determined. Table III gives the reaction conditions.

TABLE I

PURIFICATION OF THE THREE SERINE CARBOXYPEPTIDASES BY TANDEM DESALTING-METAL CHELATE AFFINITY CHROMATOGRAPHY ON SEPHADEX G-50-Cu^{2+}-IDA-SEPHAROSE 6B

Extract	*Protein (mg)*	*Activity (U)*	*Specific activity (U/mg)*	*Purification factor*	*Yield (%)*
Crude extract	6930	660	0.095	1	100
0–90% $(NH_4)_2SO_4$ extract	3132	662	0.211	2.2	100
After tandem desalting–affinity chromatography	I, 173; II, 20.5; III, 80	483 99 354	2.79 4.54 4.42	29 47 46	73 15 53
After ion-exchange chromatography	I, 39; II, 11.3; III, 28	426 98 319	10.9 8.7 11.4	115 92 120	64 15 48

RESULTS

Purification of the three serine carboxypeptidases

The purification of the three serine carboxypeptidases by ammonium sulphate precipitation, tandem desalting–affinity chromatography and anion-exchange chromatography is summarized in Table I. Instead of fractional precipitation as in ref.

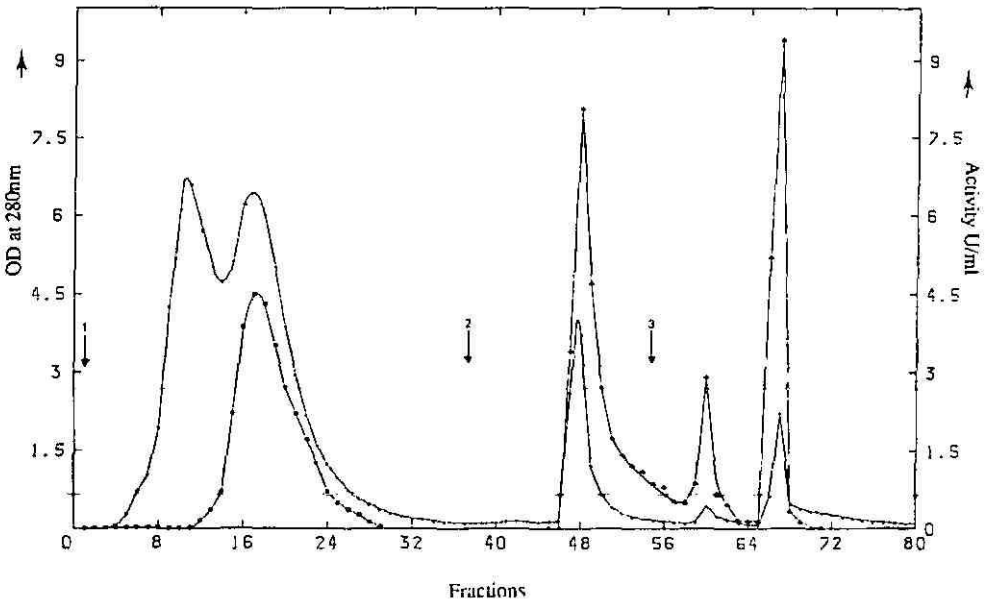

Fig. 1. Chromatography of the three carboxypeptidases and the acid protease by tandem desalting–metal chelate affinity chromatography on Sephadex G-50-Cu^{2+}-IDA-Sepharose 6B. ●, Absorption at 280 nm; ■, caseinolytic activity of the acid protease; ▲, esterase activity. Other details as in the text. Buffers: 1, 50 m*M* acetate (pH 5.3); 2, 5 m*M* Gly–HCl (pH 3.0); 3, 100 m*M* Gly–HCl (pH 3.0).

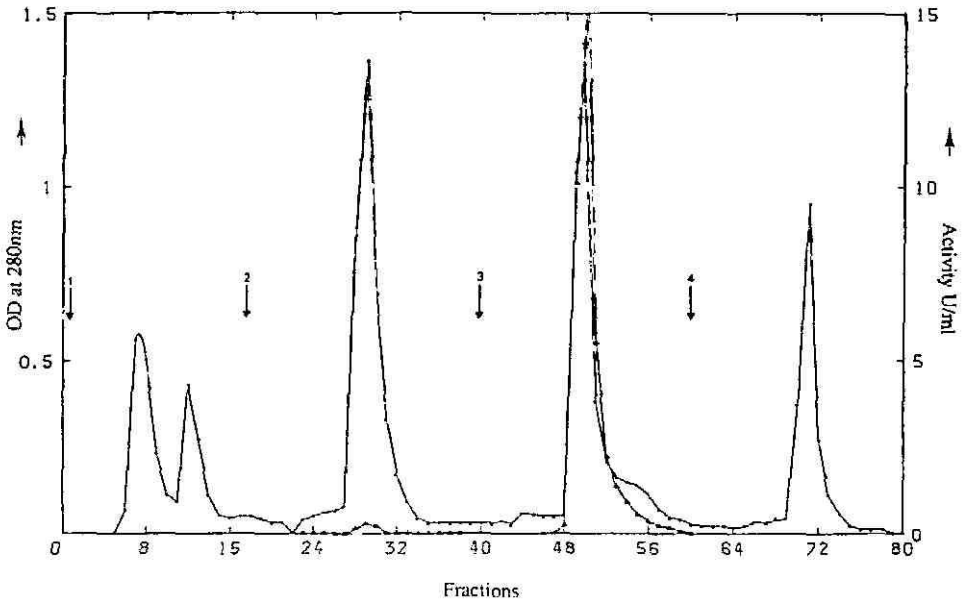

Fig. 2. Ion-exchange chromatography of carboxypeptidase I on DE-52 cellulose. Column: 16 × 2.5 cm I.D. ●, Absorption at 280 nm; ▲, esterase activity. Buffers: 1, 50 m*M* citrate (pH 5.9) (starting); 2, starting + 100 m*M* NaCl; 3, starting + 200 m*M* NaCl; 4, starting + 500 m*M* NaCl. Flow-rate, 90 ml/h (18.3 cm/h); fraction volume, 6 ml per tube.

21, a total precipitation using 90% saturation in ammonium sulphate was used. The desalting step was carried out simultaneously with the affinity step using a tandem system. Fig. 1 shows the efficiency of the affinity sorbent Cu^{2+}–IDA–Sepharose 6B for separating *endo-* and *exo*-peptidases. A stepwise gradient using Gly–HCl (pH 3.0) eluted the three enzymes in three peaks. Anion-exchange chromatography on DE-52 cellulose further purified the three carboxypeptidases (Figs. 2–4). Only a partial purification of the acid protease, by weak affinity or size-exclusion effects, was achieved (Table II and Fig. 1). The purities of the carboxypeptidases increased considerably after tandem desalting–affinity chromatography: carboxypeptidase I was 29-fold pure and II and III were each (46-fold pure). Pure extracts were obtained after the ion-exchange step. The protease obtained after IMAC, however, was only 7-fold pure. In all instances, high yields, sometimes exceeding 100%, were achieved.

Characteristics of carboxypeptidases I, II and III

Molecular weight and homogeneity. The three carboxypeptidases were found to be homogeneous electrophoretically in both the presence and the absence of SDS. The samples used were those obtained after anion-exchange chromatography. Fig. 5 shows a plot of log(molecular weight) *versus* distance from the cathode made after gradient-gel electrophoresis for the three enzymes. As is evident, all three enzymes seem to have molecular weights neighbouring that of lactate dehydrogenase (140 000). The molecular weights of the three enzymes were calculated to be carboxypeptidase I 141 000, carboxypeptidase II 135 000 and carboxypeptidase III 132 000 (Table III).

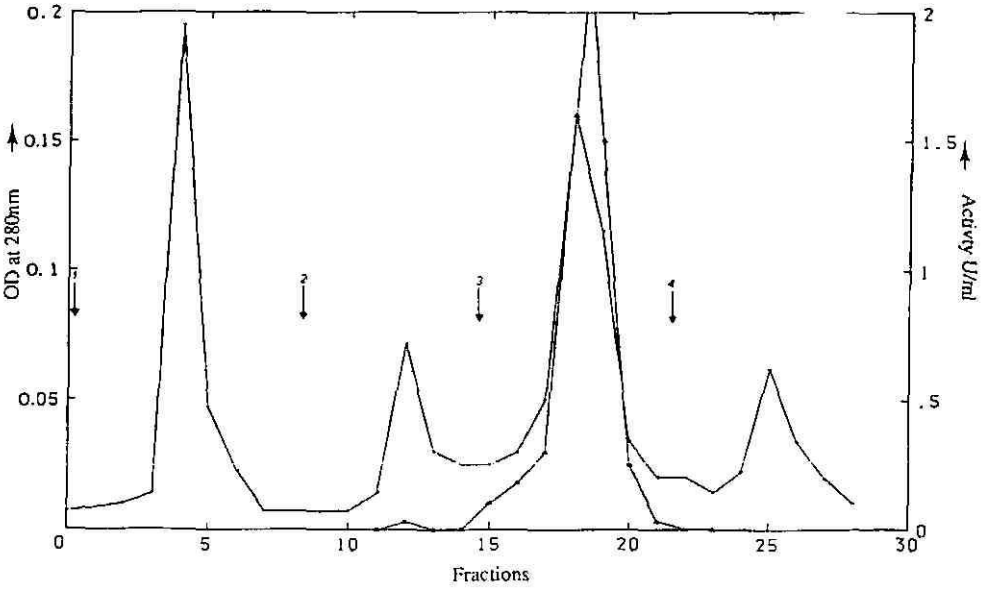

Fig. 3. Ion-exchange chromatography of carboxypeptidase II on DE-52 cellulose. Column: 7.7 × 1 cm I.D. Flow-rate, 14.1 ml/h; fraction volume, 1.7 ml per tube. Other details as in Fig. 2.

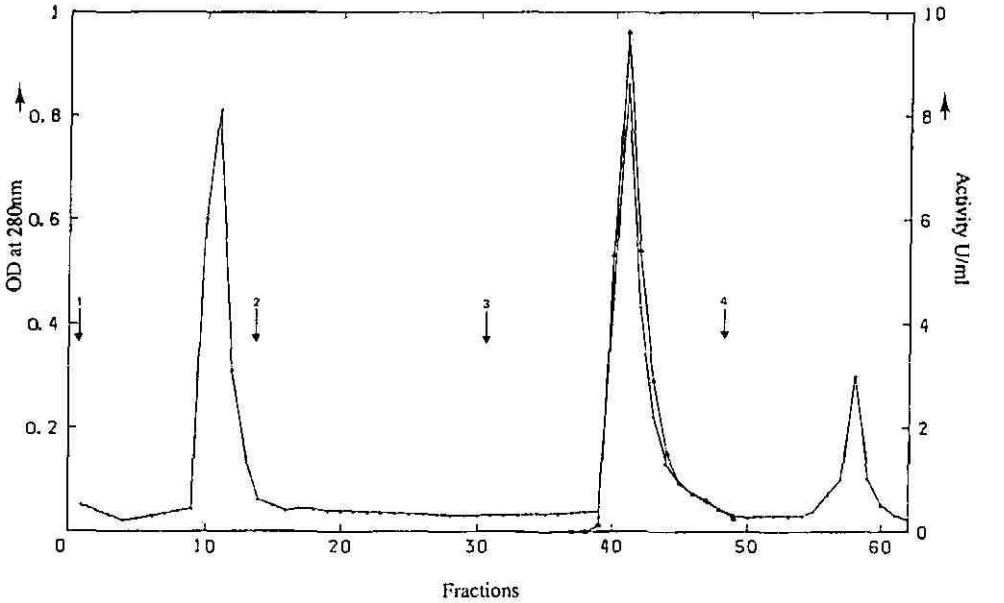

Fig. 4. Ion-exchange chromatography of carboxypeptidase III on DE-52 cellulose. Fraction volume, 4 ml per tube. Other details as in Fig. 2.

TABLE II

PURIFICATION OF THE ACID PROTEASE BY TANDEM DESALTING–WEAK AFFINITY CHROMATOGRAPHY ON SEPHADEX G-50-Cu^{2+}-IDA-SEPHAROSE 6B

Extract	*Protein (mg)*	*Activity (U/ml)*	*Specific activity (U/mg)*	*Purification factor*	*Yield (%)*
Crude extract	6930	620 840	90	1	100
0–90% $(NH_4)_2SO_4$ extract	3132	545 076	174	1.9	88
After tandem desalting–weak affinity chromatography	864	516 366	597	6.6	83

Isoelectric points. Isoelectric focusing enabled us to determine approximately the isoelectric points of the three serine enzymes. Fig. 6 shows a plot of pH *versus* distance from the cathode for the calibration proteins. The p*I* values of the three enzymes fit very close to one another on the curve. They all possess isoelectric points neighbouring pH 5.0 (Table III).

Kinetic studies. The Michaelis constants (K_m) and V_{max} values of the enzymes I, II and III determined using Lineweaver–Burk double-reciprocal plots are represented in Table IV together with the conditions of the reactions. The enzymes are distinct from one another in their affinities for the two substrates tested, namely BAEE and CGT (data given for CGT only).

Inhibition of the three carboxypeptidases by PMSF was also studied to determine conclusively the existence of a serine residue in their active sites. The effect of

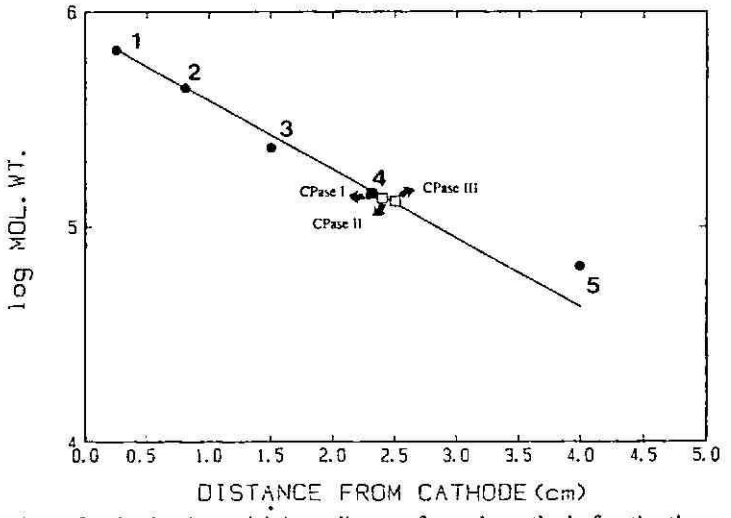

Fig. 5. Log(molecular weight) *vs.* distance from the cathode for the three carboxypeptidases and the calibration proteins. 1, Thyroglobulin (MW 669000); 2, ferritin (440000); 3, catalase (232000); 4, lactate dehydrogenase (140000); 5, bovine serum albumin (67000).

TABLE III

GENERAL SUMMARY OF SOME OF THE PROPERTIES OF THE THREE CARBOXYPEPTIDASES

Enzyme concentrations used: I, $1.435 \cdot 10^{-3}$ mg; II, $2.5 \cdot 10^{-3}$ mg; III, $5.07 \cdot 10^{-3}$ mg.

Property	*Method*	*I*	*II*	*III*	*Conditions*
Homogeneity and molecular weight	SDS-PAGE (7%)	Homogeneous	Homogeneous	Homogeneous	As in ref. 25
	Gradient-gel electrophoresis	141 000	135 000	131 000	As recommended by Pharmacia
Isoelectric point	Isoelectric focusing	5.1	4.9	4.9	As recommended by Pharmacia in their IEF p*I* calibration kit *instruction manual*
Effect of inhibitors:					
2 m*M* PMSF	Kinetically	47% residual activity after 2 h	56% residual activity after 2 h	71% residual activity after 17.2 h	Assayed for esterase activity
100 μM TPCK	Kinetically	88% residual activity after 7 h	No residual activity after 7 h	79% residual activity after 7 h	Acetate buffer 50 m*M* (pH 5.0) at 30°C
Optimum temperature (°C)	CGTase assay	50	50	50	At pH 2.7
	Esterase assay	50	50	50	At pH 5.0
Optimum pH	CGTase assay	3.4	3.4	3.4	At 30°C
	Esterase assay	5.0	5.0	5.0	At 30°C

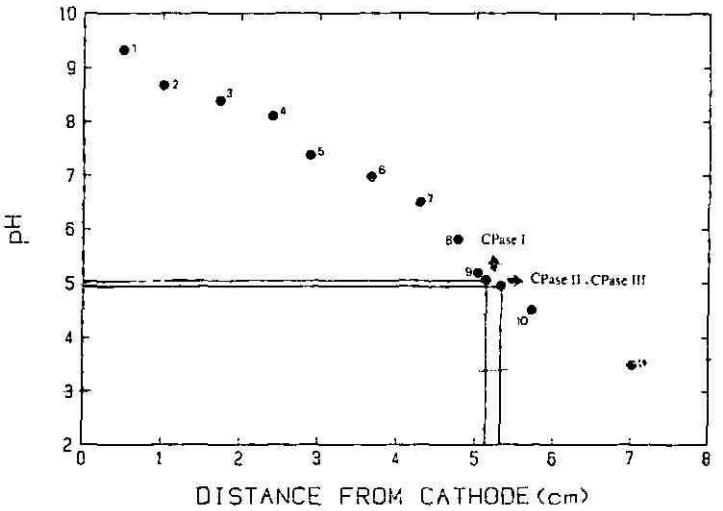

Fig. 6. pH vs. distance from the cathode for the three carboxypeptidases and the calibration proteins. 1, Trypsinogen (pH 9.3); 2, lentil lectin (8.65); 3, lentil lectin (8.45); 4, lentil lectin (8.15); 5, myoglobin (7.35); 6, myoglobin (6.85); 7, human carbonic anhydrase (6.55); 8, bovine carbonic anhydrase β (5.85); 9, β-lactoglobulin A (5.2); 10, soyabean trypsin inhibitor (4.55); 11, amyloglucosidase (3.5).

TABLE IV

KINETIC CONSTANTS OF THE THREE ENZYMES DETERMINED FOR CGT

Enzyme	K_m *(M)*	V_{max} *(min^{-1})*	*Conditions*
I	$1.9 \cdot 10^{-3}$	10 000	50 m*M* lactate
II	$4.9 \cdot 10^{-3}$	5128	buffer (pH 2.7) at
III	$6.1 \cdot 10^{-3}$	5000	30°C

the inhibitor on the enzymes is shown in the Fig. 7 whereas carboxypeptidases I and II were strongly inhibited by PMSF, only limited inhibition of the third enzyme was observed. At each time interval inhibitor-free standards were used to compare with the inhibited enzyme sample so as not to neglect any possible thermal inactivation that could have occurred, especially with carboxypeptidase III, which was incubated in the presence of the inhibitor for 17 h at 30°C. The effect of another inhibitor TPCK was also studied (Table III).

Other parameters such as the effect of temperature and pH on enzyme activity and the stabilities of the enzymes at different pHs and temperatures are also represented in Table III, the enzymes exhibited maximal activity on both BAEE and CGT at 50°C. Maximal esterase activity was at pH 5.0 and peptidyl-*L*-amino acid hydrolase activity at pH 3.4. The enzymes remained stable after incubation for 2 h at pH 7.0 and 30°C; at pH 5.0 and 60°C the residual activity was zero. There was, relatively little activity loss at pH 5.0 and 50°C after 2 h.

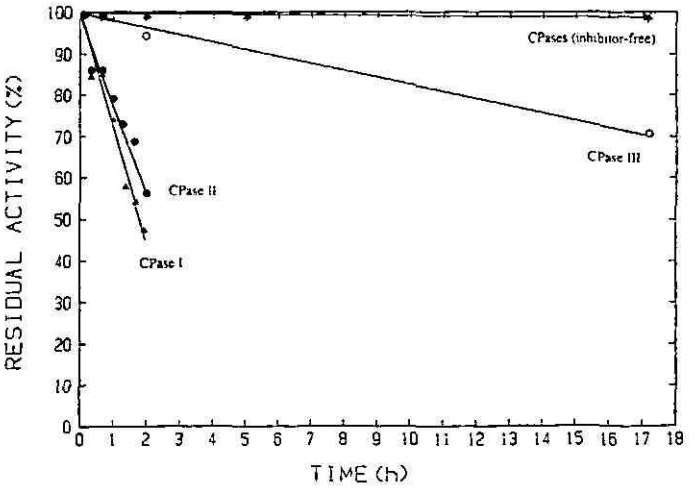

Fig. 7. Time-course inhibition of the three carboxypeptidases in the presence of 2 m*M* PMSF. ▲, I; ●, II; O, III; ★, inhibitor-free samples.

DISCUSSION

Purification

The purification method outlined here consisting of ammonium sulphate precipitation and tandem desalting–IMAC has permitted us not only to separate the *endo*-peptidase activity from the *exo*-peptidase activity but also, under appropriate conditions, to elute individually the three carboxypeptidases in the medium from the affinity column. The three enzymes were found to be isoenzymes. This is an important advantage of the method, as the enzymes could be eluted individually even though they possessed similar physico-chemical properties. Anion-exchange chromatography was used to procure pure extracts of the enzymes. As a result, carboxypeptidase I was purified 115-fold with a 64% yield, carboxypeptidase II was purified 92-fold with a 15% yield and the carboxypeptidase III specific activity was increased 120-fold with a 48% yield. In the best instances the overall yield was around 125%. Yields exceeding 100% are not rare as the removal of inhibitors during the purification could reveal some of the masked activity. The purification was 7-fold and the yield 80% for the partially purified acid protease. It should be noted that the purity of the enzymes after chromatography is dependent on the efficiency of the pre-treatment applied. Unlike in ref. 21, here the carboxypeptidases were not electrophoretically homogeneous after IMAC even though high yields were obtained and so an additional ion-exchange step was necessary.

The choice of elution conditions in affinity chromatography is of critical importance. Sulkowsky[27], based on the work of Porath and Olin[28] and others, suggested three ways of eluting proteins adsorbed in metal columns. The first is by the protonation of electron-donor groupings on the surface of the protein to reverse its coordination to IDA–M^{2+}; ligand exchange[29] is the second method and the third is chelate annihilation, normally the last resort as it involves a complete removal of metal and protein using a chelating agent stronger than IDA. The last method was our first elution procedure[21] and, although it proved effective, reloading the column with Cu^{2+} and re-equilibration became expensive and tiresome, especially for larger columns. The use of a stepwise gradient of Gly–HCl solved this problem and increased the overall performance of the entire procedure.

Ligand exchange, using imidazole, was also an effective elution method but the purifications and yields obtained from run to run were irreproducible and haphazard[30]. This method of elution, however, provided some information on the possible mechanism of interaction between Cu^{2+} and the proteins purified. Analytical chromatography using disposable columns (Bio-Rad Labs.) with imidazole-saturated Cu^{2+}–IDA–Sepharose 6B resulted in the absence of retention of the carboxypeptidases, strongly suggesting the role of histidyl residues on the protein surfaces in the interaction with Cu^{2+}. Our hypothesis was eventually confirmed by Sulkowsky[27]. Moreover, he correlated the intensity of retention of a protein on a Cu^{2+}–IDA column directly with the number of histidyl residues on the protein surfaces. Therefore, logically the number of accessible histidines on the three enzymes would decrease in the order carboxypeptidase I < II < III. Earlier we reported[21] that preliminary studies showed that Cu^{2+} competitively inhibited carboxypeptidase in solution. In the present study, however, we have shown that immobilised Cu^{2+}, although not conclusively site-directed, interacts, to different extents, with the solute depending on the accessibility of the reactive groups on its surfaces.

Properties

From the properties studied, it is evident that the three acid carboxypeptidases closely resemble those of the other *Aspergilli*. The molecular weights of carboxypeptidases I, II and III are comparable to the enzymes isolated from *Aspergillus saitoi*[13], *Aspergillus niger* var. *macrosporus*[16], carboxypeptidase I from *Aspergillus oryzae*[9] and carboxypeptidase O from *Aspergillus oryzae*[31]. These enzymes often exist in dimeric and trimeric forms. The isoelectric points of these enzymes also seem to be very close to one another. Isoelectric focusing in the pH range 3.5–9.5 revealed that all three enzymes possess p*I* values neighbouring pH 5.0.

Unlike many reported carboxypeptidases, those from *Aspergilli* exhibit maximal peptidyl-L-amino acid hydrolase activity below pH 5.0[9–13,15,32]. The three carboxypeptidases reported here also maximally hydrolyse CGT at pH 3.4. The esterase activity, however, is maximal at pH 5.0. The temperature optima also resemble those of other *Aspergillus* carboxypeptidases. Maximal esterase and peptidyl-L-amino acid hydrolase are seen at 50°C at their respective optimum pHs. The three carboxypeptidases are relatively thermostable as there is little activity loss even after incubation for 2 h at 50°C. However, there is drastic loss of activity at 60°C.

The action of inhibitors such as PMSF and DFP confirms that the carboxypeptidases from *Aspergilli* belong to the class of serine carboxypeptidase[9,32,33] like carboxypeptidase Y from yeast[34] and carboxypeptidase from *Penicillium janthinellum*[8]. In our case, whereas carboxypeptidases I and II are strongly inhibited by PMSF, carboxypeptidase III is more resistant to PMSF action. Inhibition of all three enzymes by TPCK indicates the role of a histidyl residue in their active sites[18].

What most clearly distinguishes the three enzymes from one another, however, is their affinities for the two substrates tested. In effect, although the enzymes possess very similar molecular weights and isoelectric points, conformational differences could result in differences in their substrate specificities. Thus, the K_m values of carboxypeptidases I, II and III for CGT were $1.9 \cdot 10^{-3}$ *M*, $4.9 \cdot 10^{-3}$ *M* and $6.1 \cdot 10^{-3}$ *M*, respectively, and similar differences were observed for BAEE. Indeed, in cases such as ours where the enzymes exhibit almost identical physico-chemical properties, a detailed study of their substrate specificities, as in refs. 9–12 and 35, could be the only means of distinguishing one isoenzyme from another.

Hence IMAC using Cu^{2+}–IDA–Sepharose 6B has not only permitted the complete separation of *endo-* and *exo*-peptidase activities but equally the separation of three serine carboxypeptidases exhibiting different affinities for the Cu^{2+} gel. This allows us to postulate that the technique would find wider application in the purification of proteolytic enzymes and in studying the heterogeneity of proteolytic complexes. The properties of the three enzymes are so similar that we consider them to be isoenzymes. Only their substrate specificities could differ. From this study and other references, it is evident that microbial forms secrete proteolytic "cocktails" for specific purposes of protein degradation.

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Note

Determination of micro amounts of acrolein in air by gas chromatography

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Although acrolein is an important air pollutant, there are few selective and sensitive methods for its analysis in air. Suzuki *et al.*[1] determined acrolein in air, but the analytical results involve the α,β-unsaturated aldehydes. Magin[2] analysed simple aldehydes involving acrolein in cigarette whole smoke, and Saito *et al.*[3] performed similar analysis in auto exhaust by gas chromatography (GC). On the other hand, high-performance liquid chromatographic methods have also been used for measurement of aldehydes in auto exhaust and/or polluted air[4,5]. However, the separation of acrolein and other compounds was not sufficient in these methods. We have described[6] a method of analysis of acrolein in auto exhaust based on bromination followed by GC with electron-capture detection (ECD). It was not possible to analyse acrolein in ambient air because the determination limit was about 5 ppb* with a 40-l sample of air. This paper deals with a sensitive and selective method for GC–ECD determination of micro amounts of acrolein in air as the brominated O-methyloxime derivative.

EXPERIMENTAL

Reagents and materials

Ethanol was of analytical reagent grade from Kanto Chemical (Tokyo, Japan). Methoxylamine hydrochloride (MOA · HCl), supplied by Wako Pure Chemical Industries (Osaka, Japan), was dried under reduced pressure. The other reagents used were of analytical reagent grade. A standard solution of acrolein was prepared by dissolving 100 mg of acrolein in distilled water and diluting to 100 ml. The brominated derivative of acrolein O-methyloxime was supplied by Tokyo Kasei Kogyo (Tokyo, Japan). The Sep-Pak C_{18} (SP) cartridge was from Waters Assoc. (Milford, MA, U.S.A.).

* Throughout this article the American billion (10^9) is meant.

0021-9673/86/$03.50

Preparation of standard gas

A 10-μl volume of acrolein solution was vaporized in a 1-l evacuated sampling bottle, and the bottle filled with nitrogen, yielding about 3300 ppm (v/v) of the first standard gas. A known volume of this gas was injected into a 100-l Tedlar bag filled with nitrogen to give the second standard gas. The concentration of the latter was analysed by a fluorimetric method with *m*-aminophenol[1].

Equipment, apparatus and conditions

A Gifu-Aichi Electric GA-2F handy sampler was used for sample collection. The volume of the bubbler used was about 30 ml.

An Hitachi 073 gas chromatograph with a ^{63}Ni electron-capture detector was used. The GC conditions were as follows: 2-m glass column packed with 3% silicone GE XE-60 on Chromosorb W AW DMCS (60–80 mesh); column temperature, 90°C; injection and detector temperatures, 170°C; carrier gas (nitrogen) flow-rate, 40 ml/min.

Collection and general procedure

Sample gases (3–40 l) were collected at a rate of 0.5–1.0 l/min in two bubblers containing 10 ml ethanol. After sampling, the absorption solution was made up to 25 ml with ethanol. Then 4 ml of this solution were diluted with 20 ml distilled water, 1 ml of 2 *M* sodium acetate and 1 ml of MOA · HCl (5 mg/ml) were added. The mixture was allowed to stand for 10 min at room temperature. Then, 1.2 ml of 3 *N* sulphuric acid, 0.3 ml of 0.2 *M* potassium bromate and 3 g of potassium bromide were added and dissolved by stirring. After standing for 15 min at room temperature, the excess of bromine was removed with 0.05 *M* sodium thiosulphate. After reaction, the solution was forced through an SP cartridge. The derivative in the cartridge was eluted with 1.5 ml of diethyl ether. A 4-μl aliquot of the eluate was analysed by GC.

RESULTS AND DISCUSSION

Sample derivatizations

Levine *et al.*[7] reported the GC analysis of aldehydes as their O-methyloxime. However, their method was not able to determine micro amounts of acrolein. So, an improved method involving the formation of a brominated derivative of the O-methyloxime of acrolein was investigated in this work. The use of ECD resulted in a highly sensitive and selective analysis of acrolein. The derivatization conditions were established as described in the Experimental. The efficiency of the whole reaction was about 92% for 1 mg of acrolein dissolved in 4 ml of the absorption solution.

Absorption solution and collection efficiency

Ethanol was used as an absorption solution to collect acrolein in air. Several kinds of ethanol supplied from different companies were investigated for their blank peaks. The reagent supplied by Kanto Chemical (Tokyo, Japan) showed the minimum blank peak. Fig. 1 shows the chromatograms of the blank and standard solutions.

The collection efficiencies for acrolein from the second standard gases at the levels of 4.4, 15.4 and 247 ppb were 81, 85 and 96% respectively, and the relative

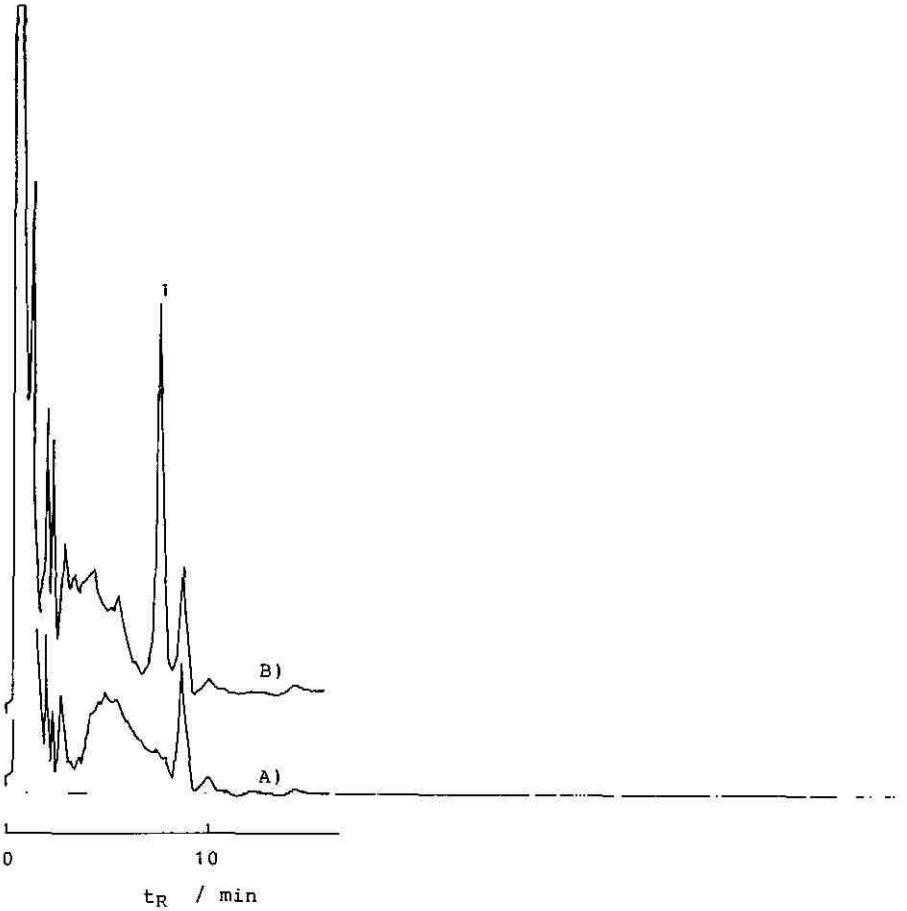

Fig. 1. The chromatograms of the products obtained from a blank (A) and a standard solution (B). Peak 1: acrolein derivative.

standard deviations of five determinations were 10.9, 9.2 and 6.1% respectively at a rate of 1.0 l/min. The collection efficiency was slightly decreased at low concentrations.

Reaction in ethanol–water

In order to prevent the precipitation of sodium sulphate and for adequate dissolution of potassium bromide, the absorption solution (ethanol) was diluted in distilled water, and the derivatization reaction was investigated in ethanol–water. The reaction efficiency for 0.2 μg acrolein in this medium was 98% of that in aqueous solution. This shows that the derivatization reaction proceeds adequately in ethanol–water.

The peak due to the acrolein derivative was sharp and separated from impurities as is seen in Fig. 1.

TABLE I

EFFECT OF DIVERSE IONS ON THE DETERMINATION OF ACROLEIN

Ion	*Amount added (μg)*	*Recovery of acrolein (%)*	
		Proposed method	*Bromination method*[6]
–*	–	100	100
Cl^-	2	98	100
NO_3^-	2	97	103
NO_2^-	2	102	88
SO_3^{2-}	2	99	96

* A 0.2-μg amount of acrolein was added to 4 ml of absorption solution.

Separation of similar compounds and effect of diverse ions

The separation of the acrolein derivative and those of similar compounds with ethylenic bonds, such as methacrolein and crotonaldehyde, was sufficient to analyse acrolein under the GC conditions proposed here.

The interference from co-existing ions was investigated. Chloride, nitrate, nitrite or sulphate ion was dissolved in 4 ml of the absorption solution, and their influence was studied. The results are shown in Table I. None of these ions interfered with the determination of acrolein in this method, though nitrite ion interfered slightly in the previous method[6].

Calibration graph

The calibration graph obtained showed a good linearity over the range 0–0.3 μg of acrolein in 4 ml of absorption solution. The regression equation was: $y = 243x + 1.25$, where x = content (μg) in 4 ml of absorption solution, y = peak height (mm) and the correlation coefficient was 0.991 ($n = 10$).

The determination limit was about 0.001 μg/ml of acrolein in the absorption solution.

Determination of acrolein in polluted air

The proposed method was applied to the determination of acrolein in ambient

TABLE II

ANALYSIS OF ACROLEIN IN VARIOUS KINDS OF AIR SAMPLES

Sample	*Acrolein found*	
	*Mean**	*Range*
Urban air A	1.0 ppb	0.9–1.1 ppb
B	1.2 ppb	1.1–1.3 ppb
Air in road tunnel	1.7 ppb	1.4–1.8 ppb
Auto exhaust A	1.7 ppm	1.5–1.9 ppm
B	3.4 ppm	3.3–3.6 ppm

* Mean of three determinations.

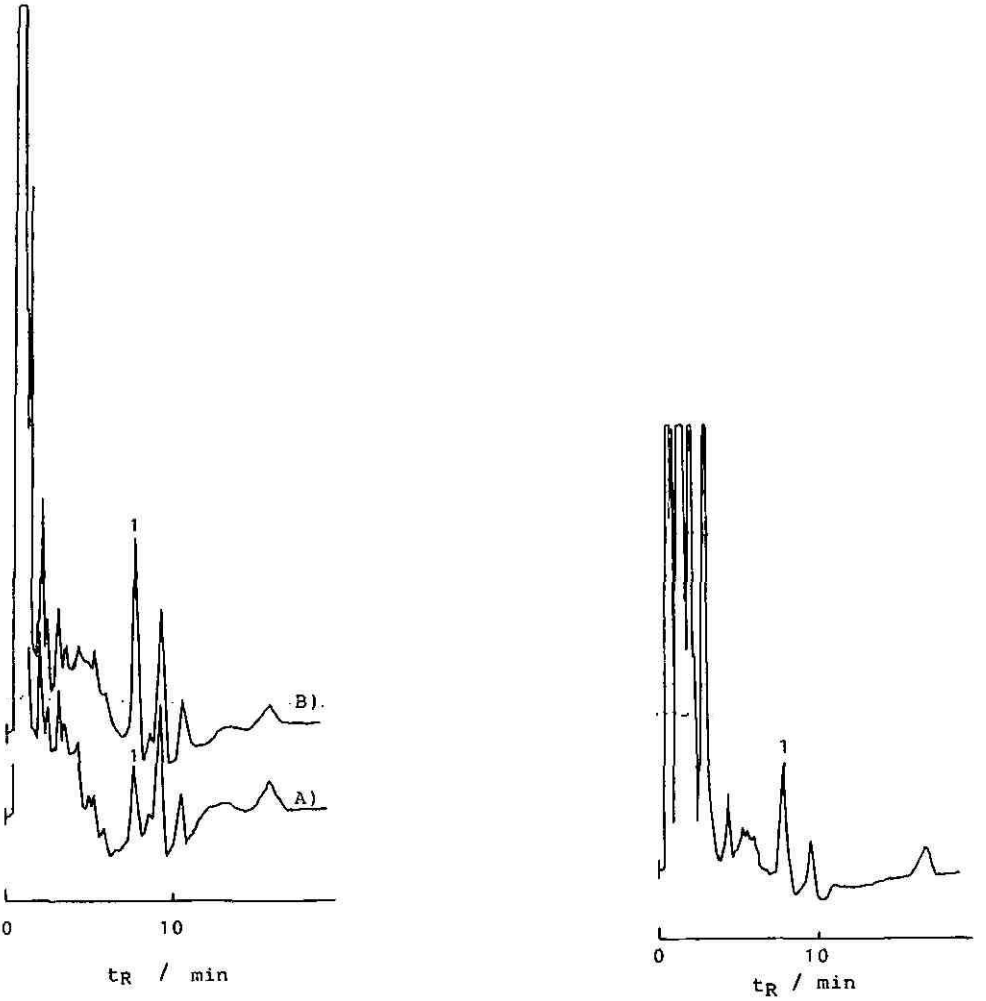

Fig. 2. Typical chromatograms of the air in a road tunnel (A) and of acrolein (0.05 μg) added to an air sample (B). Peak 1: acrolein derivative.

Fig. 3. Typical chromatogram of auto exhaust. Peak 1: acrolein derivative.

air and auto exhaust. The results are shown in Table II. The chromatograms of a sample of air from a road tunnel and of a sample from 4 ml of the absorption solution to which 0.05 μg of standard acrolein had been added are shown in Fig. 2. The peaks of acrolein in the chromatograms were coincident and the analytical results calculated from the calibration graph agreed with that obtained by addition of the standard solution. So, the proposed method is considered to be reliable. Fig. 3 shows a typical chromatogram of auto exhaust.

CONCLUSIONS

A gas chromatographic determination of micro amounts of acrolein in air was

investigated. Acrolein collected in ethanol was analysed by GC with ECD as the brominated O-methyloxime derivative. This method is highly sensitive and selective and reproducible for the determination of acrolein in air.

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Note

Chiral resolution of a carboxylic acid using droplet counter-current chromatography

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Liquid chromatographic chiral resolutions of racemic mixtures have typically employed adsorption chromatography utilizing a chiral stationary phase or a chiral mobile phase.[1] Diastereotopic interactions between the chiral element of the stationary or mobile phase and the enantiomeric components of the racemic mixture produce different adsorption behaviours for these components and subsequently different elution times. While such applications have successfully achieved baseline resolution of the enantiomers, these methods are not particularly amenable to large scale resolutions.

Two recent reports have appeared describing the use of liquid–liquid partition chromatography for the chiral resolution of norephedrine (200 mg)[2], and isoleucine[3]. The former procedure utilized the newly described rotation locular counter-current chromatography (RLCC)[4–6] with (*R,R*)-di-5-nonyltartrate as the chiral resolving agent dissolved in the mobile phase. The diastereomeric complexes formed upon solvation of the norephedrine enantiomers by the chiral tartrate ester had sufficiently different partitioning behaviour, different formation equilibria, or both to allow for resolution, though baseline resolution was not achieved.

The resolution of racemic isoleucine employed the more familiar droplet counter-current chromatography (DCCC)[7,8]. Diastereomeric mixed copper(II) complexes of the isoleucine enantiomers and N-dodecyl-L-proline were readily separated, though the quantity of isoleucine used was only 2.6 mg. The authors did state, however, that larger amounts of racemate could also be resolved. Separation of the copper(II) complexes is dependent not only upon their differing partitioning behaviours, but also upon the different ligand exchange equilibria between the N-dodecyl-L-proline–Cu(II) complexes and the two enantiomers of isoleucine.

We were recently faced with the necessity of obtaining various substituted optically pure bicyclo[2.2.1]hept-5-ene-2-carboxylic acids, such as compound 1, routinely synthesized via Diels-Alder cycloadditions (Fig. 1). Initial resolution of the racemates was attempted using more traditional methods. Fractional recrystallization of the brucine salts of the parent carboxylic acid, compound 1, and of the carboxylic acid, compound 3, formed by esterification of alcohol, compound 2, [lithium aluminium hydride (LAH) reduction of compound 1] with phthallic anhydride,[9] both failed to yield satisfactory resolution.

Esterification of compound 1 or 2 with optically pure O-acetylmandelic acid,

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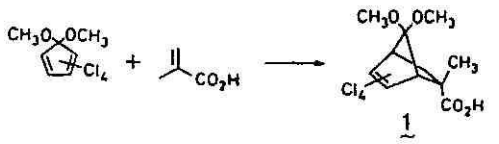

Fig. 1. Synthesis of carboxylic acid 1, via Diels-Alder cycloaddition.

compound 4, or methylmandelate, compound 5, produced diastereomeric esters. compound 6 or 7, respectively, which could be separated via high-performance liquid chromatography (HPLC)[10]. Only diastereomeric esters 6 were routinely resolvable using flash chromatography, and both esterification procedures require chemical modifications which we wished to avoid. The attempts for chemical resolution of carboxylic acid 1 are summarized in Fig. 2.

Subsequent to these efforts, we turned to liquid–liquid partition chromatography to resolve compound 1 using DCCC. We now report the successful use of DCCC in achieving this resolution utilizing (−)-(*R*)-2-aminobutanol as the chiral resolving agent.

EXPERIMENTAL

The DCCC instrument (Model DCC-300 Eyela, Tokyo Rikakikai, Tokyo, Japan) has been previously described.[5] The DCCC employed in this work utilized 225 glass columns, (40 cm × 1.9 mm I.D., 9 racks of columns with 25 columns per rack), connected in series by PTFE tubing, (0.5 mm I.D.). The flow-rate and mode of operation (ascending or descending) were optimized for each experiment; all experiments were run at ambient temperature.

The biphasic solvent systems used were equilibrated overnight prior to separation of the two phases. Partition coefficients (K') were measured by weight of sample in each phase. Values of K' reported were determined using the same ratio of the

TABLE I

EFFECT OF (−)-(*R*)-2-AMINOBUTANOL ON THE PARTITION COEFFICIENTS, K', OF CARBOXYLIC ACID 1 IN CHLOROFORM–METHANOL–WATER (pH 7, 0.01 *M* PHOSPHATE BUFFER)

Partition coefficients, K', were determined by dissolving 112 mg of compound 1 in 80 ml of the aqueous component, adding 130 ml of methanol and 70 ml of chloroform and equilibrating. The concentration of compound 1 in each phase was subsequently determined by evaporation of the solvent from the separated phases, redissolving the solute in 1 *M* hydrochloric acid and extracting with dichloromethane. Under these conditions, compound 1 is extracted entirely into the dichloromethane phase uncontaminated by the (−)-(*R*)-2-aminobutanol and the phosphate buffer, which remain in the aqueous phase. The dichloromethane was removed under reduced pressure and the weight of recovered compound 1 determined.

Solvating agent	*Concentration (mM)*	K'*
None	–	1.08
(−)-(*R*)-2-aminobutanol	20	1.44
(−)-(*R*)-2-aminobutanol	100	2.13

* K' = wt. of compound 1 in upper phase/wt. of compound 1 in lower phase.

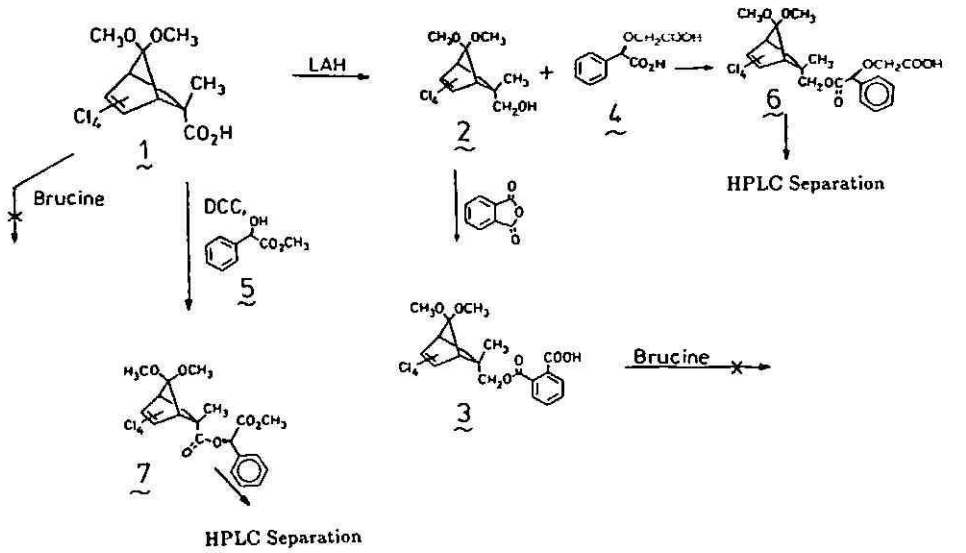

Fig. 2. Attempts at chemical resolution of carboxylic acid 1.

two solvent phases that results from the overnight equilibration rather than equal volumes of the two phases (see Table I). The samples were applied to the instruments by dissolution in a 1:1 mixture of the two phases.

All solvents were distilled prior to use. Optical rotations were measured with a Perkin-Elmer 241 polarimeter. The HPLC separations employed a Rainin Microsorb silica column (25 cm × 10 mm I.D.).

RESULTS AND DISCUSSION

Selection of the solvent system

The solvent system employed for the resolution of racemic compound 1 was selected by measuring its partition coefficient in various biphasic systems, both in the presence and the absence of the chiral resolving agent. We initially wanted a system which gave a partition coefficient of approximately unity in the absence of the chiral resolving agent. Such an appropriate system proved to be chloroform–methanol–water (pH = 7, 0.01 *M* phosphate buffer) (7:13:8) with a partition coefficient (upper phase/lower phase) of 1.08 for carboxylic acid 1. The relative volumes of the two phases which results from this solvent system is 5/2 (upper/lower).

Ideally, we desired a chiral solvating agent which would remain exclusively in the stationary phase in the absence of such solvation complex formation with compound 1. Under such circumstances, the amount of chiral resolving agent required would be reduced as more mobile phase is usually consumed in DCCC separations than stationary phase. Furthermore, possible difficulties in removing the resolving agent from the resolved carboxylic acids would be minimized as the concentration of the resolving agent in the mobile phase would be limited to that which partitions

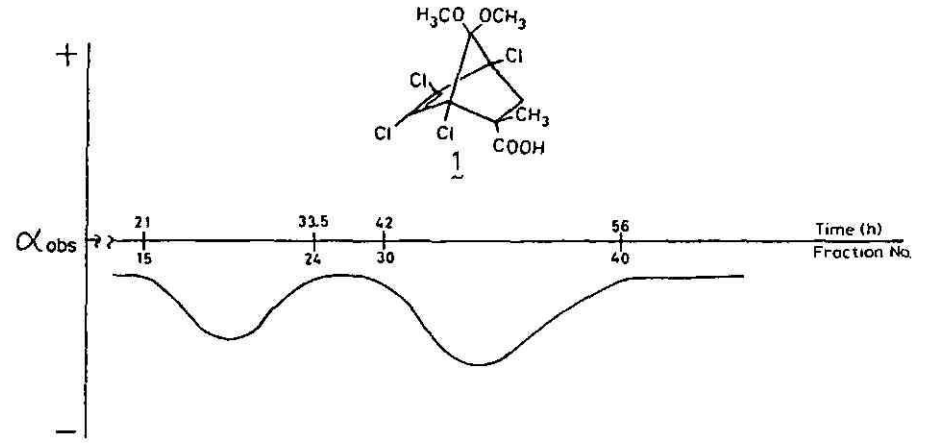

Fig. 3. DCCC chiral resolution of 100 mg of carboxylic acid 1, using chloroform–methanol–water (7:13:8) (pH 7, 0.01 *M* phosphate buffer), descending mode. 100 m*M* (−)-(*R*)-2-amnobutanol, flow-rate = 10 ml/h.

as the solvation complex with compound 1. The concentration of the resolving agent in the stationary phase, however, would not remain constant but would gradually be reduced due to loss via partitioning in the solvation complex form into the mobile phase.

Numerous potential chiral resolving agents, including sugars, amino acids, tartaric acid, and aminoalcohols, were then screened for their ability to alter this partition coefficient of compound 1. We had rationalized that variation in the partition coefficient of compound 1 could be due to the formation of a solvation complex between compound 1 and the resolving agent. Since the resolving agent employed is optically pure, the two solvation complexes formed from the two enantiomers of

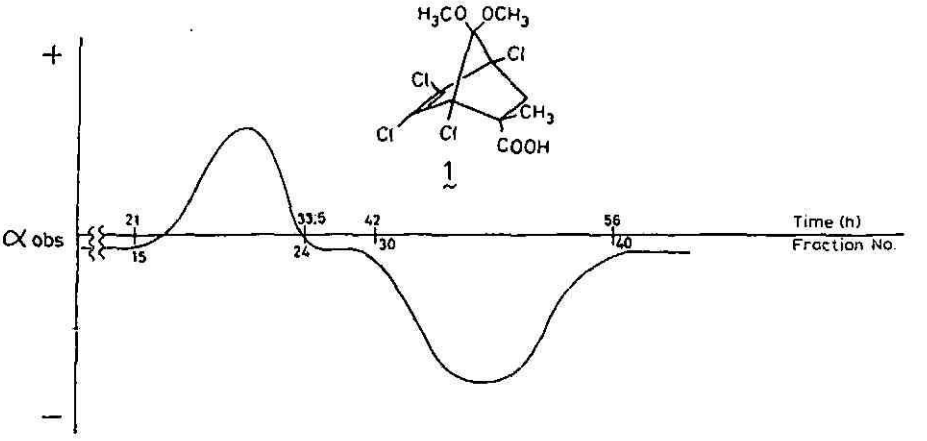

Fig. 4. Observed optical rotation of DCC fractions from chiral resolution of carboxylic acid 1, after removal of (−)-(*R*)-2-aminobutanol. For conditions of chromatography see Fig. 3.

compound 1 and the resolving agent are diastereotopic. Chiral resolution can therefore be achieved either through the different partitioning behaviour of the diastereotopic complexes, the different formation equilibria of the complexes, or a combination of these two effects. The phase which contained the lesser amount of compound 1 after equilibration was then chosen to be the mobile phase.

Only the amino alcohols showed significant alteration of the partitioning behaviour of compound 1. Of those amino alcohols tested, (−)-(*R*)-2-aminobutanol had the greatest effect (Table I) and was therefore selected as the resolving agent. Under the solvent conditions employed (pH = 7), this aminoalcohol remained exclusively in the upper layer (designated the "aqueous" phase), which was used as the mobile phase in the resolution.

Resolution of carboxylic acid 1

With the partitioning data presented in Table I in hand, we applied 100 mg of compound 1 to the DCCC system using the decending mode of operation with a flow-rate of 10 ml/h. Baseline resolution of the enantiomers of compound 1 was achieved (Fig. 3) as determined by measuring the optical rotation of each fraction (*ca.* 12 ml per fraction). The negative rotation for both solute bands was thought to be a consequence of the negative optical rotation of the (−)-(*R*)-2-aminobutanol. This was confirmed by removing the aminoalacohol via an acid wash, and remeasuring the optical rotation of each fraction. As illustrated in Fig. 4, after removal of the aminoalcohol, the (+)- and (−)-enantiomers of compound 1 show the expected signs of rotation in their respective peak with baseline resolution confirmed.

Fractions from the latter portion of peak 1 and from the beginning portion of peak 2 were reduced to the corresponding alcohol and esterified with (+)-O-acetylmandelic acid, compound 4, to produce ester 6 (Fig. 2) and subjected to HPLC analysis. The diastereomeric purity of the esters produced from each fraction confirmed baseline resolution of the enantiomers of compound 1 (Fig. 5). We have not yet assigned the absolute stereochemistry of the enantiomers of compound 1.

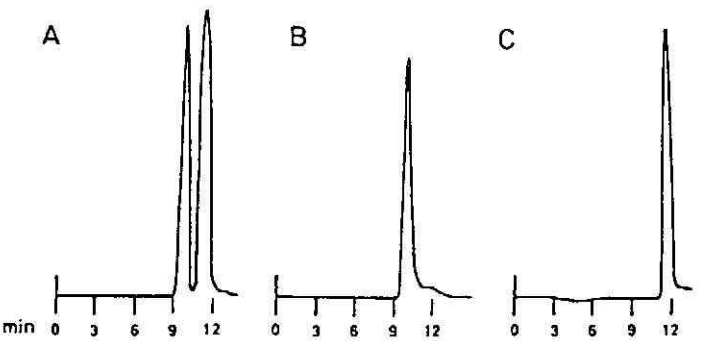

Fig. 5. Confirmation of baseline resolution of enantiomers of compound 1 following conversion to ester 6 using normal phase HPLC, dichloromethane–pet ether (1/2, v/v) flow-rate = 1.0 ml/min, UV detection 254 nm. (a) Chromatogram of diastereomeric esters 6 from racemic compound 1. (b) Chromatogram of diastereomerically pure compound 6 from optically pure (+)-compound 1 in peak 1 (see Fig. 4). (c) Chromatogram of diastereomerically pure compound 6 from optically pure (−)-compound 1 in peak 2 (see Fig. 4).

CONCLUSIONS

Chiral resolution of carboxylic acid 1 using liquid–liquid partition chromatography has been achieved using DCCC. Liquid–liquid partition chromatography using DCCC has the capability of resolving relatively large amounts of sample. We are currently examining other chiral resolving agents in hopes of successfully resolving gram quantities of material.

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Note

Solvent system for the rapid identification of phenylthiohydantoin derivatives of amino acids by high-performance liquid chromatography

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(First received July 30th, 1986; revised manuscript received August 26th, 1986)

The sequence determination of peptides and proteins using the Edman degradation remains an important technique in biochemical analysis. Various types of machines (liquid phase[1], solid phase[2] and gas–liquid–solid phase[3] are or have been commercially available to perform this technique automatically. Independent of the type of sequencer, the identification of the phenylthiohydantoin (PTH)-amino acid obtained from each cycle of degradation must be performed. Over the last 10 years, the use of reversed-phase (RP) high-performance liquid chromatography (HPLC) has become the accepted method for this analysis.

Numerous reports have appeared describing separations of PTH-amino acids, and these methods have generally consisted of RP chromatography on C_{18} column supports using isocratic[4] or gradient[5] elution with acetonitrile or methanol. An application note from Waters suggested to us that 2-propanol, a cheaper and safer solvent than acetonitrile, could be successfully used for the rapid separation of PTH-amino acids. More recently, a separation of PTH-amino acids within 35 min by isocratic elution of C_{18} columns with sodium acetate buffer–2-propanol–tetrahydrofuran was described[6]. However, PTH derivatives of Ser and Gln were not separated. Here we report that gradient elution of C_{18} columns with increasing concentrations of 2-propanol in 30 m*M* sodium acetate–acetonitrile produce a complete separation of the PTH derivatives of the commonly occurring amino acids in 13 min. The column lifetime is long, the method is applicable to sequencing at high sensitivity and the solvent system produces a good resolution of PTH-amino acids with different C_{18} columns.

EXPERIMENTAL

Acetonitrile and 2-propanol were of HPLC grade from Fluka (Buchs, Switzerland) and other chemicals were of analytical-reagent grade from either Fluka or Merck (Darmstadt, F.R.G.). Hibar LiChrocart Supersphere RP-18 columns (125 × 4 mm I.D.) were obtained from Merck, a Nova Pak C_{18} column (150 × 3.9 mm I.D.) from Waters Assoc. (Milford, MA, U.S.A.) and a Nucleosil C_{18} (5 μm particle size) column (200 × 4 mm I.D.) from Macherey, Nagel & Co. (Düren, F.R.G.).

The PTH-amino acids were purchased from either Pierce (Rockford, IL, U.S.A.) or Fluka. Methyl ester derivatives of PTH-aspartic acid, PTH-glutamic acid

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and PTH-carboxymethylcysteine were prepared by incubating the PTH derivatives at 50°C for 5 min in 1 *M* methanolic hydrochloric acid. Water used for the preparation of buffers was obtained from a Milli-Q water system (Millipore, Bedford, MA, U.S.A.).

Buffer A was prepared by adding 1.8 ml of acetic acid to 1 l of water and the solution was titrated with 4 *M* sodium hydroxide solution to the required pH. To five parts of this buffer was added 1 part of acetonitrile. Buffer B was 2-propanol–

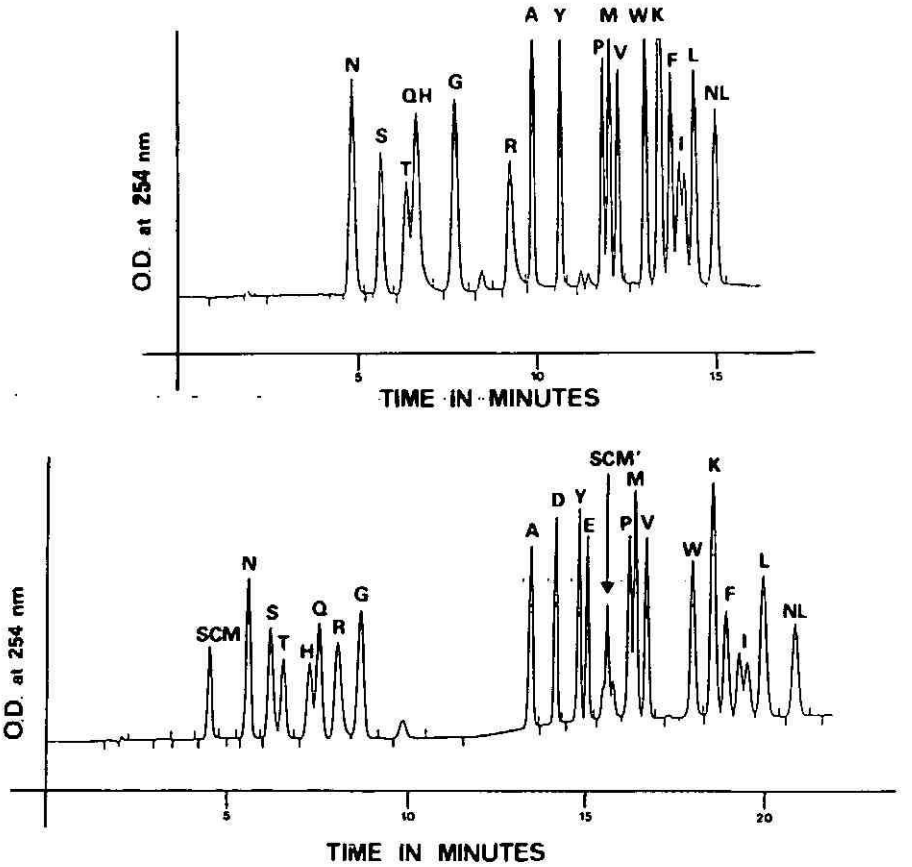

Fig. 1. Top: separation of a standard PTH-amino acid mixture on a Nova Pak C_{18} column. Buffer A was 30 m*M* sodium acetate (pH 4.8)–acetonitrile (5:1) and buffer B was 60% 2-propanol. A flow-rate of 0.7 ml/min was used and the temperature was 37°C. The programme for the elution consisted of 1.5 min of buffer A followed by an increase to 40% in B over 5 min using convex gradient No. 5. After holding these conditions for 5.5 min, the percentage of B was reduced linearly to 0% in 4 min; the cycle time was 20 min. The single-letter code for the amino acids is as recommended by the Commission on Biochemical Literature (exception: NL = norleucine). PTH-Asp and PTH-Glu are present as their methyl esters. Bottom: separation on a Nucleosil column. Buffer A was as above with the pH altered to 5.0 and equilibrating conditions were 5% in buffer B. Elution was for 6.5 min at 5% B followed by an increase to 40% B in 3.5 min using the convex gradient no. 5. Elution was continued for 8 min with 40% B and then returned to equilibrating conditions linearly over 2 min; the cycle time was 25 min. Other conditions were as described above. SCM = PTH-S-carboxymethylcysteine; SCM′ = methyl ester of PTH-carboxymethylcysteine.

water (3:2). The buffers were filtered through a 0.2 μm filter before use. Samples were injected in buffer A.

The HPLC system from Waters consisted of a WISP 710B automatic sample injector, two Model 510 pumps, a Model 680 controller and a Model 440 UV detector. Integration was performed with a Hewlett-Packard (Avondale, PA, U.S.A.) Model 3390 A integrator.

Sequence analysis was performed with a Beckman 890 C instrument modified with a cold trap and conversion to the PTH derivatives was carried out automatically using a P-6 auto-converter from Sequemat (Watertown, MA, U.S.A.). Methanolic hydrochloric acid (1 *M*) was used as the conversion reagent[7].

RESULTS AND DISCUSSION

Initial experiments with a Nova Pak C_{18} column using the manufacturer's recommended procedure failed to give an adequate resolution of a standard PTH-amino acid mixture. Sodium acetate buffers (30 m*M*) of pH varying from 4.3 to 5.5 in increments of 0.1 unit were tried and a chromatogram at the optimal pH of 4.8 is shown in Fig. 1 (top). Changing the conditions, such as temperature, salt concentration and the gradient profile, failed to achieve a better separation of PTH-His from PTH-Thr (or PTH-Gln) without sacrificing the almost complete resolution of the other PTH derivatives. This failure may arise from the different chromatographic properties often observed between supposedly identical columns. Attempts with a column of Nucleosil C_{18} (5 μm) were more successful and an almost complete separation of all derivatives was obtained within 21 min, as shown in Fig. 1 (bottom).

A newly developed chromatographic support from Merck, Supersphere (4 μm particle size), became commercially available and we tested the RP-18 cartridge (125 × 4 mm I.D.) using similar conditions to those described above. As shown in Fig. 2, a nearly complete separation of all derivatives was obtained within 13 min and with a cycle time of 17 min. The reproducibility between cartridges appears to be adequate as a similar chromatogram was obtained with a second cartridge; His and Ser were resolved only on decreasing the pH by 0.2 unit; however, this did not seriously affect the resolution of the other derivatives. This procedure was adopted as an appropriate method of analysis.

The column lifetime appears to be good. Over a period of 9 months, more than 1000 samples from the Beckman sequencer have been injected on to a single column with neither a serious loss of resolution (with the exception of His–Ser) nor a marked increase in back-pressure. A slight increase in the pH (0.1 unit) of buffer A resulted in a decrease in the elution time of His relative to Ser, and such fine tuning of the chromatogram corrected the effects of column ageing.

The sensitivity of the method is adequate for sequencing at the picomole level. Fig. 2 (bottom) shows the results for a standard mixture of 10 pmol of each PTH derivative. An increase in the baseline, although aesthetically unappealing, does not hinder the identification of PTHs at this level.

No large extraneous peaks were seen from the Beckman sequencer for the degradation of peptides at the 500 pmol level. Fig. 3 shows examples from a sequence determination of a peptide derived from a tryptic digestion of the rabbit secretory component. The major contaminant peak, presumably diphenylthiourea, was eluted

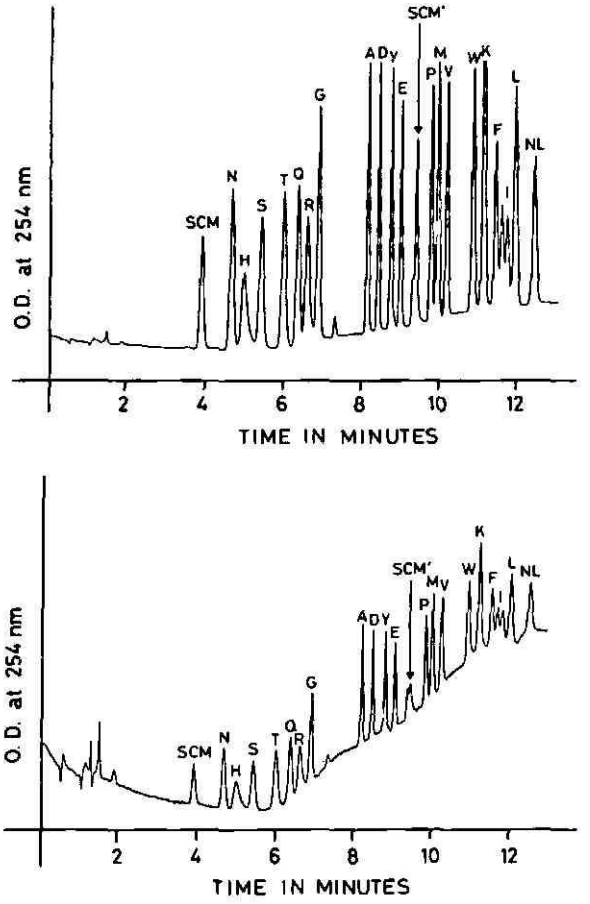

Fig. 2. Chromatography of PTH-amino acids on LiChrocart Supersphere. Amounts of 100 pmol (top) and 10 pmol (bottom) of each component were injected. Buffer A was 30 m*M* sodium acetate (pH 5.3)–acetonitrile (5:1) and buffer B was 60% 2-propanol. A flow-rate of 1 ml/min was used and the temperature was at 37°C. The programme (inject to inject time 17 min) is shown in Table I.

TABLE I

PROGRAMME FOR THE SEPARATION OF PTH-AMINO ACIDS ON LICHROCART SUPERSPHERE

Time (min)	*Flow-rate (ml/min)*	*Buffer A (%)*	*Buffer B (%)*	*Curve*
Inject	1	100	0	
1.5	1	100	0	
6.5	1	60	40	5 (convex)
11	1	60	40	6 (linear)
12	1	100	0	6

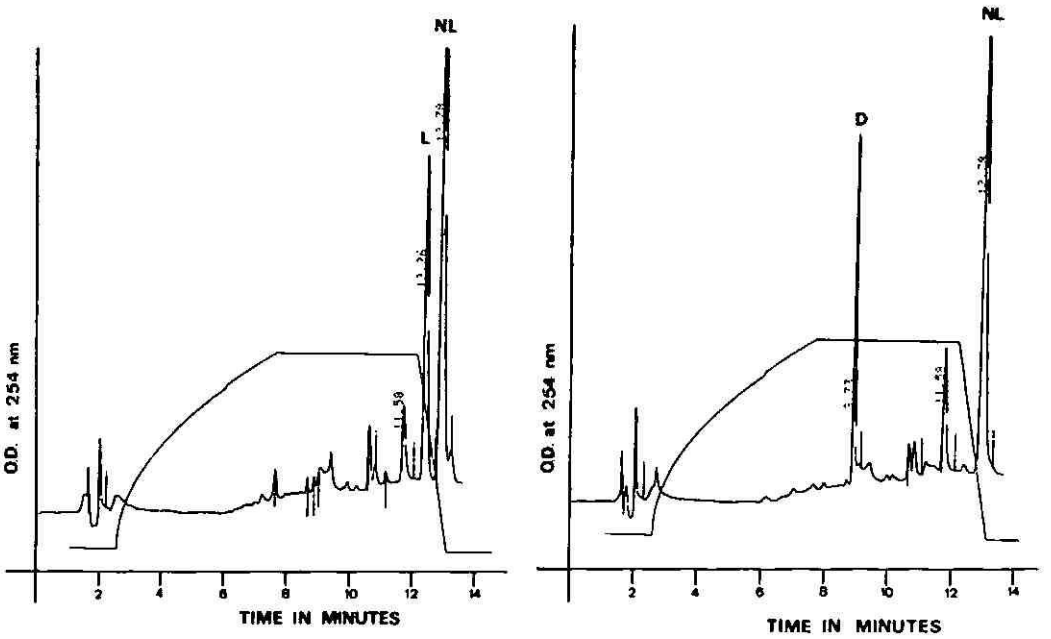

Fig. 3. Sequence analysis of a tryptic peptide derived from rabbit secretory component. The peptide (500 pmol) was degraded using a Beckman 890 C sequencer. Analyses of cycles 2 (top) and 4 (bottom) are shown; 30% of each fraction from the sequencer was injected. The gradient (see Table I) is shown superimposed.

between the Leu and Phe derivatives. When it is taken into consideration that modern gas-phase sequencers yield significantly fewer background peaks, then there should be no problem in the general application of this method.

ACKNOWLEDGEMENTS

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Note

Determination of bufexamac in cream and ointment by high-performance liquid chromatography

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Bufexamac (4-butoxy-N-hydroxybenzeneacetamide) is a drug possessing analgesic, anti-inflammatory and antipyretic effects[1–4]. It is used extensively for the treatment of contact dermatitis and acute eczema.

Some analytical procedures have been reported for the quantitation of bufexamac. In 1966, Lambelin *et al.*[5] described a colorimetric method for the determination of bufexamac in serum. This method lacks specificity because it detects not only bufexamac but also its hydroxy derivatives. Roncucci *et al.*[6] and Dell *et al.*[7] described a gas–liquid chromatographic method for the determination of bufexamac in plasma and urine. However, these methods are very tedious and time-consuming because they require a derivatization step before analysis.

In this paper, a rapid, specific and sensitive high-performance liquid chromatographic (HPLC) method for the quantitation of bufexamac in cream and ointment is described.

EXPERIMENTAL

Apparatus

The HPLC unit consisted of a JASCO pump Model BIP-I (Japan Spectroscopic Co., Tokyo, Japan), a JASCO Model UVIDEC-100 V variable-wavelength UV detector set at 280 nm and an injector Model 7125 fitted with a 20-μl loop (Rheodyne, Cotati, CA, U.S.A.). The HPLC system was linked to a Chromatopac C-R3A digital integrator (Shimadzu, Kyoto, Japan).

Chromatographic conditions

The HPLC column used was a 30 cm × 4 mm I.D. μBondapak C_{18} (Waters), particle size 8–10 μm. The mobile phase was 60% (v/v) methanol in water. The pH of the mobile phase was adjusted to 3.0 by adding phosphoric acid. It was then filtered through a Millipore membrane filter (0.45 μm; Millipore, Bedford, MA, U.S.A.) and degassed before use. The sample was chromatographed using a flow-rate of 1.0 ml/min at ambient temperature. Samples of 1 μl were injected.

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Reagents

Bufexamac (Sigma) and *n*-butyl *p*-hydroxybenzoate (Tokyo Kasei) were used as received. All other solvents used were of reagent grade (Wako). Distilled water was used when preparing the mobile phase.

Calibration curve

A stock standard solution of bufexamac was prepared in methanol (10.0 mg/ml). A solution of *n*-butyl *p*-hydroxybenzoate in methanol (approximately 10 mg/ml) was used as an internal standard solution. Aliquots of the standard stock solution, equivalent to 20, 40, 60, 80 and 100 mg, were pipetted into a 100-ml volumetric flask and aliquots of the internal standard solution, equivalent to 10 mg, were added. The resulting solutions were diluted to volume in methanol to obtain the working standard solutions. These solutions were used for calibration purposes. The calibration curve was constituted from the values of the ratio of the peak height of bufexamac to that of the internal standard.

Analysis of bufexamac samples

Approximately 1 g of sample (*ca.* 50 mg of bufexamac) was accurately weighed into a 200-ml separating funnel, 100 ml of hexane and 30 ml of methanol–water (8:2) were added and shaken well for 10 min on a shaker. The layers were allowed to separate, and the lower layer was drawn off into a 100-ml volumetric flask. The extraction was repeated with 30 ml of methanolic solution, and the methanol extracts were pooled; 1 ml of the internal standard was added, and the mixture was diluted to volume in methanol. A 1-μl aliquot of this solution was injected into the chromatograph; the amount of bufexamac present was calculated using the calibration curve.

RESULTS AND DISCUSSION

The elution characteristics of bufexamac on three reversed-phase ODS columns from different manufacturers were compared using an aqueous methanol (pH 3.0) mobile phase. The separation parameters, *i.e.*, the number of theoretical plates,

TABLE I

CALCULATED SEPARATION PARAMETERS FOR THE THREE ODS COLUMNS

Columns: A = LiChrosorb RP-18 (7 μm, 250 × 4.0 mm I.D.); B = Zorbax C_{18} (5–6 μm, 150 mm × 4.6 mm I.D.); C = μBondapak C_{18} (8–10 μm, 300 mm × 3.9 mm I.D.). Mobile phase: methanol–water (pH 3) (6:4, v/v); flow-rate 1 ml/min. Detection: UV, 280 nm. N = Number of theoretical plates per 25 cm, calculated for bufexamac; I.S. = *n*-butyl *p*-hydroxybenzoate; S = asymmetry factor, calculated as defined by Kirkland[8].

Column	N	k'		α *for bufexamac/I.S.*	S
		Bufexamac	*I.S.*		
A	136	2.8	5.5	2.34	4.13
B	152	2.9	4.4	1.68	3.37
C	4466	2.6	3.5	1.65	1.08

TABLE II

DETERMINATION OF BUFEXAMAC IN COMMERCIAL SAMPLES BY HPLC

Sample	*Formulation*	*Found**	
		mg/g	*% of declared*
1	Cream	48.6	97.2
2	Cream	51.6	103.2
3	Cream	50.0	100.0
4	Cream	48.1	96.2
5	Cream	48.7	97.4
6	Ointment	48.9	97.8
7	Ointment	49.2	98.4
8	Ointment	45.6	91.2

* Based upon three determinations for each sample. Amount declared was 50 mg/g in each case.

N, selectivity, α, peak asymmetry, S, and capacity factor, k', were calculated and are listed in Table I. It was found that columns from different manufacturers had significantly different behaviours with respect to separation parameters for bufexamac. Such variations in separation parameters among ODS columns from different manufacturers is strong evidence that column characteristics, such as the percentage of hydrocarbon loading, type of organosilanes used for the preparation of bonded hydrocarbon and the number of unsilanized hydroxy sites, can be as important as the type of the bonded hydrocarbon in determining separation parameters.

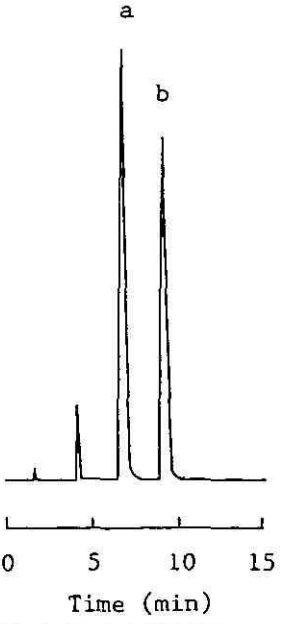

Fig. 1. Typical HPLC chromatogram of bufexamac in an ointment sample. HPLC conditions: μBondapak C_{18} column; mobile phase, 60% (v/v) methanol in water (pH 3); ambient temperature; flow-rate 1 ml/min and UV detection at 280 nm. Peaks: a = bufexamac; b = *n*-butyl *p*-hydroxybenzoate (internal standard).

The μBondapak C_{18} column was the most suitable for this analysis. The other columns did not demonstrate the efficiency required to separate bufexamac.

To optimize the system, the influences of the methanol concentration and of the pH on the separation were investigated; 60% (v/v) methanol in water and pH 3.0 were chosen as the optimum eluent parameters.

This assay method for bufexamac on μBondapak C_{18} has been developed using the internal standard technique. *n*-Butyl *p*-hydroxybenzoate is an ideal internal standard. It allows the HPLC assay to be completed in about 9 min. Both bufexamac and the internal standard have UV absorption maxima around 280 nm. The relationship between the peak-height ratio relative to *n*-butyl *p*-hydroxybenzoate and the amount of bufexamac was linear over the range selected, 0.2–1.0 mg/ml, with a correlation coefficient of 0.999.

The accuracy of the procedure was determined by spiking a placebo formulation (cream and ointment) with known concentrations of standard. Recoveries of bufexamac averaged 99.7% ($n = 6$) with a relative standard deviation (R.S.D.) of 1.02% for bufexamac levels of 50 mg/g.

Table II reports the results obtained in the analysis of commercial samples of bufexamac and Fig. 1 shows a typical chromatogram obtained from a commercial sample.

These results indicate that this method is suitable for the determination of bufexamac in cream and ointment.

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Note

Thin-layer chromatographic identification of leather dyes

II. Studies of mixtures of leather dyes

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In our earlier communication[1], the thin-layer chromatographic identification of 34 leather dyes on silica gel G layers (thickness 0.25 mm) using 86 solvent systems

TABLE I

TLC SEPARATION OF MIXTURES OF ANIONIC LEATHER DYES ON PRECOATED SILICA GEL 60 F 254

Grade in decreasing order	*Solvent systems*
I	(1) *n*-Butanol–acetic acid–water (4:1:5, upper phase)
	(2) *n*-Butanol–ethanol–water–acetic acid (6:1:2:0.05)
II	(1) Chloroform–isopropanol–water (1:3:1)
	(2) Isopropanol–ammonia (sp.gr. 0.91)–water (7:1:1)
	(3) *n*-Butanol–ethanol–ammonia (1:9) (9:2:3)
	(4) Isopropanol–ammonia (sp.gr. 0.91) (4:1)
III	(1) *n*-Butanol–acetone–water–ammonia (sp.gr. 0.91) (5:5:1:2)
	(2) Isopropanol–ammonia (sp.gr. 0.91)–water (10:1:1)
	(3) *n*-Butanol–acetic acid–water (2:1:5)
	(4) *n*-Butanol–ethanol–water (9:1:1)
	(5) Isopropanol–chloroform–water–diethylamine (50:25:20:15)
IV	(1) *n*-Propanol–ammonia (sp.gr. 0.91) (6:3)
	(2) Methanol–ethyl acetate–dilute ammonia (3 parts diluted in 7 parts water) (3:15:3)
	(3) *n*-Butanol–ethanol–water (8:1:3)
	(4) Ethyl acetate–acetic acid–water (3:1:2, upper phase)
V	(1) Isoamyl alcohol–pyridine–ammonia (sp.gr. 0.91) (8:4:1)
VI	(1) Chloroform–methanol (8:2)
	(2) Benzene–isopropanol–acetic acid (6:4:0.1)
	(3) Chloroform–ethanol–morpholine (8:1:1)
	(4) Chloroform–methyl ethyl ketone–acetic acid–formic acid (8:6:1:1)
	(5) Chloroform–methyl ethyl ketone–formic acid (6:8:1)

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was described and the solvents which gave good results for each class of dyes were reported. Leather, being a high value commodity in international commerce, is very often dyed with a mixture of dyes to get the appropriate shades and intensities to cater for aesthetic aspects and a competitive market. Earlier studies[2–5] on the thin-layer chromatography (TLC) of leather dyes were on individual dyes only and not on mixtures of dyes. As a continuation of our earlier work, studies have now been made on the TLC separation of mixtures of leather dyes with the different solvent systems which gave encouraging results, and the results are reported here.

EXPERIMENTAL

Precoated TLC plastic sheets of silica gel 60 F254 (25 cm × 20 cm, thickness 0.2 mm; E. Merck, F.R.G.) were used. The solvent systems were prepared with analytical grade reagents as reported earlier[1]. The dyes were dissolved in methanol

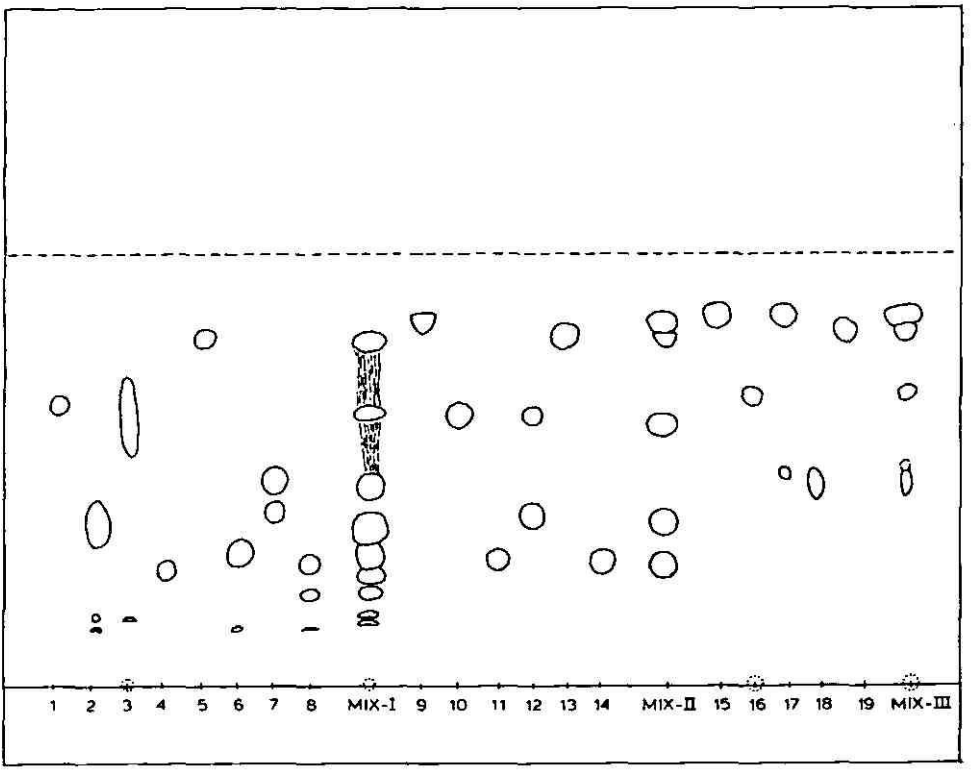

Fig. 1. TLC of anionic leather dye mixtures on precoated silica gel 60 F254 (thickness 0.2 mm) in the solvent system *n*-butanol–acetic acid–water (4:1:5, v/v, upper phase). Dyes: 1 = Polar Brilliant Blue GAW; 2 = Metalan Dark Blue; 3 = Neolan Violet Brown BX; 4 = Xyline Fast Green B; 5 = Metalan Red S-BR; 6 = Metalan Bordeaux S-B; 7 = Polar Red 3B; 8 = Metalan Brown S-GL; 9 = Cibalan Olive 3 BL; 10 = Metalan Yellow 5 RL; 11 = Ranomil Brilliant Red 3 BN; 12 = Sandopal Blue; 13 = Dermalight Yellow GLN; 14 = Derma Brilliant Red 3B; 15 = Dermalight Yellow 2RL; 16 = Chlorozol Orange Brown XS; 17 = Dermalight Orange RLN; 18 = Chlorozol Green BNS; 19 = Dermalight Grey 2 BL. Dyes 1, 4, 7, 11, 12 and 14 are acid, 2, 3, 5, 6, 8, 9, 10, 13, 15, 17 and 19 are premetallized and 16 and 18 are direct. Mixtures: I, dyes 1–8; II, 9–14; III, 15–19.

(0.1%) and each was spotted 2 cm above the edge of the TLC plates and as mixtures. and developed for 10 cm at room temperature.

RESULTS AND DISCUSSION

Generally in the leather industry, as in any other industries, mixtures of dyes are used. The leather dyes can be classified broadly into (i) anionic (acid, direct and premetallized) (ii) cationic (basic) and (iii) reactive dyes, and dyes of the same group with similar penetration power are used as mixtures for dyeing. The earlier studies carried out by Wada *et al.*[2], Sagala and Studniarski[3], Biedermann and Golz[4], Tzicas[5] and by us[1] were on individual dyes and not on mixtures of dyes. In the present studies, the TLC of 19 anionic dyes —6 acid, 2 direct and 11 premetallized— was carried out on precoated silica gel 60 F254, individually and also as mixtures consisting of acid and premetallized, and direct and premetallized, dyes. Twenty-one solvent systems[1] which gave better results for the TLC identification of various leather dyes —acid, direct and premetallized— were used. The separating power of each solvent system as indicated by its resolution of the dye mixture was studied and the solvent systems are classified in decreasing order of their resolving power for the dye components present in the mixtures (Table I). The system *n*-butanol–acetic acid–

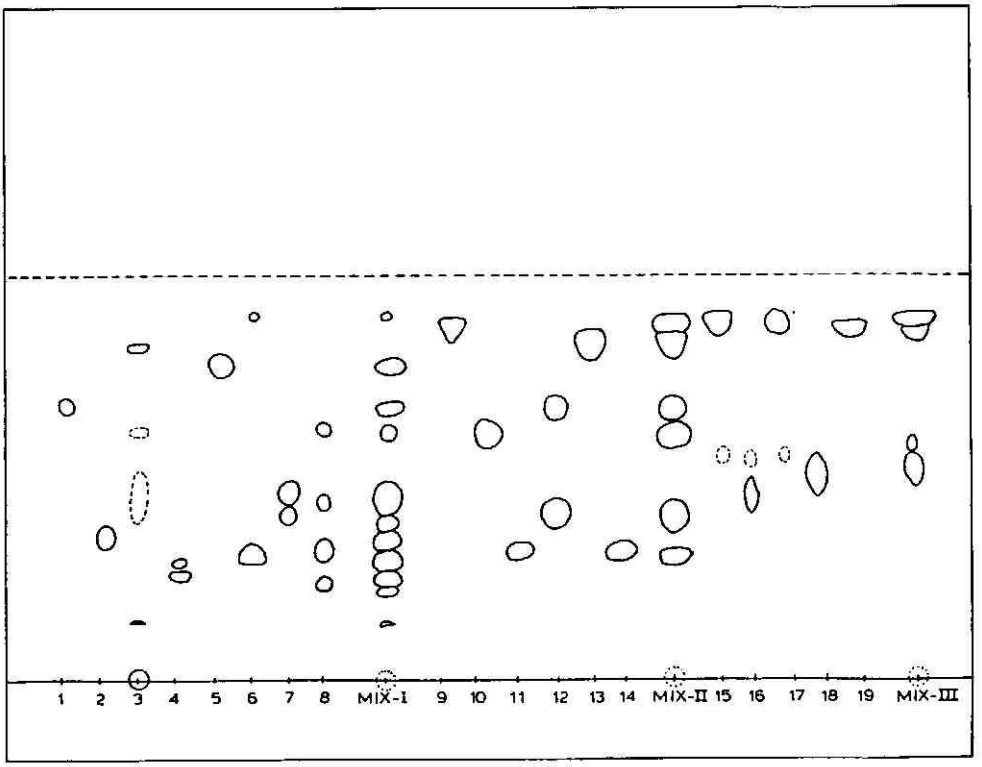

Fig. 2. TLC of mixtures of anionic leather dyes on precoated silica gel 60 F254 in the solvent system *n*-butanol–ethanol–water–acetic acid (6:1:2:0.05, v/v). Other details as in Fig. 1.

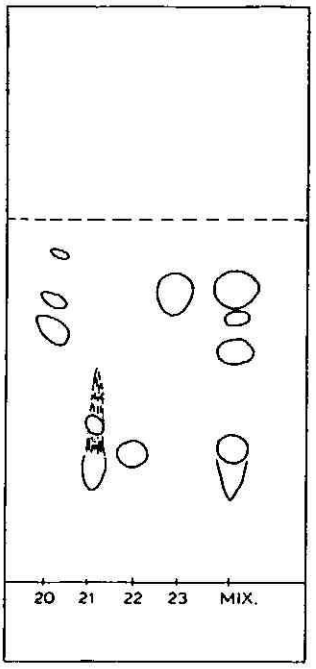

Fig. 3. TLC of a mixture of basic leather dyes on precoated silica gel 60 F254 in the solvent system *n*-butanol–acetic acid–water (4:1:5, v/v, upper phase). Dyes: 20 = Malachite Green; 21 = Methylene Blue; 22 = Fast Rubine 4 BN; 23 = Auromine O.

water (4:1:5, v/v) (upper phase) and *n*-butanol–ethanol–water–acetic acid (6:1:2:0.05, v/v) gave the best resolution of mixtures of anionic leather dyes (Figs. 1 and 2).

TLC of a mixture of four basic dyes was carried out using *n*-butanol–acetic acid–water (4:1:5, v/v, upper phase), ethyl acetate–acetic acid–water (3:1:2, v/v) and *n*-butanol–acetone–water–ammonia (sp.gr. 0.91) (5:5:1:2, v/v). Good separation of the dye components was obtained with the first of these systems (Fig. 3).

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Letter to the Editor

Static coating methods for glass capillary columns at elevated temperatures

Sir,

The continuing discussion of column preparation techniques demonstrates that more laboratories prepare their own columns than one tends to think. Today's techniques of column preparation have been developed to such perfection that they are both rapid and reliable.

Recently, Huang and Sun[1] reported on their experience with static coating at high temperature, for which we prefer the expression "free release static coating", introduced by Xu and Vermeulen[2]. I would like to add some of our experience using the same technique.

I agree with the above authors that short-term temperature stability of the heating bath is important as any relatively rapid temperature changes cause expansion or contraction of the coating solution and, as a consequence, an irregular withdrawal of the meniscus and an uneven thickness of the stationary phase film. On the other hand, slow temperature changes may be fairly drastic (many degrees) without affecting the column performance significantly. The authors suggest using a very precise thermostat, maintaining the temperature to within ± 0.05°C. We prefer a simpler system, in which the column is immersed in an only indirectly thermostated water-bath, *i.e.*, a tank of water situated in a thermostated bath. Heat transfer to the column is slow in this way, damping temperature changes in the thermostated bath. Even a thermostat of very moderate quality is good enough to give a smooth withdrawal of the meniscus.

Both Xu and Vermeulen[2] and Huang and Sun[1] reported the use of acetone in the coating solution. We hesitate to use acetone because of the observation that silicone columns suffer an increased bleeding rate. Therefore, we prefer the conventional dichloromethane–pentane mixture suggested by Kong and Lee[3] and Grob and Grob[4] or dichloromethane–isopentane mixtures.

Huang and Sun[1] mentioned a reduced coating speed if fairly concentrated coating solutions were used, but concentrations of coating solutions only up to 0.8% were tested. We observed a rather drastic reduction in the coating speed with a further increase in the concentration of the stationary phase. In fact, the speeds of solvent evaporation hardly exceeded those obtained by the classical static coating involving application of a vacuum. Further, we experienced a high risk of bumping (breakthrough) when using concentrated coating solutions, prompting us to return to the conventional method of static coating[5] at least for these applications.

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PUBLICATION SCHEDULE FOR 1986

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(Detailed *Instructions to Authors* were published in Vol. 362, No. 3, pp. 461–464. A free reprint can be obtained by application to the publisher.)

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Optimization of Chromatographic Selectivity

A Guide to Method Development

by **P. Schoenmakers,** *Philips Research Laboratories, Eindhoven, The Netherlands*

(Journal of Chromatography Library, 35)

This is the first detailed description of method development in chromatography – the overall process of which may be summarized as: method selection, phase selection, selectivity optimization, and system optimization. All four aspects receive attention in this book.

Chapter 1 gives a short introduction, describes chromatographic theory and nomenclature, and outlines the method development process. Chapter 2 describes guidelines for method selection, and quantitative concepts for characterizing and classifying chromatographic phases. Selective separation methods, from both gas and liquid chromatography are given in Chapter 3; the main parameters of each method are identified and simple, quantitative relations are sought to describe their effects. Criteria by which to judge the quality of separation are discussed in Chapter 4 with clear recommendations for different situations. The specific problems involved in the optimization of chromatographic selectivity are explained in Chapter 5. Optimization procedures, illustrated by examples, are extensively described and compared on the basis of a number of criteria. Suggestions are made both for the application of different procedures and for further research. The optimization of programmed analysis receives special attention in Chapter 6, and the last chapter summarizes the optimization of the chromatographic system, including the optimization of the efficiency, sensitivity and instrumentation.

Those involved in developing chromatographic methods or wishing to improve existing methods will value the detailed, structured way in which the subject is presented. Because optimization procedures and criteria are described as elements of a complete optimization package, the book will help the reader to understand, evaluate and select current and future commercial systems.

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