ELSEVIER

Journal of Chromatography A, 837 (1999) 201–210

JOURNAL OF CHROMATOGRAPHY A

Optimization of tandem columns for the isomer and enantiomer selective separation of toxaphenes

Ruth Baycan-Keller, Michael Oehme*

Organic Analytical Chemistry, University of Basel, Neuhausstrasse 31, CH-4057 Basel, Switzerland

Received 29 October 1998; received in revised form 19 January 1999; accepted 19 January 1999

Abstract

A tandem column system for the isomer and enantiomer selective separation of 18 toxaphenes was optimized by applying the theory of Purnell and Williams. Polymethylbiphenylsiloxane (OP) and heptakis(2,3,6-*O*-*t*-butyldimethylsilyl)-β-cyclodextrin (CD) dissolved in OV-1701 were used as stationary phases on the single columns. The isothermal separation required by the theory was modified by adding a steep temperature program rate ahead of the selected isothermal temperature to make it applicable to toxaphenes. This change had no influence on the predictability of the elution order by the theory. A length combination of 19 m CD plus 6 m of OP was most favorable. Details about the optimization process are given including the required precision of pressure measurements. A further temperature program optimization allowed separation of the most important congeners Parlar No. 26, 32, 50 and 62 on the tandem system without any interference from others isomers and enantiomers which are part of the commercial standard mixture containing 22 toxaphene congeners.

Keywords: Enantiomer separation; Coupled columns; Toxaphenes; Pesticides

1. Introduction

The chromatographic separation of the insecticide toxaphene by capillary gas chromatography (GC) is still a demanding task. It consists mainly of several hundreds of polychlorinated bornanes. Most of them are chiral. Compared to the complexity of technical toxaphene, the congener number accumulated in biota is small. Since bioaccumulation in the food chain and metabolism in biota are often enantioselective, a change of the enantiomeric ratio can be expected. Furthermore, enantiomers have often different toxic properties. Therefore, enantioselective detection is highly desirable.

At the moment, even the separation of the 22 commercially available congeners into isomers and enantiomers results in many co-elutions. Stationary phases are available which separate almost all congeners into isomers [1,2], but on enantioselective phases numerous co-elutions of isomers and enantiomers occur due to the doubling of the signals. One possibility to improve separation is to use tandem columns. The combination of stationary phases of different properties allows the achievement of a more even distribution of the signals. However, the chance of finding a suitable tandem system is rather small if the column properties (e.g. phase, length and film thickness) are selected by chance.

*Corresponding author. Tel.: +41-61-639-2301; fax: +41-61-639-2300.

E-mail address: oehme@ubaclu.unibas.ch (M. Oehme)

0021-9673/99/$ – see front matter
PII: S0021-9673(99)00112-0

Purnell et al. developed a general theory which allows optimization of the separation on columns coupled in series [3–6]. Moreover, they presented the so-called window diagram. This method enables the calculation of the length of combination necessary for the baseline separation of a compound mixture and shows the influence of the length ratio on the separation properties of the tandem system [7,8]. The theory was verified experimentally with complex mixtures, which could not be separated by single columns of any length [9–11].

The aim of this work was to optimize a tandem column system for the isomer and enantiomer selective separation of toxaphenes. The theory of Purnell et al. requires isothermal separation. However, this is not feasible for the separation of toxaphenes having relatively small differences in their retention properties. Observed problems are too long separation times and the missing solvent trapping effect at high capillary temperatures resulting in distorted peak shapes. Therefore, in this work an attempt was made to overcome this limitation of the theory by approximating isothermal conditions. A very fast heating ramp was used to reach the selected isothermal temperature as quickly as possible.

For the separation of a mixture of 18 toxaphene congeners two stationary phases were chosen. So far, a polymethylbiphenylsiloxane (Optima Delta 3, Macherey-Nagel) has produced the best isomer-selective separation [12]. Heptakis(2,3,6-*O-tert.*-butyldimethylsilyl)-β-cyclodextrin dissolved in OV-1701 allowed separation of nearly all congeners into enantiomers, but several co-elutions of isomers and enantiomers were observed.

To calculate the length of the single columns of the tandem system, the inlet and outlet pressure of the single columns have to be known precisely. A comparison was made between the results obtained by pressure measurements with high-precision instruments and the use of the pressure readouts from the gas chromatograph.

Since enantiomer separation is usually best at low temperatures (maximum of interactions with the stationary phase) [13,14], the overall length of the tandem system should be as short as possible. Therefore, calculations were aimed at obtaining the best possible isomer and enantiomer separation at the shortest possible tandem system. However, the results showed that a complete baseline separation of the 18-compound mixture into isomers and enantiomers could only be achieved with an unrealistically long tandem capillary of several hundreds of meters. The most important congeners are Parlar Nos. 26, 32, 50 and 62, which have to be determined in food [15]. Therefore, the aim was restricted to a complete separation of these congeners from the remaining toxaphenes.

2. Experimental

2.1. Standards and chemicals

A toxaphene reference standard was used containing 400 pg/μl of the congeners Parlar Nos. 11, 12, 15, 21, 25, 26, 31, 32, 38, 39, 40, 41, 42.1/42.2 (1 and 2 corresponds to the elution order of the two isomers), 44, 50, 51, 56, 58, 59, 62, 63 and 69 in cyclohexane (Ehrenstorfer, Germany). Enantiomers were marked correspondingly to their elution order as A or B. To identify the elution order, single congeners were used. Parlar Nos. 32 and 50 were obtained from Promochem (Germany, 5 ng/μl in isooctane) and the others from Ehrenstorfer (Germany, 1 ng/μl in cyclohexane). Dilutions were made with isooctane of pesticide quality (Scharlau, Germany) resulting in concentrations of about 100 pg/μl.

2.2. Instrumentation

Separations were carried out on a Hewlett–Packard 6890 gas chromatograph equipped with a ^{63}Ni electron-capture detector. Helium was used as carrier gas employing the constant flow mode. Nitrogen was used as make-up gas at a flow-rate of 100 ml/min. The temperature of the split/splitless injector was set to 160°C to avoid thermal decomposition [16] and of the electron-capture detector to 250°C. Splitless injections of 1–2 μl were performed manually at a start temperature of 100°C. The isothermal and splitless period was 1 min followed by a ramp of 100°C/min to 230°C. This temperature was kept until the last congener eluted. A heating rate of 2.5°C/min was used for the optimized separation.

Flow-rates were determined at 230°C with a soap

bubble-meter. The capillary inlet pressure was measured at 100°C with a precision pressure gauge (range 0 to 4 bar±0.6%, No. 312.20.160, Manometer, Hitzkirch, Germany) and the outlet pressure at 230°C with a MKS Baratron PDR-C-1B (range 0–10 bar±0.5%, Burlington, MA, USA) barometer. Average flow values, retention times, dead times as well as inlet and outlet pressures were obtained by repetitive measurements (n=15–30). Standard deviations were in the range of 0.15%.

2.3. Capillary columns

The following columns were employed: (1) Optima Delta 3 (OP): A capillary of 33.5 m×0.2 mm I.D., coated with 0.35 μm of polymethylbiphenylsiloxane (Macherey-Nagel, Switzerland) was used for the basic measurements outlined above. Later, it was cut into parts of 23, 6 and 4 m. (2) Enantioselective capillary (CD): A column of 30.35 m×0.25 mm I.D., coated with 0.2 μm of OV-1701-OH (14% cyanopropylphenol 86% methylpolysiloxane) with 10% heptakis(2,3,6-*O*-*t*-butyldimethylsilyl)-β-cyclodextrin [17] was selected. After measurement of the relevant parameters, it was split into lengths of 19 and 10 m. Columns were connected with a Connex Column Connector System (J&W Scientific). Lengths were measured several times after unrolling the capillary.

2.4. Calculations

Excel 7.0 under Windows NT 4.0 was used for the calculations and the corresponding macros. The following omissions were made when calculating the optimum separation properties of the tandem column system. Parlar No. 69 was not included since no co-elutions were observed for this last eluting congener. Parlar Nos. 11, 12 and 15 were not part of the calculations because they elute very early and were not available as single standards in our laboratory.

3. Results

The theory for tandem column optimization is presented in Refs. [3–11]. All equations necessary for the calculations are found in Ref. [11]. Therefore, only those essential for this work are given here. As already mentioned, this theory was originally developed for isothermal conditions. Since this is not realistic for toxaphene separations, an attempt was made to approximate the isothermal requirement by applying the shortest possible splitless time after injection (1 min) and the fastest possible heating ramp (100° C/min). However, due to the thermal mass of the gas chromatograph, 2.5 min were necessary to reach the final temperature of 230°C. With this temperature program an overall separation time of about 40 min was needed for the toxaphene mixture. Since the capillary was most of the time at 230°C, the column hold up time (t_M) measurements were performed at this temperature. Consequently, the value of the important parameter t_M is slightly too high. The results of all calculations are found in Table 1.

Table 1
Measured parameters obtained with precision gauges and from GC readouts and calculated values[a]

$t_{M,CD}$ (min)	$t_{M,OP}$ (min)	$t_{M,CD/OP}$ (min)	$t_{M,OP/CD}$ (min)	$H_{eff,CD}$ (cm)	$H_{eff,OP}$ (cm)	N_{req}	k	α		
1.001	1.055	4.64	3.81	0.042	0.0003	58983	12	1.027		
	$V^0_{M,CD}$ (ml)	$V^0_{M,OP}$ (ml)	$R_{F,CD}\cdot 10^5$ (N s m^{-3})	$R_{F,OP}\cdot 10^5$ (N s m^{-3})	p_i (kPa)	p_j (kPa)	p_o (kPa)	L_{CD} (m)	L_{OP} (m)	L_{CD+OP} (m)
Precision gauge	1.06	0.50	7.7	10.4	324	231	100	19.39	5.71	25.10
GC readouts	1.07	0.50	7.8	10.8	325	233	101	19.41	5.70	25.10

[a] CD, cyclodextrin column; OP, optima column; t_M, column hold up time; H_{eff}, height equivalent to an effective plate; N_{req}, requested number of effective plates; k, capacity ratio; α, separation factor; V^o_M, corrected mobile volume; R_F, resistance to flow; $p_{i,o,j}$, inlet, outlet and junction pressure; L, length.

3.1. R_f

The resistance to flow R_f was obtained from the slope of the equation:

$$t_M = (2LR_F/3)[(p_i^3 - p_o^3)/[(p_i^2 - p_o^2)^2]] \quad (1)$$

where L = length of column.

The parameters were obtained by measuring the column hold up time t_M for inlet pressures p_i ranging from 160 to 380 kPa ($p_i = p_o + \Delta p$; p_o, outlet pressure).

3.2. V_m

The corrected mobile volume V^{o}_{M} was obtained from the equation:

$$V_M^0 = jF_c t_M \quad (2)$$

where j = James Martin compressibility factor [18]; F_c = corrected volumetric flow-rate.

3.3. k- and α-plot.

The factor f, which ranges from 0 to 1 ($f_F + f_B = 1$), relates linearly the retention factors k of the front (F) and back (B) column with the overall retention factors k_{ov} of the tandem system:

$$k_{ov} = f_F k_F + f_B k_B \quad (3)$$

The retention factors k for all components on both single columns (see Table 2) plotted against the factor f_F leads to the $k - f_F$ plot (see Fig. 1). For better visibility, the plot was split into two parts by selecting two groups of toxaphenes whose retention factors never overlap at any length combination of the tandem system.

To optimize the length fraction of the two columns, the window diagram was constructed. The term window diagram describes a graphic plot of the factor f_F versus the resolution values α of the components, which are difficult to separate (see Fig. 2).

The maximum height of the largest window ('windows' are the white parts of the plot) delivers the f_F value necessary to calculate the pressure at the junction point of the two columns (see Eq. (5)) and the α value which allows calculation of the requested plate number N_{req} of the tandem column as follows:

$$N_{req} = 16R_s^2[a/(a-1)]^2[(1+k)/k]^2 \quad (4)$$

where R_s = peak resolution.

The plate number together with the height equivalent to an effective plate H_{eff} for the individual columns permits the determination of the required total length ($L_F + L_B$) of the tandem system.

3.4. p_j, l_F, l_f, l_b

The pressure p_j at the junction between the two columns can be calculated with the following equations:

$$p_j^3 = [p_i^3 - f_F(p_i^3 - \gamma p_o^3)]/[1 - f_F(1-\gamma)] \quad (5)$$

Table 2
Retention factors k obtained for the toxaphene congeners on the two original single columns OP and CD

	Parlar No.												
	21.A	21.B	25.A	25.B	26.A	26.B	31.A	31.B	32.A	32.B	38.A	38.B	39.A
k(CD)	11.6	12.1	9.3	10.3	8.7	8.8	10.2	11.2	13.0	13.0	17.1	17.4	12.4
k(OP)	11.7	11.7	12.7	12.7	11.9	11.9	13.9	13.9	15.9	15.9	16.7	16.7	17.4
	39.B	40.A	40.B	41.A	41.B	42.1A	42.1B	42.2A	42.2B	44.A	44.B	50.A	
k(CD)	12.4	12.1	12.4	12.0	12.4	15.0	15.0	15.0	15.4	15.2	15.4	13.0	
k(OP)	17.4	18.2	18.2	17.8	17.8	18.8	18.8	18.9	18.9	19.3	19.3	19.8	
	50.B	51.A	51.B	56.A	56.B	58.A	58.B	59.A	59.B	62.A	62.B	63.A	63.B
k(CD)	13.3	22.7	23.0	18.7	19.1	18.4	18.4	21.3	21.5	26.0	26.6	20.4	20.9
k(OP)	19.8	24.4	24.4	24.4	24.4	25.6	25.6	26.5	26.5	30.2	30.2	31.6	31.6

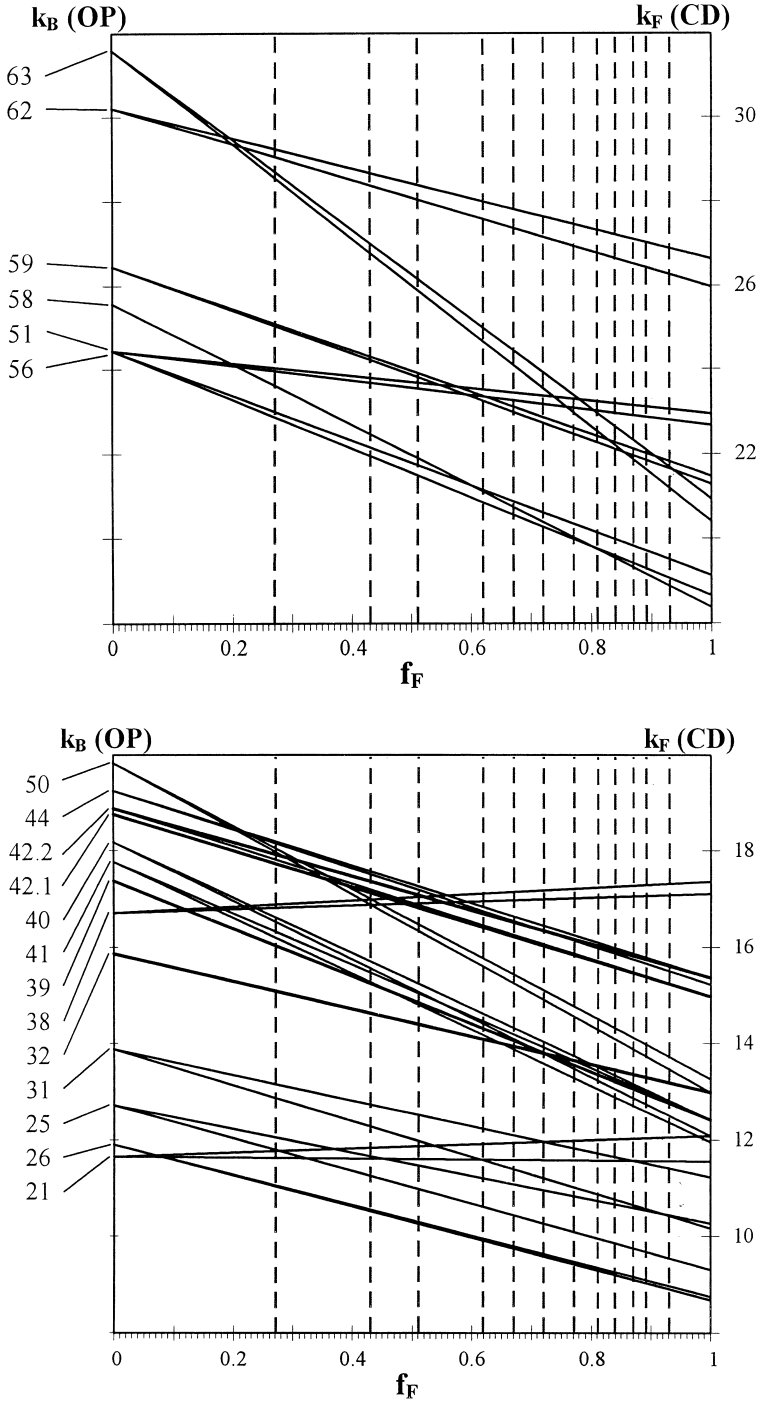

Fig. 1. Plot of the capacity ratio k of the front column (CD, secondary y-axis) and of the back column (OP, primary y-axis) versus factor f_F ($k - f_F$ plot) representing the overall capacity ratios k_{ov} for the tandem system. The f_F values for the twelve tandem combinations obtained (see Table 3) are marked with dashed lines. The intersection points of the dashed lines with the k_{ov} lines show the elution order for the corresponding tandem combination. The capacity ratios of the toxaphenes are marked with Parlar numbers.

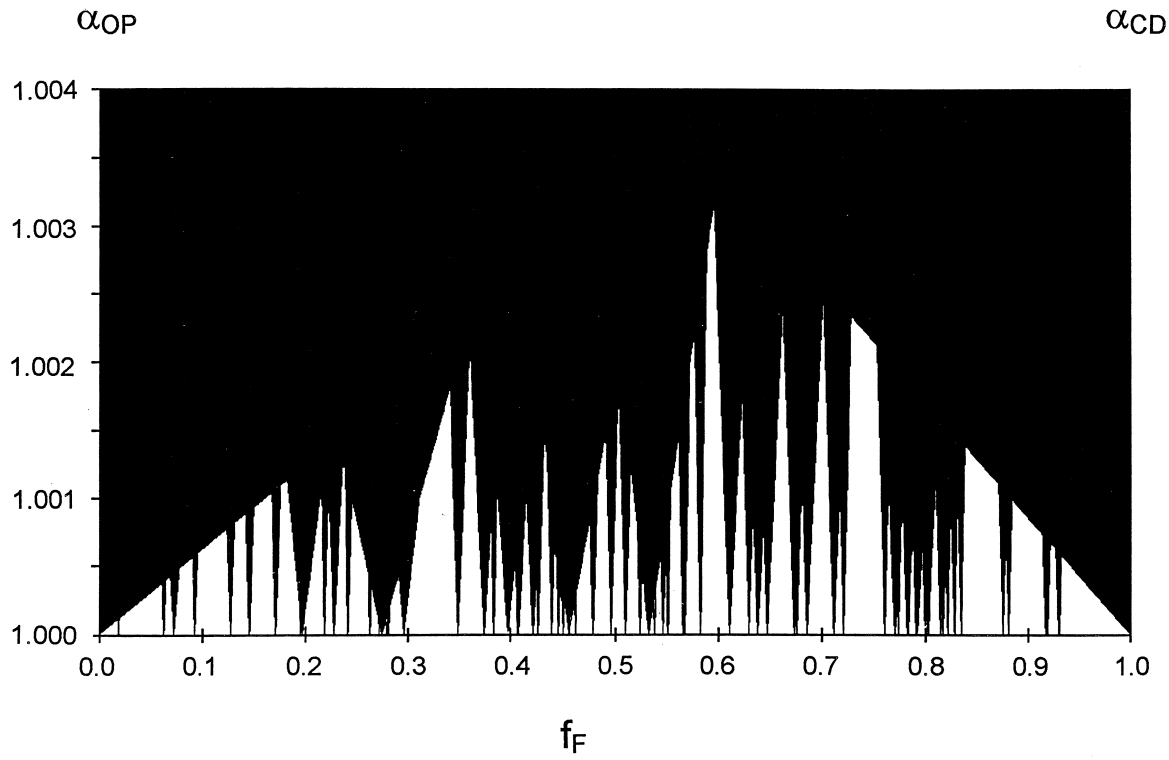

Fig. 2. Plot of α values of the single columns versus the factor f_F. The white areas are called 'windows'.

$$\gamma = (L_F R_{FF}/L_B R_{FB})(V^o_{MB}/V^o_{MF})^2 = (\overline{R_{FF}}/\overline{R_{FB}})(\overline{V^o_{MB}}/\overline{V^o_{MF}})^2 \quad (6)$$

The over-bar symbol indicates the value per unit length. The length fraction l_F ($l_F = L_F/(L_F + L_B)$) can be obtained from the following equation:

$$l_F^{-1} = [(\overline{R_{FF}V_{MB}}/\overline{R_{FB}V_{MF}})((p_j^2 - p_o^2)/(p_i^2 - p_j^2)) + 1] \quad (7)$$

l_F together with the total length ($L_F + L_B$) allows calculation of the individual column lengths for the tandem system.

3.5. Required accuracy of parameter measurements

The pressure determinations at the column inlet were carried out with a precision gauge (±0.6%). All calculations were performed with these values and compared to those obtained with the pressure readouts of the gas chromatograph. Their accuracy according to the supplier is ±2%. The small differences in measured pressures do not affect the calculated total length of the optimized tandem column (see Table 1) but a difference of a few cm in the lengths of the single columns is observed. However, this very small difference in the retention properties of the whole tandem system can easily be compensated by a minor correction of the applied temperature or pressure program. Therefore, a high precision determination of p_i and p_o is not necessary which facilitates the application of the presented approach.

4. Discussion

The use of tandem columns has been proposed several times in high-resolution gas chromatography (HRGC) to enhance the separation quality of the system. Some components of a complex mixture

may be separated on one stationary phase and the rest on another phase with very different retention properties. By combining the two, an overall improvement of the separation can be achieved.

The selectivity of the tandem system can be influenced by different methods. Interesting results were obtained by varying the pressure at the junction point of the two columns [19] and by performing heart cuts [20]. However, these methods request additional equipment of the GC. This is not the case when columns of different lengths are simply connected in series with a common connector. The selectivity of this system is influenced by the applied optimal length combinations [21].

Purnell et al. presented a well verified theory for the optimization of different length combinations for isothermal separation on tandem columns. However, it is not applicable to mixtures, which cannot be separated by isothermal conditions. In this paper the applicability is expanded to temperature-programmed gas chromatography. Moreover, it is shown that the method is also applicable to the separation of enantiomers.

4.1. Tandem column length.

The $k - f_F$ plot (see Fig. 1) shows that a baseline separation of all congeners is difficult to achieve. A lot of intersection points are observed, which means equal retention factors and hence coelution at this f_F value and corresponding length combination. Moreover, some of the congeners have nearly identical k_{ov} values making it very difficult to achieve a satisfactory separation. In addition, the windows in the $\alpha - f_F$ plot (see Fig. 2) are narrow and their maximum height is low ($\alpha \leq 1.003$). As can be seen from Eq. (4), the total tandem column length necessary for an optimal separation depends not only from the separation factor α but also from the resolution R_s. A peak resolution of $R_s = 0.8$ would require a total tandem column length of about 400 m. For a full baseline separation ($R_s = 1.5$) of all isomers and enantiomers, the length would increase to about 1600 m. This means that baseline separation of all congeners cannot be achieved with the selected capillaries at a reasonable length combination.

Therefore, a more realistic application was chosen. According to German regulations the sum concentrations of Parlar Nos. 26, 32, 50 and 62 in food should not exceed 0.1 mg/g fresh mass [22]. It is important that an interference-free isomer and enantiomer selective determination can be achieved for these congeners. Therefore, an attempt was made to obtain a separation with the available capillaries which was free of interferences from the remaining congeners in the reference mixture. Based on the information from the window diagram and the $k - f_F$ plot, the OP capillary was cut into pieces of 4 m, 6 m and 23 m and the CD capillary into lengths of 19 m and 10 m. By combining two of them, 12 different tandem systems and hence elution orders can be generated (see Table 3). Their corresponding f_F values are marked as dashed lines in the $k - f_F$ plot of Fig. 1. The intersection points of the dashed lines with the k_{ov} lines show the elution order of the respective tandem system.

4.2. Predicted and experimental elution order

Though the theory requires isothermal separation conditions, the applied approximation with a steep temperature ramp allows the prediction of the isomer and enantiomer selective separation of the toxaphene mixture very well. For the heating rate of 100°C/min the calculated elution order for the twelve tandem combinations corresponds to the experimental one.

Table 3
Length combination of twelve tandem columns and their corresponding f_F values obtained by combining OP capillaries lengths of 4 m, 6 m and 23 m with CD capillaries of 10 m and 19 m[a]

	Combinations											
	OP/CD						CD/OP					
Length (m)	23/10	23/19	6/10	4/10	6/19	4/19	10/23	19/23	10/6	10/4	19/6	19/4
f_F	0.27	0.43	0.62	0.72	0.77	0.84	0.51	0.67	0.81	0.87	0.89	0.93

[a] OP/CD means that the OP is the front and the CD the back column.

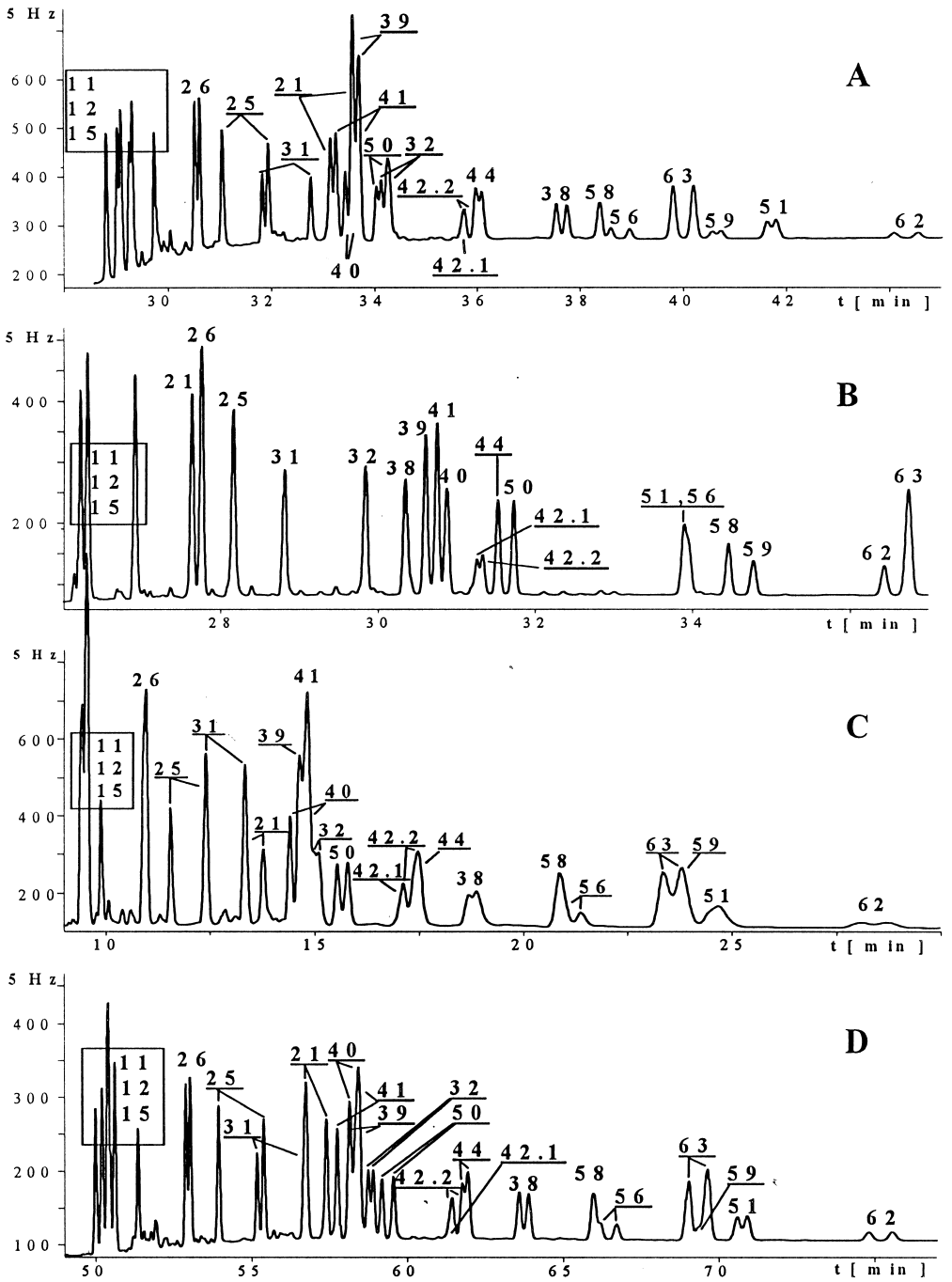

Fig. 3. Gas chromatograms of the separation of the toxaphene mixture on the single columns (A, CD column; B, OP column) and on the tandem combination CD/OP of 19 m and 6 m using a heating rate of 100°C/min (C) and of 2.5°C/min (D).

Only one deviation was observed for the combination CD/OP of 19 m/23 m (see Fig. 1, $f_f = 0.89$ and Fig. 3C). The expected elution order of 44B before 38A/B was not in accordance with the experiment where 38A/B eluted before 44B. The optimization of the heating rate to 2.5°C/min changed not more than two elution orders per tandem combination.

4.3. Achieved separation on single columns versus tandem systems

The optimized separations achieved on the single columns are shown in Fig. 3A and B. The CD capillary separated all congeners into enantiomers except Parlar nos. 58 and 42.1 (see Fig. 3A) but several co-elution of enantiomers do occur. The OP phase separates all toxaphenes including nos. 42.1/42.2 into isomers apart from nos. 51/56 (see Fig. 3B).

The combination of the capillaries of original length to a tandem system resulted in a deterioration of the separation compared to the separation with the single columns [10]. Thermal decomposition on this surface of the long tandem column was observed on both combinations (CD/OP or OP/CD), and the retention time of the last eluting congener was more than 100 min with the heating rate of 100°C/min to 230°C (isothermal hold). If a more normal heating ramp such as 5°C/min is selected the co-elutions still persist and the longer retention times lead to more thermal degradation.

The twelve tandem systems obtained after cutting the columns delivered chromatograms as expected from the $k - f_F$ plot. Good overall separations were achieved with the combinations of column pieces longer than 10 m, even though co-elution were found on all of them. This could not be avoided since a much longer overall length was requested from the calculations for an overall separation. This problem was of course even more prominent for the shorter columns.

The chromatograms of the 19 m CD/6 m OP tandem combination are shown in Fig. 3C and D. As expected, co-elutions occurred when such short column length combinations are combined with the fast heating ramp of 100°C/min. The latter particularly influences the enantioselective separation, due to a decrease of the weak interactions with the chiral surface. An optimized heating rate of 2.5°C/min improved the enantiomer as well as the isomers separation. All toxaphenes were separated into enantiomers except Parlar nos. 42.1 and 58, and the indicator congeners were not interfered by any other toxaphene. However, as predicted by the $k - f_F$ plot, several co-elutions were observed. A much longer tandem combination would be needed to improve the separation for other important congeners such as Parlar nos. 40 and 44.

5. Conclusions

The $k - f_F$ plot allows the prediction of whether a separation of the compound mixture is possible or not for the chosen tandem system. It requires only the measurements of the retention times and of the column hold up time of the single columns. To calculate the optimal length of the single columns for the tandem system, a substantial amount of calculations is necessary. However, they are mathematically trivial and simple computer programs can be made.

The optimization procedure allows the prediction of the single capillary lengths necessary for the best separation by the tandem system. Moreover, by changing the sequence of the columns, several elution orders are possible. In this way co-elution can be avoided for selected substances.

The isothermal separation requested by the theory was replaced by a steep temperature ramp. This leads to results, which are still accurate enough to predict the elution order of a tandem system and to foresee whether a separation of the substances is possible or not.

Acknowledgements

We like to thank the Swiss Science Foundation for financial support under the project nos. 2100-043399.95 and 20-50474.97

References

[1] D.C.G. Muir, J. de Boer, Trends Anal. Chem. 14 (1995) 56.

[2] J. de Boer, Organohalogen Compd. 33 (1997) 7–12.
[3] J.H. Purnell, P.S. Williams, J. Chromatogr. 292 (1984) 197–206.
[4] J.H. Purnell, P.S. Williams, J. Chromatogr. 321 (1985) 249–254.
[5] J.H. Purnell, M. Rodriguez, P.S. Williams, J. Chromatogr. 323 (1985) 402–405.
[6] J.H. Purnell, P.S. Williams, J. Chromatogr. 325 (1985) 1–12.
[7] R.J. Laub, J.H. Purnell, J. Chromatogr. 112 (1975) 71–79.
[8] R.J. Laub, J.H. Purnell, Anal. Chem. 48 (1976) 1720–1724.
[9] J.H. Purnell, M.J. Rodriguez, J. Chromatogr. 358 (1986) 39–51.
[10] J.H. Purnell, J.R. Jones, M.H.J. Wattan, J. Chromatogr. 399 (1987) 99–109.
[11] J.R. Jones, J.H. Purnell, Anal. Chem. 62 (1990) 2300–2306.
[12] R. Baycan-Keller, M. Oehme, Organohalogen Compd. 35 (1998) 229–233.
[13] M.J. Lindström, J. High Resol. Chromatogr. 14 (1991) 765–767.
[14] I. Hardt, W.A. König, J. Microcol. Sep. 5 (1993) 35–40.
[15] L. Alder, K. Bache, H. Beck, H. Parlar, Chemosphere 35 (1997) 1391–1398.
[16] R. Baycan-Keller, M. Oehme, J. High Resol. Chromatogr. 21 (1998) 298–302.
[17] M. Oehme, L. Müller, H. Karlsson, J. Chromatogr. A 775 (1997) 275–285.
[18] A.T. James, A.J.P. Martin, J. Biochem. 50 (1952) 679–690.
[19] D.R. Deans, I. Scott, Anal. Chem. 45 (1973) 1137–1141.
[20] H.J. de Geus, J. de Boer, U.A.T. Brinkman, Trends Anal. Chem. 15 (1996) 168–178.
[21] P. Sandra, F. David, M. Proot, G. Diricks, M. Verstappe, M. Verzele, J. High Resolut. Chromatogr. Chromatogr. Commun. 8 (1985) 782–798.
[22] Verordnung über Höchstmengen an Rückständen von Pflanzenschutz- und Schädlingsbekämpfungsmitteln in oder auf Lebensmitteln; Verordnung des Bundesministeriums für Gesundheit und des Bundesministeriums für Umwelt. Deutscher Bundesrat, 1994, Drucksache 252/94, Bundesanzeiger, Bonn, edition March 1998.