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(Abstracts/Contents Lists published in Analytical Abstracts, Biochemical Abstracts, Biological Abstracts, Chemical Abstracts, Chemical Titles, Current Contents/Physical, Chemical & Earth Sciences, Current Contets/Life Sciences, Index Medicus, Mass Spectrometry Bulletin, and Science Citation Index)

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Book Review

Electrodes of Conductive Metal Oxides

edited by SERGIO TRASATTI, *Laboratory of Electrochemistry, University of Milan, Italy.*

STUDIES IN PHYSICAL AND THEORETICAL CHEMISTRY 11.

This two-part work provides a general unifying introduction plus a state-of-the-art review of the physicochemical properties and electrochemical behaviour of conductive oxide electrodes (DSA). The text has been divided into two volumes – Part A dealing mainly with structural and thermodynamic properties and Part B dealing with kinetic and electrocatalytic aspects. This division came about due to the large amount of material to be treated and also because, in a rapidly developing field, difficulties arise in collecting all relevant material at one given moment.

The editor approaches the subject from a multidisciplinary angle, for example, the electrochemical behaviour of oxide electrodes is presented and discussed in the context of a variety of physicochemical properties – electronic structure, nonstoichiometry, crystal structure, surface structure, morphology and adsorption properties. For the first time the different groups of oxides are treated together in order to emphasise their similarities and differences.

This major reference work is mainly directed to electrochemists and those working on catalysis. It will also be useful to those in the fields of materials science, physical chemistry, surface and colloid chemistry and in areas where oxide surfaces may play a major role as in chromatography and photochemistry.

CONTENTS: Chapters. 1. Electronic Band Structure of Oxides with Metallic or Semiconducting Characteristics *(J. M. Honig)*. 2. Chemisorption and Catalysis on Metal Oxides *(A. Cimino and S. Carrà)*. 3. Oxide Growth and Oxygen Evolution on Noble Metals *(L. D. Burke)*. 4. Electrochemistry of Lead Dioxide *(J. P. Pohl and H. Rickert)*. 5. Properties of Spinel-Type Oxide Electrodes *(M. R. Tarasevich and B. N. Efremov)*. 6. Physicochemical and Electrochemical Properties of Perovskite Oxides *(H. Tamura, Y. Yoneyama and Y. Matsumoto)*. 7. Properties of Conductive Transition Metal Oxides with Rutile-Type Structure *(S. Trasatti and G. Lodi)*. 8. Fundamental Properties of the Oxide/Aqueous Solution Interface *(D. N. Furlong, D. E. Yates and T. W. Healy)*. 9. Reactions of Hydrogen and Organic Substances with and at Anodic Oxide Films at Electrodes *(B. E. Conway)*. 10. Oxygen and Chlorine Evolution at Conductive Metallic Oxide Anodes *(S. Trasatti and G. Lodi)*. 11. Technological Impact of Metallic Oxides as Anodes *(A. Nidola)*.

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QUANTITATIVE ANALYSIS OF COMPONENTS OF UNRESOLVED GAS CHROMATOGRAPHIC PEAKS BY ELECTRON-CAPTURE DETECTION WITH OXYGEN SENSITIZATION

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(Received January 4th, 1982)

SUMMARY

A method for the quantitative analysis of unresolved gas chromatographic peaks is described which should be useful in many instances for the trace analysis of structural isomers which are sensed by the electron-capture detector (ECD). A determination of unseparated isomeric components of a peak is made by obtaining a chromatogram of the sample without and with added oxygen in the ECD. The method is shown to be successful if the magnitude of the response enhancement caused by oxygen is dependent on the structural differences within the set of isomers. The method is applied here to mixtures of the three isomers of chloroanthracene.

INTRODUCTION

For the analysis of organic mixtures, methods which first separate components by gas chromatography have been extremely successful. Nevertheless, in dealing with complex samples such as are typically encountered in environmental analyses, incomplete separation of some of the sample components can generally be expected. Even in cases where each of the peaks in a chromatogram appear to be caused by a single chemical substance, the possibility that two or more distinct compounds have eluted with the same retention times provides an ever present source of uncertainty. In many cases the problems associated with unresolved peaks can be solved by the use of detectors which are capable of differentiating between the coeluting compounds. Mass spectrometry is often used for this purpose and is almost always successful for the cases where the coeluting chemicals are structurally quite different and their similarity of retention times is due to a coincidence of the solute–stationary phase interaction. For the cases where the equality of retention times is due to similarity in structure, however, the combined powers of gas chromatography and mass spectrometry are frequently insufficient to solve these analysis problems. This is because the conventional forms of mass spectrometry are frequently unable to differentiate between a set of structural isomers having the same molecular formula. In addressing these challenging determinations, another means is provided by the use of multiple detectors, each of which responds to the sample by a fundamentally different interac-

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tion. This approach can be successful provided that the response of at least one of the detectors is unique to each of the various components of the peak and the measured response ratio of the multiple detectors is uniquely traceable to each component.

We will demonstrate here the use of a simple electron-capture detection scheme for the analysis of unresolved chromatographic peaks where the three isomers of chloroanthracene serve as the analyte. A desirable feature of this detection method is that the two detectors used are physically the same one which is made to respond differently in repeated analysis simply by the addition of a small amount of oxygen to the nitrogen carrier gas. The cause of electron capture sensitization by oxygen has been discussed several times previously[1–4]. Briefly, for cases where oxygen sensitization occurs, the response of the oxygen-doped electron-capture detector (ECD) is thought to be proportional to the rate of reaction of O_2^- with the sample molecule, while the normal ECD response is thought to be proportional to the rate of the reaction of the gaseous electron with the analyte. We have shown in previous studies that the ratio of response of the oxygen-sensitized and normal ECDs to various sets of geometric isomers are often varied and dependent on structural detail even for cases where mass spectra offer no detectable differences. In this report the quantitative analysis of the isomers of chloroanthracene is demonstrated by this method where absolutely no separation of the isomers is provided by the chromatographic function performed by a short packed column and where only partial separation is provided by a capillary column.

EXPERIMENTAL

The gas chromatograph used is a Varian 3700 with constant-current, pulse-modulated operation of its ^{63}Ni ECD. The carrier gas is prepurified nitrogen maintained at a flow-rate of 40 ml/min. The packed column used was made from 1/8-in. stainless tubing of 1.5-ft. length and was packed with 4% OV-101 on Chromosorb W. Oxygen was added as a make-up gas after the column and immediately ahead of the detector. By combining the carrier gas with *ca.* 7 ml/min of nitrogen containing 2% oxygen, an oxygen concentration of *ca.* 3 parts per thousand is maintained in the doped detector. From one determination to another, a precise level of oxygen is fine-tuned by adjusting the make-up gas flow until a preselected magnitude of baseline frequency is observed. The temperature of the oven for packed column separations is 170°C. The injector is at 200°C.

All of the compounds studied were purchased from commercial suppliers. Standards were prepared by dilution into benzene. Aliquots of 1 μl were syringe-injected into the normal injection port of the instrument. Sample sizes sufficient to produce small, but easily measurable, peaks were chosen. These were from 1 to 10 ng per injection. Each prepared mixture of the isomers was analyzed at least three times without oxygen and at least three times with oxygen. Peak heights were reproducible to within 3–5%. The average of these repeated analyses were used for the response enhancements reported here.

An analysis is also reported here where an SE-52 capillary column (15 m × 0.25 mm I.D.) was used. The carrier gas is then He and the make-up gas is nitrogen. For the oxygen-sensitized response, the make-up gas is mixed with oxygen-containing nitrogen as described above so that the same predetermined level of baseline

frequency and oxygen concentration is selected. The oven is temperature-programmed as follows: 90°C for 3 min, 20°C increase per min to 200°C, hold at 200°C for 10 min. For capillary-column analyses, the injection port is modified to reduce its volume. A splitless injection of 0.4 μl is used where each injection contains at total of *ca.* 0.3 ng of each chloroanthracene isomer.

RESULTS AND DISCUSSION

The three isomers of chloroanthracene were chosen for this demonstration because their retention times on a short packed column will be identical and because these compounds are readily detected with an ECD. Also, this example is one where a distinction between isomers is not expected to be provided by the conventional forms of mass spectrometry. To verify this we have obtained the electron-impact mass spectrum of each of the chloroanthracene isomers. These are shown in Fig. 1. Indeed, the mass spectrum of each appears to be identical. In Fig. 2 typical chromatograms are shown from which the determinations to be reported here are obtained. The first three pairs of chromatograms are for the pure isomers of chloroanthracene and the last pair is for a mixture of all three chloroanthracenes. For each pair of chromatograms the lower one is obtained by normal electron-capture detection and the upper one is obtained with approximately 3.0 parts per thousand oxygen continuously present in the detector. It is seen that the response of each of the three pure isomers is enhanced by oxygen's presence, but by differing magnitudes. The response to the 1-isomer is enhanced by 4.0, to the 2-isomer by 6.9 and to the 9-isomer by 21.0. In addition, the data in Fig. 3 indicate that the magnitude of the response enhancements observed for each isomer is a relatively constant value over at least two orders of magnitude change in concentration above the lowest concentration levels used here.

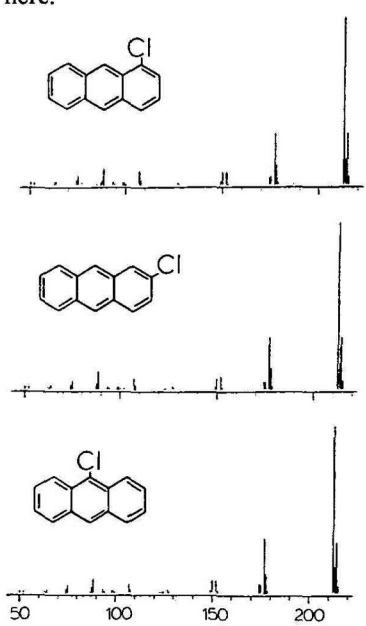

Fig. 1. Electron-impact mass spectra of three isomers of chloroanthracene.

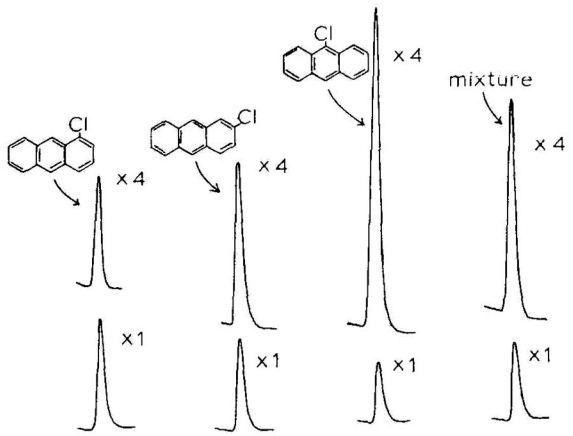

Fig. 2. ECD responses to pure isomers of chloroanthracene and a mixture of isomers without oxygen (lower chromatograms) and with 3 parts per thousand oxygen (upper chromatograms) in the detector at 300°C. Sample amounts are 2.9, 2.3 and 1.5 ng for the 1-, 2- and 9-isomers, respectively. The mixture contains molar fractions of these of 0.35, 0.28 and 0.37, respectively.

If the composition of mixed peaks are to be determined from the measured response enhancement it is necessary that the enhancement of a mixed peak, RE_{mix}, be expressable as the sum of the contributions of the individual components as shown in eqn. 1,

$$RE_{mix} = \sum_i X_i \, RE_i \tag{1}$$

where RE_i is the oxygen-caused response enhancement of the individual components, X_i is the molar fraction of substance i in the mixed peak and $\sum_i X_i = 1$. In order to test the validity of this relationship the response enhancements of many different

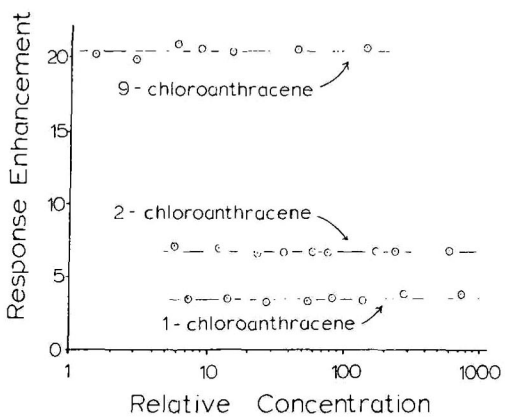

Fig. 3. Oxygen-induced response enhancements for the chloroanthracenes as a function of concentration. A relative concentration value of 1.0 corresponds to 10 pg injected.

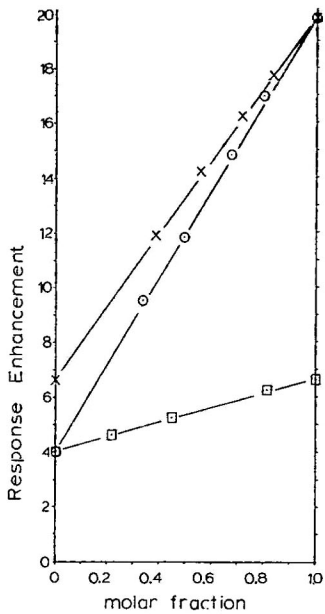

Fig. 4. Response enhancements of two-component mixtures as a function of the molar fraction of one of the components. The mixtures are composed of 1- and 2-chloroanthracene (□), 1- and 9-chloroanthracene (○), and 2- and 9-chloroanthracene (×).

mixtures of the chloroanthracenes have been measured. For the three possible sets of two-component mixtures, the results are shown in Fig. 4, where the measured enhancement is plotted as a function of the molar fraction of one of the sample components. These data indicate that the measured enhancements are linearly related to the molar fraction of each component and that eqn. 1 does, indeed, describe the response enhancement expected for a binary mixed peak. It would be relatively straightforward, therefore, to determine the relative amounts of two isomers known to be present in a mixed binary peak of unknown composition. For these cases the unknown quantities sought, X_1 and X_2, are obtained from application of eqn. 1 and the trivial relationship $X_1 + X_2 = 1$.

It is reasonable to expect that eqn. 1 will also be applicable to three component peaks. The last pair of chromatograms in Fig. 1 is seen to support this expectation, since response enhancement observed for this mixture is 11.2. From the enhancements measured for the individual isomers and from the known molar ratios of each in the mixture, a value of 11.1 is predicted ($0.35 \times 4.0 + 0.28 \times 6.9 + 0.37 \times 21.1 = 11.1$). Unfortunately, for the three-component system the relative composition of an unknown mixed peak is not uniquely determined by a single enhancement measurement as in the binary case. In this case a single RE_{mix} value has many possible solutions when applied to eqn. 1 since there are then three unknowns and only two equations relating them. For the chloroanthracenes under consideration here a solution to this problem is provided by repeating all enhancement measurements at a different detector temperature. The above measurements have indicated that at 300°C, the measured enhancements are $RE_1 = 4.0$, $RE_2 = 6.9$, $RE_9 = 21.1$ and $RE_{mix} = 11.2$. With a detector temperature of 350°C, these enhancement measure-

ments are found to be 2.5, 3.4, 15.6 and 7.6, respectively. With the data at 350°C eqn. 1 can be used a second time to provide the third equation necessary to determine uniquely the molar ratios in the three-component mixtures. Applying this procedure to the synthesized mixture of the three isomers, the data indicate that the molar ratios are $X_1 = 0.32$, $X_2 = 0.31$ and $X_9 = 0.37$. These values are in good agreement with the known composition of this sample (indicated in Fig. 2).

In solving the three-component problem it is essential to recognize that the new set of RE_i values obtained at a second detector temperature must not be proportionally related to the original set. That is, if the new set were simply half, for instance, of the original set, no additional information will be obtained by the analysis at the second temperature. The reason for this is made clear by inspection of eqn. 1 which is seen to be unchanged if all RE values are simply altered by a proportional factor. Also for this reason, no new information concerning the sample is expected by altering the amount of oxygen used in the detector for the enhancement measurement. We have previously shown[1,2] for the constant-current ECD that this change merely increases or decreases all RE values in proportion to the concentration of oxygen.

Also with this detection scheme, it is important to recognize the situations where peak height rather than peak area can be used as the measure of detector response. With the short packed column used here, the retention times of the three chloroanthracenes were indistinguishable. In this case their peak height or peak area could be used as a measure of response and the same values for RE_{mix} and RE_i are obtained (this must be as long as the normal and oxygen-sensitized responses are linearly related to sample concentration over the concentration range of interest).

Fig. 5. Capillary column gas chromatograms of the three-component mixture described in Fig. 2. The arrows indicate the point of elution of the chloroanthracene isomers. The lower chromatogram is with normal electron-capture detection and the upper is with 3 parts per thousand oxygen in the make-up gas. Detector temperature is 300°C.

However, for those cases where the components of a mixed peak are partially separated by the column, care must be taken to use peak area, only, as the measure of response. Then the method as outlined above can be used for the quantitation of a partially resolved mixed peak. For these cases the use of peak height as the measure of response will cause systematic error because at the instant of the peak maxima, the molar ratio of components in the detector differs from the molar ratios of these components in the original sample.

For chromatographic separations where partial resolution of a mixed peak is observed, the use of oxygen sensitization can also be helpful in providing an indication of nature of that partial separation. Consider, for example, the two chromatograms shown in Fig. 5. The same mixture of the three chloranthracenes as was previously considered is now partially separated into a doublet by use of a capillary column. The question one might then ask is to which portions of this doublet do the individual isomers contribute. This question could be answered by very precise measurement of the retention times of the individual components or by the analysis of several prepared standards containing varied and known amounts of each of the three isomers. Alternatively, this information is provided very clearly and simply by repeating the analysis once with oxygen in the detector. By a comparison of the two chromatograms in Fig. 5, it is evident that the second peak of the doublet is due to the 9-isomer, alone, since it is enhanced by about 21 times by oxygen. The first peak is enhanced by 5.4 times and, therefore, is a composite of approximately equimolar quantities of the 1- and 2-isomers. Since the sample analysed here is the same three-component mixture shown previously in Fig. 1, the above deductions are correct.

It is anticipated that the detection scheme described here could be useful for the analysis of many electron capture-active compounds where the relative amounts of potentially present structural isomers is of interest. In these applications it will be necessary to determine whether the set of isomers under consideration meet certain minimum requirements of the method demonstrated here for the chloroanthracenes. Firstly, the measured response enhancements of the pure isomers must be unique for each isomer. Secondly, the normal and the oxygen-sensitized responses of the ECD must be linearly related to the concentration of each analyte over the concentration range of interest.

In the experiments described here, the precision of measurement was limited mainly by the necessity of measuring the enhancement by performing separate, paired chromatographic analyses, without and then with oxygen in the detector. Since our ability to reproducibly inject samples was estimated to be of the order of a few percent, the reproducibilities of the enhancement measurements reported here are about 5% (relative standard deviations). In order to increase the precision with which the enhancement measurements can be made, we intend to explore the use of two identical ECDs placed in series where the second detector contains added oxygen. Except for the most strongly responding molecules, the ECD can be considered a non-destructive detector. Therefore, with this tandem arrangement, one can expect that each detector will receive essentially the same quantity of analyte and the reproducibility of oxygen-induced enhancement measurements may be improved significantly. This improvement should similarly improve the quantitative accuracy of the analysis method described here.

ACKNOWLEDGEMENT

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GAS CHROMATOGRAPHY OF HOMOLOGOUS ESTERS

XVI*. MONOCHLORO ALIPHATIC ESTERS

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(Received February 23rd, 1982)

SUMMARY

The retention behaviour of methyl, chloromethyl and the isomeric methyl monochloro esters of *n*-carboxylic acids and of the branched-chain C_5 carboxylic acids on Carbowax 20M and SE-30 capillary columns has been examined. The effect on retention of the position of the chlorine substituent and of branching in the acid chain is discussed and the results are compared with those of other studies of aliphatic esters.

INTRODUCTION

Several recent papers have described the retention behaviour of the methyl, methyl 2-chloro and chloromethyl esters of C_2–C_{20} *n*-carboxylic acids[1], the isomeric methyl monochloro esters of C_2–C_{18} *n*-carboxylic acids[2], the isomeric chloromethyl monochloro esters of C_3–C_{12} *n*-carboxylic acids[3] and the methyl, chloromethyl and the corresponding monochloro esters of pivalic, 2-methylbutyric, isovaleric and valeric acids[4]. The data were obtained using Carbowax 20M glass capillary columns[1–3] or a vitreous silica SE-30 wall-coated open tubular column[4]. The studies were conducted using linear temperature programming and with modest programming rates the data for homologues showed an incremental effect with the variation of a structural parameter being readily apparent.

The retention behaviour of aliphatic ester series that have been studied[5–11] where the influence of chain branching[11] and unsaturation[6,10] have been considered. This paper considers the situation where the substituent, *i.e.*, chlorine, possesses an acknowledged acceptor character. This is in complete contrast to the earlier series studied where the secondary interactive group present, either a double bond[6,10], an alkyl group[5,7,9,11] or another carbonyl group[8], is of a donor character and complementary to the effect of the ester carbonyl group.

* Part XV: J. K. Haken and D. Srisukh, *J. Chromatogr.*, 219 (1981) 45.

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EXPERIMENTAL

A Varian Model 2400 instrument with a flame-ionization detector was used. Three columns were employed: (1) a 90 ft. × 0.012 mm I.D. glass capillary column coated with 5% Carbowax 20M; (2) a 50 m × 0.3 mm I.D. glass capillary column coated with 3% Carbowax 20M; and (3) a 25 m × 0.22 mm I.D. vitreous silica SE-30 SCOT column (Scientific Glass Engineering, Melbourne, Australia).

The data in Table I were obtained using the first Carbowax 20M column with isothermal operation at 40°C for 4 min and then temperature programmed from 40 to 235°C at 8°C/min[1]. The data in Tables II and III were obtained using the second Carbowax 20M column with temperature programming from 50 to 190°C at 4°C/min[2–4]. The data were not corrected for dead volume.

RESULTS AND DISCUSSION

The retention times of the methyl, the methyl 2-chloro and the chloromethyl esters of the C_2–C_{20} *n*-aliphatic acids from the work of Korhonen[1] are shown in Table I. Fig. 1 shows plots of the data, three curvilinear plots being observed; data for several of the lower homologues have been omitted as elution was carried out under isothermal conditions rather than with temperature programming. As expected, the retention times of the chlorinated esters are higher than those of the *n*-alkyl esters,

TABLE I

RETENTION TIMES OF METHYL, METHYL 2-CHLORO AND CHLOROMETHYL ESTERS OF C_2–C_{20} *n*-CARBOXYLIC ACIDS

n-Carboxylic acid	*Retention time (min)*		
	Methyl ester	*Methyl 2-chloro ester*	*Chloromethyl ester*
C_2	1.95	5.92	3.93
C_3	2.10	4.68	5.35
C_4	2.39	6.03	6.87
C_5	3.09	7.57	8.85
C_6	4.53	9.40	10.57
C_7	6.61	11.23	12.27
C_8	8.74	12.95	13.83
C_9	10.70	14.51	15.33
C_{10}	12.43	15.95	16.70
C_{11}	14.04	17.32	18.00
C_{12}	15.54	18.63	19.27
C_{13}	16.91	19.86	20.48
C_{14}	18.23	21.10	21.68
C_{15}	19.48	22.26	22.79
C_{16}	20.73	23.31	23.85
C_{17}	21.88	24.40	24.89
C_{18}	22.98	25.45	25.91
C_{19}	24.05	26.44	26.82
C_{20}	25.12	27.41	27.96

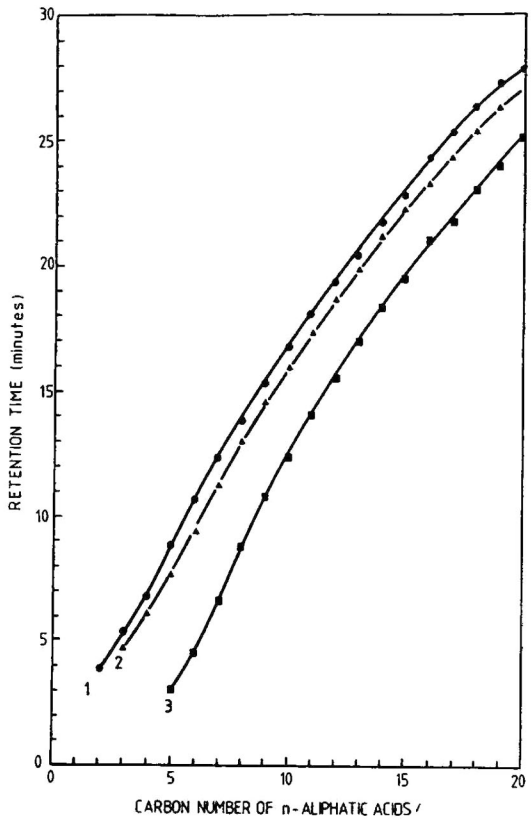


Fig. 1. Plot showing retention of methyl esters (curve 3), methyl 2-chloro esters (curve 2) and chloromethyl esters (curve 1) of C_2–C_{20} *n*-aliphatic acids.

and also the chloromethyl ester series show slight and consistently greater retention times the methyl 2-chloro esters. This effect is as previously observed with alkyl esters, where a methylene group[5,7], a methyl substituent[11] or a double bond[6,10] had a greater effect on retention when in the alcohol rather than in the acid chain. In these series donor effects due to the carbonyl group, unsaturation or methyl groups are significant and greater retention, as observed, is associated with the position of the ether link. With the chlorinated esters the substituent with an acknowledged acceptor character might be expected to reduce the overall polar effect when associated with the alcohol chain; however, this does not occur and the retentions follow the pattern previously observed.

The effect of the position of the chlorine atom in the acid chain may be observed by considering the retention of methyl and chloromethyl esters of isomeric monochloro aliphatic acids (Table II). The isomeric monochloro esters are eluted in order of increasing distance of the substituent groups from the carbonyl group although, as is evident from Fig. 2, a significant increase in retention occurs with the ω-chloro isomers. This is as observed with terminal unsaturation, which has been extensively studied with unsaturated fatty esters[12–17]. However, with the fatty acid esters a double bond adjacent to the carbonyl group, *i.e.*, equivalent here to the 2-chloro

TABLE II

RETENTION TIMES (min) OF METHYL AND CHLOROMETHYL ESTERS OF ALIPHATIC C_2–C_{12} *n*-CARBOXYLIC ACIDS

Chain length	*Methyl ester*	*Isomeric monochloro ester*										
		2-Cl	*3-Cl*	*4-Cl*	*5-Cl*	*6-Cl*	*7-Cl*	*8-Cl*	*9-Cl*	*10-Cl*	*11-Cl*	*12-Cl*
C_2	7.23	9.47										
C_3	7.28	8.81	10.77									
C_4	7.60	10.72	10.33	12.62								
C_5	7.87	11.73	12.20	12.83	15.98							
C_6	8.81	13.74	14.10	15.15	16.47	19.03						
C_7	10.12	15.79	16.70	17.41	18.92	19.53	21.86					
C_8	12.12	18.79	19.63	20.36	21.45	22.30	22.79	25.09				
C_9	14.75	21.54	22.57	23.16	24.04	24.49	25.08	25.52	27.73			
C_{10}	17.75	24.85	25.56	26.27	27.16	27.51	27.69	28.22	28.58	30.71		
C_{11}	20.66	27.47	28.10	28.92	29.54	29.75	29.96	30.16	30.78	31.00	33.06	
C_{12}	23.80	30.46	31.18	31.89	32.61	32.84	32.84	33.08	33.32	33.80	34.03	35.94
	Chloro-methyl ester											
C_3	5.19	9.88	14.40									
C_4	6.50	12.14	13.91	17.15								
C_5	7.50	13.40	15.67	16.59	19.95							
C_6	10.45	16.41	18.19	19.35	20.55	23.07						
C_7	12.35	18.52	20.22	20.95	22.24	22.90	24.98					
C_8	15.77	21.60	23.18	23.80	24.70	25.57	25.81	27.88				
C_9	17.97	23.65	25.11	25.71	26.48	26.95	27.48	27.78	29.66			
C_{10}	21.12	26.53	28.00	28.62	29.29	29.69	29.85	30.34	30.57	32.33		
C_{11}	23.25	28.44	29.90	30.53	31.13	31.46	31.58	31.70	32.17	32.39	34.22	
C_{12}	26.21	31.32	32.54	33.20	33.80	34.16	34.18	34.30	34.43	34.91	35.16	37.28

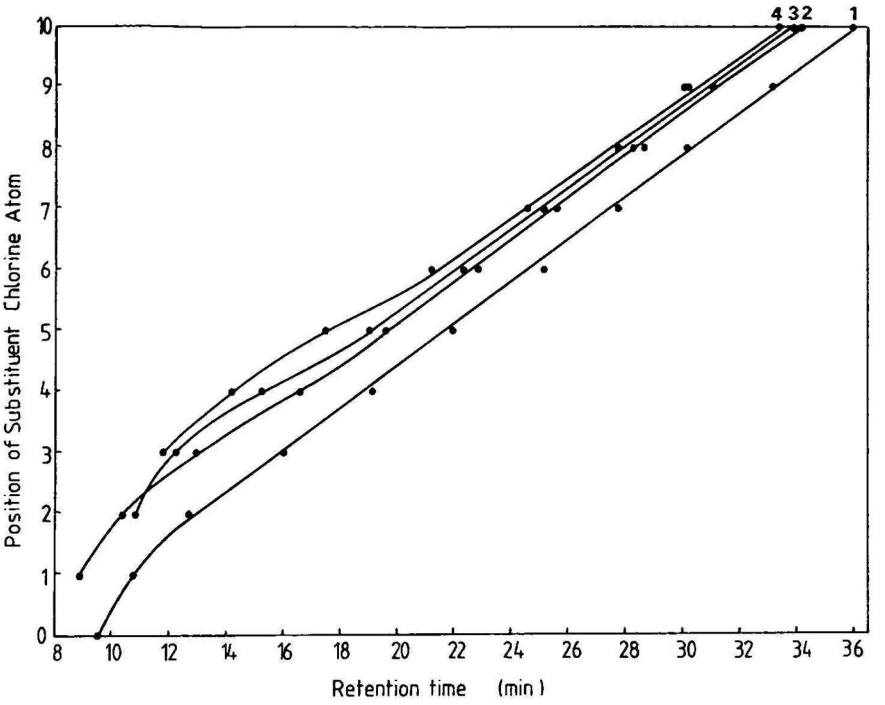


Fig. 2. Plot showing elution sequence of isomeric monochloro esters. 1, $\omega - 3$; 2, $\omega - 2$; 3, $\omega - 1$; 4, ω.

TABLE III

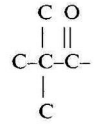
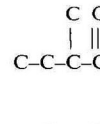
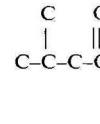
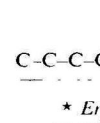
RETENTION TIMES OF METHYL, CHLOROMETHYL AND METHYLMONOCHLORO ESTERS OF ALIPHATIC C_5-CARBOXYLIC ACIDS

*Compound**	*Retention time (min)*	
	Carbowax 20M column	*SE-30 column*
Mi pivalate	4.18	3.55
Cl-Me pivalate	5.73	8.40
Me chloropivalate	6.74	10.45
Me 2-methylbutyrate	4.34	4.70
Me 2-chloro-2-methylbutyrate	6.03	9.69
Me 3-chloro-2-methylbutyrate (e)**	6.91	10.68
Cl-Me 2-methylbutyrate	7.20	11.07
Me 3-chloro-2-methylbutyrate (t)**	7.56	11.60
Me 2-chloromethylbutyrate	8.51	12.72
Me 4-chloro-2-methylbutyrate	9.30	13.68
Me isovalerate	4.36	4.74
Me 2-chloroisovalerate	6.83	10.55
Me 3-chloroisovalerate	6.55	9.49
Cl-Me isovalerate	7.70	11.07
Me 4-chloroisovalerate	10.20	14.22
Me valerate	4.60	5.91
Me 2-chlorovalerate	7.70	12.15
Me 3-chlorovalerate	9.15	13.05
Cl-Me valerate	9.37	13.63
Me 4-chlorovalerate	10.13	14.05
Me 5-chlorovalerate	15.60	18.04

* Methyl ester = Me; chloromethyl ester = Cl-Me

TABLE IV

EFFECT ON RETENTION OF BRANCHING AND OF THE POSITION OF THE CHLORINE SUBSTITUENT IN THE ACID CHAIN

Structure of parent	*Methyl esters*		*Chloromethyl esters*		*2-Chloro (methyl ester)*		*2-Chloromethyl (methyl ester)*		*3-chloro (methyl ester)*		*4-Chloro (methyl ester)*		*5-Chloro (methyl ester)*	
	Polar	*Non-polar*	*Polar*	*Non-polar*	*Polar*	*Non-polar*	*Polar*	*Non-polar*	*Polar*	*Non-polar*	*Polar*	*Non-polar*	*Polar*	*Non-polar*
	4.18	3.15	5.73	1.37			6.74	1.61						
	4.34	4.70	7.20	1.66	6.03	1.39	8.51	1.96	6.91 7.56	1.59* 1.74**	9.30	2.30		
	4.36	4.74	7.70	1.70	6.83	1.57			6.55	1.50	10.20	2.34		
	4.60	5.91	9.27	1.93	7.70	1.67			9.15	1.99	10.13	2.20	15.60	18.04

* *Erythro* form.

** *Threo* form.

isomer, or in close proximity, *i.e.*, equivalent here to the 3-chloro isomer, also produces a significant enhancement of retention owing to donor–donor effects. Such an effect is not observed with the chloro esters, as is evident in Fig. 2, where little separation occurs between the $\omega - 1$ and $\omega - 2$ esters or any of the other series which are partly overlapped with the area shown in the figure. Similarly, no significant reduction in retention of these two isomers would appear to occur due to the close proximity of the acceptor group to the carbonyl group.

The same effect is observed with the chloromethyl monochloro esters where some general increase in retention of all esters with the additional chlorine atom occurs; however, it does not appear that any undue enhancement of retention is experienced because of the additional substituent.

The retention times of chloromethyl and methylmonochloro esters of branched-chain C_5 esters are shown in Table III; in common with other simple esters, little variation of the elution order occurs with variation of the polarity of the stationary phase. The esters are assembled in Table IV according to the structure of the parent acids. Elution of the simple esters followed the established trend, with the most highly branched species, *i.e.*, the pivalate ester, having the lowest retention time. The four chloromethyl esters follow the same trend as the 2-chloromethyl esters which, as previously indicated, have lower retention times than the esters with substituents in the alcohol chain. Table III suggests a variation of this trend, as chloromethyl pivalate has a lower retention time than methyl chloropivalate; however, the structures of the two compounds are not equivalent and when the latter compound is compared (Table IV) with methyl 2-chloromethylbutyrate the expected trend is observed. The 3-chloro esters have greater retention times than the 2-chloro esters, although an anomaly occurs with methyl 3-chloro-2-methylbutyrate, where both the *erythro* and *threo* forms exhibit a higher retention time than expected owing to branching of the acid chain and a lower retention than expected owing to the position of the chlorine substituent. The 4-chloro esters follow the common pattern and have higher retention times than the 3-chloro esters, although the retention of the iso- and normal acids are very similar, while the 5-chlorovalerate ester with a terminal chlorine atom has a substantially higher retention time than any of the other esters.

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COMPUTERIZED ANALYSES OF THE DIFFUSION PROCESSES IN COMPLEXING IONITES*

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SUMMARY

A theoretical and experimental study of the diffusion processes in complex-forming ion exchangers has been made. The theoretical study is based on diffusion-type mass balance equations for the counter ions and co-ion. These equations are solved by using computers. The influence of exchange selectivity and ion mobility on the kinetics of ion exchange in a complexing ionite is discussed. The theoretical deductions have been verified experimentally for exchange in a carboxylic cation exchanger and for exchange in a complex-forming vinylpyridine cation exchanger. The experimental data agree with the theoretical deductions.

INTRODUCTION

Complex-forming ionites are often used for effective chromatographic separations. The kinetic mechanism of ion exchange accompanied by the complexing of a counter ion with a fixed exchange group of an ion exchanger has been considered qualitatively[1]. An approximate solution of this kinetic problem has been derived[2,3] for the case of a "rectangular" isotherm and numerical solutions of the equations have been presented[4–6] for arbitrary-shaped isotherms and certain combinations of individual ion difussion coefficients.

This paper discusses the influence of exchange selectivity (expressed through dissociation constants) and ion mobility on the kinetics of ion exchange in complex-forming ionites.

THEORETICAL

The equation system describing the kinetics of such an ion-exchange process along with the law of mass balance:

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$$\frac{\partial(a_i + C_i)}{\partial t} = \text{div } I_i \; (i = \text{B,A,Y}) \tag{1}$$

the condition for the absence of electric current:

$$\sum_i Z_i I_i = 0 \; (i = \text{A,B,Y}) \tag{2}$$

and the condition for electroneutrality:

$$\sum_i Z_i(a_i + C_i) = a_0 \; (i = \text{B,A,Y}) \tag{3}$$

includes an equation for the equilibrium between the bound (a_i) and free (C_i) ions in the ionite. In the ideal case, when the dissociation constants are independent of the degree of ion exchange, the equilibrium condition for the complexing reaction of singly charged ions can be written as[7]

$$K_{Ri} = \frac{C_i\,(a_0 - a_B - a_A)}{a_i} \; (i = \text{B,A}) \tag{4}$$

where C_i are the concentrations of the free A^+ and B^+ ions, C_Y is the concentration of the co-ion Y^-, a_i are the concentrations of B^+ and A^+ ions associated with fixed groups R ($a_Y = 0$), a_0 is the concentration of the fixed exchange groups, Z_i are the charges, I_i are the ion fluxes and K_{Ri} are the dissociation constants of complexes RB and RA.

With the substitution the Nernst–Planck relations for ion fluxes:

$$I_i = D_i\left(\text{grad } C_i + Z_i\, C_i \cdot \frac{F}{RT} \cdot \text{grad } \phi\right) (i = \text{B,A,Y}) \tag{5}$$

in eqn. 2, one obtains

$$\frac{F}{RT} \cdot \text{grad } \phi = \sum_i Z_i D_i \text{ grad } C_i / \left(\sum_i Z^2{}_i\, D_i\, C_i\right) (i = \text{B,A,Y}) \tag{6}$$

where D_i are the individual diffusion coefficients, F is the Faraday constant, R is the gas constant, T is the absolute temperature and ϕ is the electric potential.

From eqns. 5 and 6 we obtain equations for ion fluxes that contain concentration gradients only. One can easily obtain the relationship between grad C_i and grad a_i by differentiation of eqns. 3 and 4:

$$\sum_i Z_i \text{ grad } C_i = -\text{grad } a_B - \text{grad } a_A \; (i = \text{B,A,Y})$$

$$\text{grad } a_i = \text{grad}\left(\frac{a_0\, K_{Ri}\, C_i}{K_{RA}K_{RB} + K_{RB}\, C_A + K_{RA}\, C_B}\right) \tag{7}$$

Substitution of eqns. 5 and 6 in the mass balance eqn. 1 yields a system of two diffusion-type equations:

$$\frac{\partial(a_i + C_i)}{\partial t} = \frac{1}{r^2} \cdot \frac{\partial}{\partial r}\left[r^2\left(D_{iA} \cdot \frac{\partial C_A}{\partial r} + D_{iB} \cdot \frac{\partial C_B}{\partial r}\right)\right] (i = B,A)$$

$$D_{ij} = \frac{D_i}{D_0}\left\{\delta_{ij} + \frac{C_i\left[D_Y\left(1 + \frac{a_0 f^2}{K_{Rj}}\right) - D_j\right]}{C_A(D_A + D_Y) + C_B(D_B + D_Y) + D_Y a_0 f}\right\} \tag{8}$$

where

$$f = \frac{K_{RA}\, K_{RB}}{K_{RA}K_{RB} + K_{RA}C_B + K_{RB}C_A} \quad \left(\delta_{ij} = \begin{cases} 1; & i = j \\ 0; & i \neq j \end{cases}\right)$$

where r and t are the radial space coordinate and time, respectively, D_0 is the unit used for measuring diffusion coefficients and D_{ij} are the effective diffusion coefficients. Concentration C_Y is eliminated from eqn. 8 by substituting eqns. 3 and 7.

The equation system 2 in the case of "rectangular" isotherm can be solved approximately by the method of integral relations. Under the physically justified assumption of constancy of the co-ion Y^- concentration at the B^+/A^+ moving boundary in an ion-exchanger particle, which has been supported experimentally[8], the following equations can be obtained for a fractional conversion $F \leqslant 0.6$[2,3]:

$$F(t) = 3\sqrt{2D_{\text{eff}} \cdot \frac{c_0\, t}{a_0 r_0^2}}$$

where

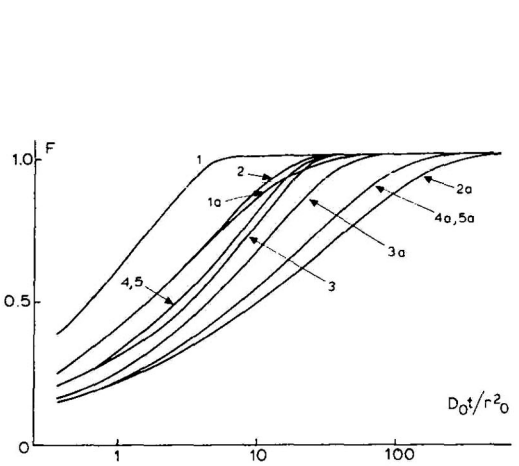
$$D_{\text{eff}} = \frac{(Z_B - Z_Y)\, D_A D_B}{Z_B D_B - Z_Y D_A} \tag{9}$$

where D_{eff} is the effective diffusion coefficient, C_0 is the solution concentration at the particle surface, r_0 is the particle radius and Z_B and Z_Y are the charges of B^+ and Y^- ions, respectively.

The system of equations 1–3 for the exchange of singly charged ions at the initial and boundary conditions corresponding to the exchange between the ionite and the continually renewed solution, when initially ionite particles are filled with A^+ ions, is calculated by using a computer.

Figs. 1–3 show the results of the numerical solution for different combinations of the individual diffusion coefficients D_B, D_A and D_Y and dissociation constants K_{RB} and K_{RA} which are given in Table I.

The values as used for the solution are $a_0 = 2.5$ mmol/cm^3 and $C_0 = 0.1$ mmol/cm^3. With a convex isotherm ($K_{RA}/K_{RB} = 5$) the exchange proceeds faster than with a concave isotherm ($K_{RA}/K_{RB} = 0.2$) (see Fig. 1).



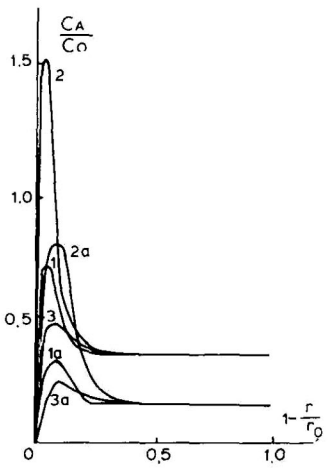
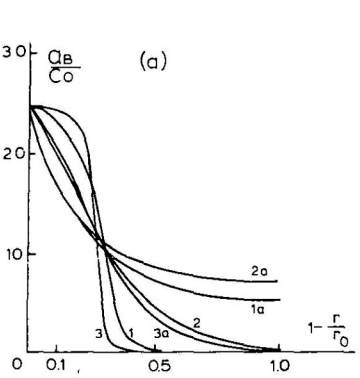


Fig. 1. Dependence of fractional conversion F on D_0t/r_0^2. Curves numbered as in Table I.

Fig. 2. Distribution of concentration C_A at $F = 0.1$. Curves numbered as in Table I.



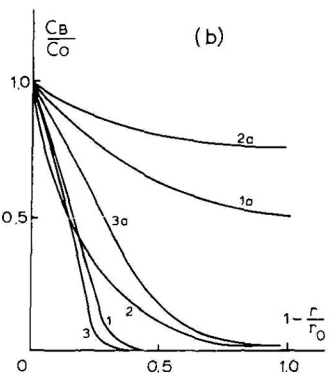


Fig. 3. (*a*) Distribution of concentration a_B at $F = 0.5$. Curves numbered as in Table I. (b) Distribution of concentration C_B at $F = 0.5$ Curves numbered as in Table I.

TABLE I

CONDITIONS FOR NUMERICAL SOLUTION

*No.**	D_B/D_0	D_A/D_0	D_Y/D_0	K_{RB}/C_0	K_{RA}/C_0
1	1.0	1.0	0.1	1.10^{-3}	$5 \cdot 10^{-3}$
1a	1.0	1.0	0.1	$5 \cdot 10^{-3}$	$1 \cdot 10^{-3}$
2	1.0	0.1	0.1	$1 \cdot 10^{-3}$	$5 \cdot 10^{-3}$
2a	1.0	0.1	0.1	$5 \cdot 10^{-3}$	$1 \cdot 10^{-3}$
3	0.1	1.0	0.1	$1 \cdot 10^{-3}$	$5 \cdot 10^{-3}$
3a	0.1	1.0	0.1	$5 \cdot 10^{-3}$	$1 \cdot 10^{-3}$
4	0.2	0.2	0.1	$1 \cdot 10^{-3}$	$5 \cdot 10^{-3}$
4a	0.2	0.2	0.1	$5 \cdot 10^{-3}$	$1 \cdot 10^{-3}$
5	0.2	0.2	1.0	$1 \cdot 10^{-3}$	$5 \cdot 10^{-3}$
5a	0.2	0.2	1.0	$5 \cdot 10^{-3}$	$1 \cdot 10^{-3}$

* Curves in Figures.

The comparison of the $F(\tau)$ curves in Fig. 1 reveals that the variation of the exchange rate as D_B varies is considerably larger with the convex than with the corresponding concave isotherm.

In contrast, the value of D_A is more important with the concave isotherm. Therefore, the exchange rate depends greatly on the diffusion coefficient of the ion absorbed selectively. The ion-exchange rate does not depend on D_Y. Finally, the dependences $F(\tau)$ for the convex isotherm and $D_A \geqslant D_B$ are described by an equation of an in-sphere diffusion with a constant coefficient; in other cases this dependence features a formal correspondence to a model whose effective diffusion coefficient varies in the course of exchange. The distribution curves (a_i, C_i; Figs. 2, 3a and 3b) characterize distinctly the features of the mass-transfer mechanism.

The results, shown in Fig. 3 (curves 1 and 3), demonstrate that with the convex isotherm and $D_A \geqslant D_B$ the ion-exchange process is accompanied by the formation of the welldefined boundary of the exchanging ions, B^+/A^+. With a ratio $D_A/D_B = 0.1$ the low mobility of the A^+ ions leads to the accumulation of A^+ in the particle. Under these conditions, the concentration of C_A can strongly affect the front width of a_B (Figs. 2 and 3, curve 2). Further, the data in Fig. 4 indicate agreement between the results of the numerical solution and the approximate analytical solution (eqn. 9) for the exchange process accompanied by the formation of the well defined boundary B^+/A^+, when $D_A \geqslant D_B$.

When $D_A < D_B$ the kinetic dependence is not described by eqn. 9 because the assumption of the constancy of the concentration of the ions Y^- and A^+ at the B^+/A^+ boundary is not verified, which can be seen from Fig. 5.

The validity of the approximate rate expression was verified by using eqn. 9 to calculate the effective interdiffusion coefficients for the experimental conditions corresponding to $D_A \geqslant D_B$ from kinetic curves of the exchange RNa–Ni, RNa–Zn, RNa–Sr, RZn–Ni, RSr–Ni, in vinylpyridinecarboxylic (VPC) ampholyte[2,3,5].

The results presented in Fig. 6 were obtained for experimental conditions corresponding to $D_A \ll D_B$ (RSr–H^+) and $D_A > D_B$ (RNa–Sr^{2+}).

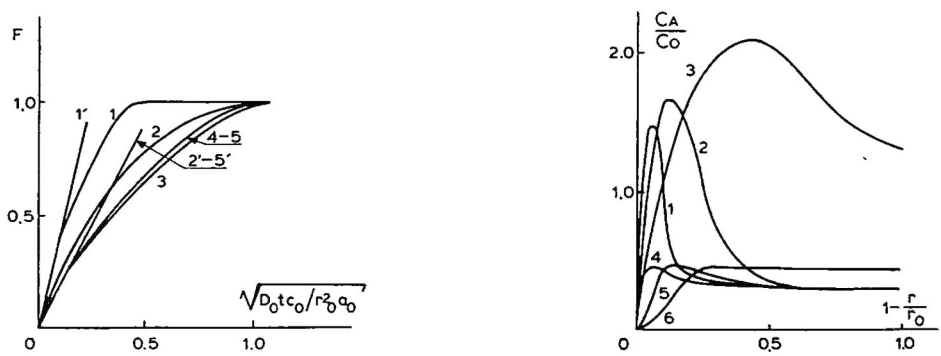


Fig. 4. Dependence of fractional conversion F on $\sqrt{D_0 t C_0 / r_0^2 a_0}$ for a convex isotherm. Curves are plotted on the basis of the numerical (curves 1–5) and approximate analytical (curves 1′–5′) solutions.

Fig. 5. Distribution of concentration C_A in the case for a convex isotherm for $D_B/D_A = 10$ (curves 1–3) and $D_B/D_A = 0.1$ (curves 4–6) at $F = 0.1$ (1), $F = 0.19$ (2), $F = 0.43$ (3), $F = 0.08$ (4), $F = 0.19$ (5) and $F = 0.38$ (6).

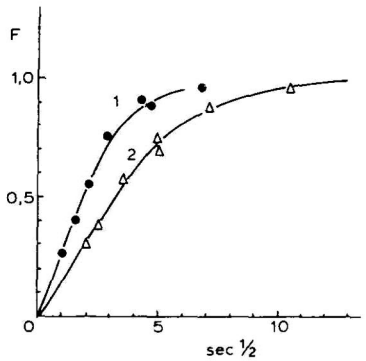


Fig. 6. Ion-exchange kinetic curves in ampholyte VPC: 1 = RSr–H^+; 2 = RNa–Sr^{2+}. T = 298°K; r^0 = 0.005 cm; $C_{0(H+)}$ = 0.05 mmol/cm^3; $C_{0(Sr)}$ = 0.025 mmol/cm^3.

The exchange rate was measured in a thin ionite layer[2,3]. If, as in the exchange RSr–H, $D_A \ll D_B$, $Z_B = 1$, $Z_Y = -1$, then the effective interdiffusion coefficient is defined from eqn. 9 as

$$D_{\text{eff (Sr–H)}} = \frac{(Z_H - Z_{NO_3}) D_{Sr} D_H}{Z_H D_H - Z_{NO_3} D_{Sr}} = 2 D_{Sr} \tag{10}$$

Further, it was generally assumed that $D_H > D_{Na} > D_{Sr}$. Thus:

$$2 D_{Sr} > D_{\text{eff}}(\text{Sr–Na}) > D_{Sr} \tag{11}$$

The ratio between the effective interdiffusion coefficients, calculated from eqn. 9 on the basis of the experimental kinetic dependences 1 and 2 (Fig. 6), is equal to

$$\frac{D_{\text{eff}}(\text{Sr–H})}{D_{\text{eff}}(\text{Sr–Na})} = 3 \tag{12}$$

The evident disagreement between eqn. 12 and the conditions 10 and 11 is the experimental manifestation of the above-found boundaries of the application of eqn. 9.

The effect of the ratio between the individual diffusion coefficients and of the exchange selectivity on the kinetic dependences in real systems has been studied for ion exchange in carboxylic (KB-4) and vinylpyridinecarboxylic (VPC) ionites[2,3,5,6,8].

The theory has been developed for singly charged ions. However, in the complexing ionites the ion exchange of multiply charged ions mostly takes place. Therefore, it was of interest to consider, for example, the ion-exchange kinetics of Ni^{2+} and Zn^{2+} ions within ampholyte VPC. We have assumed that the results for multiply charged and singly charged ions are comparable as their exchange isotherms, having different analytical expressions, can be of a similar type.

Computer simulation of the kinetics for this system was carried out with the ratio $K_A/K_B = 12$ in the case of a convex isotherm (RZn–Ni^{2+}) and $K_A/K_B = 1/12$ in the case of a concave isotherm (RNi–Zn^{2+}).

The value of K_A/K_B is identical with the relationship of the distribution coef-

ficients of Ni^{2+} and Zn^{2+} ions between the solution and the ionite under the conditions $K_{A,B} \ll 1$ and $C_0 \ll a_0$. This relationship was obtained from the experimental isotherm of the Ni^{2+}–Zn^{2+} exchange[9]. The values of K_A and K_B can be chosen arbitrarily, because their effect (when $K_{A,B} \ll 1$) on the numerical results is remarkably small.

As shown earlier[5], the individual diffusion coefficients can be characterized by the experimental kinetic curves for the systems RNa^+–Ni^{2+} and RNa^+–Zn^{2+}: $D_{Ni} \approx D_{Zn} \approx 2 \cdot 10^{-7}$ cm²/sec. The modelling of the spherical particle for the ion distribution study has been described previously[10]. The ion distribution curves calculated for the exchange kinetics of Ni^{2+} and Zn^{2+} ions are similar to the experimental ones (Figs. 7 and 8).

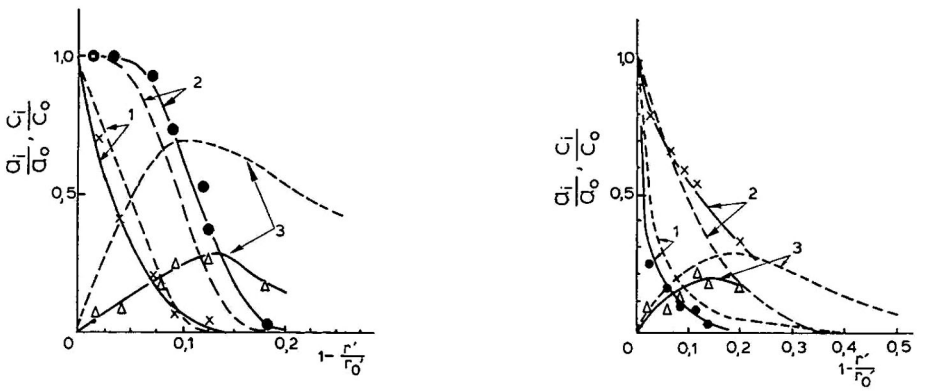


Fig. 7. Distribution of $a_{Ni}(2)$, $C_{Ni}(1)$ and $C_{Zn}(3)$ concentrations in the spherical particle sector model (r'_0 is the spherical radius) at the convex isotherm of the displacement of Zn^{2+} ions by Ni^{2+} ions from the vinylpyridine ampholyte VPC. $T = 298°K$; $C_{0(Ni)} = 0.25$ mmol/cm³; time of contact of the solution with the ampholyte, 66 h; $F_{exp} \approx 0.25$; $F_{calc.} = 0.247$. Solid lines, experimental; broken lines, calculated.

Fig. 8. Distribution of $a_{Zn}(1)$, $C_{Zn}(2)$ and $C_{Ni}(3)$ concentrations at the concave isotherm RNi–Zn. $C_{0(Zn)} = 0.25$ mmol/cm³; time of contact, 264 h; $F_{exp.} = 0.18$; $F_{calc.} = 0.176$. Solid lines, experimental; broken lines, calculated.

Some disagreement between the theoretical and experimental results can be explained mainly by the variability of the activity coefficients of ions and the dissociation constants. However, it is difficult and sometimes inadvisable to take account of these factors, because the disagreements in the distribution curves lead to only small differences in the exchange rates. For example, satisfactory agreement between the experimental and calculated values was observed on comparison of the rates of the RZn–Ni^{2+} and RNi–Zn^{2+} exchanges. The rate of exchange can be characterized by the value $\tau_{0.5}$, which is the time corresponding to a fractional conversion $F = 0.5$. The $\frac{(\tau_{0.5})\ \text{concave}}{(\tau_{0.5})\ \text{convex}}$ ratios are 7.8 and 6.5 for the experimental and calculated values, respectively.

Hence the results of computer simulation can be used for the analyses of ion-exchange kinetics accompanied by complex formation in real systems.

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CHARACTERIZATION OF POROUS POLYMER BEADS GAS CHROMATOGRAPHIC COLUMNS BY RETENTION INDEX VALUES OF ETHYLENE, ACETYLENE AND CARBON DIOXIDE

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SUMMARY

Porapak N, P, Q, R, S and T and Chromosorb 101, 102, 103, 104, 105, 106, 107 and 108 porous polymer beads (PPBs) were characterized and listed in order of increasing "polarity" by determination of the retention index values of ethylene, acetylene and carbon dioxide with respect of the first terms of the alkane series. The values of $\Delta I_Q(C_2H_2)$ (*i.e.* the difference in retention index of acetylene between any given PPB and the Porapak Q, taken as "less polar" reference term) increase in the following order: Porapak Q < Chromosorb 106 and 102 < Porapak P < Chromosorb 101 < Porapak S < Chromosorb 103 and 105 < Porapak K and N < Chromosorb 107 and 108 < Porapak T < Chromosorb 104 thus permitting a quantitative classification of the behaviour of PPB columns for the separation of gas mixtures. Values of $\Delta I_Q(C_2H_4)$ and $\Delta I_Q(CO_2)$ show similar trends, with small variations due to the chemical composition and structure of the beads.

INTRODUCTION

The gas chromatographic (GC) applications of porous polymer beads (PPBs) as synthetic sorbents are numerous and cover a wide variety of compounds having different polarities. The analysis of gas mixtures takes great advantages from the use of these stationary phases, which widely replace the "classical" packings such as silica gel and alumina and which, by the use of long columns or low analysis temperatures, permit the separation of oxygen from nitrogen and argon, previously restricted to molecular sieves. The possibility of fast temperature programming with a minimum of baseline drift due to column bleeding, allows the analysis of complex mixtures of gases to be accomplished in short times. The wide choice offered to the GC user by the numerous types of PPBs available from different producers is very useful because a column can be tailored for a given separation problem in order to perform the best resolution in the shortest time. However the wideness of the choice may be confusing as a general classification of the polarity of the various PPB has not yet been achieved.

In previous papers[1,2] the characterization of Porapak and Chromosorb "Century series" PPBs has been made by analysis of gaseous mixtures, and the values of

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ΔH^0 for the various compounds (CH_4, C_2H_2, C_2H_4, C_2H_6, CO_2) were calculated. The use of the $\alpha_{C_2H_4}$ values (retention time relative to ethylene), whose measurement is simple with respect of the determination of the thermodynamic functions, permitted the evaluation of the batch-to-batch reproducibility of the column behaviour, a semi-quantitative classification of the "polarity" of the various PPBs and a rough comparison between Porapak and Chromosorb sorbents. The use of a value relative to a unique reference substance (standard) is justified by its simplicity and by the fact that the retention time of ethylene depends less on temperature than those of other test substances (probes).

On the other hand, the validity of a unique reference standard decreases when the difference in retention times between the probes increases, and it must be taken into account that the change in $\alpha_{C_2H_4}$ values can be due either to a real change in the retention of the probes or to a change in the retention of the standard[3]. The use of a homologous series of standard substances, *e.g.* linear alkanes as suggested by Kováts[4], does not solve the latter problem, but gives a multiple reference for the determination of retention index values (I) instead of retention relative to a unique standard ($\alpha_{C_2H_4}$). The determination of the I values can also permit calculation of the ΔI value with respect to a stationary phase taken as the "non-polar" reference and can give therefore a measure of the "polarity" the various PPBs.

In order to obtain a common basis for the classification of the PPBs, the retention index values of CO_2, C_2H_4 and C_2H_2 with respect to the first members of the alkane homologous series were used, by employing a method similar to those suggested by Rohrschneider[5] and McReynolds[6] for the determination of the polarity of liquid stationary phases. The reference non-polar sorbent used was Porapak Q, because the retention index and α values for this phase were the lowest for all the probes and sorbents tested. The classical non-polar reference term of Rohrschneider's and McReynold's method, squalane, obviously cannot be used as it does not separate the light hydrocarbons used and, in any case, its gas–liquid chromatographic interaction mechanism differs from those of porous sorbents, for which at low temperatures adsorption phenomena predominate over solution mechanisms.

EXPERIMENTAL

Stainless-steel columns (3 m × 2.4 mm I.D.) were packed with known amounts of Porapak or Chromosorb "Century series" PPBs, 80–100 mesh. The types of PPBs tested are listed in Table I, with the chemical composition, surface area and pore sizes, when available[7–9].

A thermal conductivity detector (Varian 3760) was used, with helium as the carrier gas (flow-rate, 22 ml min^{-1}). The column temperature was set at temperatures ranging between 30 ± 0.1 and 70 ± 0.2°C and monitored by digital and precision mercury thermometers. Samples of gas mixtures were prepared in glass syringes and injected by using a gas-sampling valve directly connected to the column inlet, in order to reduce the dead volume. Dilution of the sample with helium split from the carrier gas line permitted the introduction of very small amounts of each test compound, in order to give small and symmetrical peaks. Fig. 1 shows the layout of the sampling system. Retention times and peak areas were measured with a Varian CDS-111 C data system. The dead time was taken as being equal to the retention time of hydro-

gen, corresponding to that of helium within the accuracy limit of the data system (± 0.01 min)[10].

RESULTS AND DISCUSSION

Figs. 2 and 3 show the behaviour of the I values on the various PPB columns over the temperature range 30–70°C. The dependence of I values on temperature was found to be linear, as a consequence of the linear dependence of ln V_N values* on temperature[1,2]. Table II shows the slope h and the intercept k of the equation

$$I = h \cdot \frac{1}{T} + k \tag{1}$$

from which the values of I at any temperature can be deduced. The intersection points, *i.e.* the temperatures at which two given compounds have the same retention index, can be calculated by solving a system of equations of the same type as eqn. 1. Of course, depending on the efficiency of the column, *i.e.* on the peak width at a given retention time (t'_R), satisfactory resolution can only be achieved when the retention index values of two compounds differ more than 5–10 index units.

The I values of the probes fall within 150 and 200, except for C_2H_2 on Porapak N, R and T and Chromosorb 104, 107 and 108 and for CO_2 on Chromosorb 104. While C_2H_2 on Porapak R and CO_2 on Chromosorb 104 are eluted so close to C_2H_6 that extrapolation of the retention index formula used (eqn. 2) gives suitable values, with the results having a deviation smaller than one index unit, the I values for C_2H_2 on Porapak N and T and on Chromosorb 104, 107 and 108 may be correctly calcu-

TABLE I

CHEMICAL COMPOSITION, SURFACE AREA AND PORE SIZE OF THE TESTED PPBS (FROM REFS. 7–9 AND FROM MANUFACTURERS' CATALOGUES)

Sty = Styrene; DVB = divinylbenzene; EVB = ethylvinylbenzene; EGDMA = ethylene glycol dimethacrylate; ACN = acrylonitrile.

PPB	*Composition*	*Surface area (m^2/g)*	*Average pore diameter (Å)*
Porapak N	Vinylpyrrolidone	225–350	–
Porapak P	Sty-DVB	100–200	–
Porapak Q	EVB-DVB	500–600	75
Porapak R	Vinylpyrrolidone	450–600	76
Porapak S	Vinylpyridine	300–450	76
Porapak T	EGDMA	250–350	91
Chromosorb 101	Sty-DVB	< 50	3500
Chromosorb 102	Sty-DVB	300–400	85
Chromosorb 103	Polystyrene	15–25	3500
Chromosorb 104	ACN-DVB	100–200	700
Chromosorb 105	Acrylic ester	600–700	500
Chromosorb 106	Polystyrene	700–800	50
Chromosorb 107	Acrylic ester	400–500	80
Chromosorb 108	Crosslinked acrylic	100–200	250

* V_N is the net retention volume calculated by multiplying the pressure gradient correction factor of James and Martin by the adjusted retention volume V'_R.

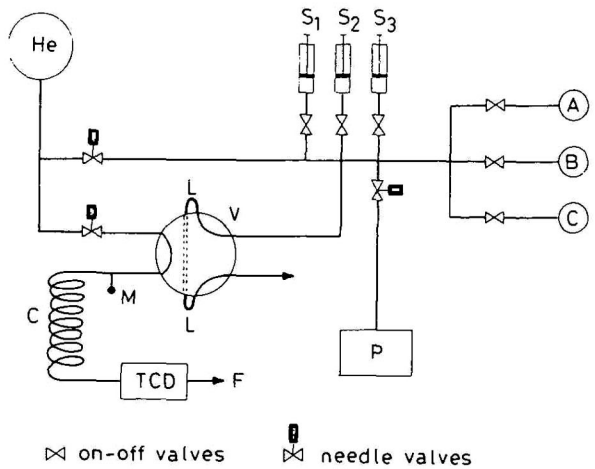

Fig. 1. Schematic diagram of the manifold used for preparation and injection of samples. He = carrier gas; V = rotary-type gas-sampling valve; LL = calibrated loop; M = connection point of the measure of inlet pressure; C = column; TCD = detector; F = carrier gas outlet to flow meters; A, B, C = reservoirs of standard gases; S_1, S_2, S_3 = gas-tight and calibrated glass syringes for preparation and dilution of standard mixtures; P = vacuum pump. Thermostatting ovens and electronic modules are not shown.

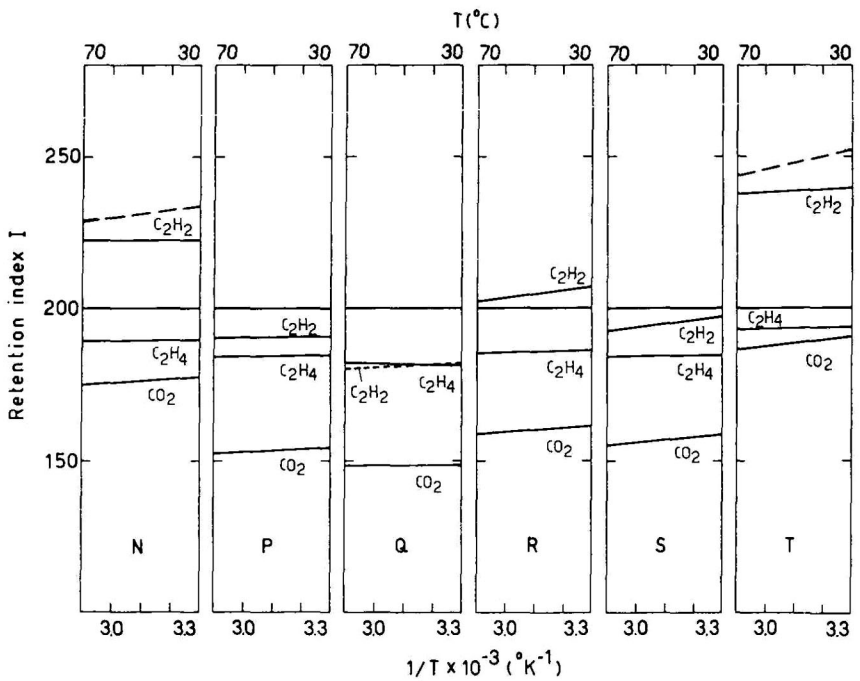

Fig. 2. Retention index values (interpolated between CH_4 and C_2H_6) of CO_2, C_2H_4 and C_2H_2 as a function of temperature on various Porapak PPBs (80–100 mesh). Column 3 m × 1/8 in. I.D.; helium flow-rate, 22 ml min^{-1}; dashed lines, *I* values of C_2H_2 interpolated between C_2H_6 and C_3H_8.

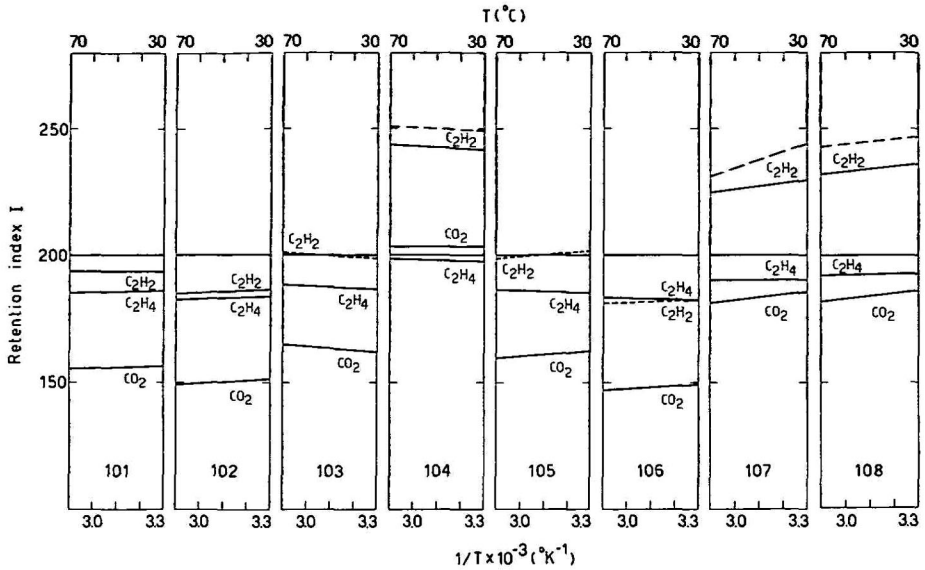

Fig. 3. Retention index values of CO_2, C_2H_4 and C_2H_2 as a function of temperature on various Chromosorb "century series" PPBs. Conditions and symbols as in Fig. 2.

lated by using C_2H_6 and C_3H_8 as standard compounds. The linearity of the plot of ln t'_R as a function of the number of C atoms in a homologous series of compounds often shows deviations when the lowest members on the series are considered; this phenomenon was also previously observed for *n*-alcohols of PPB columns[7]. The deviation

TABLE II

VALUES OF THE SLOPE (h) AND INTERCEPT (k) OF THE EQUATION $I = h \cdot 1/T + k$, FOR CO_2, C_2H_4 AND C_2H_2, ON VARIOUS PORAPAK AND CHROMOSORB COLUMNS, IN THE RANGE 30–70°C

Carrier gas, helium; flow-rate, 22 ml min^{-1}.

Column	CO_2		C_2H_4		C_2H_2	
	h	k	h	k	h	k
Porapak N	7.7	152.6	0.047	189.7	10.9	187.7
Porapak P	7.2	130.7	4.3	171.0	2.9	181.7
Porapak Q	0.1	148.4	−5.5	199.2	4.3	168.2
Porapak R	8.8	132.2	0.0	186.1	10.9	171.0
Porapak S	9.0	128.8	3.4	173.7	9.8	165.3
Porapak T	12.3	150.5	0.0	193.3	0.0	238.5
Chromosorb 101	4.4	141.9	2.2	178.8	1.4	189.3
Chromosorb 102	0.0	150.4	1.8	177.7	−2.8	194.9
Chromosorb 103	−8.6	190.4	−4.2	200.5	−4.4	213.5
Chromosorb 104	−0.08	203.8	−2.2	204.9	−4.7	257.3
Chromosorb 105	7.9	136.3	−3.5	196.8	7.9	175.7
Chromosorb 106	0.02	147.6	−1.7	188.3	4.9	166.6
Chromosorb 107	0.16	181.5	0.13	189.6	0.13	185.5
Chromosorb 108	11.05	149.4	9.12	196.3	−9.37	205.6

from linearity of the ln t'_R values of CH_4, C_2H_6 and C_3H_8 is very small over the temperature range considered (Fig. 4 shows the dependence on temperature and on number of carbon atoms for Porapak N and T and Chromosorb 104, 107 and 108) but large enough to cause appreciable differences in the I values for C_2H_2, depending on the reference compounds used as standards.

$$I(x) = \frac{\ln t'_R(x) - \ln t'_R(CH_4)}{\ln t'_R(C_2H_6) - \ln t'_R(CH_4)} \cdot 100 + 100 \qquad (2)$$

The dashed lines in Figs. 2 and 3 show the I values of C_2H_2 calculated by interpolation between C_2H_6 and C_3H_8.

A correct application of the Kovats' retention index concept requires the interpolation between two members of the alkane series, and therefore the $I(C_2H_2)$ values calculated by using C_2H_6 and C_3H_8 should be choosen for Porapak N and T and for Chromosorb 104, 107 and 108, but, owing the long retention time of C_3H_8, only reference to CH_4 and C_2H_6 is rapid and precise enough for practical purposes.

From the I values, values of ΔI_Q (the difference between the I value of a particular PPB and that of Porapak Q) were calculated. These are shown (at 30 and 70°C) in Table III, in order of increasing $\Delta I_Q(C_2H_2)$, and correspond reasonally well with the empirical order of polarity given by the manufacturers and found by several authors for the two series of PPBs.

Except for Porapak R and Chromosorb 103, 105, 107 and 108, which have very similar values, $\Delta I_Q(CO_2)$ follows the same order as $\Delta I_Q(C_2H_2)$ while $\Delta I_Q(C_2H_4)$ shows fluctuations at lower values and is generally smaller than the ΔI_Q value of other probes. This is in accord with the small influence that changing the analytical parame-

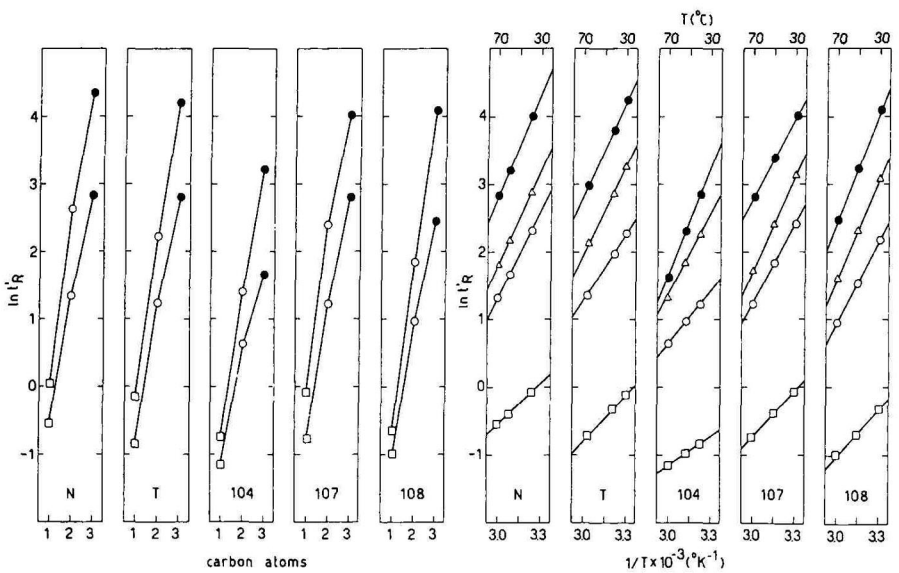

Fig. 4. Linearity of ln t'_R (min) values as a function of the number of carbon atoms for light paraffins (left) and as a function of temperature for light paraffins and acetylene (right). Conditions as in Fig. 2. Plots: □ = methane; ○ = ethane; ● = propane; △ = acetylene.

TABLE III

ΔI_Q VALUES WITH RESPECT TO PORAPAK Q (REFERENCE "LESS POLAR" COLUMN) FOR C_2H_2, C_2H_4 AND CO_2 AT 30 AND 70°C

All I values calculated by reference to CH_4 and C_2H_6 retention times. In parentheses are the $\Delta I_Q(C_2H_2)$values referred to C_2H_6 and C_3H_8.

Porapak or Chromosorb type	C_2H_2		C_2H_4		CO_2	
	30°C	*70°C*	*30°C*	*70°C*	*30°C*	*70°C*
Q	0	0	0	0		0
106	0.46	0.44	1.77	0.92	0.04	0.02
102	4.52	4.80	2.83	0.33	2.51	0.31
P	8.62	9.77	4.01	1.81	5.72	3.56
101	11.3	13.2	5.08	2.77	8.07	6.19
S	14.9	12.4	3.70	1.93	9.99	6.12
103	16.5	20.8	5.56	6.04	13.4	16.1
105	19.4	18.4	4.53	3.90	13.9	10.6
R	24.8	22.2	5.37	3.04	12.8	9.4
N	40.0	42.1	8.74	7.18	29.3	26.4
	(51.6)	(48.5)				
107	47.0	43.5	8.33	7.52	37.6	32.6
	(57.1)	(49.3)				
108	51.5	51.7	10.6	9.44	36.8	32.7
	(63.3)	(61.8)				
T	57.4	57.1	12.5	10.8	42.2	38.1
	(69.6)	(62.5)				
104	59.3	63.0	16.9	15.9	55.0	54.7
	(66.6)	(70.5)				

ters has on the retention behaviour of C_2H_4, as has been previously observed, and justifys the choice of this hydrocarbon as reference compound for the calculation of the $\alpha_{C_2H_4}$ values[1,2,10].

Fig. 5 shows the $\Delta I(C_2H_2)$ and $\Delta I_Q(C_2H_4)$ values plotted at 30°C as a function of $\Delta I_Q(CO_2)$.

Linear correlation were also found by plotting $\Delta I_Q(C_2H_2)$ as a function of $\Delta I_Q(C_2H_4)$. Table IV shows the intercept a and the angular coefficient b of eqns. 3–5

$$\Delta I_Q(C_2H_2) = a_1 + b_1 \cdot \Delta I_Q(CO_2) \quad (3)$$

$$\Delta I_Q(C_2H_4) = a_2 + b_2 \cdot \Delta I_Q(CO_2) \quad (4)$$

$$\Delta I_Q(C_2H_2) = a_3 + b_3 \cdot \Delta I_Q(C_2H_4) \quad (5)$$

at 30 and 70°C, and the corresponding values of the correlation coefficient r, calculated by using the ΔI_Q values of the Porapak, the Chromosorb and all the PPBs together. Values in parentheses are obtained by using in the least-squares calculation the $I(C_2H_2)$ values interpolated between C_2H_6 and C_3H_8. Notwithstanding the small number of experimental points, the linearity of the plots, as shown by the values of the correlation coefficient r, is good and does not change appreciably with temperature. The values of r calculated when the ΔI_Q values obtained from both types of PPB

TABLE IV

VALUES OF INTERCEPT (a), SLOPE (b) AND CORRELATION COEFFICIENT (r) OF EQNS. 3–5

In parentheses are values calculated with ΔI_Q values interpolated between C_2H_6 and C_3H_8.

Parameter	*Porapak*		*Chromosorb*		*Porapak + Chromosorb*	
	30°C	*70°C*	*30°C*	*70°C*	*30°C*	*70°C*
a_1	3.56 (0.22)	6.04 (5.07)	1.23 (1.41)	4.66 (4.04)	2.72 (1.54)	4.88 (4.20)
b_1	1.28 (1.68)	1.36 (1.55)	1.17 (1.36)	1.16 (1.35)	1.19 (1.45)	1.23 (1.42)
r_1	0.99 (0.996)	0.995 (0.995)	0.98 (0.97)	0.98 (0.97)	0.98 (0.97)	0.98 (0.97)
a_2	2.05	0.54	1.93	0.82	1.66	0.60
b_2	0.24	0.26	0.24	0.26	0.25	0.27
r_2	0.99	0.997	0.96	0.98	0.97	0.99
a_3	−6.71 (−13.15)	3.38 (2.09)	−3.69 (−5.30)	2.01 (1.10)	−3.06 (−4.90)	2.29 (1.93)
b_3	5.23 (6.86)	5.11 (5.84)	4.30 (5.06)	4.27 (4.92)	4.43 (5.32)	0.99
r_3	0.98 (0.99)	0.990 (0.990)	0.93 (0.90)	0.96 (0.94)	0.94 (0.92)	0.96 (0.95)

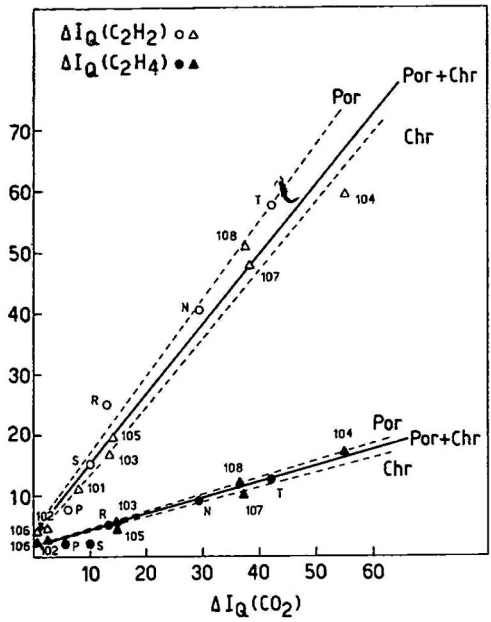

Fig. 5. $\Delta I_Q(C_2H_2)$ and $\Delta I_Q(C_2H_4)$ values at 30°C plotted as a function of the $\Delta I_Q(CO_2)$ values. Straight lines are calculated by the method of least squares [eqns. (3) and (4)]. Dashed lines, calculated from ΔI_Q values obtained on Porapak (○,●) and Chromosorb (△,▲); full lines, calculated from ΔI_Q values obtained on both PPBs.

are fitted together are lower, owing to the slight difference between the slopes on the Porapak and Chromosorb beads. C_2H_2 is in fact better retained by Porapak-type beads than by the equivalent Chromosorb ones, in respect of CO_2. Use in the calculations of the values of $\Delta I_Q(C_2H_2)$ obtained by interpolation yields greater slopes, as a consequence of the slight deviation from linearity of the plot of retention time *vs.* number of carbon atoms (see Fig. 4).

Comparison of Tables I and II shows that the lowest values of the retention index and, as a consequence, of ΔI_Q, are shown by EVB–DVB polymers (Porapak Q). In the field of aromatic polymers, EVB–DVB beads are followed by Sty–DVB (Porapak P and Chromosorb 101 and 102) and cross-linked polystyrene (Chromosorb 103 and 106). When similar chemical compositions are given by the manufacturer, as in the case of Sty–DVB Chromosorb 101 and 102, of Polystyrene Chromosorb 103 and 106, and of acrylic Chromosorb 105, 107 and 108, the ΔI_Q values increase with decreasing surface area and, except for Chromosorb 105, with increasing pore diameter.

No regular correlation was found between the ΔI_Q values and the amount of crosslinking, equivalent to DVB content, as determined by pyrolysis gas chromatography of some Sty–DVB and crosslinked polystyrene polymers[11]: this gave the following values for copolymerized divinylbenzene: Chromosorb 101, 3%; Chromosorb 102, 3.5%; Porapak P, 4%; Chromosorb 103, 5%. The nitrogen-containing polymers show increasing ΔI_Q values in the order Porapak S (vinylpyridine) < Porapak R and N (vinylpyrrolidone) < Chromosorb 104 (ACN–DVB). Also the two vinylpyrrolidone polymers show increasing ΔI_Q values with decreasing surface area.

The two most polar PPBs (Porapak T and Chromosorb 104) have very similar behaviour, notwithstanding the different chemical composition, from the point of view of elution of acetylene and ethylene, while Chromosorb 104 is the only PPB which elutes CO_2 after ethane and ethylene.

CONCLUSIONS

Taking into account the fact that the calculation of the retention index values of CO_2, C_2H_4 and C_2H_2 can be accomplished easily at room temperature, the use of ΔI_Q values permits the "polarity" of the various PPBs, previously known in a non-quantitative way, to be expressed with numerical values, in order to give a reproducible classification of the packings belonging to the same series or to different commercial types. Moreover, the similarities and differences between the Chromosorb and Porapak PPBs can be quantitatively measured and used for choosing the best packing for the analysis of a given gas mixture and for the calculation of mixed columns.

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GLASS CAPILLARY COLUMNS WITH MIXED STATIONARY PHASES

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SUMMARY

Deactivation of the glass capillary and coating of the stationary phase can be performed in one operation. Static coating with a solution of both 95% methyl 5% phenyl silicone and poly(ethylene glycol) gives capillaries of superior quality than by successive treatments.

INTRODUCTION

In order to change the selectivity of chromatographic systems, mixed stationary phases are often used. Capillary chromatography is a form of chromatography which has partition abilities so great that the selectivity, and even the polarity, of the stationary phase are of secondary importance. Increasing the selectivity as well as subtle modulation of polarity by mixing the solvents may therefore seem pointless. However, the main deficiency of glass capillary gas chromatography is the preparation of the column itself, and especially the formation of a stationary-phase homogenous film which always requires proper preparation of the capillary interior surface, *i.e.* its etching and deactivation.

Deactivation, which improves the wettability of the glass surface by the non-polar stationary phase, is carried out by means of binding silanes[1] or Carbowax 20M[2].

In the present work it was shown that using a mixed stationary phase, composed of poly(ethylene glycol) (Carbowax 20M) and non-polar 95% methyl 5% phenyl silicone oil (SE-52), the surface deactivation step may be omitted. Deactivation already occurs in the coating process itself owing to the preferential adsorption of Carbowax 20M. With regard to efficiency, the columns prepared are equivalent to those with pure SE-52 deposited on the previously silanized surface and are of higher efficiency than columns with pure SE-52 deposited on the previously silanized surface and are of higher efficiency than columns with pure Carbowax 20M.

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EXPERIMENTAL

Capillary tubes (0.3 mm I.D.) were drawn from tubes (1 m × 3 mm I.D.) made of Polish soda glass (Krosno Glass Works) using a drawing machine made in our laboratories. The internal capillary surface was increased by treatment with dilute hydrochloric acid[3].

All capillaries were coated by the static method with solutions of SE-52 and Carbowax 20M in the ratios 3:1 and 1:3 and with pure Carbowax 20M and pure SE-52 in dichloromethane. The concentration of the solution was adjusted to give a film thickness of 0.15, 0.3 and 0.5 μm for each kind of stationary phase (for Carbowax 20M also 0.25 μm). Two other columns were prepared: column 11 (Carbowax 20M coated) and column 1 (with pure SE-52 on the hexamethyldisilane (HMDS)-treated surface)[1].

A Pye Unicam GCD chromatograph was used for the work with capillary columns, in which the cage-located capillaries were removed, and the column endings were directly introduced into the injector and a flame-ionization detector (FID) burner. The samples were of 1 μl, with a splitting ratio of 1:30, and the concentration of the solutions corresponded to the concentration of the Grob test mixture[4].

The specific retention volumes (V_g) of various compounds were measured on pure Carbowax 20M, on pure SE-52 and on their mixtures in order to determine the stationary-phase film thickness. Gas-Chrom CLZ coated with 3% SE-52, Carbowax 20M, and their mixtures in ratios of 3:1, 1:1, and 1:3 were used as packing materials. The measurements were performed on a W. Giede GChF 18.3 (Leipzig, G.D.R.) gas chromatograph equipped with a katarometer. Nitrogen was used as the carrier gas. The temperature was 100°C. The stationary-phase film thickness was calculated from the retention data (V_g and k) of the methyl esters of C_{10} and C_{12} fatty acids.

RESULTS AND DISCUSSION

The properties of all capillary columns with the mixed stationary phases and individual solvents SE-52 and Carbowax 20M are given in Table I.

Columns 1 and 2 have an SE-52 film of the same thickness, but in the preparation of column 2 the silanization stage was omitted. The resolution of this column is almost five-fold smaller than that of column 1. In fact it is so small that the column can be regarded as completely useless. Column 2 was made only for underlining the contrast between its properties and the properties of the columns with mixed stationary phases, which were also prepared without predeactivation of the surface (columns 5–10 and 12–14).

Columns 11 and 12 with Carbowax 20M are characterized by their almost identical (within the limits of experimental error) properties despite the fact that column 11, before coating, underwent the deactivation stage. As can be seen in the case of the polar stationary phase, deactivation is not necessary since it does not improve the quality of the column.

Columns with mixed stationary phases are characterized by relatively high resolution values ($R_s = \frac{1}{2} R_s$ obtained for these esters). The highest resolution can be observed at the predominance of SE-52 (columns 5–7) and its value of EPN (effective peak number) is close to the EPN of the columns with pure SE-52. When Carbowax

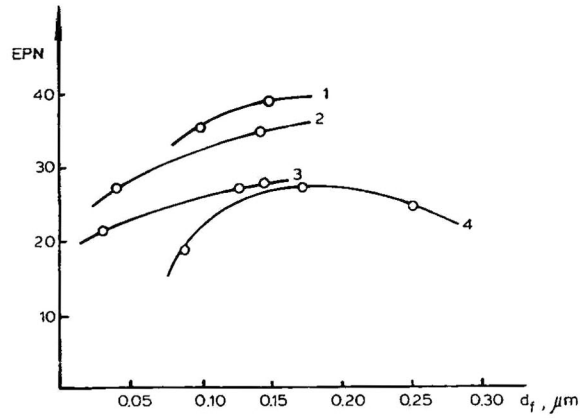

Fig. 1. Effective peak numbers (EPN) as a function of film thickness (d_f). Stationary phases: 1 = SE-52; 2 = SE-52 + Carbowax 20M (3:1); 3 = SE-52 + Carbowax 20M (1:3); 4 = Carbowax 20M.

20M predominates, EPN is comparable to the one obtained on Carbowax 20M itself, whereas the values of the capacity ratio (k) for all columns with the mixed stationary phase remain at the same k as in the columns with pure SE-52. This means that in the columns with mixed stationary phases we observe a non-linear dependence of the retention data as a function of the stationary phase composition. A lengthening of the retention time can be observed which occurs without simultaneous broadening of chromatographic bands; thus the high resolution (and also efficiency) of these columns is preserved.

The resolution of the peaks and their retention remain a function of the

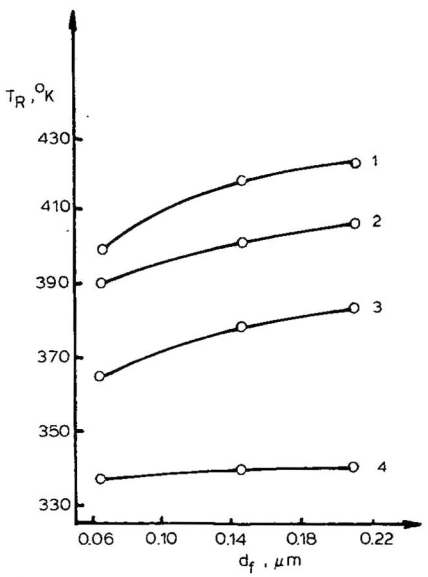

Fig. 2. Variation of retention temperatures with stationary phase film thickness for Carbowax 20M columns. Initial temperature, 333°K; programming rate, 4°/min. Curves: 1 = 2,3-dimethylnaphthalene; 2 = 2,6-dimethylaniline; 3 = methyl decanoate; 4 = n-decane.

TABLE I

CHARACTERISTICS OF GLASS CAPILLARY COLUMNS WITH SILICONE SE-52, CARBOWAX 20M AND THEIR MIXTURES

Characteristic	*Stationary phase and column no.*													
	SE-52				*SE-52 + Carbowax 20M (3:1)*			*SE-52 + Carbowax 20M (1:3)*			*Carbowax 20M*			
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*
Column length (m)	24.0	24.5	22.3	24.5	24.5	24.5	23.5	24.5	24.5	24.5	24.5	24.5	28.9	24.5
Film thickness (μm)														
provided	0.15	0.15	0.30	0.50	0.15	0.30	0.50	0.15	0.30	0.50	0.15	0.15	0.25	0.30
obtained	0.14	0.14	0.11	0.18	0.04	0.14	0.14	0.03	0.12	0.13	0.08	0.07	0.16	0.23
Capacity ratio, k', for methyl dodecanoate at 100°C	58.0	49.6	40.0	63.0	16.7	61.0	61.3	12.6	46.7	51.6	10.8	8.8	21.1	28.3
EPN for methyl esters	39.0	8.0	20.2	25.1	26.0	35.8	27.0	21.6	29.0	26.0	18.8	17.3	27.8	23.0

TABLE II

RETENTION INDICES AND RELATIVE COLUMN POLARITY

Characteristic	*Stationary phase and column no.*							
	SE-52		*SE-52 + Carbowax 20M (3:1)*		*SE-52 + Carbowax 20M (1:3)*		*Carbowax 20M*	
	1	*packed*	*5*	*7*	*8*	*10*	*14*	*packed*
Film thickness, μm	0.14		0.04	0.14	0.03	0.13	0.23	
1-Octanol (retention indices)	1079	1065	1165	1168	1347	1397	1550	1474
1-Octanol (relative polarity %)	0.0	–	18.3	19.7	56.9	67.5	100.0	–

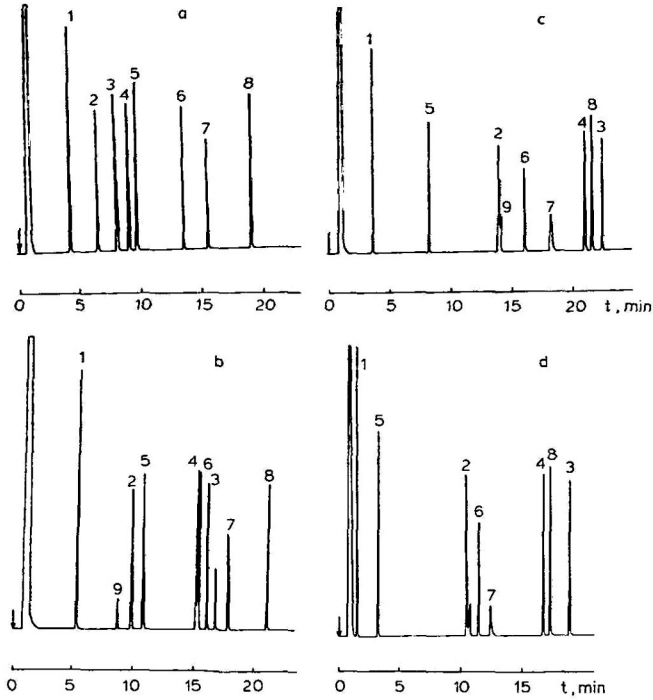

Fig. 3. Grob's tests for columns coated with SE-52, Carbowax 20M and their mixtures. Initial temperature, 333°K; programming rate, 4°/min. Sample, 1 μl; carrier gas, H_2. Columns: a = column 2 with SE-52; b, = column 6 with SE-52 + Carbowax 20M (3:1); c, = column 10 with SE-52 + Carbowax 20M (1:3); d, = column 13 with Carbowax 20M. Peaks: 1 = *n*-decane; 2 = 1-octanol; 3 = 2,6-dimethylphenol; 4 = 2,6-dimethylaniline; 5 = *n*-dodecane; 6 = methyl decanoate; 7 = dicyclohexylamine; 8 = methyl dodecanoate; 9 = 2,3-butanediol.

stationary phase film thickness (Figs. 1 and 2). The peak resolution reaches its maximum value at d_f = 0.13–0.17 μm (Fig. 1). The dependence of the retention temperature (T_R) in the analysis with the programmed temperature on the film thickness is different for different types of compounds (Fig. 2). The aliphatic hydrocarbon (C_{10}) is least sensitive to the changes in the film thickness; the aromatic hydrocarbon (2,3-dimethylnaphthalene) is the most sensitive to these changes.

Chromatograms of the Grob text mixture[4], shown in Fig. 3, show that the columns with the mixed stationary phase do not exhibit adsorptive properties, despite the fact that they were not predeactivated. The peaks of the polar compounds, 1-octanol, 2,6-dimethylphenol, and 2,6-dimethylaniline, retain *ca.* 100% of their height[4].

It can be seen from Table I that the obtained film thickness is considerably different from the planned one. As the static-coating method was used this result is very surprising, and was probably caused by "creeping" of the stationary phase out of the capillary in the course of prolonged (24-48 h) vacuum evaporation of the solvent. This "creeping out" certainly changes the composition of the mixed stationary phase. A new absolute determination of the components is very difficult, and therefore an indirect method was used.

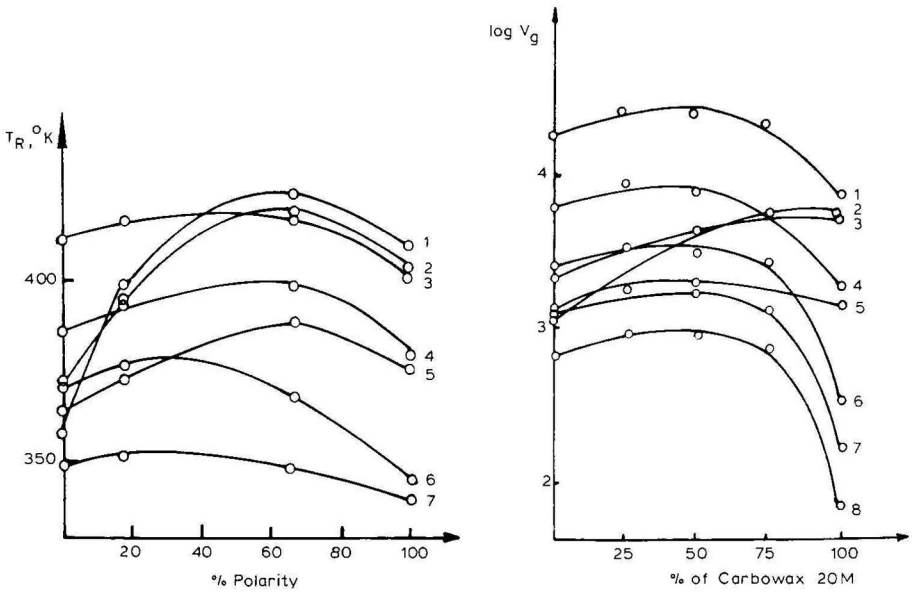

Fig. 4. Retention temperatures of various solutes as a function of polarity of capillary columns. Columns and conditions as in Fig. 3. Solutes: 1 = 2,6-dimethylphenol; 2 = 2,6-dimethylaniline; 3 = methyl dodecanoate; 4 = methyl decanoate; 5 = 1-octanol; 6 = *n*-dodecane; 7 = *n*-decane.

Fig. 5. log V_g of various solutes *versus* stationary phase composition on packed columns with SE-52, Carbowax 20M and their mixtures. Solutes: 1 = methyl dodecanoate; 2 = 2,6-dimethylphenol; 3 = 2,6-dimethylaniline; 4 = methyl decanoate; 5 = 1-octanol; 6 = *n*-dodecane; 7 = *n*-undecane; 8 = *n*-decane.

The polarity of the column with the mixed stationary phase, relative to the column with pure components, was determined. The differences of retention indices of 1-octanol (calculated from the retention temperature[5]) (ΔI) were used as the basis of the determination.

The polarity of a given column was determined by relating the difference in the retention indices on this column and on the column with pure SE-52 to the difference of the indices on the columns containing Carbowax 20M and SE-52 (Table II).

The relative polarity of the columns is slightly smaller than one would predict from the composition of the coating solution. The retention indices obtained from capillaries are higher than the values measured in the packed columns, which is probably due to the influence of the glass surface on the retention data and also to higher adsorption occurring at the surface of the coating than in packed columns.

Fig. 4 shows the retention temperatures of compounds of different polarity as a function of the relative polarity of the stationary phase, for the column with d_f = 0.15 μm, and Fig. 5 shows log V_g as a function of the stationary phase composition for the same compounds chromatographed on the packed columns. Owing to the different form of the retention expression it is difficult to speak about a quantitative comparison between these relations. One can see, however, that for polar compounds, there is a greater deviation from linearity in the capillary columns than in the packed ones.

A mixture of polycyclic aromatic hydrocarbons was chromatographed in order

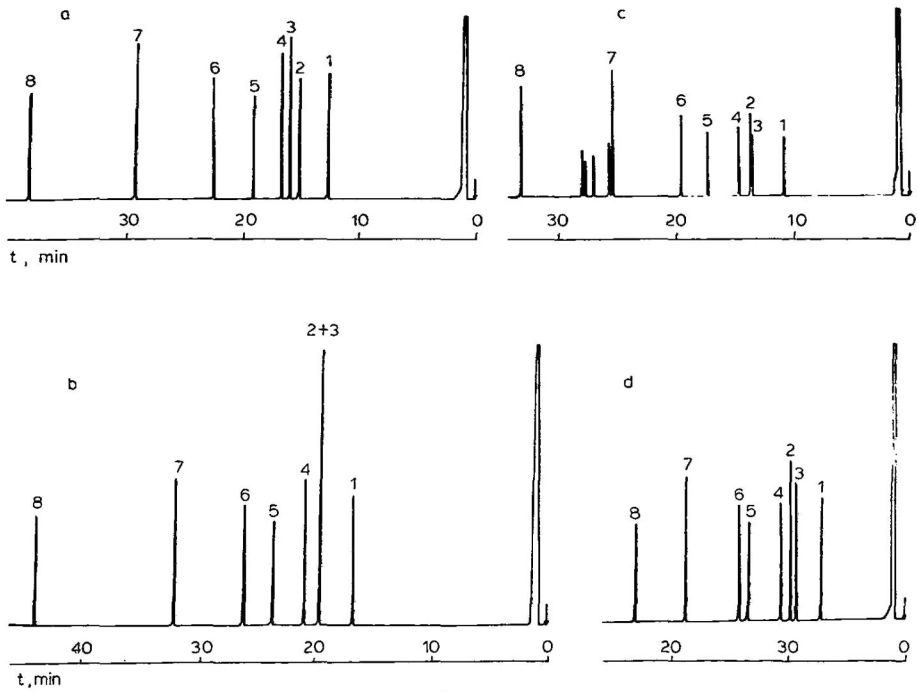

Fig. 6. Chromatograms of mixtures of polynuclear aromatic hydrocarbons on (a) column 13 (Carbowax 20M, $d_f = 0.16$ μm), (b) column 6 [SE-52 + Carbowax 20M (3:1), $d_f = 0.14$ μm], (c) column 5 [SE-52 + Carbowax 20M (3:1), $d_f = 0.04$ μm], (d) column 1 (SE-52, $d_f = 0.14$ μm). Initial temperature, 333°K; programming rate, 4°/min; carrier gas, H_2; sample, 1 μl. Peaks: 1 = 2-methylnaphthalene; 2 = 2,6-dimethylnaphthalene; 3 = biphenyl; 4 = 2,3-dimethylnaphthalene; 5 = 2,3,6-trimethylnaphthalene; 6 = fluorene; 7 = phenanthrene; 8 = pyrene.

to test the analytical abilities of columns with mixed stationary phases. Best results were obtained when the initial composition of the stationary phase was SE-52: Carbowax 20M in the ratio of 3:1, *i.e.* at 19% relative polarity.

Columns with thin films were very useful for the analysis of polycyclic aromatic hydrocarbons. Fig. 6c shows a chromatogram run on a column having the same composition of the stationary phase as in Fig. 6b, but with $d_f = 0.04$ μm. As can be seen, the resolution of the column does not decrease while, at the same time, considerable shortening of the analysis time is achieved.

CONCLUSION

The preparation of capillary columns with a mixed stationary phase composed of non-polar silicone oil and poly(ethylene glycol) (Carbowax 20M) allows one to omit the deactivation stage in the laborious and time-consuming preparation of columns.

Although the process of solvent evaporation in the static-coating procedure is accompanied by loss of a certain amount of the stationary phase, the polarity of these columns can be estimated using the retention indices, or even on the basis of the

position of the peaks of the polar components of the Grob test mixture on the chromatogram.

The columns obtained are characterized by good analytical properties, *viz.*, high resolution ability (to 40 units of EPN for 24 m of the column length).

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LARGE BORE COATED COLUMNS IN ANALYSIS FOR TRACE ORGANIC POLLUTANTS IN WATER

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SUMMARY

Large bore coated (LBC) columns were used in model systems for concentration and recovery of trace organic chemicals in water. The chemicals used represent various types of possible pollutants, with emphasis on aromatic hydrocarbons. The LBC column is used to concentrate the trace organics from water simply by passing a sample of water through this device. The trapped organics are then themally desorbed onto a short porous polymer or charcoal pre-column. After the residual moisture is driven off, the trapped material is eluted onto the analytical gas chromatographic column and analyzed under programmed temperature conditions. The chemicals used in the model analyses were benzene, dodecane, naphthalene, *o*-cresol, dibenzyl and fluorene; three concentrations between 50 ppb (ng/ml) and 5 ppm (μg/ml) were examined. Only 20 ml of sample was needed for analysis. Manipulation of sample volume and number of passes through the LBC column resulted in good quantitative recovery for most of these chemicals. Recovery for benzene, however, was poor. The LBC column, coated with SE-30, showed different retention efficiencies for each compound at different concentrations. The lowest concentration gave best recoveries. The polynuclear compounds gave particularly good results and quantitative recoveries of these components were possible at the 50-ppb level.

The results obtained with the LBC column compared favorably with those obtained by headspace nitrogen sparging or solvent extraction. Reference is also made to the analysis of petroleum fractions dissolved in water (10 ppm) and to the influence of dissolved substances in affecting recovery efficiency. Some discussion of the effect of column dimensions upon performance is also given.

INTRODUCTION

Since the first use of large bore coated (LBC) columns in the trapping of airborne volatiles and those from breath was described[1], several applications for these devices have been reported for the trapping of trace amounts of flavorants in water and beverages[2,3]. Analyses in both cases can be performed at the ppb level or below, though recoveries of flavors can be poor from some aqueous systems in the presence of fixation phenomena probably due to colloidal components.

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Water pollution is an important problem, and analyses for water pollutants have commanded considerable attention. The diversity of pollutants has required the use of varied techniques for trapping these chemicals, which are usually at trace levels. The techniques reported are numerous for specific pollutants, but less so for generalized methods which seek comparable concentration of all foreign compounds whether known or not. Direct trapping on activated charcoal[4] was for many years the only general technique, but more recently these techniques have expanded to include resins[5,6], porous polymers[5,7,8] and stripping of volatiles followed by trapping[9]. LBC columns are very simple devices, consisting of long lengths of open metal tubing which are wall-coated with standard chromatographic substrates. The substrate of choice has been silicone (SE-30) rubber, but this choice is based primarily on its thermal stability in the desorption phase of the analysis rather than solvent efficiency in the trapping phase. Of all the substrates examined, only SE-30 has shown great reliability and consistency in providing the minimum number and amount of artifacts upon thermal stripping of the LBC column. This stripping, performed with a backwards flush of nitrogen at 250°C for 12 min, effects the transfer of the absorbed organics from the LBC column to the gas chromatograph itself, usually via the medium of an intermediate short pre-column of porous polymer sized to fit into the injector heater of the gas chromatographic unit, although other transfer media may at times be used.

This paper deals with the specific application of LBC columns in water pollution analysis, whereby they are used as traps for the trace organics. Model pollutants, representing various classes of chemicals, were used in the analyses. The chemicals used were benzene, dodecane, naphthalene, *o*-cresol, dibenzyl and fluorene. The trapping efficiency of the LBC column was demonstrated by recovery of traces of the model pollutants in water when an aliquot of the spiked water was sampled directly on the column. The recovered amounts were compared to those obtained from direct injection of the same amounts as in the water sample.

In addition to analysis for model pollutants, trace gasoline was analyzed for in water. Comparison of performance of LBC columns to other techniques was carried out by analyses for the model pollutants at similar concentration by solvent extraction and by headspace purge and trap technique.

Reproducibility of the LBC column technique was checked by repetitive analyses of identical samples over a period of 3 weeks.

EXPERIMENTAL

Gas chromatographic conditions are shown in Table I.

Materials

The pre-column packing was Tenax GC (60–80 mesh; Applied Science Labs., State College, PA, U.S.A.). The LBC coating material was SE-30 methyl silicon rubber (Analabs, North Haven, CT, U.S.A.).

The pre-columns were short lengths of Pyrex glass tubing, 15 × 0.6 cm (0.38 cm I.D.). The packing (200 mg Tenax) was contained between glass wool plugs to ensure it was always within the hot zone of the pre-column heating oven used for sample desorption. The pre-column was conditioned at 275°C for a minimum of 3 h, with a nitrogen flow of 50 ml/min prior to use.

TABLE I

GAS CHROMATOGRAPHIC CONDITIONS

Gas chromatograph	Perkin-Elmer Model 3920 with FID
Column	8 ft. aluminum, 0.25 in. O.D. (0.186 in. I.D.) packed with 10% Carbowax 20M on Chromosrob W 80–100 mesh (acid washed and DMCS-treated)
Carrier	Nitrogen at 50 ml/min
Column oven temperature	4 min at 70°C, then programmed at 8°/min from 70 to 230°C and held at the final temperature
Injector and interface temperatures	250°C
Integrator	Perkin-Elmer M-1
Recorder	0–5 mV with a chart speed of 0.5 in./min

LBC columns

An aluminum tube [25 ft. × 0.25 in. O.D. (0.186 in. I.D.)] with a coating of 2.1 g SE-30 was used. The calculated film thickness was 19 μm. The technique for coating and conditioning the column, which may be sectioned or joined in series to obtain other lengths, was as described previously[2]. The effect of varying column diameter or length with a given weight of substrate, or of varying coating thickness for a given column diameter or column length, has also been reported[2], but with a major emphasis on sampling air rather than aqueous systems.

Sampling

An aliquot of 20 ml or more of the aqueous solution was poured into the LBC column and allowed to pass through with gravity. A gentle nitrogen flow can also be used to assist in the flow of the sample. The emerging sample was collected in a beaker and then poured through the column up to four times more; if needed, it can be centrifuged or filtered prior to sampling.

The column was washed with 3 × 50-ml portions of distilled water. This step is not necessary if the sample has no dissolved materials which will cause interference if they decompose with heat during the elution step. The bulk of residual moisture is then removed from the LBC column using nitrogen at 500 ml/min for 10 min. The LBC column temperature is ambient, or the column can be slightly warmed at this point to speed water removal.

Sample desorption

The LBC column is transferred to a heating oven for reverse flushing with nitrogen. The trapped volatiles are eluted by heating the oven to 250°C and passing a nitrogen flow of 50 ml/min for 12 min through the LBC column onto a Tenax-GC pre-column, which is at ambient temperature just outside the oven. The pre-column may also be cooled, if desired. The pre-column is then dried with nitrogen at 100 ml/min at ambient temperature until the condensed moisture is no longer obvious.

The sample is eluted for analysis directly onto the analytical column by heating the pre-column at 250°C for 6 min in a special tube oven (Chromalytics 1022) which

fits on the injection port of the gas chromatograph. The temperature of the tube oven is controlled with Chromalytics' power supply and temperature controller (Model 1047). The carrier gas is rerouted with a toggle valve to pass through the pre-column while eluting the sample. The chromatographic analysis is initiated concurrently with the initiation of sample desorption. The sample may also be eluted directly from the LBC trap into a small volume of organic solvent, by bubbling into a cooled aliquot, and after subsequent concentration analyzed by direct injection into the gas chromatograph. In fact, 3–5 ml of cooled methylene chloride in a small test tube can replace the porous polymer pre-column, allowing direct transfer from the LBC column into methylene chloride. In this case, the test tube will also contain condensed water which can be easily separated.

RESULTS AND DISCUSSION

The factors which were shown previously[2,3] to affect the efficiency of trapping by LBC columns are length and diameter of the column, size of sample and number of sampling passes through the column. The number of sampling passes is very important to achieve equilibrium of the trace solute between the aqueous solution and the wall coating. Five passes were adopted for convenience and as a compromise between time and maximum recoveries. The time required to promote equilibrium differs for each component. The original concentration of the trace organics in water

TABLE II

RECOVERIES OF TRACE MODEL POLLUTANTS FROM WATER

Pollutant concentrations: 50 ppb, 200 ppb and 5 ppm; trapping column: LBC, 25 ft. × 0.186 in. I.D., with 0.019 mm SE-30 wall coating; the effluent of the trapping column was subsequently eluted onto a Tenax-GC pre-column. Samples of 20 ml were analyzed. The recoveries are relative to direct injection of equivalent amounts.

Compound	*Amount in 20-ml sample (μg)*	*Recovery (%)*
Benzene	1.0	0.1
	3.9	4.3
	97.9	2.9
Dodecane	1.1	28.9
	4.3	23.8
	106.3	24.9
Naphthalene	1.0	114.2
	4.0	85.8
	99.3	86.9
o-Cresol	1.0	51.2
	4.1	31.4
	103.2	24.0
Dibenzyl	1.0	91.5
	4.1	80.2
	101.4	62.4
Fluorene	1.0	112.8
	4.0	82.7
	99.8	28.4

also affects the efficiency of their trapping and recovery by the LBC column. Table II shows recoveries of the model pollutants when present in the same volume samples but at different concentrations (0.05, 2 and 5 μg/ml). The recoveries for most of the components, except benzene and dodecane, are better in the lower concentration samples. Very good recovery of naphthalene was achieved at all concentrations examined. Other wall coatings need to be examined for selectivity of certain kinds of compounds or for broad based non-specific absorption characteristics.

Table III shows a comparison of the LBC method with other techniques, based on recoveries of the model compounds when samples of similar concentrations were analyzed by direct trapping on the LBC column, purging with nitrogen and trapping on Tenax-GC, and by extracting with methylene chloride and concentrating the extract to small volume. The results obtained by LBC sampling, except for benzene and dodecane, especially in the most dilute samples, are closer to the results of solvent extraction.

TABLE III

RECOVERIES OF TRACE MODEL POLLUTANTS WITH VARIOUS ENRICHMENT TECHNIQUES

(A) Direct sampling on 25 ft. × 0.186 in. I.D. LBC column with 0.019-mm SE-30 coating. (B) 20-ml sample of 10% sodium sulfate solution containing the indicated amount purged with 12 l of nitrogen at 100 ml/min on Tenax-GC precolumn. (C) Sample extracted with 2 ml methylene chloride and concentrated to a final volume of 0.3 ml.

Compound	*Amount in 20-ml sample (μg)*	*Recovery (%)**		
		A	*B*	*C*
Benzene	104.6	2.9	16.9	70.2
	1.1	7.1	22.4	79.6
Dodecane	102.4	24.9	56.6	107.6
	1.0	17.0	50.9	72.8
Naphthalene	101.4	86.9	98.1	112.8
	1.0	97.1	79.0	99.4
o-Cresol	112.7	24.0	21.2	62.7
	1.1	84.3	50.1	85.0
Dibenzyl	109.4	62.4	92.4	93.7
	1.1	80.4	73.2	109.7
Fluorene	105.2	28.3	66.6	116.2
	1.1	87.1	73.2	116.8

* Relative to direct injection of the same amounts as in the sample.

Direct extractions of the aqueous model pollutant system using Tenax-GC (200 mg) or activated charcoal (10 mg) resulted in poor recoveries and were not pursued. In both, the headspace sparging and LBC sampling methods, the recovery of *o*-cresol especially from the more concentrated sample is quite low. The low recoveries of benzene and dodecane by headspace sparging is due to their low breakthrough volumes from the Tenax trap. This same factor probably accounts for some of the low recovery of these compounds via LBC sampling since the sample transfer is carried out on Tenax.

TABLE IV

REPRODUCIBILITY OF ANALYSES FOR TRACE MODEL POLLUTANTS FROM WATER BY DIRECT TRAPPING ON LBC COLUMN

Column: 25 ft. × 0.186 in. I.D.; 0.019-mm wall coating.

Compound	*Amount in 20-ml sample (μg)*	*Recovery (%)*				*S.D.*
		1	*2*	*3*	*4*	
Benzene	1.0	0.1	6.9	7.1	0	4.0
Dodecane	1.1	28.9	13.6	17.0	27.8	7.6
Naphthalene	1.0	114.2	92.2	97.1	97.6	9.6
o-Cresol	1.0	51.2	83.7	84.3	40.2	22.6
Dibenzyl	1.0	91.5	68.4	80.4	97.5	12.9
Fluorene	1.0	112.8	87.1	87.1	110.2	14.1

Table IV shows the extent of reproducibility in LBC sampling. The analyses were performed on identical samples over a three-week period. Except for the wider variation in recoveries of *o*-cresol and low recoveries of benzene and dodecane, the reproducibility is good, as indicated by the standard deviations, considering the extremely low concentrations (0.05 ppm) involved.

Fig. 1 is a gas chromatographic analysis of the model pollutants in water at 0.05 ppm each when extracted directly on the LBC column, which is similar to the direct analysis of 0.1 μl of stock standard solution containing the same amounts of the model compounds except for the much more enhanced peaks of benzene and dodecane in the direct analysis.

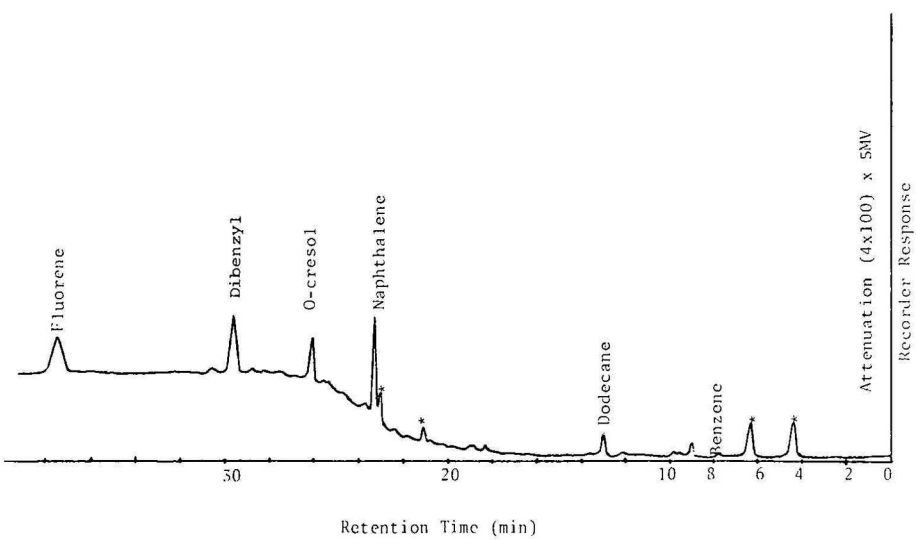

Fig. 1. Analysis for model pollutants spiked in distilled water at 0.05-ppm concentration by direct sampling on LBC column (25 ft. × 0.186 in. I.D.; 0.019-mm SE-30 wall coating). A 20-ml aliquot was recirculated 5 times through the column. Amount of each component in sample is 1 μg. *, Artifact and/or trace impurity.

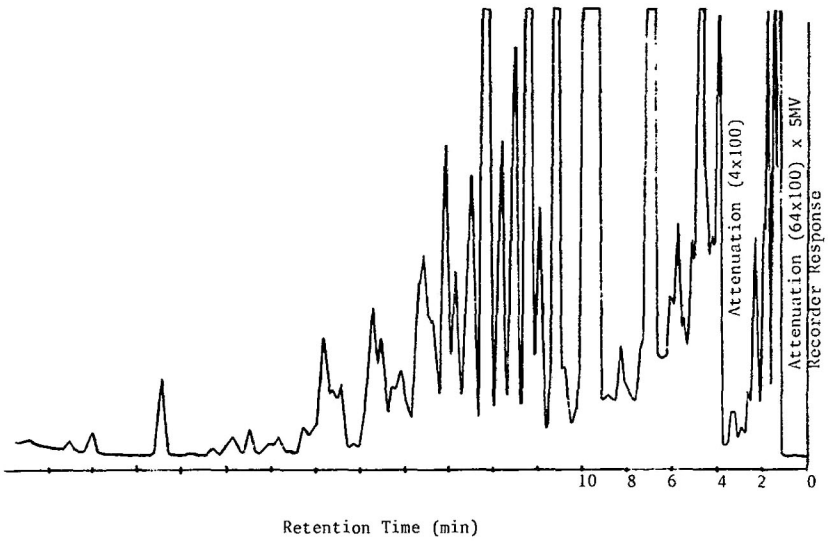

Fig. 2. GC analysis of 1.0 μl gasoline by direct injection.

Fig. 2 shows a chromatogram of gasoline by direct injection while Fig. 3 shows a chromatogram of an LBC analysis of 100 ml of water containing 1 μl of the same gasoline. While the bulk of the highly volatile components is lost from the LBC sample, the pattern of the remaining components is very similar to that obtained by direct injection. The relationship, however, is not quantitative.

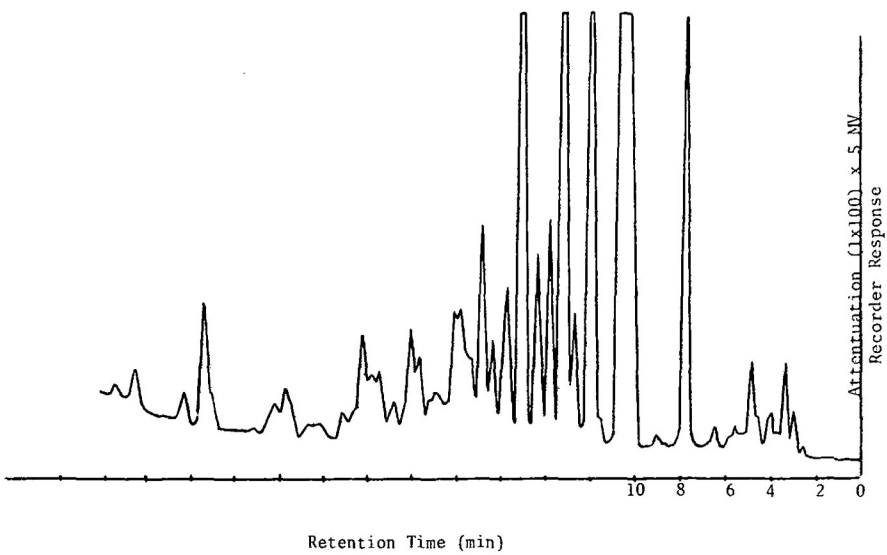

Fig. 3. GC analysis for trace gasoline (0.001%) in water by sampling directly on LBC column. A 100-ml sample was recirculated 5 times through the column (25 ft. × 0.19 in. I.D.; 0.0019-mm SE-30 wall coating). Sample was then desorbed onto Tenax-GC precolumn and onto the analytical column.

The effect of prolonged residence time of the sample in the column was checked by leaving a 50-ml sample of water, which contained 0.2 ppm each of the model pollutants, in a capped 25-ft. LBC column for 17 h. Appreciable improvement in recovery of dodecane was noted. Up to 75% recovery was obtained for dodecane, but no appreciable improvement in recovery of *o*-cresol was achieved. Good recoveries for naphthalene, dibenzyl and fluorene were still obtained. Improvement in recovery of benzene was only slight.

Recycling the sample through the column with a peristaltic pump for 45 min at 300 ml/min also resulted in improved recoveries of dodecane. However, extraneous trace contaminants from the rubber tubing of the peristaltic pump were noted.

CONCLUSION

LBC columns can be effective trapping devices for analysis of trace organic pollutants in water. A potential exists in varying the selectivity of the trap by varying the wall-coating material of the LBC. Improved recoveries may be achieved by manipulating column length, residence time of the sample, sample size and concentration.

ACKNOWLEDGEMENT

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PULSE METHOD FOR THE RADIO-CHROMATOGRAPHIC DETERMINATION OF THE SORPTION CAPACITY OF CATALYSTS*

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SUMMARY

A radio-gas-chromatographic method is presented for determination of the quantities of components sorbed irreversibly on catalysts and adsorbents. The method is illustrated with measurements on the chemisorption of H_2/HT on Wilkinson's catalyst.

INTRODUCTION

In investigations of the mechanisms and kinetics of heterogeneous catalytic reactions, it is indispensable to study the adsorption properties of the catalyst including the extents to which the reactants are chemisorbed on the catalyst.

A number of methods are known for the determination of the adsorption capacities of solid or dissolved catalysts. In laboratories where the necessary equipment (volumetric adsorption apparatus, sorptometer, etc.) is not available, use can be made of the "pulse dynamic method" described by Paryjczak *et al.*[1,2] which can be carried out on a normal gas chromatograph. Our method differs from this in using a radiation detector, a natural choice for the detection of labelled gas components, instead of a thermal conductivity detector, which was used by Paryjczak *et al.*

For application of the method, the radiograph itself is in fact sufficient; it is in essence a flow-proportional counter (supplemented with feed-gas supplies and electronic amplifying and recording units).

Description of method

The principle of the measurement is very simple. Impulses of known volumes of the gaseous or vapour-phase component labelled with ^3H or ^{14}C are passed into

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the radiograph, and the count for one impulse is determined. Next, a reactor (or column) packed with a known amount of catalyst is connected in the path of the carrier gas (by means of a suitable valve), and impulses of the labelled component are passed, one after another, through the column into the detector, until the impulses are unchanged by passage through the catalyst (by comparison with the previously determined count). The difference in total activity, ascribable to the impulses admitted to the catalyst and the impulses measured, is proportional to the quantity of the component that is chemisorbed.

The method can be employed unambiguously only in the case of irreversible adsorption (chemisorption).

EXPERIMENTAL

Measurements were made on a radio gas chromatograph obtained by coupling a Perkin-Elmer RGC-170 radiograph and a Hewlett-Packard 5710 A gas chromatograph, with an appropriate gas-mixing apparatus and a gas-feed valve. The equipment is depicted in Fig. 1. An account of the theoretical and practical problems of radio-gas-chromatographic measurements involving a proportional counter as detector is to be found in ref. 3.

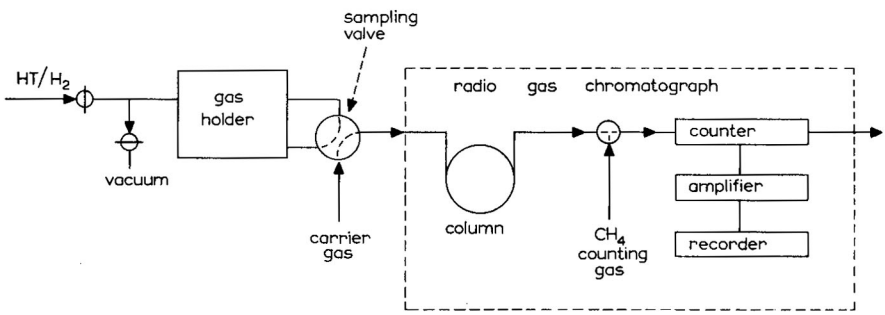

Fig. 1. Experimental apparatus.

Measurements were carried out with ^{3}H-labelled dihydrogen, the specific activity of which had been adjusted to the desired value of *ca.* 10^{12} Bq mol^{-1} by dilution with inactive dihydrogen. At room temperature the volume (V) of the gas burette containing the labelled dihydrogen was 128.5 cm^3. From this, gas impulses of volume $v = 0.5$ cm^3 were added to the carrier gas going into the gas-chromatograph. The flow-rate of the nitrogen carrier gas was varied from 0.5 to 2.0 cm^3 sec^{-1}.

The catalyst to be examined was packed into the column of the gas chromatograph. In the present measurements, a study was made of the hydrogen-binding capacity of Wilkinson's hydrogenation catalyst[4]; this consists of tris(triphenylphosphine)rhodium(I) chloride [$(TPP)_3RhCl$] in 1,1-diphenylethylene on a solid support. Before the experiments, the catalyst charge was evacuated at 10^{-2} Pa for 30 min at 333°K. The packed column (2 m × 3 mm) in the gas chromatograph was thermostatted to the desired value between 273 and 333°K.

The total activity of the tritiated dihydrogen impulse used in the measurements was determined before the measurements themselves. The impulses were led through

the empty column directly into the detector, and the numbers of counts per impulse were recorded. The activity of the β-emitting tritium was measured with a flow-proportional counter with a volume of 10.0 cm^3, in which methane fed in at a flow-rate of 0.2–1.0 $cm^3\ sec^{-1}$ was used as counter gas.

During the actual measurement, labelled sorbate (tritiated dihydrogen) impulses were admitted at definite intervals onto the column containing the adsorbent (the catalyst) and the activities of the leaving impulses were detected. If the adsorption capacity of the catalyst is high, no leaving impulse is observed initially. However, when the amount of adsorbate irreversibly bound by the catalyst approaches the chemisorption capacity, increasingly greater gas impulses leave the column, and finally only impulses unchanged in magnitude, with the activity determined in the preliminary experiments, are observed.

A very important feature in the planning of the experiments is the suitable choice of the quantity of the adsorbent, or the catalyst to be studied (the charge of the column). If this is relatively large, very many impulses will be required for saturation to be attained, and the measurement may last an inconveniently long time. However, if the adsorption capacity of the column charge is small compared to the volume of the impulse, then the measurement will be completed within a few impulses; this is disadvantageous, for the statistical fluctuation in the detection of the radioactivity in the case of a small number of experimental points may cause a large error.

RESULTS

In order to show the capabilities of the method, the chemisorption of dihydrogen ($^3H_2$) was measured on a Wilkinson-type catalyst at 333°K. The column packing consisted of 15% (w/w) of a solution of $(TPP)_3RhCl$ in 1,1-diphenylethane (17 mmol of the salt dissolved in 1 dm^3 solvent) on a Porolith support. From this column filling, 4.35 g was actually used. The first impulse contained $2.69 \cdot 10^{-7}$ mol H_2 and its total activity corresponded to $A_1^0 = 236{,}200$ counts.

The experimental results are summarized in Table I and Fig. 2. It is seen that the observed number of counts per pulse increases from the first to the seventh pulse, but decreases after the eighth. This is due to the decrease in the material content of the gas burette. It is obvious that, disregarding statistical fluctuations, the experimental points increasingly tend to lie on the line calculated from the volume ratio v/V. In the given case

$$v/V = \frac{0.50\ (cm^3)}{128.5\ (cm^3)} = 0.003891$$

Also, the amount of H_2 in each successive pulse is reduced by 0.3891 %. Consequently the activities of the pulses can be computed from the equation

$$A_n^0 = A_1^0\,(1 - v/V)^{n-1}\ (n = 2, 3, \ldots) \tag{1}$$

where n is the serial number of the given pulse. (The values A_n^0 are marked by + in Fig. 2.) It follows from the series expansion

TABLE I

EXPERIMENTAL RESULTS

Pulse number (n)	*Count number/pulse*				
	Measured	*Calculated*		*Differences*	
	(A_n)	A_n^0	A_n^*	$A_n^0 - A_n$	$A_n^* - A_n$
1	219300	236200	236200	16900	16900
2	226950	235281	235281	8331	8331
3	227700	234365	234362	6665	6662
4	228900	233454	233443	4554	4543
5	230100	232545	232524	2445	2424
6	230400	231640	231605	1240	1205
7	229650	230739	230686	1089	1036
8	230400	229841	229767	− 559	− 633
9	228900	228947	228848	47	− 52
10	227850	228056	227929	206	79
11	229200	227167	227010	− 2033	− 2190
12	224400	226285	226091	1885	1690
13	225750	225404	225172	− 346	− 578
14	224250	224527	224253	277	3
15	223200	223653	223334	453	134
16	−	222783	222415	−	−

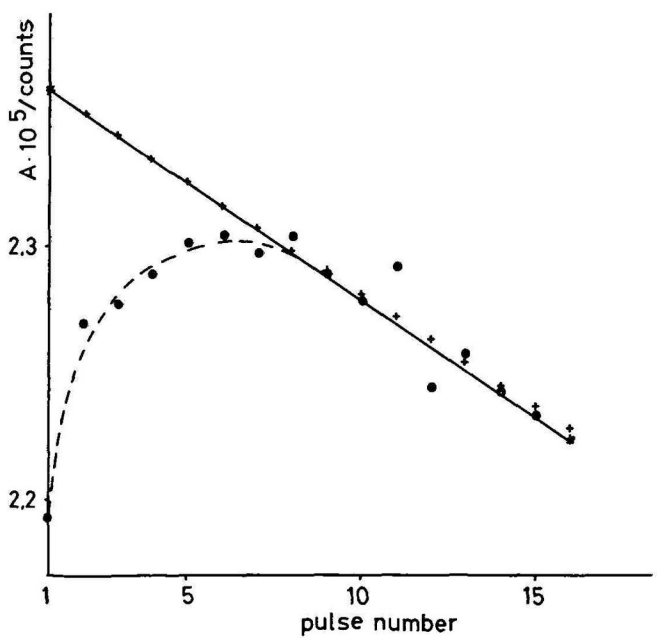

Fig. 2. Determination of the chemisorbed amount of hydrogen on a sample of Wilkinson's catalyst. Temperature: 333°K; flow-rate of the carrier gas: 2 cm^3 sec^{-1}; material contant of a pulse: $2.69 \cdot 10^{-7}$ mol H_2; ● = measured count number (A_n); + = calculated count number (A_n^0).

$$\frac{A_n^0}{A_1^0} = \sum_{i=0}^{n-1} \binom{n-1}{i} (-v/V)^i = 1 - (n-1)\, v/V + \frac{(n-1)(n-2)}{2} (v/V)^2 - \ldots + (v/V)^{n-1}$$

that instead of eqn. 1 the simple relationship

$$A_n^0 = A_n^* = A_1^0 [1 - (n-1)\, v/V] \tag{2}$$

holds for the decrease in the activities of the successive pulses, provided v/V is sufficiently small. It is clearly seen from the Fig. 2 that the straight line (*) drawn according to eqn. 2 fits the points computed from eqn. 1 especially well at low counts per pulse.

For the application of the pulse dynamic method, provided nearly 10 pulses are sufficient to saturate the sorbent, no correction is necessary in the case of $v/V < 10^{-4}$ ($A_n^0 \equiv A_1^0$); the simple correction eqn. 2 is satisfactory if $10^{-4} < v/V < 10^{-2}$; in every other case the activities have to be corrected according to eqn. 1 if greater precision is needed.

The chemisorbed amount can be computed from the sum of the differences [counts (fed) − counts (observed)] for each pulse; finally this total count should be divided by the specific activity of dihydrogen (in the same units).

From Table I it follows that

$$\sum_{n=1}^{7} (A_n^0 - A_n) = 41224; \qquad \sum_{n=1}^{7} (A_n^* - A_n) = 41101$$

$$\sum_{n=1}^{15} (A_n^0 - A_n) = 41154; \qquad \sum_{n=1}^{15} (A_n^* - A_n) = 39555$$

In other words this means that in the given example the linear correction is completely satisfactory. This is more true if the sum is taken only up to that pulse where, in Fig. 2, the dashed curve reaches the straight line (seventh pulse).

From the specific activity $8.78 \cdot 10^{14}$ counts mol^{-1} H_2 of the dihydrogen (as computed from the first pulse), it follows that the chemisorbed amount of H_2 on the Wilkinson-type catalyst at 333°K corresponds to

$$\frac{41224 \text{ counts}}{4.35 \text{ g } 8.78 \cdot 10^{14} \text{ counts mol}^{-1} \text{ H}_2} = 1.079 \cdot 10^{-8} \text{ mol H}_2 \text{ g}^{-1}$$

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CHROM. 14,863

PRÄPARATIVE ISOLIERUNG VON OLIGOGUANOSINPHOSPHATEN AUS DNA-PARTIALHYDROLYSATEN MIT HILFE DER TEMPLATE-CHROMATOGRAPHIE

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SUMMARY

Preparative isolation of oligoguanosine phosphates from partial hydrolyzates of DNA using template chromatography

DNA from herring sperm is partially hydrolyzed to oligoguanosine phosphates. High-molecular-weight fragments are isolated from the partial hydrolyzate using DEAE-cellulose and afterwards QAE-Sephadex. The mixture of guanosine oligonucleotides is separated into complementary and non-complementary fragments by template chromatography on two differently substituted polyvinyl alcohol-p$(dC)_n$-DEAE-celluloses. Influences of pH as well as the kind of the immobilized oligocytidylic acids upon the results of the template chromatographic separations are discussed. The complementary oligoguanosine phosphates are enzymatically dephosphorylated. Fractionation on QAE-Sephadex yields single substances of the serie $(dG)_{3-8}$ on preparative scale, which are chromatographically pure except $(dG)_8$. Rechromatography of $(dG)_8$ on paper yields a pure product (fingerprint).

EINLEITUNG

Desoxyribonucleinsäure (DNA) kann chemisch zu einem Gemisch von Oligoguanosinphosphaten partialhydrolysiert werden. Mit den gebräuchlichen chromatographischen Methoden, die zu einem Trennungsgang kombiniert werden, sind aus dem Hydrolysat bisher Oligoguanosinphosphate mit bis zu 5 Monomereinheiten in präparativen Mengen zugänglich[1]. Unter Einbeziehung der Template-Chromatographie[2], in der die Spezifität des Basenpaarungsmechanismuses nach Watson und Crick zur Trennung genutzt wird, lässt sich der Trennungsgang wesentlich vereinfachen. Ausserdem sind auf diesem Weg Oligoguanosinphosphate mit bis zu 8 Monomereinheiten zugänglich, wie im folgenden gezeigt wird.

EXPERIMENTELLES

Material

Chemikalien. Chemikalien werden in "chemisch reiner" Form verwendet.

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DEAE-Cellulose (Whatman DE-23, W. & R. Balston, Maidstone, Great Britain); QAE-Sephadex A-25 (Pharmacia, Uppsala, Schweden); Membranen (Amicon, Lexington, MA, U.S.A.); Chromatographiepapier 2316 (Schleicher & Schüll, Dassel. B.R.D.); Enzyme: Alkalische Phosphatase, Orthophosphorsäure-Monoester-Phosphohydrolase (alkalisches Optimum) (EC 3.1.3.1.), Phosphodiesterase I. Oligonucleotid-5′-Nucleotidhydrolase (EC 3.1.4.1.) (Boehringer, Mannheim, B.R.D.); DNA, Nucleinsäurebausteine: dG, pdG (PWA Waldhof, Mannheim, B.R.D.). $(dG)_5$ wird in unserem Labor synthetisiert. 2,4,6-Triisopropylbenzolsulfonsäurechlorid (TPS) (EGA-Chemie, Steinheim, B.R.D.); Polyvinylalkohol (PVAL; MG ≈ 70,000) (C. Roth, Karlsruhe, B.R.D.); Pyridin 1 × über KOH destilliert und über Molekularsieb 4 Å aufbewahrt; Hexamethylphosphorsäuretriamid (HMPT): über Molekularsieb 4 Å getrocknet; LiChrosorb RP-18, 5 μm (Merck, Darmstadt, B.R.D.).

Methoden

(A) Synthese der PVAL-p $(dC)_n$-DEAE-Cellulosen (Fig. 1). Typ A der PVAL-p$(dC)_n$-DEAE-Cellulose wird auf dem beschriebenen Weg in drei Schritten dargestellt[3]. Typ B wird in zwei Schritten wie folgt synthetisiert. 1.3 g PVAL (MG ≈ 70,000) werden portionsweise in 65 ml *ca.* 100°C warmes HMPT eingerührt, in der Wärme vollständig gelöst und dann mit 65 ml trockenem Pyridin versetzt. Zu der auf Raumtemperatur abgekühlten Lösung gibt man 2.4 g (4.6 m*M*) Pyridinium-N-anisoyldesoxycytidin-5′-phosphat (pandC)[4]. Die Suspension wird zur Entfernung eventuell vorhandener Wasserspuren mehrmals mit trockenem Pyridin an der Ölpumpe eingeengt. Unter Feuchtigkeitsausschluss werden dann *ca.* 1.4 g (4.6 m*M*) TPS und eine Lösung von teilweise polykondensiertem p(andC)$_n$ zugegeben. Diese Lösung enthält 2.4 g (4.6 m*M*) pandC das in 20 ml trocknem Pyridin mit 4.1 g (13.6 m*M*) TPS 1 h bei Raumtemperatur polykondensiert wird. Die vereinigten Lösungen werden gut verschlossen weitere 12 h polykondensiert und danach mit 150 ml $NH_{3conz.}$ unter Eiskühlung versetzt. Nach einwöchiger Ammonolyse wird der Ammoniak am

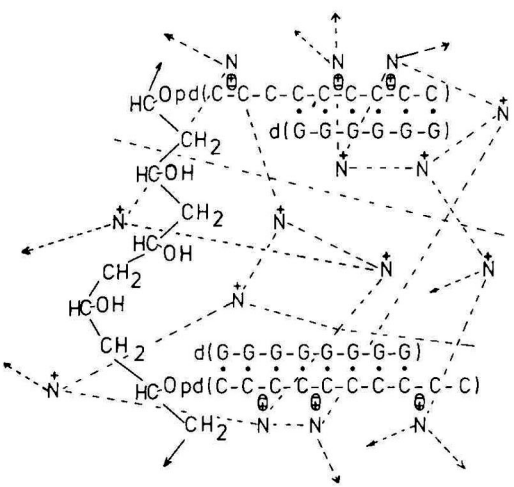

Fig. 1. Schematischer Aufbau der PVAL-p$(dC)_n$-DEAE-Cellulosen.

Rotationsverdampfer entfernt. Die wässrige Lösung, die *ca.* 85,000 A_{260}-Einheiten* aufweist, wird an einer UM 2 Membran 3 Tage ultrafiltriert. Das Retentat, in dem dann noch *ca.* 51,000 A_{260}-Einheiten verbleiben, wird lyophilisiert und ergibt *ca.* 3.2 g eines gelben Pulvers.

Das Lyophilisat wird in 100 ml Wasser gelöst, mit 75 g, in Wasser gequollener DEAE-Cellulose, versetzt und langsam 12 h bei Raumtemperatur gerührt. Während der "Beschichtung" nimmt das Quellvolumen der DEAE-Cellulose stark zu. Anschliessend füllt man die erhaltene PVAL-p(dC)$_n$-DEAE-Cellulose in die zuverwendende Säule, die danach mit 1 *M* NaCl so lange eluiert wird, bis die Absorption unter 0.1 A_{260}-Einheiten fällt. Hierbei schrumpft die Säulenfüllung auf das Volumen der DEAE-Cellulose vor ihrer "Beschichtung". Von den zur "Beschichtung" eingesetzten 51,000 A_{260}-Einheiten verbleiben nach der Elution *ca.* 10,000, die unter den Bedingungen der Template-Chromatographie irreversibel gebunden bleiben.

(B) Säulenchromatographische Fraktionierung der höhermolekularen DNA-Fragmente an QAE-Sephadex A-25. Auf dem früher beschriebenen Weg[1] wird 1 kg Heringsspermen-DNA zu Oligoguanosinphosphaten partialhydrolysiert, die an DEAE-Cellulose säulenchromatographisch in nieder- und höhermolekulare Fragmente vorgetrennt werden. Hierbei erhält man *ca.* 40 g der höhermolekularen Fragmente, die für die folgende Fraktionierung in 5 l Wasser gelöst, auf eine QAE-Sephadex A-25 Säule (55 × 6.5 cm I.D.) mit *ca.* 400 ml/h gepumpt werden. Die Säule ist mit 0.05 *M* Tris–HCl pH 7.5 äquilibriert. Nach dem Auftragen der Lösung wird bei einer Laufgeschwindigkeit von *ca.* 700 ml/h zunächst mit 8 l Wasser, dann im dreistufigen NaCl-Gradienten, der mit 0.05 *M* Tris–HCl (pH 7.5) gepuffert ist, wie folgt eluiert: (1) 10 l 0.3 *M* NaCl; (2) 10 l 0.5 *M* NaCl; (3) 3 l 2.0 *M* NaCl. Die Produkte der 0.3-, 0.5- und 2-*M* Fraktion werden am Rotationsverdampfer konzentriert und anschliessend an einer UM 2 Membran so lange ultrafiltriert, bis das Eluat mit Silbernitrat keine Trübung zeigt. Die salzfreien Retentate werden lyophilisiert, wobei von der 0.3-*M* Fraktion *ca.* 26 g, von der 0.5-*M* Fraktion *ca.* 11 g und von der 2-*M* Fraktion *ca.* 1 g Lyophilisat erhalten werden. Zur weiteren Aufarbeitung wird im folgenden nur das Lyophilisat der 0.5-*M* Fraktion verwendet.

(C) Template-Chromatographie des Lyophilisats der 0.5-M Fraktion aus (B) an den PVAL-p(dC)$_n$-DEAE-Cellulosen (vgl. Fig. 2). Die zur Template-Chromatographie verwendeten PVAL-p(dC)$_n$-DEAE-Cellulose Säulen (40 × 2 cm I.D.) sind mit Kühlmänteln versehen, die mit einem Thermostaten verbunden sind. Die verschiedenen Proben (vgl. Tabelle I) werden in 1–10 ml Elutionspuffer gelöst und bei 30°C auf die Säule aufgetragen. Nachdem die Probenlösung in das Gelbett eingezogen ist, wird die Säule auf 0°C gekühlt und dann so lange bei dieser Temperatur eluiert, bis die Absorption des Eluats nach einem steilen Anstieg (Peak I der Fig. 2) auf *ca.* 0.1 A_{260}-Einheiten fällt. Dieser Wert wird nach 1.0–1.5 l erreicht, wobei der Durchfluss auf *ca.* 50 ml/h eingestellt wird. Die Elution erfolgt in Fig. 2a mit 0.5 *M* NaCl, 0.01 *M* Na_2HPO_4 (pH 7); in Fig. 2b zuerst mit 0.5 *M* NaCl, 0.01 *M* Natriumacetat (pH 5), dann mit 0.5 *M* NaCl, 0.01 *M* Tris–HCl (pH 9). Unter diesen Bedingungen verlässt in Fig. 2b nach Peak I eine zweite Fraktion (Peak Ia) die Säule, wozu nochmals *ca.* 1.0 l Elutionspuffer benötigt werden. Nachdem Peak I bzw. I und Ia

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* A_{260}-Einheit = Nucleotid-Menge in 1 cm^3 Solvens, die bei 260 nm die Absorption 1 ergibt (Schichtdicke 1 cm).

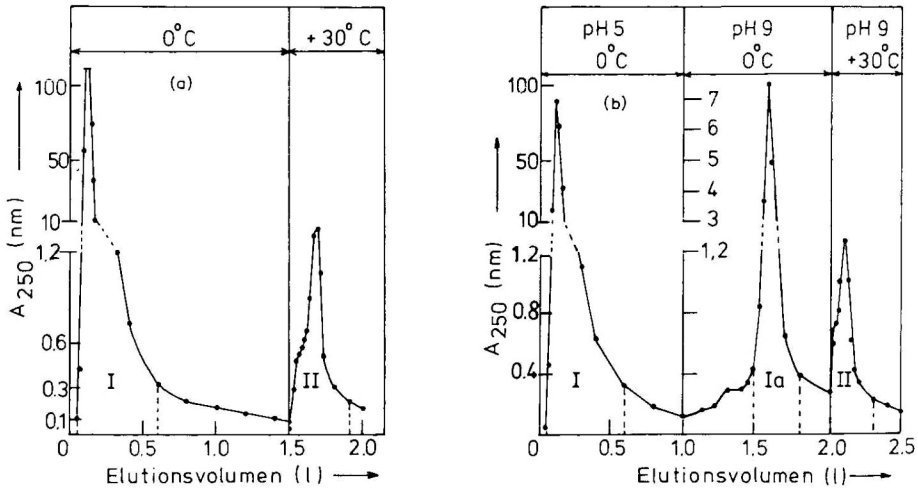

Fig. 2. Elutionsprofile der Template-Chromatographie von jeweils 130 mg (2800 A_{250}-Einheiten) einer Oligoguanosinphosphatmischung (0.5-*M* Fraktion) an PVAL-p(dC)$_n$-DEAE-Cellulose (Typ A). Säulenmasse: 40 × 2 cm I.D. Fliessgeschwindigkeit *ca.* 50 ml/h. Die Säulen wurden im zweistufigen Temperaturgradienten (0 und 30°C) eluiert. Die Elution erfolgt in (a) mit 0.5 *M* NaCl, 0.01 *M* Na_2HPO_4 (pH 7). In (b) wird zunächst mit 0.5 *M* NaCl, 0.01 *M* Natriumacetat (pH 5) eluiert. Nachdem Peak I die Säule verlassen hat, wird die Elution mit 0.5 *M* NaCl, 0.01 *M* Tris–HCl (pH 9) fortgesetzt. Fraktionen innerhalb der senkrechten Strichelung werden vereinigt und deren Produkte isoliert.

eluiert sind, stoppt man die Elution und erwärmt die Säule auf 30°C. Bei dieser Temperatur wird die Elution dann fortgesetzt, bis die Absorption nach einem 2. Anstieg (Peak II der Fig. 2a) bzw. nach einem 3. Anstieg (Peak II der Fig. 2b) wieder auf 0.1 A_{260}-Einheiten gesunken ist. Dieser Wert ist nach *ca.* 0.5 l erreicht. Die Elution bei 30°C erfolgt in Fig. 2a weiterhin mit 0.5 *M* NaCl–0.01 *M* Na_2HPO_4 (pH 7), während in Fig. 2b 0.5 *M* NaCl–(0.01 *M* Tris–HCl) (pH 9) als Elutionspuffer verwendet wird. Fraktionen zu 20 ml werden gesammelt. Die Elution wird automatisch bei 254 nm registriert. Zusätzlich werden die Absorptionen einiger Fraktionen photometrisch bei 250, 260 und 280 nm nachgemessen. Die Auftragung der bei 250 nm erhaltenen Messwerte gegen das Elutionsvolumen führt zu den Elutionsprofilen der Figuren. Die Fraktionen von Peak I, Ia und II werden innerhalb der senkrechten Strichelung vereinigt, an einer UM 2 Membran entsalzt und anschliessend lyophilisiert. Die Lyophilisate von Peak Ia und II aller Läufe werden vereinigt. Zur Isolierung von Einzelsubstanzen werden bspw. 50 mg (1700 A_{250}-Einheiten) der vereinigten Lyophilisate von Peak Ia und II zur Entfernung terminaler Phosphatgruppen mit alkalischer Phosphatase behandelt[5]. Anschliessend wird der Reaktionsansatz in *ca.* 30 ml 7 *M* Harnstoff aufgenommen und wie folgt an einer QAE-Sephadex Säule fraktioniert.

(D) Säulenchromatographische Fraktionierung des dephosphorylierten Lyophilisats von Peak Ia, II der Fig. 2 an QAE-Sephadex A-25 (vgl. Fig. 3). Die enzymatisch dephosphorylierten Oligoguanosinphosphate (*ca.* 1700 A_{250}-Einheiten) aus (C) werden in 7 *M* Harnstoff auf eine mit 0.1 *M* NaCl, 0.05 *M* Tris–HCl (pH 7.5), 7 *M* Harnstoff äquilibrierte QAE-Sephadex-Säule (40 cm × 2 cm I.D.) aufgetragen. Die Säule wird bei Raumtemperatur im linear steigenden NaCl-Gradienten eluiert: 2 l 0.1 *M* NaCl, 0.05 *M* Tris–HCl (pH 7.5), 7 *M* Harnstoff im Mischgefäss; 2 l 0.5 *M* NaCl,

TABELLE I

BEDINGUNGEN UND ERGEBNISSE DER TEMPLATE-CHROMATOGRAPHIE VON OLIGOGUANOSINPHOSPHATEN AN PVAL-p(dC)$_n$-DEAE-CELLULOSEN (VGL. FIG. 2)

Säulenfüllung Typ	*Aufgetragene Probenmenge*		*Elutionsbedingungen*		*Eluierte Mengen*		%
	mg	A_{250}-*Einh.*	*Puffer**	*Temperatur (°C)*	*Peak Nr.*	A_{250}-*Einh.*	
A	65	1400	1	0	I	1240	88.6
			1	+ 30	II	130	9.3
B	65	1400	1	0	I	1280	91.4
			1	+ 30	II	100	7.1
A	130	2800	1	0	I**	2490	88.9
			1	+ 30	II**	260	9.3
B	130	2800	1	0	I	2580	92.1
			1	+ 30	II	190	6.8
A	260	5600	1	0	I	4980	88.9
			1	+ 30	II	510	9.1
B	260	5600	1	0	I	5090	90.9
			1	+ 30	II	390	7.0
A	520	10800	1	0	I	10020	92.8
			1	+ 30	II	710	6.6
B	520	10800	1	0	I	10170	94.1
			1	+ 30	II	530	4.9
A	780	15800	1	0	I	14800	93.7
			1	+ 30	II	920	5.8
B	780	15800	1	0	I	14980	94.8
			1	+ 30	II	720	4.6
A	130	2800	2	0	I***	1750	62.5
			3	0	Ia***	680	24.2
			3	+ 30	II***	290	10.4

* 1 = 0.5 *M* NaCl, 0.01 *M* Na_2HPO_4 pH 7; 2 = 0.5 *M* NaCl, 0.01 *M* Natriumacetat (pH 5); 3 = 0.5 *M* NaCl, 0.01 *M* Tris–HCl (pH 9).

** Siehe Fig. 2a.

*** Siehe Fig. 2b.

0.05 *M* Tris–HCl (pH 7.5), 7 *M* Harnstoff im Vorratsgefäss. Die Elutionsgeschwindigkeit wird mit einer Schlauchpumpe auf *ca.* 300 ml/h eingestellt. Nach dem Gradienten wird die Säule mit *ca.* 400 ml 1 *M* NaCl eluiert. Fraktionen zu 20 ml werden gesammelt, die von Peak a–f der Fig. 3 innerhalb der senkrechten Strichelung vereinigt, an einer UM 2 Membran entsalzt und anschliessend lyophilisiert. Die Ergebnisse sind in Tabelle II zusammengefasst.

(E) Hochdruckflüssigkeitschromatographie (HPLC) der enzymatisch hydrolysierten Oligoguanosinphosphate aus Peak a–f der Fig. 3. *Ca.* 1 A_{260}-Einheit des jeweiligen Oligoguanosinphosphats wird in 20 µl Wasser, 5 µl 1 *M* Tris–HCl (pH 8.1), 5 µl 0.1 *M* $MgCl_2$ gelöst und mit *ca.* 5 µl der käuflichen Phosphodiesterase aus Schlangengift 6 h bei 37°C inkubiert. Anschliessend wird der Reaktionsansatz an einer RP-18 5 µm-Säule (250 × 4.6 mm) mittels eines Spectra-Physics SP 8000 Hochdruckflüssigkeitschromatographen mit folgendem linearen Gradienten bei Raumtemperatur fraktioniert. Lösung A = 0.01 *M* KH_2PO_4 (pH 5–6); Lösung B = 60% Methanol, 40% Wasser. Innerhalb von 20 min geht A in 60% A, 40%

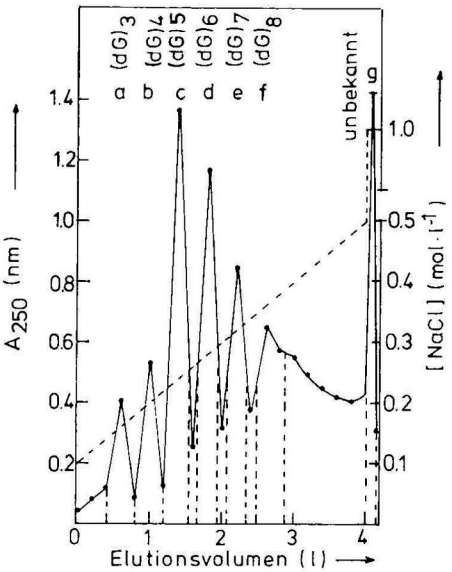

Fig. 3. Elutionsprofil der säulenchromatographischen Nachtrennung von *ca.* 1700 A_{250}-Einheiten einer dephosphorylierten Oligoguanosinphosphatmischung (Peak Ia, II der Fig. 2) an QAE-Sephadex A-25. Säulenfüllung: 40 × 2 cm I.D. Die Säule wird bei Raumtemperatur im steigenden NaCl-Gradienten eluiert, der mit 0.05 *M* Tris–HCl auf pH 7.5 gepuffert und mit 7 *M* Harnstoff versetzt ist. Fliessgeschwindigkeit *ca.* 300 ml/h. Ergebnisse siehe Tabelle II.

B über. Danach wird die Elution isokratisch mit 60% A, 40% B fortgesetzt. Der Durchfluss beträgt 1 ml/min. Die Retentionszeiten stimmen mit denen von käuflichem dG und pdG überein und liegen für pdG bei 9.7 min, für dG bei *ca.* 20 min.

(F) Homochromatographie und Fingerprint der ^{32}P-markierten Oligoguanosinphosphate. Die 5'-Hydroxylgruppe des jeweiligen Oligoguanosinphosphats (0.05–0.1 A_{260}-Einheiten) wird mit Hilfe von [γ-^{32}P]ATP und T_4 Polynucleotid-Kinase enzymatisch phosphoryliert[12]. Anschliessend wird der Enzymansatz auf eine Dünnschicht-Fertigfolie (Polygram, Cel 300 DEAE/HR-2/15; Macherey und Nagel) aufgetragen. Die Platte wird in einem für zwölfer Oligonucleotide bereiteten Partialhydrolysat einer RNA (Homomix)[12] bei 60°C aufsteigend *ca.* 6–12 h chromatographiert. Die Homochromatographie wird beendet, wenn der blaue Farbstoff einer Farbstoffmischung (1% Xylen Cyanol FF, 1% Säurefuchsin, 1% Methylorange in Wasser) *ca.* 3/4 der Laufstrecke gewandert ist. Die durch Autoradiographie sichtbar gemachten markierten Oligoguanosinphosphate werden eluiert und anschliessend mit Phosphodiesterase aus Schlangengift partialhydrolysiert. Zur Bestimmung der Kettenlänge wird die eine Hälfte des Partialhydrolysats erneut unter den obigen Bedingungen der Homochromatographie unterzogen.

Zur Sequenzierung der markierten Oligoguanosinphosphate wird die zweite Hälfte der partialhydrolysierten Oligoguanosinphosphate zweidimensional chromatographiert (Fingerprint). In der 1. Dimension erfolgt die Auftrennung des Partialhydrolysats auf Celluloseacetatstreifen (*ca.* 30 × 550 mm, Schleicher und Schüll) mittels Hochspannungselektrophorese. Die Elektrophorese wird in Pyridin–Eisessig–Wasser

TABELLE II

ERGEBNISSE DER SÄULENCHROMATOGRAPHISCHEN NACHTRENNUNG VON OLIGOGUANOSINPHOSPHATEN AN QAE-SEPHADEX (VGL. FIG. 3), DIE AN DEN PVAL-p(dC)$_n$-DEAE-CELLULOSEN HYBRIDISIERT UND ANSCHLIESSEND DEPHOSPHORYLIERT WERDEN

Es werden *ca.* 1700 A_{250}-Einheiten fraktioniert.

Peak	*Eluiert bei Natriumchlorid-Konz. (M)*	*Eluierte Mengen*		*Bezeichnung*	R_F*-Werte* relativ zu dem von pdG*
		A_{250}*-Einh.*	*%*		
a	0.14–0.18	86	5.1	$(dG)_3$	0.70
b	0.18–0.24	112	6.6	$(dG)_4$	0.62
c	0.24–0.25	276	16.2	$(dG)_5$	0.36
d	0.26–0.29	228	13.4	$(dG)_6$	0.26
e	0.31–0.34	176	10.4	$(dG)_7$	0.18
f	0.35–0.38	115	6.8	$(dG)_8$	0.09
g	1.00	295	17.4	unbekannt	Bande

* Papier: Schleicher & Schüll Chromatographiepapier 2316, absteigend. Laufmittel: 1-Propanol–konz. NH_4OH–Wasser (55:10:35, v/v/v).

Fig. 4. Autoradiogramm der ^{32}P markierten, partialhydrolysierten Oligoguanosinphosphate aus Peak f der Fig. 3. Die Auftrennung des Partialhydrolysats erfolgt in der 1. Dimension durch Elektrophorese bei pH 3.5, in der 2. Dimension durch Homochromatographie. Der Fingerprint bestätigt die erwartete Sequenz $(dG)_8$.

(1:10:189, v/v/v) pH 3.5 bei 1000 V begonnen. Wenn die erste Trennung des als Marker aufgetragenen obigen Farbstoffgemisches erkennbar ist, wird die Spannung auf 3000 V erhöht. Die Elektrophorese wird abgebrochen, sobald der rote Säurefuchsinfarbstoff etwa die Hälfte der Laufstrecke erreicht hat. Je nach der Oligonucleotidsequenz muss die optimale Laufstrecke von Fall zu Fall empirisch ermittelt werden. Die in der Elektrophorese getrennten Substanzen werden anschliessend vom Celluloseacetatstreifen auf eine Dünnschichtfertigfolie übertragen, indem der aufgelegte Streifen mit feuchtem Papier überschichtet und so eluiert wird. Die auf die Dünnschichtfolie übertragenen Produkte werden dann unter den oben angegebenen Bedingungen homochromatographiert. Das Autoradiogramm (Fingerprint) des zweidimensional chromatographierten Partialhydrolysats von Peak f der Fig. 3 ist in Fig. 4 abgebildet.

ERGEBNISSE

DNA aus Heringsspermen wird nach dem bereits früher beschriebenen Verfahren[1], das in Schema I zusammengefasst ist, durch Hydrazinolyse zunächst depyrimidiniert und dann durch alkalische Hydrolyse selektiv zu Oligoguanosinphosphaten abgebaut.

DNA (100 g)
(1) ↓ (a) Hydraziniumhydrat, 4 h, 60°C
↓ (b) Ultrafiltration
Depyrimidinierte DNA (≈37 g)
(2) ↓ (a) 7 *M* KOH, 1 h, 100°C
↓ (b) Neutralisation ($HClO_4$)
↓ (c) Ultrafiltration
Oligoguanosinphosphatgemisch (≈ 14 g)

Schema I. Chemischer abbau einer DNA zu oligoguanosinphosphaten.

Aus dem erhaltenen Oligoguanosinphosphatgemisch können nach dem in Fig. 5 aufgeführten Weg Einzelsubstanzen der Reihe $(dG)_{3-8}$ in präparativen Mengen isoliert werden.

Zunächst wird der grosse Überschuss der niedermolekularen Verbindungen an DEAE-Cellulose säulenchromatographisch im zweistufigen NaCl-Gradienten von den höhermolekularen DNA-Fragmenten weitgehend abgetrennt. Die höhermolekularen Fragmente werden dann an QAE-Sephadex mit einem dreistufigen NaCl-Gradienten (0.3, 0.5, 2.0 *M* NaCl) in 3 Fraktionen getrennt. Die 0.3-*M* und 2.0-*M* Fraktion werden in der vorliegenden Arbeit nicht näher untersucht. Zur Isolierung der Oligoguanosinphosphate wird ausschliesslich die 0.5-*M* Fraktion verwendet. Von dieser Fraktion verbleiben nach Entsalzen und Lyophilisieren *ca.* 1.1 g, wenn *ca.* 100 g DNA aus Heringsspermen der Partialhydrolyse unterworfen und auf dem beschriebenen Weg fraktioniert werden. Die 0.5-*M* Fraktion, die zu *ca.* zweidrittel unerwünschte Spaltprodukte und teilweise abgebaute Oligoguanosinphosphate aufweist, kann mit Hilfe der Template-Chromatographie[2] aufgetrennt werden, während herkömmliche Trennverfahren versagen. Nach diesem Trennschritt, in dem hybridisierende von nicht hybridisierenden DNA-Fragmenten über den Basenpaarungsmechanismus getrennt werden, wird das erhaltene Gemisch der hybridisierenden Oligogua-

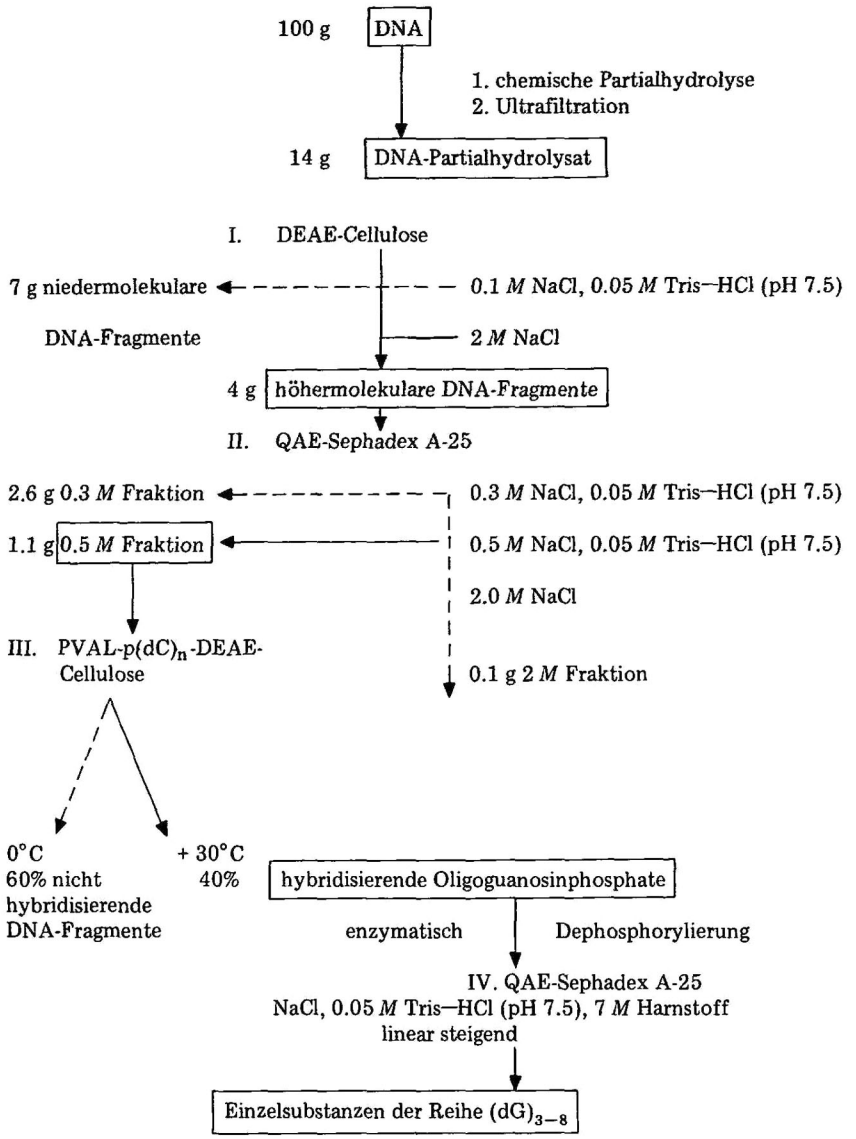

Fig. 5. Trennungsgang zur Isolierung von Oligoguanosinphosphaten der Reihe $(dG)_{3-8}$ aus 100 g partialhydrolysierter Heringsspermen-DNA.

nosinphosphate enzymatisch dephosphoryliert[5]. Das Gemisch kann anschliessend mühelos an QAE-Sephadex in Einzelsubstanzen der Reihe $(dG)_{3-8}$ fraktioniert werden.

Die Template-Chromatographie erfolgt an den von uns synthetisierten PVAL-$p(dC)_n$-DEAE-Cellulosen. Bei diesem Trennmaterial, dessen Aufbau in Fig. 1 schematisch dargestellt ist, handelt es sich um käufliche DEAE-Cellulose, die mit kovalent an PVAL gebundenen, synthetischen Oligomeren der Desoxyribocytidylsäure $p(dC)_n$ "beschichtet" ist. Ionische Kräfte und Wasserstoffbrückenbindungen, die sich

zwischen den polymergebundenen Oligonucleotiden und den funktionellen Gruppen der DEAE-Cellulose-Matrix zustande kommen, reichen aus, um die "Oligonucleotidbeschichtung" unter den angewendeten chromatographischen Bedingungen aufrecht zuerhalten.

Zur Template-Chromatographie werden zwei unterschiedliche Typen (A, B) der PVAL-p(dC)$_n$-DEAE-Cellulose verwendet. Die Darstellung von Typ A erfolgt in einer dreistufigen Synthese[3]. Zunächst werden nach der Diestermethode durch Polykondensation von N-Anisoyl-desoxycytidin-5'-phosphat geschützte Oligomere der Desoxyribocytidylsäure dargestellt. Die Homologen mit vier und mehr Monomereinheiten werden im zweiten Reaktionsschritt über ihre 5'-Phosphatgruppen an freie Hydroxylgruppen von PVAL kondensiert. Nach der ammoniakalischen Entfernung der Nucleobasenschutzgruppen werden im dritten Reaktionsschritt die polymergebundenen Oligodesoxycytidylsäuren an DEAE-Cellulose irreversibel adsorbiert. Der dreistufige Syntheseweg gewährleistet die Immobilisierung längerkettiger Desoxyribocytidylsäuren, da kurzkettige Fragmente an mehreren Stellen der Synthese ausgesondert werden. Typ B der PVAL-p(dC)$_n$-DEAE-Cellulose wird in Analogie zur Darstellung der Oligonucleotid-Cellulosen[6–9] in einer zweistufigen Synthese dargestellt. Hierzu wird im 1. Schritt N-Anisoyl-desoxycytidin-5'-phosphat nach der Diestermethode in Anwesenheit von PVAL polykondensiert. Im 2. Schritt werden die polymergebundenen Oligodesoxycytidylsäuren nach Entfernung der Basenschutzgruppen, wie bei Typ A ausgeführt, an die DEAE-Cellulose fixiert. Die Darstellung von Typ B ist zwar einfacher durchzuführen, hat aber den Nachteil, dass vor allem mono-trimere Oligocytidylsäuren immobilisiert werden, da längerkettige Cytidylsäuren bei der Polykondensation nur in geringen Mengen gebildet werden. In der zur Füllung einer Säule (40 × 2 cm I.D.) verwendeten PVAL-p(dC)$_n$-DEAE-Cellulose sind bei Typ A *ca.* 6000 A_{260}-Einheiten Oligocytidylsäuren mit 4 bis vermutlich 12 Monomereinheiten immobilisiert, während Typ B in der gleichen Menge *ca.* 10,000 A_{260}-Einheiten an Oligocytidylsäuren mit 1 bis vermutlich 12 Monomereinheiten aufweist, wobei der Anteil der Mono-Trimeren sicherlich über 50% liegt. Mit Hilfe der unterschiedlich derivatisierten PVAL-p(dC)$_n$-DEAE-Cellulosen wird untersucht, wie sich die Beladung und der erhöhte Anteil längerkettiger immobilisierter Oligocytidylsäuren auf die Trenneigenschaften auswirken. Da im Partialhydrolysat einer Heringsspermen-DNA Oligoguanosinphosphate mit über 8 Monomereinheiten in präparativen Mengen vermutlich nicht auftreten, wird die 0.5-*M* Fraktion im volgenden zweistufigen Temperatur-Gradienten an den PVAL-p(dC)$_n$-DEAE-Cellulosen fraktioniert. Zur Adsorption von Oligomeren mit 3 und mehr Monomereinheiten wird die Mischung bei 0°C aufgetragen. Zur Desorption der hybridisierten Oligomeren wird die Säule bei 30°C eluiert, da basengepaarte Oktamere in diesem Temperaturbereich "schmelzen".

Ein zweistufiger Temperaturgradient hat gegenüber mehrstufigen oder linear ansteigenden Temperaturgradienten, die bisweilen in der Template-Chromatographie angewendet werden[6–9], verschiedene Vorteile. Die thermisch labilen DNA-Fragmente werden nur kurzfristig belastet und verlassen in einem kleinen, schnell zu entsalzenden Volumen die Säule. Das erhaltene Gemisch lässt sich anschliessend an Anionenaustauschern im steigenden Salzgradienten wesentlich effektiver in Einzelsubstanzen fraktionieren, als dies in der Template-Chromatographie mit einem langsam steigenden Temperaturgradienten möglich ist.

Zur Template-Chromatographie wird die jeweilige Menge des Lyophilisats der 0.5-*M* Fraktion, von dem bis zu 780 mg pro Lauf chromatographiert werden (vgl. Tabelle I), im Elutionspuffer gelöst auf die 30°C warme Säulenfüllung aufgetragen. Dann kühlt man die Säule auf 0°C und eluiert im 1. Schritt alle DNA-Fragmente, die keine Basenpaarung mit den immobilisierten Oligocytidylsäuren eingehen. Oligoguanosinphosphate, die bei 0°C basengepaart sind, verlassen im 2. Schritt bei 30°C gemeinsam die Säule, da bei dieser Temperatur die Basenpaarung zwischen komplementären Tri-Octanucleotiden aufgehoben wird. Der Elutionsvorgang aller Läufe wird photometrisch verfolgt. Da sich die Elutionsprofile nicht prinzipiell unterscheiden, ist als stellvertretendes Beispiel in Fig. 2a das Elutionsprofil der Trennung abgebildet, in der 130 mg des Lyophilisats chromatographiert werden.

Der Anteil der hybridisierten DNA-Fragmente steigt mit der Erhöhung der aufgetragenen Lyophilisatmenge unterschiedlich. Im Bereich von 65–260 mg (1400–5600 A_{250}-Einheiten) nimmt die Menge an adsorbierten Oligoguanosinphosphaten proportional mit der aufgetragenen Lyophilisatmengen zu, da jeweils *ca.* 9% bei 30°C in Peak II eluiert werden. Bei weiterer Erhöhung der aufgetragenen Menge werden zwar noch mehr, prozentual aber weniger A_{250}-Einheiten adsorbiert. Aus der Menge der adsorbierten Oligoguanosinphoshate lässt sich abschätzen, dass die verwendete Gelmenge von Typ A bis zu 1000 A_{250}-Einheiten über ihre immobilisierten Oligocytidylsäuren adsorbiert. Die höhere Kapazität der PVAL-p(dC)$_n$-DEAE-Cellulose von Typ A entspricht dem bekannten Befund, dass längerkettige Oligonucleotide die Basenpaarung begünstigen, während immobiliserten Mono- und Dimere nur unwesentlich daran beteiligt sind. Obwohl Typ A der PVAL-p(dC)$_n$-DEAE-Cellulose im Vergleich zu Typ B eine deutlich höhere Kapazität aufweist, ist die aufwendigere Synthese hiermit kaum zu rechtfertigen.

Bei der chemischen Partialhydrolyse einer DNA werden bevorzugt Fragmente mit terminalen Phosphatgruppen gebildet, die eine Hybridisierung der kurzen Oligomeren erschweren oder gar verhindern. Da in Peak I kurze Oligoguanosinphosphate mit terminalen Phosphatgruppen zu vermuten sind, wird das Lyophilisat von Peak I mit alkalischer Phosphatase zur Entfernung terminaler Phosphatgruppen inkubiert[5] und erneut der Template-Chromatographie unterworfen. Von bspw. 2800 A_{250}-Einheiten des enzymatisch behandelten Lyophilisats werden an Typ A der PVAL-p(dC)$_n$-DEAE *ca.* 800 A_{250}-Einheiten adsorbiert, während vor der Phosphatasebehandlung im gleichen Lyophilisat keine hybridisierenden Produkte mehr gefunden werden. Aus dem Lyophilisat der 0.5-*M* Fraktion sind *ca.* 9% hybridisierende Oligoguanosinphosphate direkt erhältlich, während weitere *ca.* 30% erst nach enzymatischer Dephosphorylierung hybridisieren. Die restlichen 60% sind nicht näher bestimmbare DNA-Fragmente, die im Molekülverband vermutlich so viele zerstörte Nucleobasen aufweisen, dass eine ausreichend stabile Basenpaarung verhindert wird. Da sich diese Fragmente aufgrund ihrer intakten Polymerhauptkette in ihrer Gesamtladung allenfalls geringfügig von gleichlangen hybridisierenden Oligoguanosinphosphaten unterscheiden, ist es naheliegend, dass mit herkömmlichen chromatographischen Methoden (bspw. Ionenaustauschern) die präparative Auftrennung der 0.5-*M* Fraktion nicht gelingt.

Bei der Wechselwirkung von Poly(G) mit Oligo(C) in Lösung werden, in Abhängigkeit vom pH-Wert, neben den komplementären G · C Assoziaten, die im Sinne von Watson und Crick basengepaart sind, auch Assoziate von 2 C · G oder 2 G · C

beobachtet[10,11]. Zur Prüfung, ob derartige Komplexe auch während der Template-Chromatographie auftreten, wird der Einfluss des pH-Wertes auf die Trenneigenschaften der PVAL-p(dC)$_n$-DEAE-Cellulosen untersucht. Hierzu werden bspw. 130 mg (*ca.* 2800 A_{250}-Einheiten) des Lyophilisats der 0.5-*M* Fraktion auf die PVAL-p(dC)$_n$-DEAE-Cellulose (Typ A) bei 0°C aufgetragen. Anschliessend werden bei dieser Temperatur in Peak I der Fig. 2b mit 0.5 *M* NaCl, 0.01 *M* Natriumacetat (pH 5) nicht hybridisierende DNA-Fragmente eluiert. Im 2. Schritt, der ebenfalls bei 0°C erfolgt, werden mit 0.5 *M* NaCl, 0.01 *M* Tris–HCl (pH 9) in Peak Ia Oligoguanosinphosphate eluiert, die vermutlich bei pH 5 und 0°C mit den basengepaarten Oligonucleotiden höherkoordinierte Assoziate ausbilden, die bei pH 9 dissoziieren. Hierbei bleibt aber die Watson–Crick-Basenpaarung zwischen den Oligoguanosinphosphaten der mobilen und den Oligocytidylsäuren der stationären Phase im wesentlichen erhalten. Bei 30°C verlassen die restlichen Oligoguanosinphosphate die Säule, da dann die Basenpaarung vollständig aufgehoben ist. Die Mengen der bei 0°C, pH 9 und 30°C, pH 9 eluierten DNA-Fragmente (24 bzw. 10%) lassen keine eindeutige Aussage über die Koordination dieser pH abhängigen Assoziate zu. In Peak Ib und II werden in etwa die gleichen Produkte eluiert.

Zur Isolierung definierter Einzelsubstanzen werden die, in der Template-Chromatographie hybridisierenden Produkte aus Peak Ib bzw. II der Fig. 2 durch Ultrafiltration entsalzt und lyophilisiert. Bei der papierchromatographischen Untersuchung zeigt sich, dass in beiden Peaks im wesentlichen die gleichen Produkte enthalten sind. Die Lyophilisate von Peak Ia und II werden daher vereinigt und anschliessend an QAE-Sephadex fraktioniert. Zur Vereinfachung der Trennung werden vorher eventuell noch vorhandene terminale Phosphatgruppen der Oligoguanosinphosphate mit alkalischer Phosphatase enzymatisch entfernt. Hierbei resultieren Homologe der Reihe (dG)$_n$, die ohne Schwierigkeiten auf dem folgenden Weg an QAE-Sephadex A-25 in Einzelsubstanzen getrennt werden (vgl. Fig. 3, Tabelle II).

Die Lösung der dephosphorylierten Oligoguanosinphosphate (bspw. 1700 A_{250}-Einheiten) wird auf eine QAE-Sephadex A-25 Säule aufgetragen und bei Raumtemperatur im linear steigenden NaCl Gradienten eluiert, der mit Tris–HCl auf pH 7.5 gepuffert und mit 7 *M* Harnstoff versetzt ist. Unter diesen Bedingungen verlassen Peak a–f der Fig. 3 die Säule. Nach dem Gradienten werden mit 1 *M* NaCl restliche, nicht näher untersuchte Produkte in Peak g eluiert. Fraktionen innerhalb der senkrechten Strichelung werden vereinigt, die Menge darin enthaltener Oligoguanosinphosphate photometrisch bestimmt, durch Ultrafiltration entsalzt und lyophilisiert. Bei der Ultrafiltration können, vor allem bedingt durch eine Depolymerisation der Oligoguanosinphosphate, bis zu 20% verloren gehen. Bei kleineren Salzmengen empfiehlt es sich daher, die Entsalzung säulenchromatographisch bspw. an Sephadex G-10 durchzuführen. Die Trennergebnisse sind in Tabelle II zusammengefasst.

Zur Identifizierung der in Peak a–g eluierten Oligoguanosinphosphate werden Aliquote der Lyophilisate mit (dG)$_5$ als Referenz auf Papier chromatographiert. Die Lyophilisate von Peak a–e bilden einheitliche Flecken. Die Produkte aus Peak f wandern in einer teilweise aufgelösten Bande, während die von Peak g eine nicht aufgelöste Bande bilden und daher nicht näher untersucht werden. Aus den R_F-Werten (siehe Tabelle II), UV-Absorptionsverhältnissen und Ergebnissen der enzymatischen Hydrolyse mit Phosphodiesterase aus Schlangengift; Daten, die üblicherweise zur eindeutigen Identifizierung von homologen Oligonucleotiden ausreichen,

folgt, dass in Peak a–e nacheinander $(dG)_3$, $(dG)_4$, $(dG)_5$, $(dG)_6$ und $(dG)_7$ in chromatographisch reiner Form die Säule verlassen, während Peak f neben verschiedenen Verunreinigungen $(dG)_8$ als Hauptprodukt enthält. Die Verunreinigungen sind vermutlich Oligoguanosinphosphate, die in ihrem Molekülverband neben intakten auch in den Nucleobasen zerstörte Bereiche aufweisen. Für diese Annahme spricht, dass die UV-Absorptionsverhältnisse merklich von denen für Oligoguanosinphosphate entsprechenden Werten abweichen. Die teilweise zerstörten Oligoguanosinphosphate hybridisieren über ihre intakten Segmente mit den immobilisierten Oligocytidylsäuren und gelangen somit in die Fraktionen der hybridisierenden Oligoguanosinphosphate.

Zur Überprüfung der getroffenen Identifizierung werden mit den papierchromatographisch gereinigten Lyophilisaten von Peak a–f, die vom Papier eluiert werden, folgende zusätzliche Bestimmungen durchgeführt. Die Spaltprodukte, die bei der Hydrolyse der Oligoguanosinphosphate mit Phosphodiesterase aus Schlangengift anfallen, werden an einer RP-18-Säule mit Hilfe Hochdruckflüssigkeitschromatographie getrennt. Hierbei zeigt sich, dass über 99% der Hydrolysate aus pdG und dG bestehen und keine anderen Monomereinheiten nachweisbar aus den Oligoguanosinphosphaten freigesetzt werden.

Zur eindeutigen Bestimmung der Kettenlänge werden Aliquote der papierchromatographisch gereinigten Lyophilisate von Peak a–f mit T_4-Polynucleotidkinase in der 5'-Position mit Hilfe von [γ-^{32}P]ATP phosphoryliert und anschliessend der Homochromatographie unterzogen[12]. Die Autoradiogramme, die nach der Homochromatographie erhalten werden, bestätigen, dass in den Lyophilisaten von Peak a–e reine Oligoguanosinphosphate der Reihe $(dG)_{3-7}$ vorliegen, während Peak f ausser einem Hauptprodukt noch geringfügige Verunreinigungen ($< 5\%$) aufweist, die oberhalb und unterhalb des Hauptprodukts wandern. Der ^{32}P-markierte Hauptfleck im Homochromatogramm von Peak f wird zur Sequenzierung aus der Dünnschichtplatte isoliert, mit Phosphodiesterase aus Schlangengift partialhydrolysiert und anschliessend nach der Fingerprintmethode[13] zweidimensional chromatographiert. Das Autoradiogramm des Fingerprints (vgl. Fig. 4) bestätigt eindeutig, dass aus dem Lyophilisat von Peak f durch papierchromatographische Reinigung $(dG)_8$ erhalten wird.

Bei der hier beschriebenen, für den Labormassstab dimensionierten Methode, werden Oligoguanosinphosphate mit bis zu 8 Monomereinheiten in Mengen erhalten, mit denen in der chemisch-enzymatischen Gensynthese üblicherweise gearbeitet wird. Der Massstab lässt sich aber nach Belieben erweitern. Der Vorteil der Methode gegenüber der chemischen Synthese liegt vor allem darin, dass die Isolierung weitaus einfacher zu praktizieren ist, als der Syntheseweg. Der chemische Aufbau eines Oligonucleotids aus Monomereinheiten ist in der Regel Spezialisten vorbehalten. Falls die Synthese gelingt, steht in jedem Fall am Ende eine umfangreiche Reinigung bevor, in der das gewünschte Oligonucleotid von einer Vielzahl sehr ähnlicher Nebenprodukte mit falschen und/oder unvollständigen Sequenzen getrennt werden muss. Die chromatographische Isolierung als Alternative zur chemischen Synthese ist allerdings auf natürliche DNA-Sequenzen beschränkt, die einerseits gehäuft in einer DNA auftreten und andererseits unversehrt aus dem Molekülverband durch Partialhydrolyse freigesetzt werden können. In diesen Fällen stellt die Isolierung eine einfache Alternative zur chemischen Synthese dar.

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ZUSAMMENFASSUNG

DNA aus Heringsspermen wird chemisch zu Oligoguanosinphosphaten partialhydrolysiert. Aus dem Partialhydrolysat werden zunächst mit DEAE-Cellulose, dann mit QAE-Sephadex höhermolekulare Fragmente isoliert. Das Gemisch der höhermolekularen Oligoguanosinphosphate wird mit Hilfe der Template-Chromatographie an zwei unterschiedlich substituierten PVAL-p$(dC)_n$-DEAE-Cellulosen in hybridisierende und nicht hybridisierende Oligoguanosinphosphate getrennt. Einflüsse des pH-Wertes sowie der immobilisierten Oligocytidylsäuren auf die Ergebnisse der Template-Chromatographie werden untersucht. Die hybridisierenden Oligoguanosinphosphate werden enzymatisch dephosphoryliert, an QAE-Sephadex in Einzelsubstanzen der Reihe $(dG)_{3-8}$ fraktioniert und hierbei bis auf $(dG)_8$ chromatographisch rein in präparativen Mengen erhalten. $(dG)_8$ wird papierchromatographisch nachgetrennt und hierbei chromatographisch rein erhalten, was durch "Fingerprint" bestätigt wird.

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CONFIGURATION OF THE OLEFINIC BONDS IN THE HETEROOLEFINIC SIDE-CHAINS OF JAPANESE LACQUER URUSHIOL

SEPARATION AND IDENTIFICATION OF COMPONENTS OF DIMETHYLURUSHIOL BY MEANS OF REDUCTIVE OZONOLYSIS AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The configuration of the olefinic bonds in the heteroolefinic side-chains of Japanese lacquer urushiol components has been determined. Dimethylurushiol is resolved into ten components by combined high-performance liquid chromatography (HPLC) with Unisil QC_{18} and Hitachi 3043Ag gel columns due to the difference in the degree of unsaturation and in the *cis* and *trans* configuration. Each resolved di- or triolefinic dimethylurushiol component was partially reduced with hydrazine, and the monoolefinic side-chain dimethylurushiol formed was separated by HPLC on a Unisil QC_{18} gel column and submitted to reductive ozonolysis, followed by derivatization of the resulting aldehydes into the 2,4-dinitrophenylhydrazones. The hydrazones were identified by HPLC using a Hewlett-Packard 1084B chromatograph with a Develosil ODS-3 gel column.

INTRODUCTION

Since the appearance of the first paper on the constituents of the sap of lacquer trees (*Rhus vernicifera*)[1], much work has been carried out on the characterization of the urushiol not only in the sap of Japanese lacquer trees[2,3], but also in oak nuts[4] and ivy leaves[5,6]. Urushiol is now the subject of research related to its total synthesis[7–10], allergenic activity[11–13] and behaviour in the polymerization of Japanese lacquer[14–16].

After clarifying contradictory procedures and interpretations published in the past six decades, Majima[2], Sunthanker and Dawson[3] and Hashimoto and Minami[17,18] established that urushiol is composed of five components of 3-substituted catechol derivatives with pentadecyl, 8-pentadecenyl, 8,11-pentadecadienyl, 8,11,13-pentadecatrienyl and 8,11,14-pentadecatrienyl groups.

Of the possible configuration of the olefinic bonds in the side-chains of urushiol, only the *cis* configuration has been established for 3-(8′-pentadecenyl)catechol[3]. Difficulties occur in the separation of the heteroolefinic urushiol components.

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Progress has been made recently in the development of gels for use in high-performance liquid chromatography (HPLC). Some of the heteroolefinic urushiol components have since been resolved without chemical modification by HPLC on a μBondapak C_{18} gel column[19] and on a Hitachi 3053 gel column[20]. Despite the resolution of urushiol diacetate into 16 components by HPLC on a silver nitrate-coated LiChrosorb Si-60 gel column[21], the acetyl group of the diacetate hindered the observation of the IR bands characteristic of *cis*- and *trans*-olefinic arrangements of the side-chain.

The Unisil QC_{18} gel column is specific for the resolution of long-chain fatty acid esters owing to the differences in chain length, degree of unsaturation and position of the olefinic bond[22–24], and silver-containing silica gel is available for the separation of *cis*- and *trans*-isomers of olefins[25] by HPLC.

In this work, the configuration of the olefinic bonds in the side-chains of urushiol components has been determined by applying HPLC to the separation and identification of dimethylurushiol and related compounds. Chromatographically pure dimethylurushiol which had been obtained by gel permeation chromatography (GPC) was separated into heteroolefinic components of dimethylurushiol by HPLC on a Unisil QC_{18} gel column. The separated di- and triolefinic dimethylurushiols were reduced partially with hydrazine, and the monoolefinic *cis*- and *trans*-monoolefinic dimethylurushiol components formed were separated by consecutive HPLC on Unisil QC_{18} and Hitachi 3043Ag gel columns, subsequently being examined for the configuration and position of the olefinic bond by means of IR spectroscopy and reductive ozonolysis. HPLC has been applied successfully to the identification of 2,4-dinitrophenylhydrazones of the aldehydes formed in the reductive ozonolysis.

EXPERIMENTAL

Reagents

All of the solvents and reagents were of extra-pure grade, except methylene chloride used as the solvent for ozonolysis (spectrally pure grade), and were used without further purification.

Dimethylurushiol

Crude urushiol, obtained as the acetone-soluble part of the sap of lacquer trees (*Rhus vernicifera*), was treated with potassium carbonate[26] and chromatographically pure dimethylurushiol was separated from the oligomeric product by GPC (two TSK G2000HG packed columns, 60 × 2.2 cm I.D.; Toyo Soda, Tokyo, Japan; eluent, chloroform; flow-rate, 3.5 ml/min; loading of 0.7 g in 3 ml of chloroform).

Absorbents and column packing

Unisil QC_{18} gel (5 μm, 20% ODS loading; Gasukuro Kogyo, Tokyo, Japan), Hitachi 3043Ag gel (10 μm, 10% silver loading; Hitachi, Tokyo, Japan) and Develosil ODS-3 gel (3 μm, 20% loading; Nomura Chemical Co., Seto City, Aichi Prefecture, Japan) were used as received. They were packed in our laboratory into stainless-steel columns (25 cm × 8 or 7.6 mm I.D., 25 cm × 4 mm I.D. and 15 cm × 4.6 mm I.D.; Nihon Seimitsu, Tokyo, Japan) using hexanol-1–methylene chloride (1:1) as a slurry medium[27].

HPLC system

The preparative or analytical HPLC system for dimethylurushiol consisted of a Type SF-0369-57 pump (Milton-Roy, Philadelphia, PA, U.S.A.), gel-packed stainless-steel columns (see above), a 350 kg/cm^2 pressure gauge (Umetani Seiki), a Rheodyne Model 7125 20- or 500-μl syringe-loading sample injector, a Type UV log 5-III detector (Oyobunko, Tokyo, Japan) at 274 or 362 nm (λ_{max} of 2,4-dinitrophenylhydrazones of aliphatic aldehydes in acetonitrile) and a Type RI-2 refractive index (RI) detector (JAI, Tokyo, Japan).

For HPLC analysis of 2,4-dinitrophenylhydrazones of the aldehydes prepared from dimethylurushiol, a Model 1084B liquid chromatograph (Hewlett-Packard, Washington, DC, U.S.A.) equipped with a Develosil-3 gel column (15 cm × 4.6 mm I.D.) and a Hewlett-Packard variable-wavelength UV detector (operated at 362 nm) was used under the following conditions: volume injected, 5 μl; column pressure, 146 kg/cm^2; column temperature, 40°C; flow-rate, 1.20 ml/min. All samples were injected on to the column automatically with a gradient eluent from acetonitrile–water (55:45) to 100% acetonitrile for 12 min, and then with 100% acetonitrile for 2 min and acetonitrile–water (55:45) for 4 min to prepare the column for the next run.

Spectral measurements

A Hitachi RMU-6E mass spectrometer, a High Sens SM 401 UV spectrometer (Union Giken, Osaka, Japan) and an IRA-1 grating IR spectrometer (JASCO, Tokyo, Japan) were used.

Separation of dimethylurushiol components by HPLC

As can be seen in Fig. 1, dimethylurushiol is resolved into peaks 1–5 with a 100–150 mg loading in 0.5 ml of acetonitrile by reversed-phase HPLC on the Unisil QC_{18} gel column (25 cm × 8 mm I.D.), using acetonitrile as an eluent. The compounds in the peaks were obtained in the following yields: peak 1, 56.5 mg; 2, 9.5 mg; 3, 16 mg; 4, 1.3 mg; and 5, 4.2 mg.

By HPLC on the Hitachi 3043Ag gel column (25 cm × 7.6 mm I.D.), peak 1 in Fig. 1 was resolved into three peaks, 1-1 (1.1 mg), 1-2 (33 mg) and 1-3 (4.4 mg) (see Fig. 2a), using *n*-hexane–ethyl acetate (92.5:7.5) as the eluent at a flow-rate of 2.0 ml/min, peak 2 was resolved into peaks 2-1 (4.5 mg) and 2-2 (3.0 mg) (see Fig. 2b) and peak 3 was separated into peaks 3-1 (9.1 mg) and 3-2 (1.0 mg) (see Fig. 2c), using *n*-hexane–ethyl acetate (95:5) as the eluent.

The *m/e* values (M^+) for the parent ions obtained by mass spectrometry and IR and UV spectral data were obtained for the compounds in the HPLC peaks resulting from 100–150-mg loadings; the results are summarized in Table I.

Partial reduction with hydrazine of polyolefinic dimethylurushiol components and separation of the resulting monoolefinic dimethylurushiol components

No migration of olefinic bonds in reductive ozonolysis was established in a study with unsaturated fatty acids[28].

A mixture of the finally obtained tri- or diolefinic dimethylurushiol components (1–5 mg) and 5 ml of a 10% solution of hydrazine in ethanol, previously mixed with 0.01% of propyl gallate as an antioxidant, was stirred magnetically at 40°C until a maximum concentration of the monoolefinic dimethylurushiol component was

given by reversed-phase HPLC on the analytical Unisil QC_{18} gel column (25 cm × 4 mm I.D.), using acetonitrile as the eluent and an RI detector, and the peak of the monoolefinic dimethylurushiol component formed was fractionated on the Unisil QC_{18} gel column (25 cm × 8 mm I.D.) under the same conditions as for the separation of the heteroolefinic dimethylurushiol components. The compounds in the peak that had been obtained by removal of the volatile materials from the fraction was examined for IR bands in the range 900–1000 cm^{-1} and a UV spectrum was also obtained.

Reductive ozonolysis of monoolefinic side-chain dimethylurushiol and identification of 2,4-dinitrophenylhydrazones of the resulting aldehydes

Reductive ozonolysis was performed according to the Beroza and Bierl method[29]. Ozone was passed into a solution of the fractionated monoolefinic dimethylurushiol component in 1.0 ml of methylene chloride, cooled at −70°C until excess of ozone gas passing through in 5% potassium iodide–starch solution could be detected. Nitrogen was bubbled through the ozonide solution to replace oxygen and the solution was then allowed to react with triphenylphosphine (1.0 mg). The resulting solution was mixed with 1.0 ml of 2 *N* hydrochloric acid saturated with 2,4-dinitrophenylhydrazine, previously filtered with a Sartorius membrane filter (type SM 113), and stirred magnetically for 2 h at room temperature (the above filtration is necessary to remove compounds that interfere in the HPLC of the 2,4-dinitrophenylhydrazones derived from the aldehydes). The oily layer was separated from the aqueous layer, the former was evaporated to dryness at 35°C under vacuum, and the residue obtained was shaken with a mixture of 2 *N* hydrochloric acid (1.0 ml) and *n*-hexane (2.0 ml). The oily layer was separated from the aqueous layer and the former was evaporated to dryness to give the 2,4-dinitrophenylhydrazones as the residue.

Identification of 2,4-dinitrophenylhydrazones by HPLC

Standard 2,4-dinitrophenylhydrazones of normal C_1–C_{11} aliphatic aldehydes were prepared according to the method in the literature[30]. The observed melting points of these 2,4-dinitrophenylhydrazones (with literature[30,31] values in parentheses) are as follows: from formaldehyde, 168–169°C (166°C); acetaldehyde, 162–165°C (164–165°C); *n*-propionaldehyde, 148–149°C (142–148°C); *n*-butyraldehyde, 122°C (123°C); *n*-valeraldehyde, 107–110°C (104°C); *n*-caproldehyde, 107–108°C (104°C); *n*-heptaldehyde, 106–107°C (108°C); *n*-octylaldehyde, 106–107°C (106–110°C); *n*-nonylaldehyde, 108°C (106°C); *n*-capraldehyde, 103–104°C (104°C); and *n*-undecylaldehyde, 103–104°C.

Fig. 4 shows a chromatogram of a mixture of the standard 2,4-dinitrophenylhydrazones: the peaks appeared separately in order of increasing carbon number. Their retention times (minutes) and peak areas (as a percentage of the whole peak area) are indicated on each peak. HPLC was repeated three times for each standard mixture every 2 h. A high reproducibility was ascertained for the retention times, with a standard deviation of 0.01 min, and for the peak areas, with a maximum standard deviation of 1%, which is adequate for identification and quantitative analysis of the compounds in the peaks.

The 2,4-dinitrophenylhydrazones were dissolved in 1 ml of acetonitrile, and 5 μl of the solution were subjected to HPLC. In the chromatograms obtained, two

peaks with the same area should appear, corresponding to the equimolar formation of an aliphatic and a 2,3-dimethoxyphenyl-containing aldehyde from a monoolefinic side-chain dimethylurushiol. However, the experimental peak areas were not always identical, presumably owing to loss of the aldehyde or its incomplete derivatization into the corresponding 2,4-dinitrophenylhydrazone in the procedure. Further, the former peak appeared earlier than the latter, reflecting the greater hydrophilicity of the former in the gradient elution system used. These correlations are very informative for deciding whether a peak in HPLC is due to a major component or to a contaminant. In the chromatograms of the 2,4-dinitrophenylhydrazones, the retention time (minutes) and peak area (per cent) are indicated for each peak, and in the discussion, the retention time is first noted for the aliphatic aldehyde and then for its 2,3-dimethoxyphenyl-containing aldehyde in parentheses. The peaks with retention times of 0.75–0.81 and 1.42–4.45 min, which appeared in most of the chromatograms, were discarded, except for peaks 1-2-2-1 and 1-1-1, as their counter peaks did not appear. Moreover, peaks with peak areas of less than 1% were also neglected, although some of them might be meaningful.

RESULTS AND DISCUSSION

Chromatograms of the various compounds are shown in Figs. 1–5.

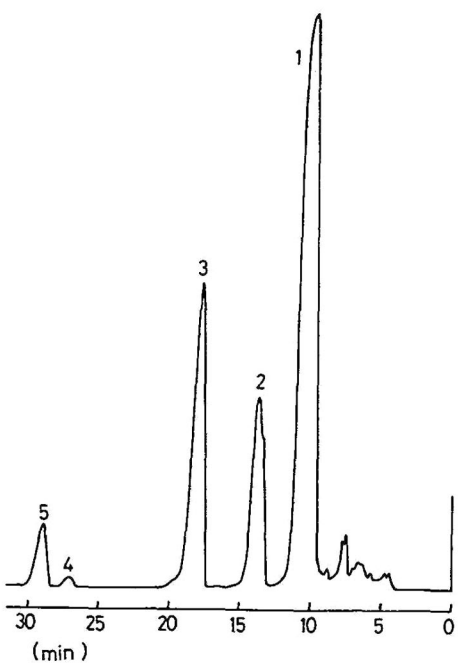

Fig. 1. Chromatograms of dimethylurushiol with acetonitrile as eluent. Column, Unisil QC_{18}, 5 μm, 25 cm × 8 mm I.D.); flow-rate, 2.0 ml/min; RI detector.

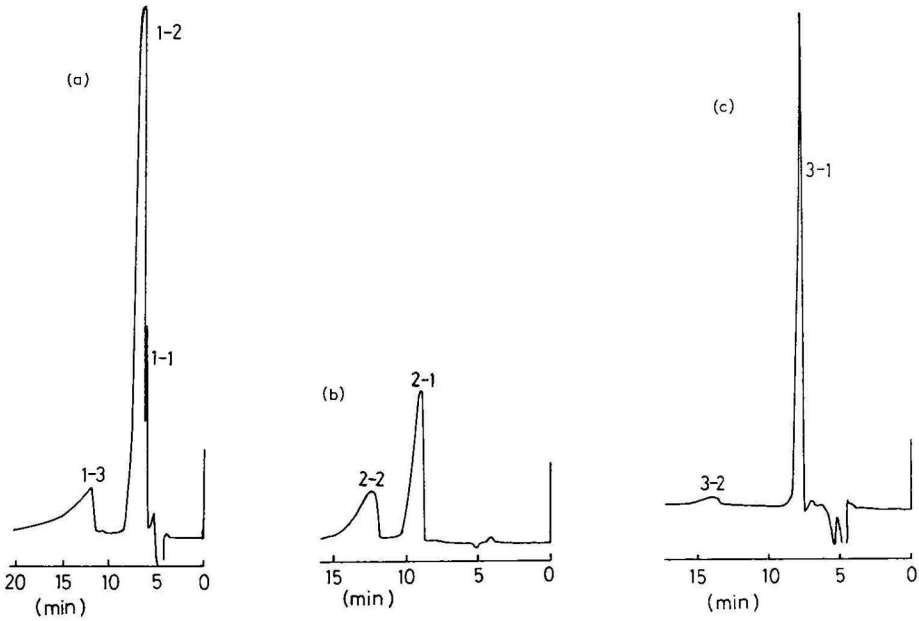

Fig. 2. Chromatograms of heteroolefinic dimethylurushiol (peak 1-3 in Fig. 1) with *n*-hexane–ethyl acetate (55:45) as eluent. Column, Hitachi 3043Ag, 10 μm, 25 cm × 7.6 mm I.D.; flow-rate, 2.0 ml/min; RI detector. (a) Peak 1; (b) peak 2; (c) peak 3.

Resolution of dimethylurushiol by HPLC

From the mass and IR spectral data in Table I, it is clear that, as can be seen in Fig. 1, dimethylurushiol is resolved into peaks 1–5 by HPLC on the Unisil QC_{18} gel column, using acetonitrile as an eluent, appearing in the order tri-, di- and mono-olefinic C_{15} side-chain dimethylurushiol components, C_{17} side-chain dimethylurushiol and 3-(pentadecyl)veratrole; also, each of the di- and triolefinic peaks is further resolved into two or three peaks owing to the difference in concentration of the olefin or chain length of dimethylurushiol on the Hitachi 3043Ag gel column, using *n*-hexane–ethyl acetate (95:5) as the eluent. It should be noted that peak 3 was resolved into 3-1 (pentadecenylveratrole) and 3-2 (heptadecadienylveratrole) (see Fig. 2c).

Constituents of Japanese lacquer urushiol

Carbon skeleton of Japanese lacquer urushiol

Each peak was hydrogenated made over palladium–charcoal in ethanol and a mixed melting point determination was made using an authentic sample. In this way, peak 1-1 in Fig. 2a and peak 3-1 in Fig. 2c were identified as 3-pentadecylveratrole (m.p. 35–36°C; lit.[3], 35–36°C), peak 3-2 in Fig. 2c and peak 4 in Fig. 1 as 3-heptadecylveratrole (m.p. 41–42°C; lit.32, 43–44°C) and peak 5 also as 3-pentadecylveratrole (m.p. 35–36°C).

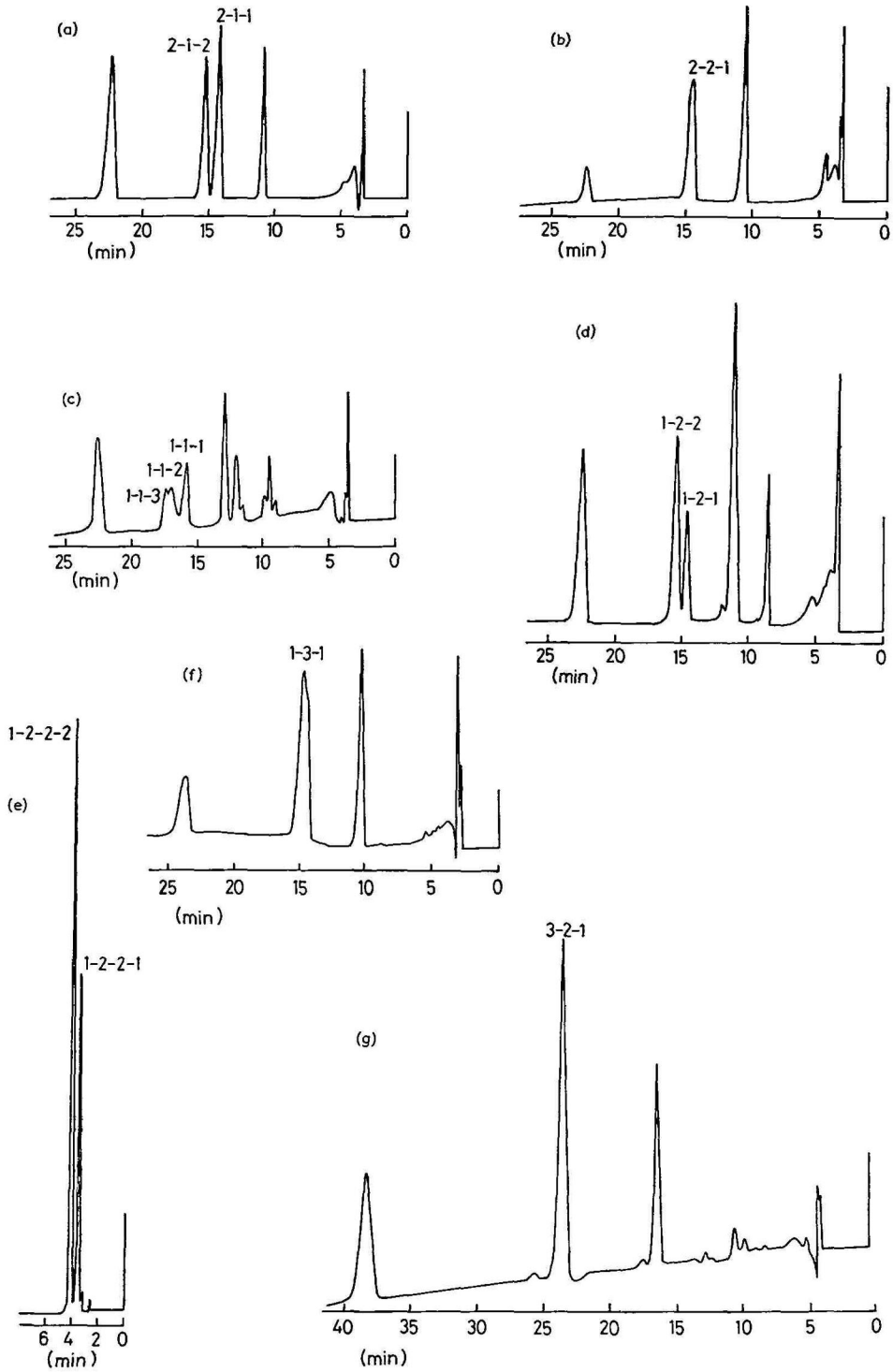

Fig. 3. Chromatograms of partially reduced di- or triolefinic dimethylurushiol of the following peaks: (a) 2-1; (b) 2-2; (c) 1-1; (d) 1-2; (e) 1-2-2; (f) 1-3; (g) 3-2. Conditions as in Fig. 1.

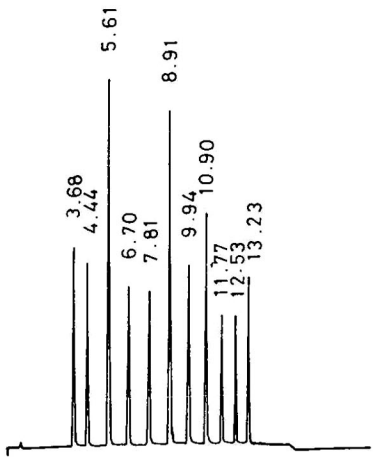

Fig. 4. Chromatograms of a mixture of standard 2,4-dinitrophenylhydrazones of C_1–C_{11} *n*-aliphatic aldehydes with a Hewlett-Packard 1084B chromatograph with gradient elution from acetonitrile–water (55:45) to acetonitrile in 12 min. Column, Nomura Develosil ODS, 3 μm, 15 cm × 4.6 mm I.D.; flow-rate, 1.2 ml/min; UV detector (362 nm). The numbers on the peaks indicate retention times in minutes.

Identification of configuration of the olefinic bond in heteroolefinic side-chain dimethylurushiol

C_{15} side-chain dimethylurushiol. (a) Monoolefinic components. Peak 3-1 in Fig. 2c showed no recognizable IR band in the range 900–1000 cm^{-1}, and therefore it has a *cis*-olefinic bond in the side-chain. Fig. 5a, shows the chromatogram for the 2,4-dinitrophenylhydrazones of the aldehydes as the reductive ozonolysis products of peak 3-1. The major peak with a retention time of 9.94 min (11.17 min) agreed with that (9.94 min) of the standard 2,4-dinitrophenylhydrazone of heptaldehyde (see Fig. 4). Further, a minor peak with a retention time of 7.83 min (12.45 min) is in good agreement with 7.81 min for the standard valeraldehyde derivative. From these results, it is concluded that peak 3-1 is composed mainly of 3-[8′(*Z*)-pentadecenyl]veratrole and a trace of 3-[10′(*Z*)-pentadecenyl]veratrole.

(b) Diolefinic compounds. Peak 2-1 in Fig. 2b showed an IR band at 965 cm^{-1}, characteristic of an isolated *trans*-olefinic bond. Each of the monoolefinic peaks 2-1-1 and 2-1-2, prepared by reduction with hydrazine of peak 2-1, was fractionated by HPLC (see Fig. 3a); in the range 900–1000 cm^{-1} the former showed no recognizable IR band, whereas the latter exhibited an IR band at 965 cm^{-1} due to a *trans*-olefinic bond.

As can be seen in Fig. 5b, 2,4-dinitrophenylhydrazones of the aldehydes of peak 2-1-1 showed a peak with a retention time of 9.96 min (11.91 min), which can be assigned to the 2,4-dinitrophenylhydrazone of heptaldehyde in the same manner as already shown for peak 3-1. Similarly, the peak in Fig. 5c with a retention time of 6.73 min (13.03 min) can be assigned to the 2,4-dinitrophenylhydrazone of butyraldehyde, which had a retention time of 6.70 min for the standard. Therefore, it is concluded that peak 2-1 is composed of 3-[8′(*Z*),11′(*E*)-pentadecadienyl]veratrole.

Peak 2-2 in Fig. 2b showed no noticeable IR band in the range 900–1000 cm^{-1}, indicating that the two olefinic bonds in the side-chain have a *cis* configuration. The

peak was partially reduced with hydrazine for 3 h, and the monoolefinic peak 2-2-1 formed was fractionated (see Fig. 3b), ozonized and reduced, then derivatized into the corresponding 2,4-dinitrophenylhydrazones.

In Fig. 5d, the major peaks with retention times of 6.70 min (13.04 min) and 9.96 min (11.20 min) can be identified as the 2,4-dinitrophenylhydrazones of butyraldehyde and heptaldehyde, respectively, by comparing their retention times with those of the corresponding standards in Fig. 4. Similarly, a minor peak with a retention time of 7.82 min (12.48 min) can be ascribed to the 2,4-dinitrophenylhydrazone of valeraldehyde. Thus peak 2-2 was identified as 3-[8′(*Z*),11′(*Z*)-pentadecadienyl]-veratrole, probably contaminated with 3-[8′(*Z*),10′(*Z*)-pentadecadienyl]veratrole.

(c) Triolefinic components. Peak 1-1 in Fig. 2a showed only an IR band at 990 cm^{-1}, indicating the presence of a conjugated *trans–trans*-olefinic bond, isolated from the above conjugated diene, judging from the UV band at 232 nm for peak 1-1 (see Table I).

Peak 1-1 was partially reduced with hydrazine for 5 h, and a mixture of the monoolefinic component peaks 1-1-1, 1-1-2 and 1-1-3 formed (see Fig. 3c) was fractionated, ozonized and reduced. The resulted aldehydes were converted into the corresponding 2,4-dinitrophenylhydrazones, which showed three peaks with retention times of 4.45 min (14.12 min), 6.71 min (13.03 min) and 9.96 min (11.21 min), as can be seen in Fig. 5e. These peaks can be ascribed to the 2,4-dinitrophenylhydrazones of

TABLE I

MASS, UV AND IR SPECTRAL DATA FOR THE DIMETHYLURUSHIOL COMPONENTS RESOLVED BY HPLC

*Peak No.**	*Parent peak*	*λ_{max} (nm) in n-hexane*	*IR band (cm^{-1})*	*No. of olefinic bonds*	*No. of side-chain carbon atoms*
1-1	342(356**)	232 ($1.0 \cdot 10^4$) 272 ($6.7 \cdot 10^3$)	990	3	15
1-2	342(356**)	231 ($2.5 \cdot 10^4$)	990, 950 and 930	3	15
1-3	342	228 ($1.5 \cdot 10^3$) 274 ($5.3 \cdot 10^2$)	920	3	15
2-1	344	228 ($8.1 \cdot 10^3$) 274 ($3.0 \cdot 10^3$)	965	2	15
2-2	344	227 ($5.2 \cdot 10^3$) 274 ($2.3 \cdot 10^3$)		2	15
3-1	346	224 ($2.5 \cdot 10^3$) 274 ($7.4 \cdot 10^2$)		1	15
3-2	374	229 ($3.2 \cdot 10^3$) 274 ($1.2 \cdot 10^3$)		2	17
4	372	229 ($1.0 \cdot 10^3$) 274 ($6.0 \cdot 10^2$)		1	17
5	348	224 ($3.3 \cdot 10^3$) 274 ($9.6 \cdot 10^2$)		0	15

* Peak numbers in chromatograms in Figs. 1–5.

** A very small peak which might occur as a result of oxidation of the peak in the procedure.

acetaldehyde, butyraldehyde and heptaldehyde, respectively, as the reductive ozonolysis products of the side-chain of peak 1-1. Therefore, peak 1-1 can be identified as 3-[8′(*Z*),11′(*E*),13′(*E*)-pentadecatrienyl]veratrole.

Peak 1-2 in Fig. 2 showed IR bands at 930, 950 and 990 cm^{-1}, indicating the presence of a conjugated *cis–trans*-diene as well as a *cis*-olefinic bond in the side-chain. It was reduced partially with hydrazine for 3 h, and the monoolefinic dimethylurushiol components formed, peaks 1-2-1 and 1-2-2, were separated by HPLC (see Fig. 3d).

Peak 1-2-1 showed no recognizable IR band in the range 900–1000 cm^{-1}, indicating that it has a *cis*-olefinic bond in the side chain. On the other hand, peak 1-2-2, with a moderate IR band of a *trans*-olefinic bond at 960 cm^{-1}, was found to be resolved into peaks 1-2-2-1 and 1-2-2-2 by HPLC on the Hitachi 3043Ag gel column

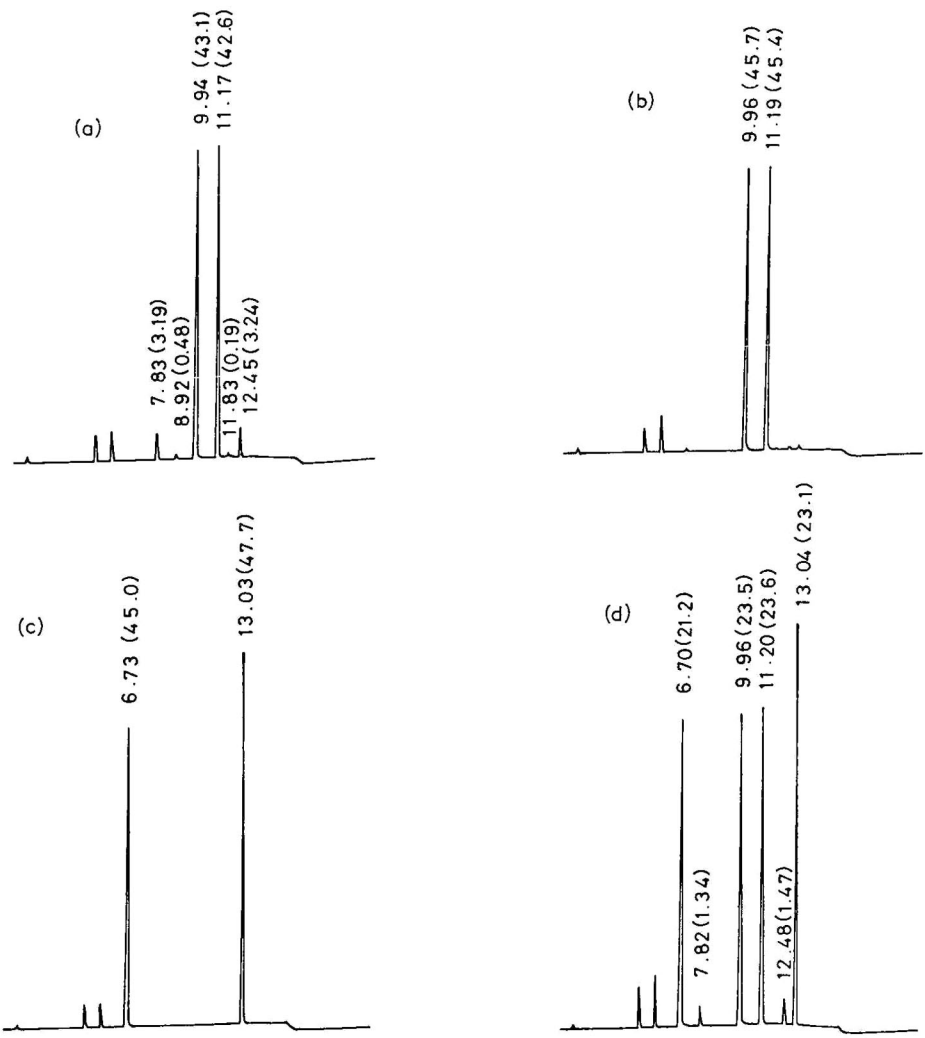

Fig. 5.

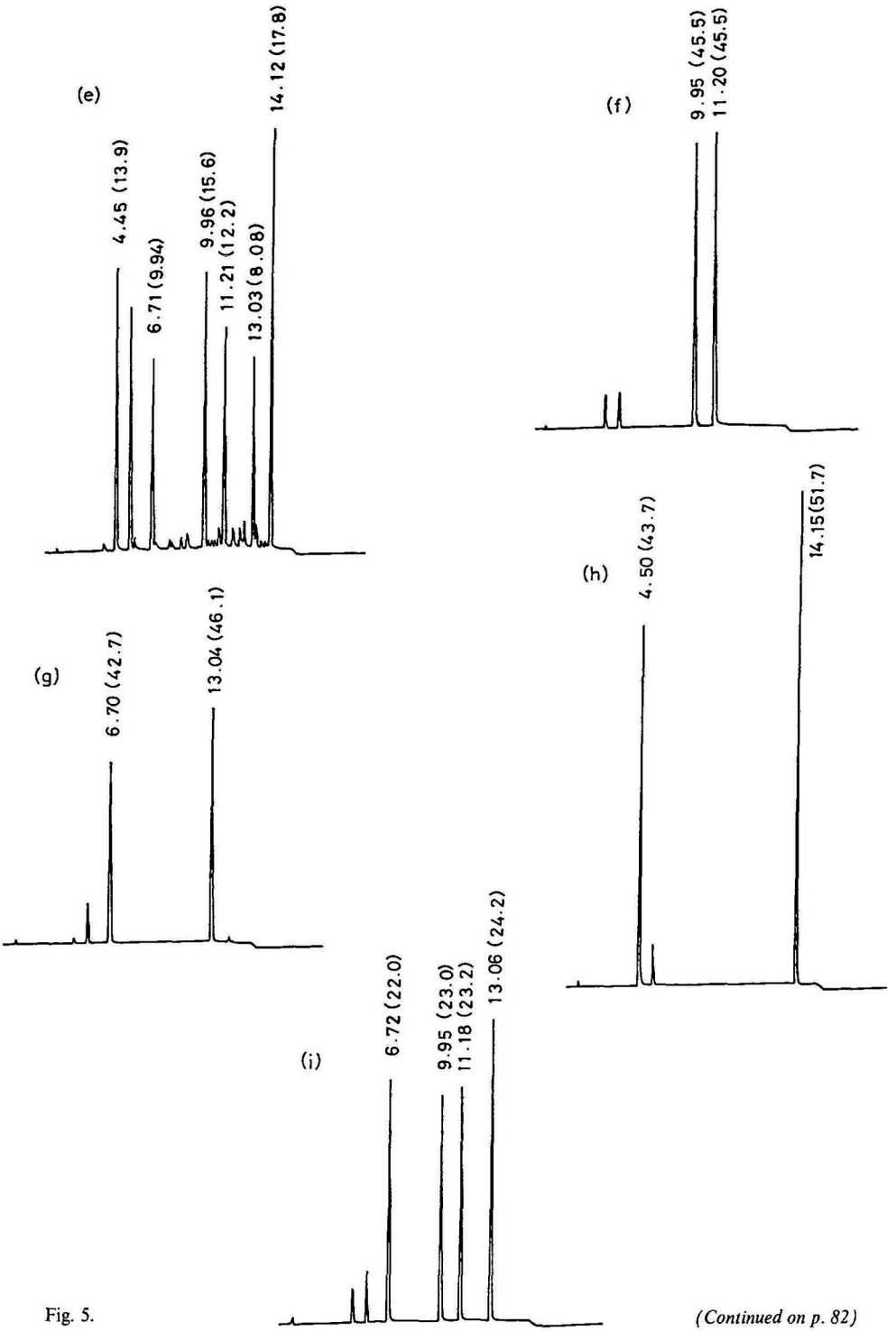

Fig. 5.

(Continued on p. 82)

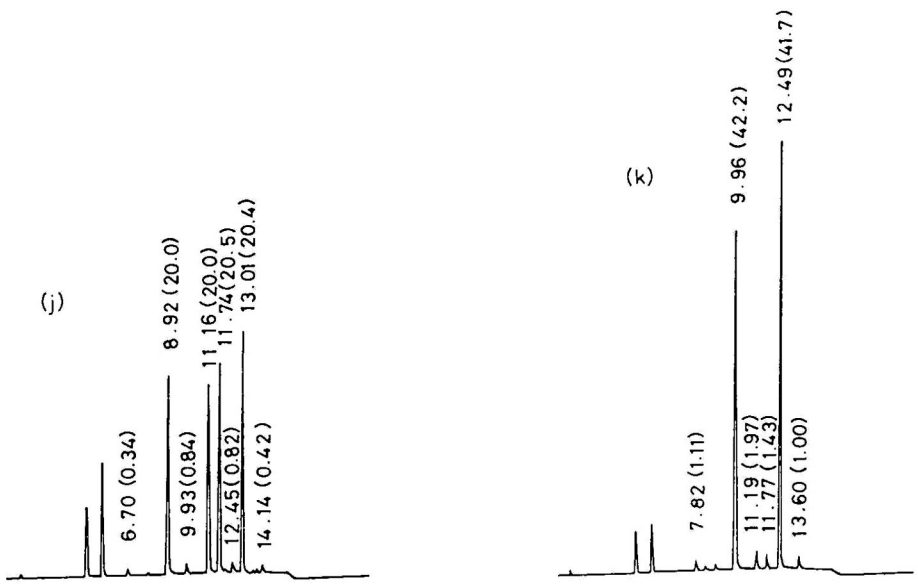

Fig. 5. Chromatograms of 2,4-dinitrophenylhydrazones of aldehydes as reductive ozonolysis products of monoolefinic dimethylurushiol of the following peaks: (a) 3-1; (b) 2-1-1; (c) 2-1-2; (d) 2-2-1; (e) 1-1-1 + 1-1-2 + 1-1-3; (f) 1-2-1; (g) 1-2-2-1; (h) 1-2-2-2; (i) 1-3-1; (j) 3-2-1; (k) 4. Conditions as in Fig. 4. Numbers with and without parentheses indicate peak area as a percentage of the whole peak area and retention time (minutes), respectively.

(25 cm × 7.6 mm I.D.) using *n*-hexane–ethyl acetate (95:5) as the eluent (see Fig. 3e); the former peak showed a strong IR band characteristic of a *trans*-olefinic bond at 960 cm^{-1}, whereas the latter showed no noticeable IR peak in the range 900–1000 cm^{-1}, indicating that the olefinic bond in the side-chain has a *cis* configuration.

Fig. 5f shows a chromatogram of the 2,4-dinitrophenylhydrazone of the reductive ozonolysis products of peak 1-2-1. The peak with a retention time of 9.95 min (11.20 min) correlates with that of the standard 2,4-dinitrophenylhydrazone of heptaldehyde (9.94 min). Similarly, the peak in Fig. 5g with a retention time of 6.70 min (13.04 min), derived from peak 1-2-2-1 in Fig. 3e, and that in Fig. 5h with a retention time of 4.50 min (14.15 min), derived from peak 1-2-2-2 in Fig. 3e, were identified as the 2,4-dinitrophenylhydrazones of heptaldehyde and acetaldehyde, respectively. From these results, it is evident that peak 1-2 is 3-[8′(*Z*),11′(*E*),13′(*Z*)-pentadecatrienyl]veratrole.

Peak 1-3 showed only a strong IR band at 920 cm^{-1} characteristic of a terminal vinyl group in the range 900–1000 cm^{-1}, and therefore the other olefinic bonds are assigned to a *cis* configuration. The monoolefinic dimethylurushiol component peak of 1-3-1 derived from peak 1-3 by its partial reduction with hydrazine showed a doublet peak (see Fig. 3f), resulting from the equimolar formation of the 2,4-dinitrophenylhydrazones of butyraldehyde (retention time 9.95 min) and heptaldehyde (retention time 6.70 min) as the reductive ozonolysis products of the peak (see Fig. 5i). However, the peak corresponding to the 2,4-dinitrophenylhydrazone of formaldehyde derived from the terminal vinyl group could not be found, as the terminal vinyl group is preferentially reduced rather than an internal olefinic bond, as demon-

TABLE II

CONSTITUENTS OF URUSHIOL

Type	*Compound*	*Concentration (%)**
C_{15} side-chain urushiol	3-(Pentadecyl)catechol	4.5
	3-[8′(*Z*)-Pentadecenyl]catechol	15.0
	3-[10′(*Z*)-Pentadecenyl]catechol	1.5
	3-[8′(*Z*),11′(*E*)-Pentadecadienyl]catechol	6.5
	3-[8′(*Z*),11′(*Z*)-Pentadecadienyl]catechol	4.4
	3-[8′(*Z*),11′(*E*),13′(*E*)-Pentadectrienyl]catechol	1.8
	3-[8′(*Z*),11′(*E*),13′(*Z*)-Pentadecatrienyl]catechol	55.4
	3-[8′(*Z*),11′(*Z*),14′-Pentadecatrienyl]catechol	7.4
C_{17} side-chain urushiol	3-[11′(*Z*)-Heptadecenyl]catechol	1.5
	3-[8′(*Z*),11′(*Z*)-Heptadecadienyl]catechol	1.8

* These values are based tentatively on the peak heights in the chromatograms.

strated by Mori *et al.*[33]. These results indicate that peak 1-3 is identical with 3-[8′(*Z*),11′(*Z*),14-pentadecatrienyl]veratrole.

C_{17} side-chain dimethylurushiol. Peak 3-2 in Fig. 3g, 3-(heptadecadienyl)-veratrole, showed no characteristic olefin IR band in the range 900–1000 cm^{-1}, indicating that the two double bonds in the side-chain have a *cis* configuration. Partial reduction with hydrazine of peak 3-2 for 3 h gave a monoolefinic dimethylurushiol, peak 3-2-1 (see Fig. 3g), which gave a mixture of 2,4-dinitrophenylhydrazones of caproldehyde and nonylaldehyde as its reductive ozonolysis products, with retention times of 8.92 min (11.61 min) and 11.74 min (13.01 min), respectively (see Fig. 5). From these results, peak 3-2 was identified as 3-[8′(*Z*),11′(*Z*)-heptadecadienyl]veratrole.

Peak 4 in Fig. 1, heptadecenylveratrole, showed no noticeable IR *trans*-band, indicating that the olefinic bonds in the side-chain have a *cis* configuration. The position of the olefinic bond in the side-chain of peak 4 was decided by assigning the peak in Fig. 5k with a retention time of 8.92 min (13.01 min) to the 2,4-dinitrophenylhydrazone of caproldehyde with a retention time of 8.91 min as a standard, concluding that peak 4 is identical with 3-[11′(*Z*)-heptadecenyl]veratrole.

CONCLUSION

The urushiol components identified in this work are listed in Table II, together with those already reported.

3-[10′(*Z*)-Pentadecenyl]catechol and 3-[8′(*Z*),11′(*E*)-pentadecadienyl]catechol have been newly identified in addition to the already known components, 3-[8′(*Z*)-pentadecenyl]catechol and 3-[8′(*Z*),11′(*Z*)-pentadecadienyl]catechol.

Of triolefinic urushiol components, 3-[8′(*Z*),11′(*Z*),13′(*Z*)-pentadecatrienyl]-catechol has previously been believed to be a major component of urushiol without adequate evidence. However, from the present work, it became evident that this configurational structure of the side chain is erroneous, and it should be replaced by two urushiol components, 3-[8′(*Z*),11′(*E*),13′(*Z*)-pentadecatrienyl]catechol and 3-

[8′(*Z*),11′(*E*),13′(*E*)-pentadecatrienyl]catechol. 3-[8′(*Z*),11′(*Z*),14′-Pentadecatrienyl]-catechol has also been confirmed to exist in a relatively large amount in Japanese lacquer urushiol, as already reported by Hashimoto and Minami[17].

Of the components of Japanese lacquer urushiol, only C_{15} side-chain urushiol has been discussed. It is clear that two urushiol homologues with a C_{17} side-chain, 3-[11′(*Z*)-heptadecenyl]catechol and 3-[8′(*Z*),11′(*Z*)-heptadecadienyl]catechol should also be taken into consideration as Japanese lacquer urushiol homologues, although they are minor components. Thus the components of Japanese lacquer urushiol are seen to be similar to those of poison ivy, contrary to Sunthanker and Dawson's proposal[3].

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SIMULTANEOUS DETERMINATION OF TRACE AMOUNTS OF BROMIDE AND IODIDE BY METHYLATION WITH DIMETHYL SULPHATE AND ELECTRON-CAPTURE GAS CHROMATOGRAPHY

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SUMMARY

A specific and sensitive gas chromatographic method is described for the simultaneous determination of trace quantities of bromide and iodide. Bromide and iodide are methylated with dimethyl sulphate at 70°C to form methyl bromide and methyl iodide, respectively. The resulting methyl derivatives are simultaneously determined by gas chromatography with electron-capture detection. The detection limits are 0.1 μg/ml bromide and 0.5 ng/ml iodide. The interferences of several anions were investigated. The application of the method to the determination of bromide and iodide in spring-water is demonstrated. The results reveal that the recoveries are over 90% for both halides.

INTRODUCTION

Inorganic halide anions (*i.e.*, chloride, bromide, iodide, etc.) play important rôles in biological and environmental sciences. Therefore, their determination is very important, and various methods have been reported. Of these methods, several based on colorimetry[1] and ion-selective electrodes[2] have often been used. Each of these methods can be used for the determination of only one of the halide anions, but none can be used for the simultaneous determination of different halide anions. Recently, several techniques for the simultaneous determination of organic and inorganic anions by high-performance liquid chromatography have been published[3–9]. "Ion chromatography" developed by Small and co-workers[3–5] is very effective for the simultaneous determination of halide anions.

On the other hand, several gas chromatographic (GC) techniques have been reported for the determination of halide anions[10–14]. These methods are based on derivatization of halide anions to organic compounds detectable by GC. However, only one such method, developed by Stephen and co-workers[10,11], has been applied

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to the simultaneous determination of different halide anions. The method is based on the reaction of halide anions with trifluoromethylmercury(II) nitrate[10] or phenylmercury(II) nitrate[11] to form the corresponding organic mercury(II) halides which are subsequently determined by GC. Recently, we reported a new method to determine inorganic anions by flame-ionization GC[15,16]. The method is based on methylation or ethylation of the inorganic anions with reagents such as dimethyl or diethyl sulphate, methyl or ethyl *p*-toluenesulphonate and trimethyl or triethyl phosphate.

In this work, we have adapted the method to the simultaneous determination of trace quantities of bromide and iodide by using an electron-capture detector (ECD). In the previous studies[15,16], we reported that dimethyl sulphate is the most suitable reagent for the derivatization of bromide, whereas diethyl sulphate is the most suited for that of iodide. In this work, however, both bromide and iodide were methylated with dimethyl sulphate in order to determine them simultaneously.

EXPERIMENTAL

Apparatus

A Shimadzu (Kyoto, Japan) GC-4BM gas chromatograph equipped with a ^{63}Ni ECD was used. A stainless-steel column (1 m × 3 mm I.D.) was packed with Porapak P (80–100 mesh). Nitrogen was used as the carrier gas at a constant flow-rate of 50 ml/min. The detector, injection port and column temperatures were maintained at 250, 250 and 125°C, respectively. The peak areas were measured by a digital integrator (Shimadzu Chromatopac ElA).

Materials

Dimethyl sulphate was a commercial grade reagent purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Benzene, a special grade reagent for liquid chromatography, was obtained from Wako (Osaka, Japan). Deionized water was distilled before use. All other reagents were of analytical reagent grade. The column packing material, Porapak P, was obtained from Waters Assoc. (Milford, MA, U.S.A.).

Procedure

The recommended procedure for the simultaneous determination of bromide and iodide at trace levels (below 25 μg/ml for Br^- and 0.1 μg/ml for I^-) was as follows. Dimethyl sulphate (0.05 ml) was added to a 1.0-ml aliquot of neutral aqueous sample in a reaction vessel (ca. 10 ml) with a glass stopper. The vessel used was brown to protect the contents from the light. It was sealed tightly with the stopper and shaken mechanically for 20 min in an incubator at 70°C. After cooling the reaction solution in an ice-bath, 1.0 ml of benzene was added. Then the derivatized methyl bromide and methyl iodide were extracted by shaking the vessel for 10 min at room temperature (18°C), and the organic layer was separated from the aqueous layer. An aliquot of the organic layer (0.3 μl) was injected into the gas chromatograph equipped with an ECD. In the case of the analysis of basic or acidic samples, a neutralization step is necessary before analysis.

When a sample contained bromate (BrO_3^-) and iodate (IO_3^-) ions, these species were reduced to bromide and iodide by sodium thiosulphate as follows. A 0.10-ml

volume of sodium thiosulphate ($5.0 \cdot 10^{-2}$ *M*) was added to the sample (1.0 ml) in the reaction vessel, and allowed to stand for a few minutes at room temperature (18°C). The methylation and GC measurements were performed as described above, and total bromine ($Br^- + BrO_3^-$) and total iodine ($I^- + IO_3^-$) in the sample were thus determined.

RESULTS AND DISCUSSION

Optimum methylation conditions

In order to perform the methylation under optimum reaction conditions, the effects of reaction temperature, pH and reaction time on the methylation yields of bromide and iodide were investigated. In these studies, 25 μg/ml bromide and 0.10 μg/ml iodide solutions were used as the samples, and other reaction conditions used were identical to those described in the Experimental section.

The effects of reaction temperature on the peak areas of derivatized methyl bromide and methyl iodide were investigated. The peak areas do not vary between 50 and 70°C. However, when methylation was performed at temperatures lower than 50°C, the column deteriorated and the baseline fluctuated. Therefore, the reaction temperature was fixed at 70°C.

The effect of pH was examined as follows. To 1.0 ml of each aqueous solution of bromide and iodide, 0.1 ml of KOH or H_2SO_4 aqueous solution of different concentrations was added before the addition of dimethyl sulphate, and then the recommended procedure was performed. Fig. 1 shows the effects of KOH and H_2SO_4 concentrations on the peak areas of methyl bromide and methyl iodide. The maximum peak area of methyl bromide derivatized from bromide (25 μg/ml) or methyl iodide from iodide (0.10 μg/ml) was arbitrarily assigned a value of 100 throughout this paper. In Fig. 1, the peak areas decrease with increasing KOH concentration, but do not vary with H_2SO_4 concentration. Similar behaviour was observed in the case of methylation of these anions at 0.10 *M*[15]. On the basis of these results, dimethyl sulphate was directly added to the sample, as long as the sample was not

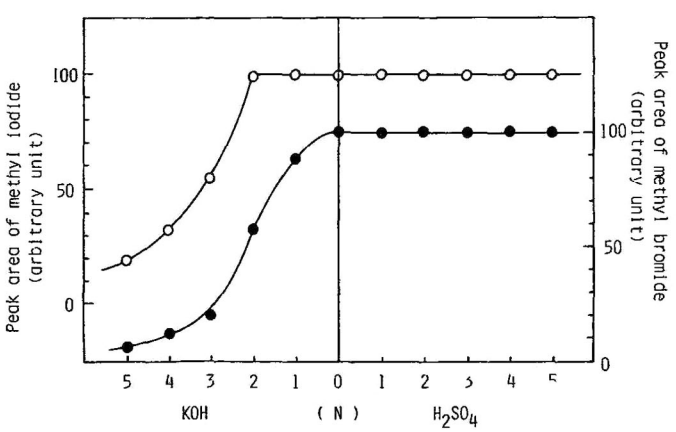

Fig. 1. Effect of normality of added acid or base on methylation of bromide (●, 25 μg/ml) and iodide (○, 0.10 μg/ml).

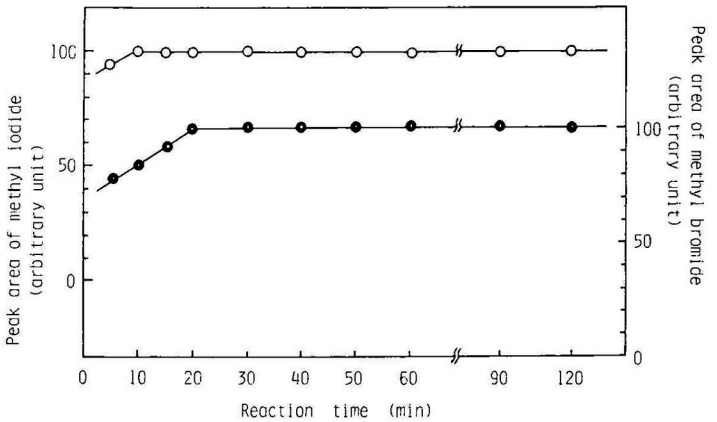

Fig. 2. Effect of reaction time on methylation of bromide (●) and iodide (○).

basic or acidic. The effects of reaction time were also examined, and the results are shown in Fig. 2. The times required to obtain methylated products at constant yields are around 20 min for bromide and 10 min for iodide. The reaction time, therefore, was fixed at 20 min.

From these results, the procedure described in the Experimental section was adopted. The methylation yield of iodide was determined by a similar method to that reported in our previous papers[15,16]. A yield of 76.3 ± 0.4% was found for $7.87 \cdot 10^{-7}$ *M* (= 0.10 μg/ml) iodide. This value agrees satisfactorily with that obtained when 0.10 *M* iodide was methylated and its yield evaluated by flame-ionization GC (73.2 ± 1.8%)[15]. The yield of bromide cannot be estimated exactly because of the difficulty in preparing standard solutions of methyl bromide (b.p. 3.6°C).

Analytical calibration

Calibration curves were constructed by plotting the peak areas of methyl derivatives *vs.* the concentrations of bromide and iodide (Fig. 3). Good linear relationships are obtained in the concentration ranges of 2.5–25 μg/ml bromide and 0.01–0.10 μg/ml iodide. The calibration curve for bromide passes through the origin, whereas that for iodide does not. This is due to the very small unknown peak given by the blank without iodide and to the difficulty in separating the blank peak from the peak of methyl iodide. A commonly accepted definition of the detection limit is the concentration of analyte giving a signal twice the average noise. According to this definition the detection limit of bromide was 0.1 μg/ml. On the other hand, the detection limit of iodide could not be obtained by the above definition, because the peak of methyl iodide overlapped the small blank peak. A detection limit of 0.5 ng/ml iodide was obtained as follows. The mean value (M) and standard deviation (S.D.) of the blank peak area were obtained by performing five replicate analyses. The detection limit in this case was defined as the concentration of iodide giving a peak area of (M + 2 S.D.). It is possible to detect several times lower concentrations of bromide and iodide than the detection limits by injecting a volume of the benzene extract slightly higher than 0.3 μl into the gas chromatograph or by decreasing the volume of benzene added in the extraction procedure. The determination level and the detection limit of bromide are a few hundred times higher than those of iodide. This behaviour is based

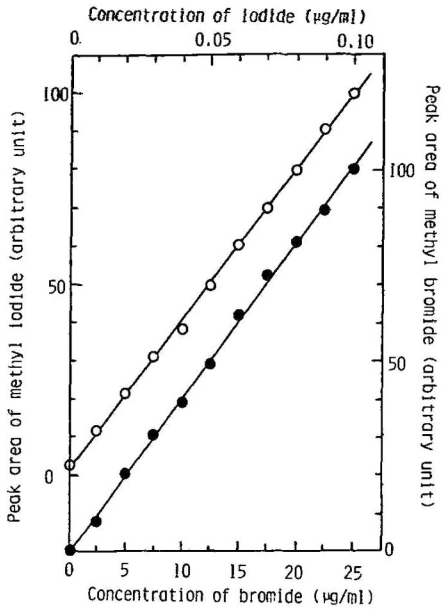

Fig. 3. Calibration curves for bromide (●) and iodide (○).

on the difference in the responses of methyl bromide and methyl iodide to an ECD. Generally, the concentration of bromide is several hundred times higher than that of iodide in environmental and biological samples, which is all the more convenient for applying the proposed method to the simultaneous determination of bromide and iodide in those samples.

Interference study

The interferences of several anions in the determination of bromide and iodide were investigated (see Table I). The anions selected are those normally found with bromide and/or iodide in environmental and biological samples. The concentrations of the anions added to the standard solution containing bromide (25 μg/ml) and iodide (0.10 μg/ml) are much higher than those in environmental and biological samples. Except for chloride and nitrate, the anions do not interfere with this analysis. Chloride at a very high concentration (20,000 μg/ml = 2.0%; corresponding to the concentration in sea-water) gives a slightly negative interference with the bromide determination. In this case, the gas chromatogram shows a small peak of methyl bromide on the tailing of the peak of methyl chloride produced by methylation of the large amount of chloride. Therefore, the peak area of methyl bromide seems to be slightly underestimated by the integrator. However, such interference is not observed at a chloride concentration of 100 μg/ml. On the other hand, the interference of nitrate (100 μg/ml) in the iodide determination is due to a small peak of a by-product derivatized from nitrate. The retention time of this by-product is the same as that of methyl iodide. The by-product is probably methyl nitrate, although it has not been identified. This interference is negligible when the nitrate concentration is reduced to 10 μg/ml. In addition, nitrite and acetate are also methylated with dimethyl sulphate

TABLE I

INTERFERENCE STUDY

Concentrations of bromide and iodide are 25.0 μg/ml and 0.10 μg/ml, respectively.

Anion	*Concentration (μg/ml)*	*Added as*	*Peak area**	
			Methyl bromide	*Methyl iodide*
Standard	–	–	100.0 ± 1.2	100.0 ± 1.7
Cl^-	20,000	NaCl	91.6 ± 2.6	100.6 ± 2.7
Cl^-	100	NaCl	101.3 ± 2.2	98.4 ± 1.0
F^-	100	NaF	98.1 ± 1.6	99.3 ± 0.9
Br^-	100	NaBr	–	100.4 ± 2.0
I^-	100	NaI	98.0 ± 1.2	–
CH_3COO^-	100	CH_3COOK	99.4 ± 2.4	99.9 ± 2.9
SO_4^{2-}	100	Na_2SO_4	98.7 ± 1.6	100.6 ± 0.8
CO_3^{2-}	100	K_2CO_3	101.2 ± 1.3	102.5 ± 1.6
NO_3^-	100	$NaNO_3$	100.3 ± 1.9	**
NO_3^-	10	$NaNO_3$	–	99.3 ± 2.0
NO_2^-	100	$NaNO_2$	100.4 ± 2.2	100.1 ± 3.6

* Mean ± S.D. of five replicate analyses.

** The peak area of unidentified product from nitrate is not negligible.

in low yields[15]. In the test of nitrite interference, two new peaks were observed in addition to the peaks from the standard solution (Fig. 4A), but these do not interfere with the analysis because of the good separation of the two peaks from the peaks of methyl bromide and methyl iodide. On the other hand, the peak of methyl acetate derivatized from acetate was not observed because of its very low ECD response.

Applications

This method was applied to the determination of bromide and iodide in spring-water. Recovery tests for bromide and iodide added to the spring-water samples were

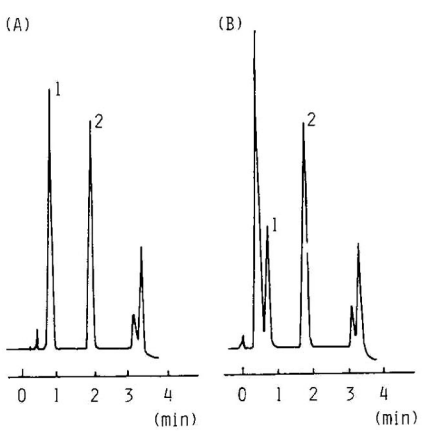

Fig. 4. Typical gas chromatogram obtained from standard solution (A), and gas chromatogram obtained in the analysis of spring-water sample (B). Peaks: 1 = methyl bromide; 2 = methyl iodide.

TABLE II

RESULTS OF ANALYSES OF SPRING-WATER SAMPLES WITH BROMIDE AND IODIDE RECOVERY TEST

Spring-water samples were diluted four-fold with water before analyses.

Sample No.	*Amount added (μg/ml)*		*Amount found (μg/ml)*		*Recovery (%)*	
	Bromide	*Iodide*	*Bromide*	*Iodide*	*Bromide*	*Iodide*
1	0.0	0.000	10.8* (0.38)	0.037* (0.0020)	–	–
	5.0	0.050	15.4	0.088	92.0	102.0
	10.0	0.100	19.8	0.136	90.0	99.0
	15.0	0.150	26.8	0.189	106.7	101.3
	20.0	0.200	30.6	0.236	99.0	99.5
2	0.0	0.000	7.7* (0.31)	0.034* (0.0013)	–	–
	5.0	0.050	12.3	0.081	92.0	94.0
	10.0	0.100	16.7	0.131	90.0	97.0
	15.0	0.150	22.4	0.186	98.0	101.3
	20.0	0.200	27.1	0.235	97.0	100.5

* Mean of five replicate analyses, with S.D. in parentheses.

performed. The results are shown in Table II, and Fig. 4B shows the gas chromatogram obtained in the analysis of the spring-water sample. The recoveries are all over 90% for both anions and the relative standard deviations of the method are less than 6% for both cases. Fig. 4A shows the gas chromatogram obtained from a standard solution prepared by dissolving potassium bromide and potassium iodide in water. In the gas chromatogram in Fig. 4B, the largest peak whose retention time is identical with that of methyl chloride appears very closely to the methyl bromide peak; the spring-water samples tested contain a large amount of chloride (*ca.* 3%). Therefore, it seems possible to determine chloride together with bromide and iodide by this method. However, chloride cannot be determined precisely and sensitively because of the high volatility of methyl chloride (b.p. −24°C) and its low ECD response.

Determination of total bromine and total iodine

A sample may contain bromate and iodate ions together with bromide and iodide. In that case, it is impossible to determine accurately only bromide and iodide, if bromate and iodate are simultaneously derivatized to methyl bromide and methyl iodide, respectively. However, total bromine ($Br^- + BrO_3^-$) and total iodine ($I^- + IO_3^-$) seem to be analyzable by reducing them completely to bromide and iodide and then methylating. We have, therefore, investigated the reduction of bromate and iodate by using sodium thiosulphate as the reducing agent, and the results are given in Table III. A $1.5 \cdot 10^{-4}$ *M* bromate and $8.0 \cdot 10^{-7}$ *M* iodate solution was analysed by the procedure described in the Experimental section. The peak areas of methyl bromide and methyl iodide obtained from a solution containing bromide ($1.5 \cdot 10^{-4}$ *M* = 12 μg/ml) and iodide ($8.0 \cdot 10^{-7}$ *M* = 0.10 μg/ml) without adding the reducing agent were arbitrarily assigned a value of 100 in Table III. The direct derivatization

TABLE III

DERIVATIZATION OF BROMATE AND IODATE

Concentration of Br^- or BrO_3^- is $1.5 \cdot 10^{-4}$ *M*, and that of I^- or IO_3^- is $8.0 \cdot 10^{-7}$ *M*.

Anion	*Peak area**	
	$Na_2S_2O_3$ *added*	*Not added*
Br^-	100.0 ± 3.4	100.0 ± 1.2
BrO_3^-	100.0 ± 2.8	11.4 ± 1.4
$Br^- + BrO_3^-$	200.8 ± 3.2	115.3 ± 2.6
I^-	100.0 ± 1.0	100.0 ± 1.7
IO_3^-	99.3 ± 3.2	4.7 ± 0.2
$I^- + IO_3^-$	200.6 ± 4.2	105.3 ± 0.6

* Mean ± S.D. of five replicate analyses.

(no addition of sodium thiosulphate) of bromate and iodate to methyl bromide and methyl iodide proceeds in low yields. On the other hand, bromate and iodate are quantitatively derivatized by the addition of sodium thiosulphate. Therefore, the method proposed in this paper is applicable to the simultaneous determination of total bromine and total iodine.

ACKNOWLEDGEMENT

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ELECTRON-CAPTURE CAPILLARY GAS CHROMATOGRAPHY AND MASS SPECTROMETRY OF TRIFLUOROACETYLATED CYTOKININS

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SUMMARY

Trifluoroacetylated derivatives of cytokinins, including N^6-(Δ^2-isopentenyl)-adenine, N^6-(Δ^2-isopentenyl)adenosine, N^6-fufuryladenine (kinetin), N^6-benzyl-adenine, *trans*-zeatin, dihydrozeatin and *trans*-zeatin riboside, were chromatographed on fused silica capillary columns and investigated by electron impact mass spectrometry. By using the "on-column" injection technique and electron capture detection, quantities as low as 1 pg of cytokinins can be detected.

INTRODUCTION

Cytokinins are potent cell division factors occurring in plant tissues and microorganisms in submicrogram quantities. Owing to their closely related structures (Fig. 1) and low concentrations, their isolation, identification and quantification raises several problems.

Various chromatographic procedures have been applied in recent years for the detection of cytokinins, including high-performance liquid chromatography (HPLC)[1–5] and gas chromatography (GC)[6–11], in some cases in combination with mass spectrometry[4–6,9–11]. In spite of this extensive work sufficient separation as well as sensitive detection of cytokinins has been achieved only rarely. The limit of detection by UV-monitoring of HPLC separations is in the range of 10 ng for pure zeatin. In GC, trimethylsilyl (TMS) derivatives, packed columns and flame ionization detection are usually employed, yielding a detection limit in the nanogram range. Even when using selected ion monitoring in mass spectrometry, 1 ng of a cytokinin was the lowest detectable amount[10]. Improved GC separations were achieved by use of glass capillary columns[12,13].

In an attempt to improve further the separation and identification procedure for natural and synthetic cytokinins, we combined the newly developed on-column injection method[14,15] with highly inert fused silica capillary columns and electron capture detection of trifluoroacetyl (TFA) derivatives of cytokinins.

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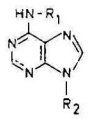


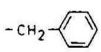
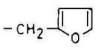
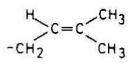
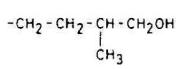
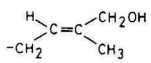
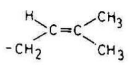
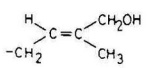
R_1	R_2	Name
Synthetic cytokinins		
	H	Benzyladenine
	H	Kinetin
Natural cytokinins		
	H	N^6-(Δ^2-Isopentenyl) adenine
	H	Dihydrozeatin
	H	trans-Zeatin
	Ribose	N^6-(Δ^2-Isopentenyl) adenosine
	Ribose	Zeatinriboside

Fig. 1. Structures of synthetic and natural cytokinins.

EXPERIMENTAL

Formation of derivatives

Amounts of 10–500 ng of pure cytokinins (Sigma, St. Louis, MO, U.S.A.) dissolved in methanol or ethanol were transferred into 1-ml vials (Microproduct L 125, Wheaton) and dried with nitrogen. After adding 200 μl dichloromethane and 50 μl trifluoroacetic anhydride, the tightly closed vials were heated for 35 min at 95°C. The excess of reagent was then removed with a gentle stream of nitrogen and the residue was dissolved in 500 μl of cyclohexane. The solution (0.1–1.0 μl) was injected into the gas chromatograph.

Gas chromatography

For GC separations, fused silica capillaries (25 m × 0.33 mm I.D.) coated with CpSil 5 (Chrompack, Berlin, G.F.R.), Carlo Erba Model 4160 and Packard 429 gas chromatographs, both equipped with an on-column injection system, and a ^{63}Ni-electron capture detector were used. The injector temperature was kept at 50–60°C; the column temperature was 180°C and was increased to 260°C at a rate of 3°C/min.

The detector temperature was 310°C. Hydrogen was used as carrier gas and nitrogen was used for the detector at a flow-rate of 20–30 ml/min.

Gas chromatography–mass spectrometry (GC–MS)

For GC–MS a Varian MAT 311 double-focusing mass spectrometer, equipped with a Carlo Erba Model 2101 gas chromatograph and Pt/Ir-interface (open coupling), was used. Electron impact (70 eV) mass spectra of TFA derivatives were recorded with a Varian MAT SS 500 data system.

RESULTS AND DISCUSSION

Although excellent separations of TMS derivatives of cytokinins can be obtained on fused silica capillary columns[13], halogenated derivatives would be preferable for electron capture detection, in order to decrease the detection limit. Several authors attempted to use perfluoroacylated derivatives but obtained unsatisfactory results[6,16], due to the extreme sensitivity of perfluoroacyl derivatives to moisture. However, from earlier MS studies on trifluoroacetylated nucleosides, it was known that TFA derivatives can be obtained in good yields[17,18]. In fact we found that all the cytokinins could be derivatized to yield single peaks in the gas chromatogram (Fig. 2). Only the retention times of zeatin and dihydrozeatin were found to be identical and resolution was not possible under the conditions described. However, these compounds can be separated by HPLC[3,19], for example on Bondapak C_{18} columns[20]. Since intensive purification of plant extracts by HPLC is advisable prior to GC, the combination of both procedures resolves this problem without consuming additional time.

In comparing the trifluoroacetyl, pentafluoropropionyl and heptafluorobutyryl derivatives, the TFA derivatives proved to be most stable, only negligible amounts of by-products being formed. Due to the high separation efficiency of the

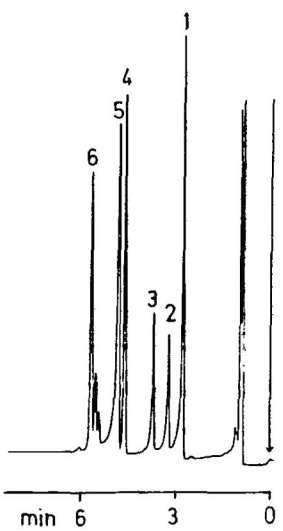


Fig. 2. Gas chromatographic separation of a standard solution of cytokinin TFA derivatives; 1 ng of each component before derivatization. Peaks: 1 = N^6-(Δ^2-isopentenyl)adenine; 2 = kinetin; 3 = zeatin; 4 = N^6-(Δ^2-isopentenyl)adenosine; 5 = N^6-benzyladenine; 6 = zeatin riboside. Electron capture detection, attenuation 512; separation conditions as in Experimental.

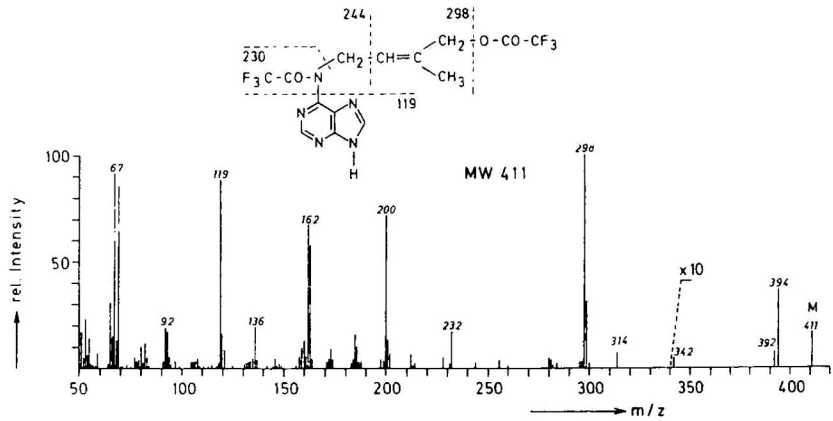


Fig. 3. 70-eV mass spectrum and fragmentation scheme of zeatin TFA derivative, GC–MS introduction.

fused silica capillary column, the excellent reproducibility of the on-column injection technique and the high sensitivity of the electron capture detector, the detection limit for all cytokinins is in the picogram range. As an example, a dose response curve for N^6-(Δ^2-isopentenyl)adenine was linear from 0.1 to 10 pg.

Although the derivatives are stable in cyclohexane solution for at least 8 h, GC analysis should be carried out shortly after derivatization.

For confirmation of the structure of the derivatives formed, mass spectra were investigated via the capillary GC inlet of the cytokinin derivatives. The mass spectra showed that the purine bases are not acylated at N-9, as indicated by the intense m/z = 119 ion of the free purine residue in the zeatin mass spectrum (Fig. 3). Intense fragment ions appear at m/z = 298 (loss of $CF_3COO\cdot$), m/z = 232 (loss of the side chain from N-6 and transfer of two hydrogen atoms to the ion), m/z = 200 (loss of CF_3COOH and CF_3CO) and m/z = 162 (loss of CF_3 from m/z = 231). In the upper mass range a molecular ion at m/z = 411 and fragments at m/z = 394 (M − OH), m/z = 392 (M − F) and m/z = 314 (M − $CF_3CO\cdot$) are observed.

The MS fragmentation was confirmed by exact mass measurements at high resolution. The mass spectrum of trifluoroacetylated zeatin riboside (Fig. 4) is very similar to the fragmentation of adenosine and adenosine derivatives[17,18]. Major fragments arise from cleavage of the base moiety B (m/z = 410) by loss of 97 mass units (B + H − $CF_3CO\cdot$; m/z = 314) and loss of 113 mass units (B + H − $CF_3COO\cdot$; m/z = 298). Further loss of a trifluoroacetic acid molecule from m/z = 314 gives rise to m/z = 200. The sugar residue (m/z = 421) easily loses two molecules of trifluoroacetic acid to yield m/z = 193. The molecular ion at m/z = 831 splits off a fluorine radical (m/z = 812) and $CF_3COO\cdot$ (m/z = 718). From the molecular ion, a $CF_3CO\cdot$ radical (m/z = 734) and an additional CF_3COOH (m/z = 620) are also lost. The ions at m/z = 232 and m/z = 162 originate from a fragmentation similar to that for the zeatin derivative.

Promising results were obtained when this new GC procedure was applied in conjunction with electron capture detection to the identification and quantification of cytokinins from plant extracts. Fig. 5 shows a gas chromatogram from 14 ml of sunflower exudate obtained by decapitating young seedlings and collecting the liquid from the cut surface of the hypocotyl stump. The exudate was prepurified by

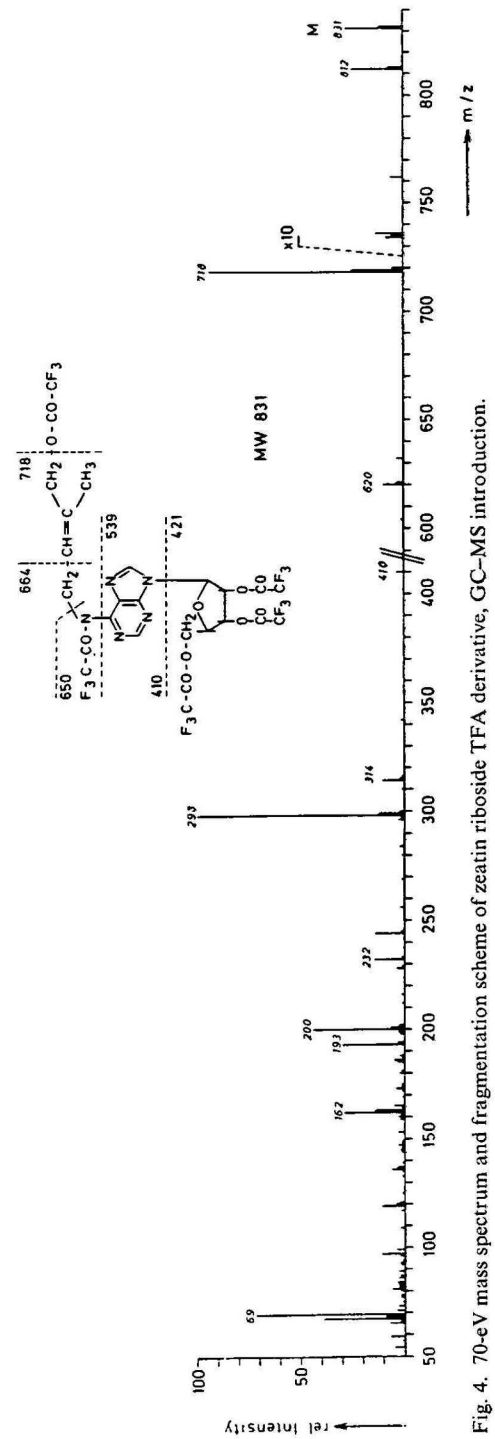


Fig. 4. 70-eV mass spectrum and fragmentation scheme of zeatin riboside TFA derivative, GC–MS introduction.

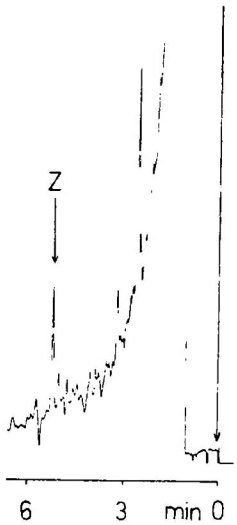


Fig. 5. Detection of zeatin (Z) from 14 ml of sunflower exudate, TFA derivative; signal corresponds to 15 pg zeatin. Electron capture detector, attenuation 16; separation conditions as in Experimental. Column temperature: 180°C.

HPLC[20]. Besides zeatin, N^6-(Δ^2-isopentenyl)adenine and the ribosides of the two cytokinin bases were identified.

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PENTAZOCINE TABLET ANALYSIS USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A rapid extraction ion-pair reversed-phase system has been demonstrated for analysis of pentazocine hydrochloride in tablets. This high-performance liquid chromatographic method was also shown to separate other members of a series of benzomorphans. A favorable comparison was found between results by this method and a normal-phase HPLC method for pentazocine. The effects of ion-pairing agent and of concentration were investigated in view of possible retention mechanisms. A review of previous analytical methods for pentazocine is given.

INTRODUCTION

Benzomorphan analgesics have been widely studied in human and animal species following their introduction in the 1960's. Notable among these has been pentazocine, an analgesic agonist with certain degrees of antagonist activity. A variety of analytical techniques have been employed by workers mainly in the field of biological disposition and pharmacokinetics of pentazocine. Spectrofluorometric methods[1–4] were introduced early as were gas chromatographic methods. Initially flame ionization detection was used, with and without derivatization of pentazocine[5–9]. A nitrogen detector found some application[10] however more recent work has been done using the sensitive electron capture detector[11–13]. Sensitive and specific radioimmunoassay procedures have been used for analysis of pentazocine in human and dog plasma and urine[14,15].

The use of high-performance liquid chromatography (HPLC) for pharmaceutical analysis gives sensitive, selective, rapid and stability-indicating results. These considerations become important when large numbers of samples are encountered routinely. This method eliminates the need and time for derivatization, possible hazards associated with the use of radioactivity and time consuming antibody production. Pentazocine has been studied by HPLC in a series of narcotic agonists using a reversed-phase, phosphate buffer–methanol system[16]. While a normal-phase method was used to determine pentazocine absorption from aqueous suspensions[17], the results of analysis for pentazocine in one dosage form have been published[18]. This was for repackaged pentazocine hydrochloride solution. The HPLC method used was

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TABLE I

PENTAZOCINE ANALYTICAL METHODS

Materials analyzed: p = plasma; u = urine; b = blood; d = solution dosage form; c = cerebrospinal fluid; br = brain.

Method	*Ref.*	*Recovery (%)*	*Sensitivity*	*Linear range*	*Statistics*
Fluorometric	1 (p)		0.03 μg/ml		
	2	90	0.2 μg/ml		
	3,4 (u)(p)(c)		0.15–5 μg/ml		±10%
Gas chromatography					
F.I.D.	5,6 (br)	96		25–500 ng	
	7 (b)	98	0.2 μg/ml	20–40 ng/ml	5% S.D.
	(u)	93	1 μg/ml		3% S.D.
	8,9 (u)	97	0.5 μg/ml	0.1–30 μg/ml	4% S.D.
Nitrogen detector	10 (b)	100.3	0.119–2 μg/ml	2.4–40 ng	4.3% S.D.
Electron capture	11,12 (p)		25 ng/ml		7% C.V.
			5 ng/ml	1–50 ng/ml	5.5% C.V.
	13 (u)	91–104 (no hydrolysis)	5 ng		5–6% S.D.
		97–100 (with hydrolysis)			3–4% S.D.
	(p)	92–95	100 ng/ml		11–13% S.D.
		72–100	25 ng/ml		14–44% S.D.
Radioimmunoassay	14 (p)	91–118	5 ng/ml		
	15 (p)			1–100 ng/ml	8.2–11.6% C.V.
HPLC	18 (d)	99	0.2 mg/ml		0.0035 S.D.
					1.8% C.V.

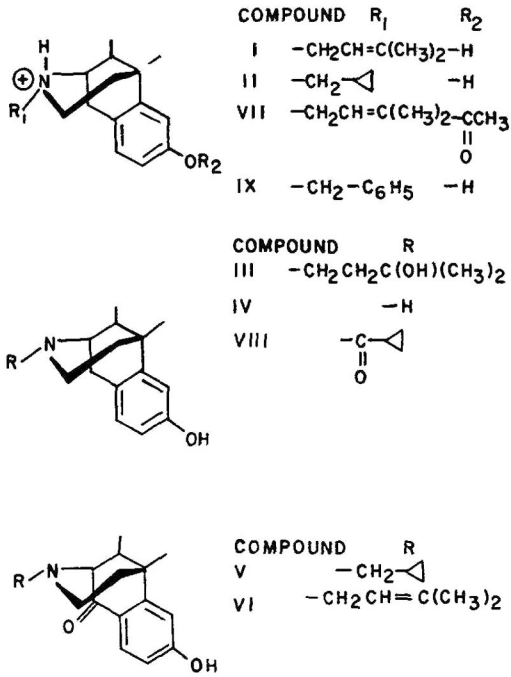


Fig. 1. Structures of benzomorphans studied.

reversed-phase with a heptanesulfonic acid salt pairing ion. Table I summarizes previous analytical methods with an indication of the sensitivity, recovery and linear range obtained where available. The present study reports the application of an ion-pairing reversed-phase HPLC method for pentazocine in tablets as well as its use for other members of the benzomorphan series. In addition the reversed-phase ion-pairing retention mechanism of benzomorphans is investigated. Studies concerning the effect of size and concentration of the pairing ion on benzomorphan retention are presented.

EXPERIMENTAL

Reagents and materials

Methanol (MCB, glass dist., LC grade), chloroform (MCB, glass dist., LC grade), isopropylamine (MCB), water was house distilled and filtered (Millipore, 0.45 μm), sodium octanesulfonate, sodium heptanesulfonate, sodium hexanesulfonate, sodium pentanesulfonate, sodium decyl sulfate and sodium dodecyl sulfate (Eastman-Kodak). Benzomorphans studied (Sterling-Winthrop): I, pentazocine hydrochloride (2α,6α,11*R**)-(±)-1,2,3,4,5,6-hexahydro-6,11-dimethyl-3-(3-methyl-2-butenyl)-2,6-methano-3-benzazocin-8-ol hydrochloride; II, cyclazocine hydrochloride, (2α,6α,11*R**)-(±)-3-(cyclopropylmethyl)-1,2,3,4,5,6-hexahydro-6,11-dimethyl-2,6-methano-3-benzazocin-8-ol hydrochloride; III, pentazocine hydrate, (2α,6α,11*R**)-(±)-1,4,5,6-tetrahydro-8-hydroxy-α,α,6,11-tetramethyl-2,6-methano-3-benzazocine-3(2*H*)-propanol; IV, pentazocine nor-base, (2α,6α,11*R**)-(±)-

1,2,3,4,5,6-hexahydro-6,11-dimethyl-2,6-methano-3-benzazocin-8-ol; V, ketocyclazocine, (2α,6α,11*S**)-(±)-3-(cyclopropylmethyl)-3,4,5,6-tetrahydro-8-hydroxy-6,11-dimethyl-2,6-methano-3-benzazocin-1(2*H*)-one; VI, ketopentazocine, (2α,6α,11*S**)-(±)-3,4,5,6-tetrahydro-8-hydroxy-6,11-dimethyl-3-(3-methyl-2-butenyl)-2,6-methano-3-benzazocin-1(2*H*)-one; VII, pentazocine acetate (2α,6α,11*R**)-(±)-1,2,3,4,5,6-hexahydro-6,11-dimethyl-3-(3-methyl-2-butenyl)-2,6-methano-3-benzazocin-8-ol acetate (ester) hydrochloride; VIII, Win 20722, (2α,6α,11*R**)-(±)-3-(cyclopropylcarbonyl)-1,2,3,4,5,6-hexahydro-6,11-dimethyl-2,6-methano-3-benzazocin-8-ol; IX, Win 28389, (2α,6α,11*R**)-(±)-1,2,4,5,6-hexahydro-6,11-dimethyl-3-(phenylmethyl)-2,6-methano-3-benzazocin-8-ol hydrobromide.

The above names conform with current Chemical Abstracts nomenclature.

Chromatography

Reversed-phase ion-pairing. The mobile phase was prepared following a modification of a method for pentazocine lactate. 600 ml of a 0.005 *M* sodium octanesulfonate solution (1.0814 g/l water) was added to a 1-l mixing cylinder, diluted to 1 l with methanol and acidified with 1.0 ml phosphoric acid. The solution obtained having a pH of 2.4 was degassed before use by ultrasonication. An Altex Model 110A pump was used at 1.5–2.0 ml/min along with a μBondapak C_{18} column (Waters) (30 cm × 3.9 mm I.D.). The detector was an Altex Model 153 at 280 nm with an absorbance of 0.08 a.u.f.s. The analytical wavelength was chosen by use of a Perkin-Elmer LC-75 UV detector with stopped-flow scanning capabilities. A Micromeritics 725 Autoinjector or a Rheodyne 7125 sample injector was used along with a Varian A-25 recorder.

Standard preparation. Approximately 28 mg of previously dried pentazocine hydrochloride equivalent to 25 mg pentazocine base were weighed accurately and transferred to a 50-ml volumetric flask. This was dissolved in and diluted to the mark with mobile phase.

Sample preparation. For the reversed-phase linearity study, pentazocine hydrochloride samples equivalent to 0%, 80%, 100% and 120% of tablet content were added in duplicate sets to placebo mixtures contained in 50-ml glass-stoppered graduated cylinders. These were extracted by adding 50.0 ml mobile phase, sonicating for 1 min and shaking intermittently for 15 min. The suspension was then filtered (Whatman No. 1 paper) and 20-μl samples were injected.

For reversed-phase replicate, analysis, pentazocine hydrochloride equivalent to 100% average tablet content and placebo mixture were intimately ground using a mortar and pestle. Weights of this mixture were extracted following the above procedure.

Normal phase. The mobile phase was prepared by adding 960 ml chloroform to a 1-l mixing cylinder, diluting to 1 l with methanol, adding 2.0 ml isopropylamine and degassing before use. A Waters M6000 pump was used at 1.2 ml/min with a Partisil 5/25 5-μm silica column (25 cm × 4.6 mm I.D.) (Whatman). The detector was a Waters 440 at 280 nm with an absorbance of 0.02 a.u.f.s. A Micromeritics 725 Autoinjector was used along with a Fisher Recordall 5000 recorder at 0.1 in./min.

Normal phase replicate analysis. Samples of pentazocine hydrochloride tablet mixture equivalent to one tablet weight were accurately weighed and placed in 50-ml glass-stoppered graduated cylinders. 50.0 ml of 0.035 *N* sulphuric acid–methanol

(1:1) was added to each, shaken 15 min intermittently, sonicated 2 min and filtered (Whatman No. 1). 10.0 ml were pipetted into 125-ml separatory funnels followed by the addition of 30 ml water and 5 ml 10% Na_2CO_3 solution. These were shaken briefly and extracted by the addition of 60 ml chloroform for 1 min. These were filtered (Whatman No. 1) into 100-ml volumetric flasks and filled to the mark with chloroform. A 20-μl sample was chromatographed.

Standard preparation. Approximately 28 mg of pentazocine hydrochloride standard, accurately weighed and dried, were placed in a 100-ml volumetric flask, dissolved in and diluted to the mark with 0.035 *N* sulphuric acid–methanol (1:1). 10.0 ml were pipetted into a 125-ml separatory funnel and extracted following the above procedure.

Minimum quantifiable limit reversed-phase study. Pentazocine hydrochloride standard in mobile phase was serially diluted using mobile phase giving 1:10, 1:100 and 1:1000 dilutions. These were analysed using the most sensitive detector setting of 0.005 a.u.f.s.

Benzomorphan analog reversed-phase chromatography. Compounds I–VII and IX were dissolved in mobile phase at the 0.5 mg/ml level. Compound VIII was dissolved in mobile phase at the 0.1 mg/ml concentration. Samples of these solutions were chromatographed and capacity factors determined.

Pairing ion concentration effect. Standard solutions of pentazocine hydrochloride and cyclazocine hydrochloride (I and II) were chromatographed in the reversed-phase ion-pairing mode described above. Concentrations of octanesulfonate pairing ion ranged from 0 to 18 m*M* in the mobile phase with no additional supporting electrolyte. Runs were also made with mobile phases containing sodium chloride to maintain constant ionic strength of 18 m*M* with the octanesulfonate.

Effect of pairing ion carbon chain length. Mobile phases were prepared as described above for sodium octanesulfonate using the following pairing ions: sodium pentanesulfonate, sodium hexanesulfonate, sodium heptanesulfonate, sodium decyl sulfate and sodium dodecyl sulfate. Samples of pentazocine hydrochloride and cyclazocine hydrochloride were chromatographed and capacity factors determined.

Quantitative determinations

Calculations of pentazocine content were carried out by use of the external standard technique of bracketting samples with standards. A Hewlett-Packard 3354 computer system was used to calculate peak heights and areas. Calculations were also done by the manual peak height method. A BASIC least squares program was run on linearity samples giving slopes, *y* intercept, average percent recovery, percent R.S.D. recovery, accuracy, precision and bias.

RESULTS AND DISCUSSION

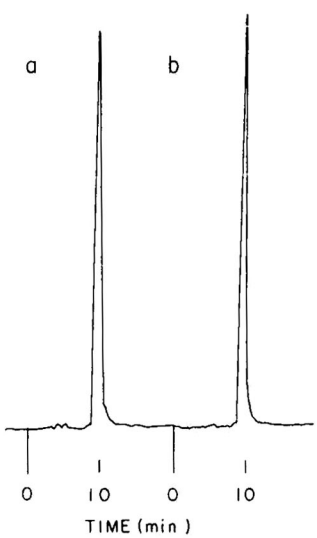
Linearity of recovery was obtained by the rapid extraction reversed-phase technique. Table II shows the results of duplicate sample analysis by peak height and peak area measurements. Peak heights generally proved to be most useful having percent recoveries in the 99.6–99.8% range and were consequently used in subsequent procedures. No interference from excipient peaks is seen in either the reversed-phase or the normal phase systems as seen in Figs. 2 and 3. The results of replicate analysis

TABLE II

LINEARITY OF PENTAZOCINE RECOVERY BY REVERSED-PHASE HPLC

Sample	*mg Pentazocine hydrochloride contained*	*mg Pentazocine hydrochloride found*	
		Peak height	*Peak area*
0%-1	0.0	0.0	0.0
0%-2	0.0	0.0	0.0
80%-1	24.2	24.4	24.3
80%-2	23.8	24.4	24.1
100%-1	28.4	28.4	29.1
100%-2	28.3	28.3	28.3
120%-1	32.2	31.3	30.2
120%-2	32.0	31.1	30.4
	Average % recovery	99.6	98.7
	% R.S.D. recovery	2.19	3.69
	Slope	0.985	0.972
	Intercept	+0.636	+0.921

of tablet mixtures by reversed-phase ion-pairing and by normal phase adsorption techniques show excellent correlation, Table III. While this correlation was obtained in terms of accuracies and precisions of recovery, overall simplicity and rapidity of the reversed-phase method makes it more practicable.



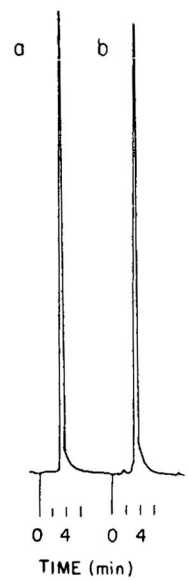


Fig. 2. Reversed-phase ion-pairing chromatograms of (a) pentazocine hydrochloride standard and (b) extracted tablet mixture of pentazocine hydrochloride. Mobile phase: sodium octanesulfonate (0.005 *M*)–methanol–H_3PO_4 (600:400:1). Stationary phase: Microbondapak C_{18}.

Fig. 3. Normal phase chromatograms of (a) pentazocine hydrochloride standard and (b) extracted tablet mixture of pentazocine hydrochloride. Mobile phase: chloroform–methanol–isopropylamine (960:40:2). Stationary phase: 5 μm silica.

TABLE III

REPLICATE ANALYSIS OF PENTAZOCINE, 25 mg

Reversed-phase ion-pairing			*Normal phase*		
mg Pentazocine hydrochloride		*Percent recovery*	*mg Pentazocine hydrochloride*		*Percent recovery*
Added	*Found*		*Added*	*Found*	
28.4	27.7	97.5	28.2	28.1	99.6
28.2	28.1	99.6	28.1	27.9	99.3
28.3	28.1	99.3	28.4	27.9	98.2
28.4	28.6	100.7	28.1	27.8	98.9
28.2	27.5	97.5	28.2	27.4	97.2
Average % recovery		98.9			98.6
% R.S.D. recovery		1.41			0.97

It was possible with the inexpensive UV detector utilized in the reversed-phase studies to detect a pentazocine peak at 1:1000 standard dilution. This had a peak height ratio of approximately 2 × baseline noise. This signal however was in the non-linear portion of the range. The linear range extended at least to the 1:100 standard dilution or 5.6 μg/ml of injected solution. Table IV shows these results along with information on the linear portion of the dilution plot. While the sensitivity of the present procedure could have been increased considerably by use of a lower wavelength of detection, such as 229 nm, or by a derivatization procedure, either is unwarranted in routine dosage form analysis of this compound.

Capacity factors for benzomorphan derivatives chromatographed are shown in Table V. These ranged from 1.4 to 5.8 having retention times from 4.8 to 13.5 min. These extreme values were found for the more polar norpentazocine and the less polar ketopentazocine respectively with pentazocine itself having a capacity factor of 4.0. Fig. 4 is a chromatogram of a mixture of pentazocine and pentazocine hydrate. This latter compound is one of the few known degradation products of pentazocine[19–21]. The separation achieved for various benzomorphans under the stated reversed-phase ion-pairing conditions is seen in Fig. 5.

TABLE IV

MINIMUM QUANTIFIABLE LIMIT

Correlation line using first three points of log peak height *vs.* log dilution factor +3: slope = 0.935; intercept = 2.29; $r = 0.999$; % R.S.D. of $y = 1.27$.

Pentazocine hydrochloride standard dilution	*Computer peak height observed (computer units)*
Initial (0.561 mg/ml)	130,805
1:10	13,183
1:100	1765
1:1000	542

TABLE V

CAPACITY FACTORS FOR BENZOMORPHANS

Solvent front retention time 1.8–2.0 min.

Benzomorphan	*k'*
nor-Pentazocine	1.4
Pentazocine hydrate	1.7
Cyclazocine hydrochloride	2.4
Ketocyclazocine	3.4
Pentazocine hydrochloride	4.0
Pentazocine acetate	4.0
Win 20722	4.4
Win 28389	4.5
Ketopentazocine	5.8

The variation in capacity factor for pentazocine hydrochloride and cyclazocine hydrochloride with number of carbons in the pairing ion is shown in Fig. 6. Here a break in the curves occurs between eight and ten carbons. Behavior of this nature has been recently reported for codeine, morphine and ethylmorphine[22]. Ion-pair formation in the mobile phase was postulated with increased retention being due to an increase in hydrophobicity of the ion-pairs. This results when the pairing ion of more than eight carbons length extends beyond the surface of the morphine derivative to which it was paired. This overlap of pairing ions can be similarly visualized in the case of benzomorphans by use of molecular models. The carbon chain of the pairing ion fits well on the α side of the ring system *trans* to the nitrogen bridge. Pairing ion carbons of eight or more extend beyond the benzene ring allowing for additional

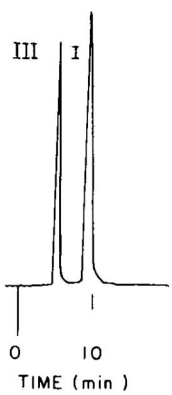


Fig. 4. Reversed-phase ion-pairing chromatogram of mixture of pentazocine hydrochloride (I) and its degradation product pentazocine hydrate (III). Chromatographic conditions given in Fig. 2.

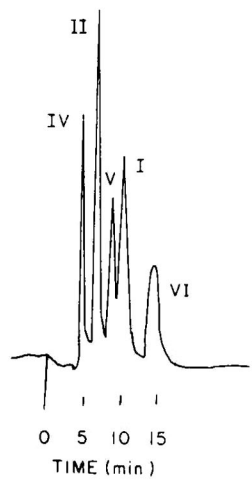


Fig. 5. Reversed-phase ion-pairing chromatogram of benzomorphan mixture including nor-pentazocine (IV), cyclazocine hydrochloride (II), ketocyclazocine (V), pentazocine hydrochloride (I) and ketopentazocine (VI). Chromatographic conditions given in Fig. 2.

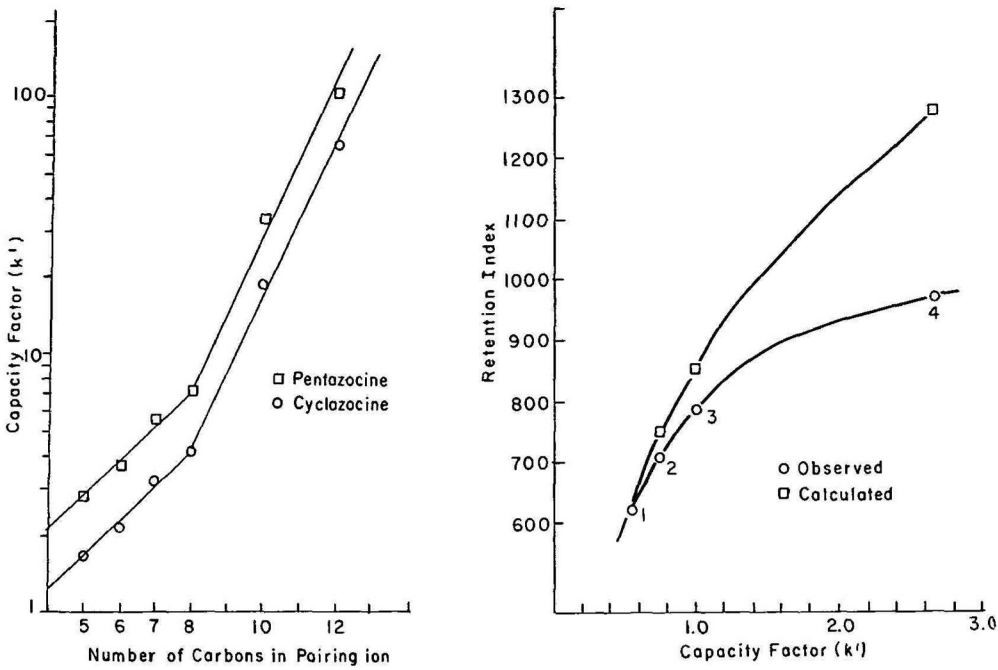


Fig. 6. Relationship between log capacity factor and number of carbons in pairing ion for pentazocine and cyclazocine. Mobile phase: sodium salt of pairing ion (0.005 *M*)–methanol–H_3PO_4 (600:600:1). Stationary phase: octadecylsilane.

Fig. 7. Relationship between calculated and observed retention indices (ref. 16) and measured capacity factors. 1 = Morphine, 2 = codeine and 3 = ethylmorphine (ref. 22), 4 = pentazocine, this study. Mobile phase: 0.005 *M* sodium octanesulfonate and 0.01 *M* ammonium nitrate in acetonitrile–water (375: 625). pH adjusted to 3.3 with acetic acid.

hydrophobic interaction with non-polar bonded phases. Ion-pairing of this nature has similarly been proposed by Loh *et al.*[23] between opiates and cerebroside sulfate, a model for the opiate receptor.

A decrease in capacity factors for pentazocine and cyclazocine to 2.7 and 1.8 respectively was obtained when chromatographed under the conditions of the earlier work[22] using the octanesulfonate pairing ion. This gives the same rank ordering as the retention index values calculated and measured in the non-pairing-ion reversed-phase study of Baker *et al.*[16]. In that study the calculated retention index, based on lipophilicity, of pentazocine was greater than the observed value. This indicates that partitioning behavior into the C_{18} bonded phase was less important under the conditions of their analysis. The non-linear nature of the relation between retention indices and observed capacity factors obtained by ion-pairing seen in Fig. 7 shows that different mechanisms are operative. The increased hydrophobicity of the formed ion-pair and a possible ion exchange component would contribute to this increased retention.

The results of the pairing ion concentration effect are seen in Fig. 8 where the customary "hyperbolic" relation between capacity factors for pentazocine and cycla-

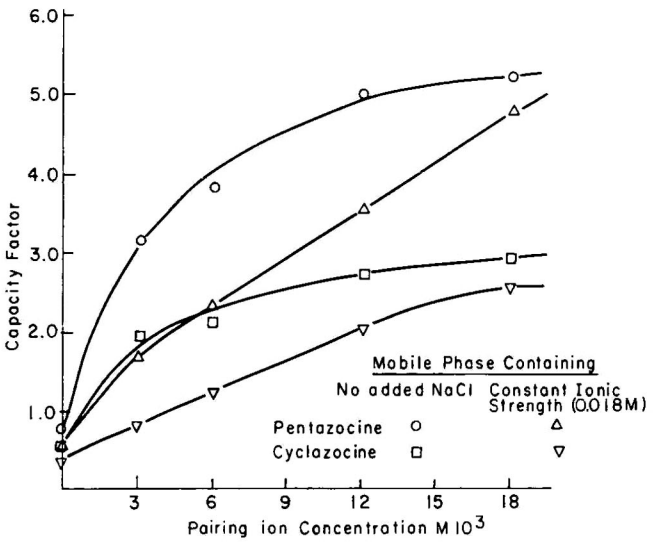


Fig. 8. Effect of sodium octanesulfonate pairing ion concentration on capacity factor for: pentazocine hydrochloride (○) and cyclazocine hydrochloride (□); pentazocine hydrochloride (△) and cyclazocine hydrochloride (▽) at constant ionic strength of 18 m*M* with added NaCl. Other chromatographic conditions as in Fig. 2.

zocine and the pairing ion concentration is apparent. While this relation can be explained by either ion-pair formation in the mobile phase or an ion exchange retention mechanism[24], it is umimportant to the actual chromatographic process according to other authors[25]. The decrease in capacity factor with increased ionic strength apparent in Fig. 8 has been previously observed for a series of benzoic acids[26]. This behavior can be explained by a decrease in the ion-pair formation constant at increased ionic strength, possibly due to a shielding effect of the added ions. This could support a retention mechanism such as the dynamic complex exchange of Melander and Horvath[24].

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AUTOMATED PURIFICATION OF HUMAN ERYTHROCYTIC 6-PHOSPHOGLUCONATE DEHYDROGENASE

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SUMMARY

Human 6-phosphogluconate dehydrogenase (6PGD) was purified from hemolyzate by group affinity chromatography on 2′,5′-ADP-Sepharose, followed by buffer exchange chromatography on Sephadex G-25 and finally salting-out chromatography on Sepharose 6B. An apparatus was assembled from commercially available elements, in which the purification procedure can proceed and be completed unattended and under fully automatic control. The weekly production of the set-up is at present 10 mg of pure 6PGD. The choice of the purification procedure and the advantages of automation are discussed.

INTRODUCTION

6-Phosphogluconate dehydrogenase (D-6-phosphogluconate: $NADP^+$ 1-oxidoreductase, E.C. 1.1.1.44) (6PGD) is an enzyme of the pentose shunt pathway. This dimeric enzyme (molecular weight of the monomer 53,000 daltons) catalyzes the transformation of 6-phosphogluconate into D-ribulose 5-phosphate. Numerous variants of human 6PGD have been described based on electrophoretic and functional studies of 6PGD from blood cells[1,2]. Human 6PGD has often been found to occur in post-translational modifications[3]. More information on the covalent structure of the native enzyme is required for the study of 6PGD variants or of post-translational modifications on a structural basis. If such a study of the enzyme is to be undertaken, relatively large amounts, that is several tens of milligrams, of protein must be purified, even though modern techniques of protein sequencing have reduced, by several orders of magnitude, the actual amount of material needed[4].

Since blood is easy to obtain, it is the preferred starting material for the purification of human 6PGD. Initially, two methods were considered for the purification of large quantities of 6PGD. The first was to start from a large amount of blood (20 or 30 l). The second was to purify the protein from a smaller volume of blood (that is less than 1 l), but repeat the procedure many times. The first choice was discarded because we did not possess the required equipment, namely centrifuges accommodating large volumes, or high-capacity chromatography columns. However, the second method is

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very time-consuming. The solution was to automate nearly all the purification procedure.

In this paper we describe our automated purification procedure. It is based on a technique described by Morelli[5], but with substantial modifications.

EXPERIMENTAL

Materials

Most chemicals (reagent grade) were purchased from E. Merck (Darmstadt, G.F.R.); NADP was obtained from Boehringer (Mannheim, G.F.R.). Chemicals for Dns derivatization were purchased from Pierce (Rockford, IL, U.S.A.) and polyamide plates from Schleicher & Schüll (Dassel, G.F.R.). The 2′,5′-ADP-Sepharose, Sephadex G-25, DEAE-Sephadex A-25 and Sepharose 6B were purchased from Pharmacia (Uppsala, Sweden), DEAE-Trisacryl M from l'Industrie Biologique Française (Gennevilliers, France) and DE-22 from Whatman (Maidstone, Great Britain). Electromagnetic valves were obtained from ASCO (Scherpenzeel, The Netherlands), small-bore Kel F valves and their pneumatic actuators from Gilson (Villiers le Bel, France). Poly(tetrafluoroethylene) or polyethylene tubings were connected with standard 1/4-in. 28-thread fittings (Gilson), Swagelok fittings from Crawford Fitting (Solon, OH, U.S.A.) or with fittings made in the laboratory from parts of perfusion systems supplied by Vermed (Neuilly en Thelle, France). Polyethylene two- or three-way stopcocks were also obtained from Vermed. The columns were from Whatman or made in the laboratory. Most of the glassware used in the automatic chromatography system were custom-made, according to our design, with Sovirel SVL parts (Sovirel, Levallois Perret, France). The pumps used in the chromatographic system were purchased either from Gilson or from Ismatec (Zurich, Switzerland).

Top 2000 electric timers were obtained from Crouzet (Valence, France), relays, switches, electric plugs and sockets, as well as wiring elements, from local retailers. Human blood, from CNTS (Paris, France), consisted of freshly outdated hemoconcentrates.

Enzymatic assay

6PGD was assayed, at 30°C, in 50 m*M* triethanolammonium chloride buffer, pH 7.5, containing 0.1 *M* $MgCl_2$, 0.25 m*M* NADP and 10 m*M* 6-phosphogluconate.

Protein assay

The hemoglobin concentration was determined according to the method of Drabkin[6]. The protein concentration of pure 6PGD solutions was determined from the absorbance at 280 nm ($E^{\%}_{1\,cm}$ was found to be 10 with precisely weighed, pure, lyophilized 6PGD).

Polyacrylamide gel electrophoresis

10% Acrylamide slab gel electrophoresis containing sodium dodecyl sulphate (SDS) was performed according to Laemmli's technique[7].

N-terminus study

The Dns derivatization of several samples of purified 6PGD (each 0.15 mg) was performed according to the method of Weiner *et al.*[8]. The acid hydrolysis of Dns-

protein was carried out with 5.7 *N* HCl, or with 3 *N p*-toluenesulphonic acid containing 0.2% 3-(2-aminoethyl)indole[9] in order to avoid the destruction of an eventual Dns-N-terminal tryptophan. The hydrolysis products of Dns-6PGD were submitted to thin-layer chromatography on polyamide plates[10].

Buffers used in the purification procedure

The buffers used in the purification procedure contained 10^{-3} *M* ethylenediaminetetraacetic acid, 10^{-2} *M* β-mercaptoethanol and 10^{-3} *M* ε-aminocaproic acid. They were prepared in large volumes and either stored at 4°C, or frozen in suitable aliquots if they contained labile substances like NADP or urea. Buffer A was 0.2 *M* potassium phosphate, pH 6.00; B was 0.2 *M* potassium phosphate, pH 7.85; C was 0.1 *M* potassium phosphate buffer, pH 7.85, containing 0.1 *M* KCl; D was 0.05 *M* potassium phosphate, pH 7.85.

The elution buffer was prepared by dissolving 400 mg of NADP in 100 ml of buffer D (final concentration of NADP, *ca.* 5 m*M*). Washing buffers containing 6 *M* urea and 1 *M* KCl were adjusted to either pH 6.5 (acid buffer) or pH 8.5 (basic buffer). Buffer E was 0.04 *M* potassium phosphate, pH 5.6, 45% saturated with ammonium sulphate; F was 0.04 *M* potassium phosphate, pH 5.6, 30% saturated with ammonium sulphate.

The amount of ammonium sulphate needed for the preparation of the buffers was calculated from tables[11].

Valves

Many valves were used in the chromatography system. They were: (a) four stainless-steel two-way valves (one normally open, three normally closed); (b) four three-way slide valves and two four-way slide valves made of acetal resin, their positioning being ensured by pneumatic activators which were connected either to compressed air or to the atmosphere, by electromagnetic three- or four-way brass valves.

Purification procedure

The procedure consisted of three chromatographic separations following the preparation of hemolyzate.

The first chromatogram was carried out in a 2′,5′-ADP-Sepharose column. The 6PGD was eluted with an NADP-containing buffer. The second chromatogram, on Sephadex G-25, was performed to exchange the elution buffer with buffer E (which contained ammonium sulphate at 45% saturation). During the third chromatogram on a Sepharose 6B column, the 6PGD was salted out at 45% saturation with ammonium sulphate, and eluted in a pure form with a linear gradient of decreasing ammonium sulphate concentration. The three chromatography columns were interconnected by tubings and valves. The operation of the valves and pumps was controlled by a programmer, and the whole chromatographic procedure was, thus, entirely automatic. A flow chart of the system is shown in Fig. 1, a photograph of the purification set-up placed in a cold room is shown in Fig. 2 and Fig. 3 shows the electric wiring diagram of the programmer.

The entire purification procedure shall now be described step by step.

Preparation of the hemolyzate. Two bags of packed red cells were pooled and two volumes of cold distilled water were added. The mixture was quickly frozen in a

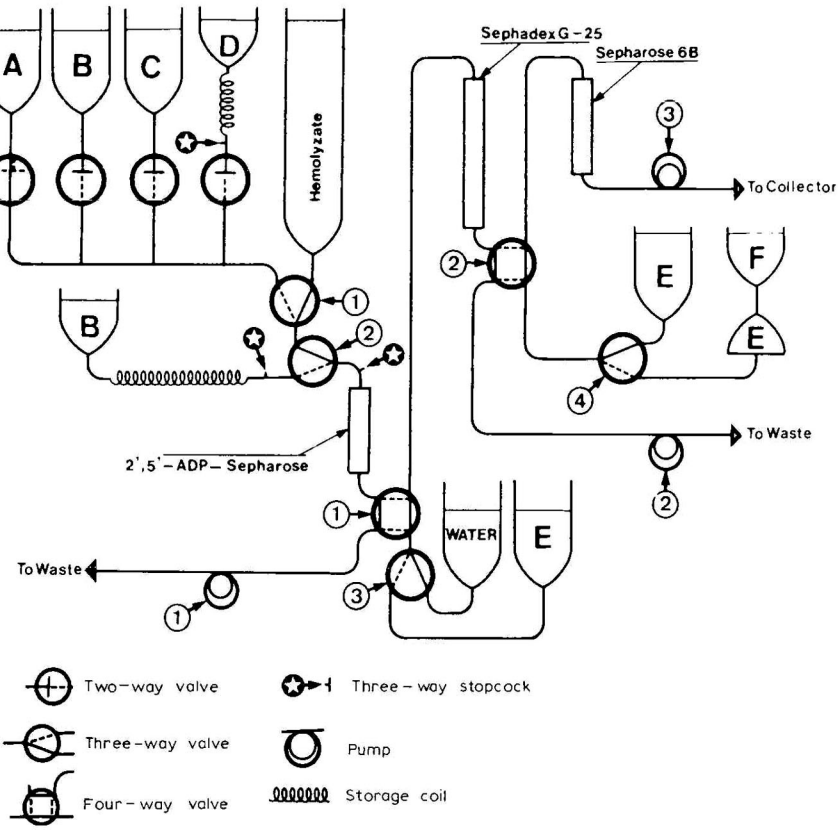

Fig. 1. Diagram of the chromatographic system. The two positions of the valves are shown: the solid line through the valve shows the inactivated position, while the dotted line indicates the activated position. The three- and four-way valves, as well as the pumps, are numbered as in the text. The absorbance monitor and the magnetic stirrer are not shown. Numerous two- and three-way stopcocks have also been deleted, as they are not absolutely necessary to operate the set-up, but simply convenient for cleaning or flushing the tubings and the buffer reservoirs.

large polyethylene basin floating on a dry ice–methanol bath. After 1 h, the polyethylene basin was removed from the bath and allowed to float in a sink filled with running tap-water. As the hemolyzate thawed, the liquid was removed with a peristaltic pump and pumped into a graduated cylinder surrounded with crushed ice. After all the hemolyzate had been transferred, it was diluted with an equal volume of buffer A–water (1:1). After homogenization, the hemolyzate was centrifuged for 45 min at 15,000 *g*. The supernatant, or part of it, was used in the first step of purification.

2',5'-ADP-Sepharose chromatography. When the automatic program was initiated by pressing the start button (Fig. 3), the hemolyzate was pumped on the column (2.5 cm wide, 2 cm long) through three-way valve 1 (Fig. 1) by peristaltic pump 1 (working at high flow-rate, 90 ml/h). Twelve hours later, the position of the three-way valves was changed, and the peristaltic pump was set at low flow-rate, 60 ml/h. The column was then rinsed with buffers A, B and C for 1, 1 and 2.5 h respectively. The buffer selection was achieved through appropriate switching of the stain-

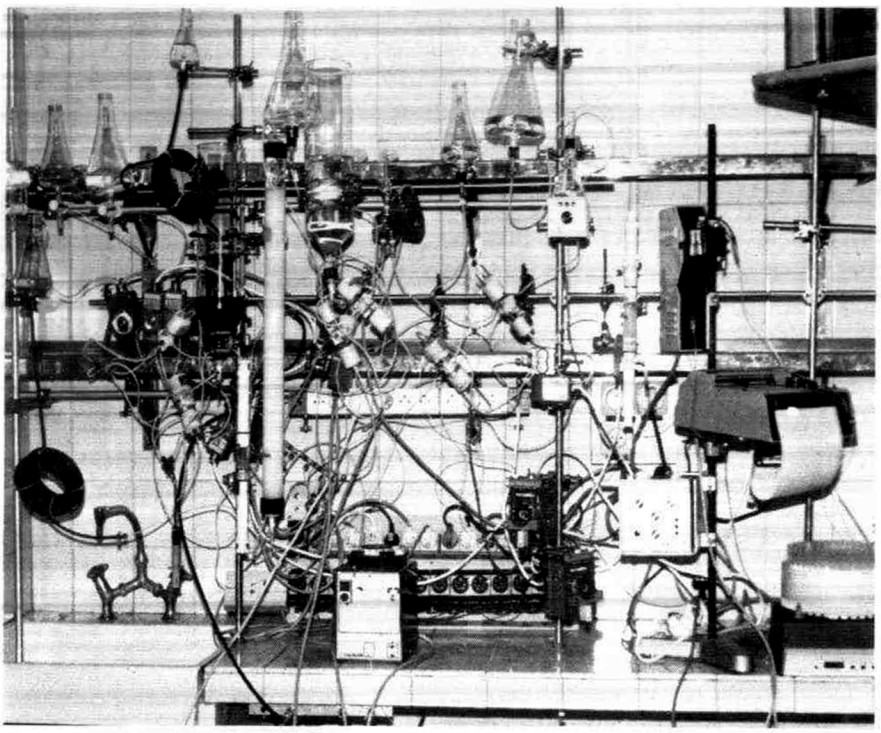

Fig. 2. Photograph of the purification set-up.

less-steel two-way valves situated ahead of the column. (The two-way valve for buffer A is normally open while the other two are normally closed.) The 6PGD was eluted by pumping the NADP-containing buffer on the column. Before the initiation of the program, using a syringe and a three-way stopcock, this buffer was placed in a storage coil (made of polyethylene tubing, 0.4 cm I.D.) connected with a glass reservoir filled with buffer D. In this way, all the NADP-containing buffer can be flushed on to the column with buffer D. When purified 6PGD was present in the effluent of the 2′,5′-ADP-Sepharose column, a four-way valve (1) in the effluent line was activated, and the outlet of the 2′,5′-ADP-Sepharose column thus connected to the inlet of the G-25 column and disconnected from the waste reservoir.

After sufficient time for the deposition of the 6PGD on top of the G-25 column, the four-way valve 1 was deactivated and the effluent line again connected to the waste reservoir. The 2′,5′-ADP-Sepharose column was then automatically rinsed sequentially with acid and alkaline urea-containing buffers, then with buffer B by actuation of three-way valve 2. The polyethylene coil situated ahead of this valve was filled successively with buffer B, alkaline and acid urea-containing buffers, using a syringe and a three-way stopcock as previously described for the NADP-containing elution buffer. This elegant method of programming buffer changes without any valve, by simply using storage tubing of small inside diameter, has been described and used for amino acid analysis by Hare[12]. The column was then re-equilibrated with buffer A by suitable positioning of the three-way valve 2 and of the two-way valves.

Sephadex G-25 chromatography. The purpose of the chromatography on the

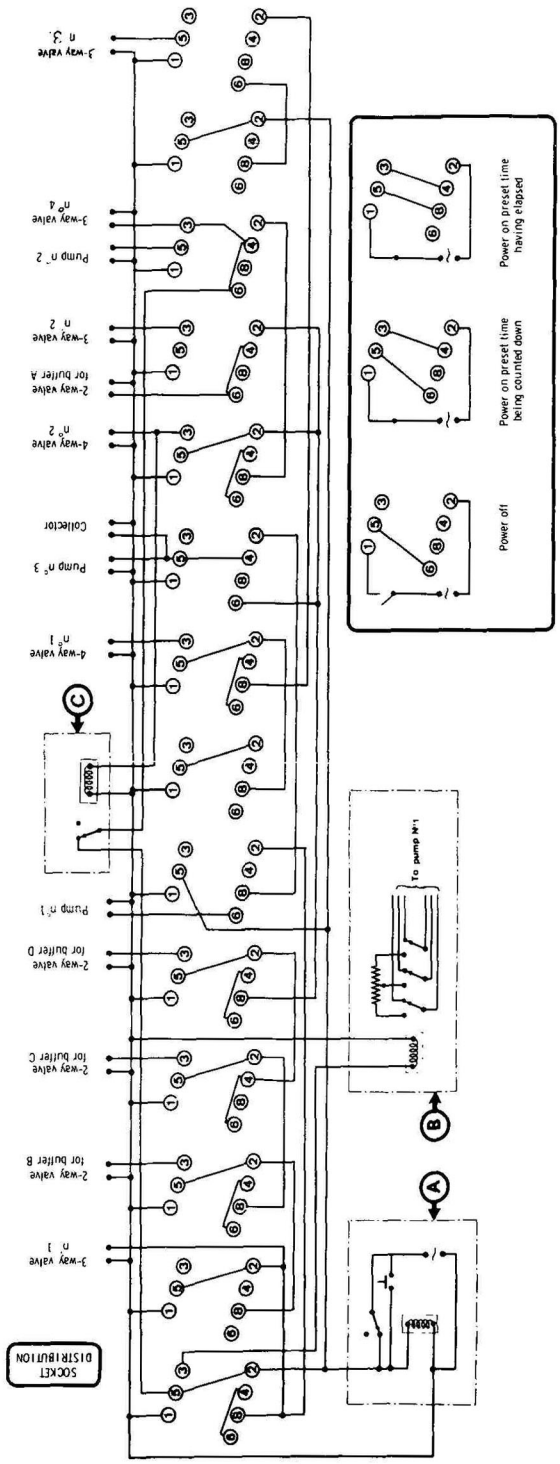

Fig. 3. Diagram of the wiring of the programmer. The main part shows the wiring: only the connections added to the timers are shown; the internal connections of terminal 5 to terminals 6 or 8, or of 3 to 4, are not indicated. The boxes show the three auxiliary assemblies whose functions are explained in the text. Assembly A is shown as running, that is with the relay excited. Some switches used for the manual activation of auxiliary assemblies, and some pilot lights, have been deleted to simplify the diagram. The insert shows the internal connections of the timers and how they work: when the electric power is connected to terminals 1 and 2, the preset time begins to be counted down while 3 and 4 are shorted together; when the preset time has elapsed, the contact between 5 and 6 is broken but 5 and 8 are shorted together. A reset of the timer is obtained by switching off the power to 1 or 2.

G-25 column equilibrated in buffer E is to exchange the 6PGD-containing buffer: the protein must be dissolved in buffer E before chromatography on Sepharose 6B. This chromatography was carried out at a constant flow-rate of 60 ml/h (peristaltic pump 2 or 3), on a column 3.6 cm wide and 65 cm long. The proteins, eluted from the 2′,5′-ADP-Sepharose, were deposited on top of the G-25 column after flowing through four-way valve 1. The four-way valve, in the effluent line of G-25 column, was activated in due course in order to let the proteins, eluted in the void volume of the G-25, be deposited on top of the Sepharose 6B column, while four-way valve 2 was activated, pump 2 turned off and the flow on both G-25 and Sepharose 6B columns ensured by pump 3 only.

It should be noted that the G-25 column must be rinsed with water after the buffer exchange. If this is not done, a retention of NADP on the column is observed, and large volumes of buffer E as well as a long time would be necessary to wash out completely the NADP. The washing of the G-25 column with water as well as its re-equilibration with buffer E for the next chromatographic step was effected through three-way valve 3. Of course, during washing and re-equilibration, the four-way valve 2 directs the G-25 effluent to the waste reservoir.

Sepharose 6B chromatography. The chromatography on Sepharose 6B was carried out at a constant flow-rate of 60 ml/h (peristaltic pump 3), in a column 1.6 cm wide and 15 cm long. The Sepharose 6B column was equilibrated in buffer E when the proteins eluted from the G-25 column were deposited on top of it due to the movement of four-way valve 2. After the automatic completion of sample application, the Sepharose 6B column was washed isocratically with buffer E for 15 min, then a linear gradient, made with 100 ml of buffer E and 100 ml of buffer F, was initiated through activation of three-way valve 4. The shape of the gradient, as well as the automatic rinsing of the column with limiting buffer, was simply obtained with an erlenmeyer flask containing 100 ml of initial buffer. The outlet of the flask was connected with the top of the column by three-way valve 4. The inlet was connected to a reservoir containing limiting buffer. Hence the flask is refilled with limiting buffer at the same rate as it is pumped on the column; magnetic stirring permits an efficient mixing of the initial and the final buffers.

The effluent from the Sepharose 6B column flows first through the cuvette of an absorbance monitor, then towards a fraction collector. The Sepharose 6B column is re-equilibrated with buffer E during the next chromatographic step.

Pure 6PGD was eluted nearly at the end of the gradient as a symmetrical optical density peak. The contents of the enzyme-containing tubes were pooled and the protein was concentrated to 4 ml with a Millipore immersible filter CX30. A 1-ml aliquot of glycerol was added, and the enzyme solution stored at 4°C.

Programmer for the automatic purification

The programmer comprised a number of inexpensive electric timers. Their mode of functioning is shown in the insert of Fig. 3.

The wiring of the fourteen timers is shown in Fig. 3. Three auxiliary assemblies were added to the timers. The function of assembly A is to prevent an automatic restart at the beginning of the program if the electric current were to be shut down for a time. Instead, in case of temporary electric power failure, the pumps will remain turned off and the purification will proceed only if the start push button is pressed

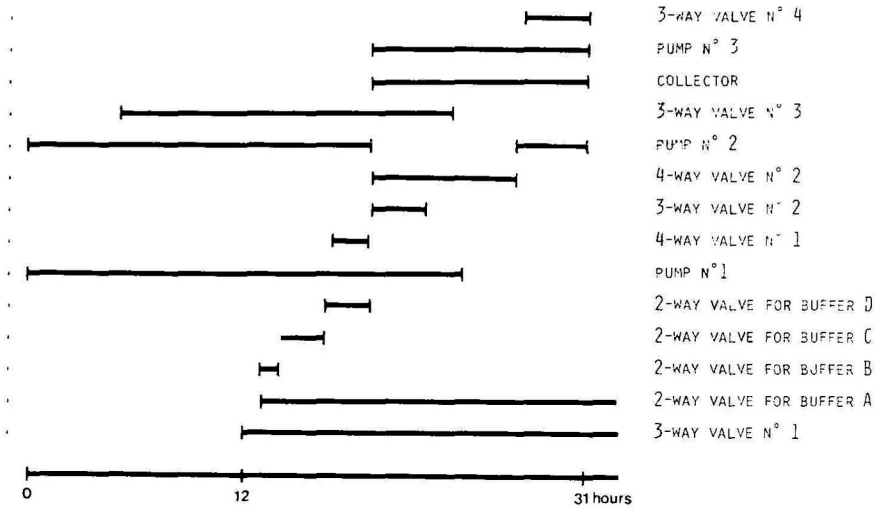

Fig. 4. Diagram of the program routinely used for the automatic purification of 6PGD. The solid lines parallel to the time axis show when the power is brought to the electric sockets feeding the valves, the pump or the collector in operation. For instance, buffer B flows on the 2′,5′-ADP-Sepharose column if the power is brought to peristaltic pump 1 and if the two-way valve for buffer B (normally closed) is activated together with the two-way valve for buffer A (normally open) and the three-way valve 1 (four-way valve is inactivated).

manually after the necessary adjustments of the preset times of the fourteen timers. Auxiliary assembly B is used to control the high flow-rate of pump 1; C turns off pump 2 while four-way valve 2 is activated. The programmed functions, with the exception of the one depending on auxiliary assembly B, are obtained by switching on the current from the electrical sockets in which are plugged the power cords of the electromagnetic valves pumps or of the collector.

Fig. 4 shows a diagrammatic representation of the program used for the purification set-up.

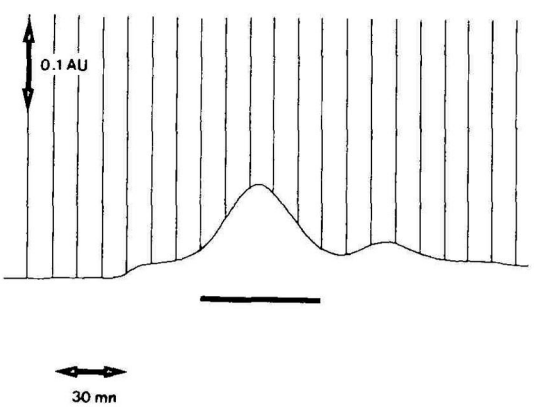

Fig. 5. Recorder tracing of the absorbance monitor. The chart paper speed was 4 cm/h. The vertical lines intersecting the recorder tracing are made by the event marker of the collector. The horizontal bar shows the 6PGD-containing tubes to be pooled.

RESULTS

Fig. 5 shows a part of a typical recorder tracing of the absorbance monitored at the Sepharose 6B outlet.

The contents of the tubes corresponding to the optical density peak, identified by the horizontal bar, were pooled. They contained the 6PGD activity; the smaller peak which follows the 6PGD peak corresponds to a protein as yet unknown. In the sodium dodecyl sulphate (SDS) gel system, it migrates slightly more slowly than 6PGD.

The overall yield of the purification is usually between 45 and 55%. The specific activity of the purified enzyme is of 25 IU/mg. This corresponds to a 5600-fold purification. SDS slab gel electrophoresis is used routinely to check the purity of each batch of automatically purified 6PGD.

Fig. 6 shows a photograph of one such slab gel which was loaded with 50 μg of 6PGD from eight different batches.

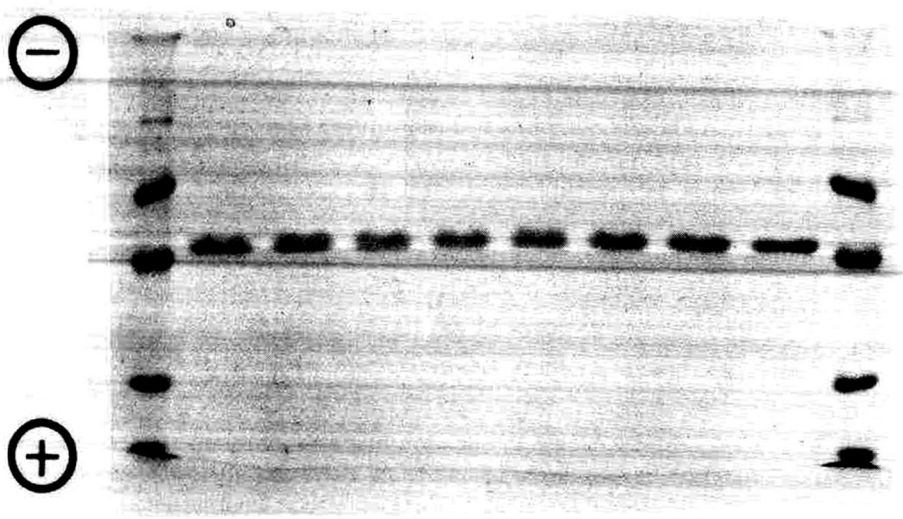

Fig. 6. SDS slab gel. Aliquots of 50 μg of purified 6PGD were loaded on each lane, with the exception of the left and right one where standards of known molecular weight were placed. The gel was coloured with Coomassie blue.

After Dns derivatization and acid hydrolysis of 6PGD, no Dns-amino acid, other than Dns-O-tyrosine and ε-Dns-lysine, was found.

DISCUSSION

The use of 2′,5′-ADP-Sepharose chromatography for purification of human 6PGD was first described by Morelli[5]. Actually, he described a complete procedure based on a 2′,5′-ADP-Sepharose chromatography followed by an ion-exchange step on DEAE-Sephadex A-25. The end product was said to be a pure protein, based on the results of SDS gel electrophoresis. In our hands this procedure did not work very

well: the resulting 6PGD was always contaminated with at least one slower migrating band. Other ion exchangers, DE-22 and DEAE-Trisacryl M, were tried in either manual or automated procedures.

Several modifications of the elution conditions were also tried. However, it was not possible to obtain reproducibly 6PGD with a single band on SDS gels. It should be noted, however, that Morelli prepared the 2′,5′-ADP-Sepharose employed and it is possible that we failed to reproduce his results because we used commercial 2′,5′-ADP-Sepharose with a different degree of substitution and, therefore, different chromatographic properties.

Since we did not succeed in obtaining a pure protein by ion exchange after group affinity chromatography, we decided to adopt, for the next purification step, a completely different technique. We tried salting-out chromatography. In order to define the precise conditions of this purification step we followed the guidelines of Von der Haar[13,14]: at pH 5.6, 6PGD precipitated in a 55% saturated ammonium sulphate solution. When 6PGD, dissolved in 45% ammonium sulphate, at the same pH, was applied to a Sepharose 6B column, the enzyme was adsorbed by the Sepharose. The protein could then be desorbed from the column by a decreasing ammonium sulphate gradient. The enzyme obtained after this salting-out chromatography was pure, as shown by SDS gel electrophoresis and N-terminal end group determination (no free N terminus could be detected).

Salting-out chromatography on unsubstituted Sepharose has been used for preparative purposes by several authors[13–15]. In the particular case of the purification of halophilic enzymes extracted from Halobacterium, this technique was thought to be particularly satisfactory since the preservation of these enzymatic activities requires the use of high salt concentrations throughout the whole purification procedure[15]. The work of Von der Haar[13,14], as well as our own, shows that salting-out chromatography on unsubstituted Sepharose can be a useful alternative to other, more common, chromatographic procedures, even for proteins which do not share the special properties of the halophilic enzymes. It should be noted that our yield from salting-out chromatography on Sepharose 6B is somewhat low (ranging from 50 to 60%), compared to the yields published by other users of this method; the salting-out chromatography on Sepharose of halophilic enzymes (ref. 15 and relevant references cited therein) resulted in yields of 74% or higher. Von der Haar[13] obtained 88 and 94% for two different proteins, but only 59% for a third one. The missing enzymatic activity in the pooled peak of 6PGD cannot be found in any other fraction, and cannot be desorbed from the Sepharose 6B column by rinsing it with low ionic strength buffer. It should be pointed out that when 6PGD activity in effluent of G-25 column is searched manually, it appears that the activity peak is eluted later than the excluded volume of the G-25 column measured when the latter is equilibrated with water. The activity peak is also somewhat tailing. It can be postulated that such an abnormal behaviour of 6PGD on G-25 at high ammonium sulphate concentration results from the same mechanism which governs salting out on Sepharose 6B. The yield from the G-25 chromatography is nevertheless quantitative.

We feel that the automation of the major part of the purification procedure constitutes a very important improvement: the purification set-up is started three times a week, thus producing nearly 10 mg of pure enzyme per week. Two and a half hours are sufficient to: pool the contents of 6PGD-containing tubes from the preced-

ing purification, prepare a fresh hemolyzate and set the purification machine in starting conditions. The preparation of the hemolyzate is made as simple as possible; the centrifugation before placing the hemolyzate on the 2′,5′-ADP-Sepharose is necessary to avoid clogging the column with cellular debris. Our attempts to substitute centrifugation by filtration were unsuccessful; of course, loading the centrifuge and collecting the supernatants remains a manual procedure. Before starting the purification procedure, one must check the level of the buffer reservoirs and refill them if necessary. (As mentioned in Experimental all buffers were prepared in bulk, thus reducing the work of operating the set-up.) It is necessary to fill the two storage coils and the gradient generator, to put new clean tubes in the fraction collector, to fill the cleaned hemolyzate reservoir with the new hemolyzate and to flush the tubing between the hemolyzate reservoir and the 2′,5′-ADP-Sepharose column by use of a syringe and a three-way stopcock (see Fig. 1). The start button is then pushed, and the purification can proceed unattended for 31 h, as shown in Fig. 4.

The success of the automation was made possible by the small number of chromatographic steps. If a precipitation in the liquid phase had been one of the necessary steps of purification, automation would have been considerably more difficult. In addition, the fact that all columns could be operated at the same flow-rate of 60 ml/h was advantageous. Of course, the possibility of using the columns several times without repacking makes automation more attractive by decreasing the maintenance work. To date, our 2′,5′-ADP-Sepharose column has not needed repacking although it has been used three times a week for 3 months; G-25 and Sepharose 6B columns have been used for 8 months without repacking.

To summarize, we have described a purification procedure for 6PGD from human erythrocytes, based on group affinity chromatography on 2′,5′-ADP-Sepharose followed by salting-out chromatography on Sepharose 6B. The automation of the chromatographic procedure permits the production of 10 mg of pure native enzyme per week, with a minimum amount of time and work.

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CHROM. 14,839

DETERMINATION OF DIAMINE OXIDASE ACTIVITY BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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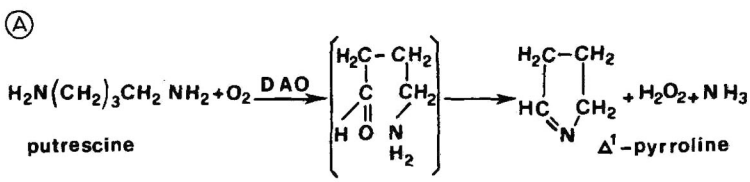
SUMMARY

A sensitive method for the assay of diamine oxidase is described, based on the separation by reversed-phase high-performance liquid chromatography of the products arising from the oxidation of the reagents (A) homovanillic acid, (B) 3,5-dichloro-2-hydroxybenzenesulphonic acid–4-aminoantipyrine and (C) pyridoxine–4-amino-N,N-diethylaniline. The analysis time is short and the assay is quantitative and reproducible. Because of its speed and high sensitivity and selectivity, the method is a useful alternative to the standard spectrophotometric assay.

INTRODUCTION

Diamine oxidase (diamine:oxygen oxidoreductase, deaminating, E.C. 1.4.3.6) (DAO) from pea seedlings oxidizes short-chain aliphatic diamines[1], such as putrescine, to give an aminoaldehyde, ammonia and hydrogen peroxide. The aminoaldehyde cyclizes to give Δ^1-pyrroline and the overall reaction is



Many methods have been reported for the assay of DAO based on manometric[2] and colorimetric[3–6] techniques. All of these methods are time consuming, of low sensitivity or not adequately reproducible and quantitative.

High-performance liquid chromatography (HPLC) has potential for enzyme assays, because it is rapid, selective and sensitive. In a previous paper[7] we described a reversed-phase HPLC method for the assay of oxalate oxidase by coupling the en-

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zymatically produced hydrogen peroxide with peroxidase and homovanillic acid. This approach has been extended to the determination of DAO activity using both the fluorogenic homovanillic acid (A) and the chromogenic 3,5-dichloro-2-hydroxybenzensulphonic acid–4-aminoantipyrine (B)[8] and pyridoxine–4-amino-N,N-diethylaniline (C)[9].

Reagent B has been widely employed in clinical chemistry after its application by Trinder[8] to the enzymic determination of glucose. As alternative we investigated also reagent C, which is oxidized by hydrogen peroxide/peroxidase to a strongly UV-absorbing quinoneimine.

The sequences of the reactions are shown in Fig. 1.

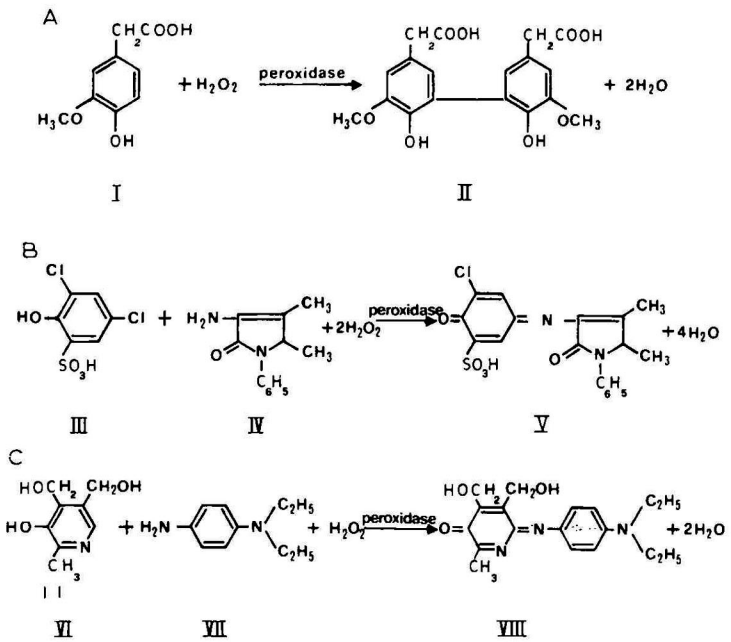


Fig. 1. Reagent A: I = homovanillic acid; II = 2,2′-dihydroxy-3,3′-dimethoxybiphenyl-5,5′-diacetic acid. Reagent B: III = 3,5-dichloro-2-hydroxybenzenesulphonic acid; IV = 4-aminoantipyrine; V = 4′-aminoantipyryl-3-chloro-5-sulphonic acid-1,4-benzoquinoneimine. Reagent C: VI = pyridoxine; VII = 4-amino-N,N-diethylaniline; VIII = 4′-amino-N,N-diethylanilyl-3,4-dihydroxymethyl-6-methyldihydropyridine-2,5-dioneimine.

The oxidized products are separated by reversed-phase HPLC and determined by fluorometric and ultraviolet detection.

EXPERIMENTAL

Apparatus

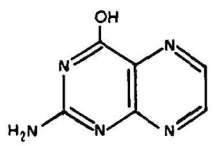
The chromatographic unit consisted of a Model 6000 solvent delivery system, a Model U6K Universal Injector, a Model 440 ultraviolet detector, a Model 730 Data Module (Waters Assoc., Milford, MA, U.S.A.) and a Jasco FP-110 spectrofluorimeter.

Ultraviolet absorption was measured at 254 nm; the fluorescence excitation and emission maxima were 315 and 425 nm, respectively.

A pre-packed stainless-steel column containing Bondapak C_{18} (30 cm × 4 mm I.D.; particle size 10 μm) was obtained from Waters Assoc. A pre-column of Bondapak C_{18} Corasil (2 cm × 4 mm I.D.) was used.

Materials

Reagent-grade diammonium hydrogen phosphate, 4-aminoantipyrine, pyridoxine hydrochloride, hydrogen peroxide, tris(hydroxymethyl)aminomethane were obtained from Merck (Darmstadt, G.F.R.), 3,5-dichloro-2-hydroxybenzenesulphonic acid (DHBS) from BDH (Poole, Great Britain), 4-amino-N,N-diethylaniline sulphate from Koch-Light (Colnbrook, Great Britain), homovanillic acid (HVA), nicotinic acid, 4-aminoantipyrine and peroxidase (Type II) from Sigma (St. Louis, MO, U.S.A.) and 2-amino-4-hydroxypteridine (AHP) from Fluka (Buchs, Switzerland).



2-Amino-4-hydroxypteridine

(AHP)

DAO was purified from 10-day germinated pea seedlings according to the method of Srivastava and Prakash[10]. Water was deionized and distilled and was filtered through Millipore membrane filters, pore size 0.45 μm (Millipore, Bedford, MA, U.S.A.). Methanol was of HPLC grade (Chromasolv; Riedel-De Haen, Hannover, G.F.R.).

Reagent A

Working reagent. HVA (0.17 mg/ml) and peroxidase (0.05 mg/ml) were dissolved in 0.1 *M* tris(hydroxymethyl)aminomethane buffer (pH 7.8). The reagent should be used within 12 h.

Reagent B

Buffer–peroxidase–4-aminoantipyrine reagent. 4-Aminoantipyrine (0.032 mg/ml) and peroxidase (0.6 mg/ml) were dissolved in 0.01 *M* diammonium hydrogen phosphate (pH 7.0).

DHBS reagent. 3,5-Dichloro-2-hydroxybenzenesulphonic acid (0.5 mg/ml) was dissolved in water.

Working reagent. The buffer–peroxidase–4-aminoantipyrine and DHBS reagents were mixed in the ratio 1:1. The resulting mixture should be used within 12 h.

Reagent C

Buffer–peroxidase–pyridoxine reagent. Pyridoxine hydrochloride (2 mg/ml) and peroxidase (1.72 mg/ml) were dissolved in 0.05 *M* diammonium hydrogen phosphate buffer (pH 7.0).

4-Amino-N,N-diethylaniline reagent. 4-Amino-N,N-diethylaniline sulphate (0.75 mg/ml) was dissolved in water.

Working reagent. The buffer–peroxidase–pyridoxine and 4-amino-N,N-diethylaniline reagents were mixed in the ratio 0.5:1. The mixture should be used within 12 h.

Nicotinic acid solution. Nicotinic acid (0.15 mg/ml) was dissolved in water.

AHP solution. 2-Amino-4-hydroxypteridine (0.082 mg/ml) was dissolved in 0.01 *M* sodium hydroxide solution (stable at 4°C for 4 weeks). A 1:100 dilution of this stock solution with 0.02 *M* sodium phosphate buffer (pH 7.25) was made before use.

Chromatographic conditions

Chromatography was performed at room temperature using 0.01 *M* diammonium hydrogen phosphate buffer–methanol. Before mixing, the buffer was adjusted to pH 4.9 with 20% phosphoric acid and filtered through a 0.45 μm membrane filter (Type HA, Millipore). The buffer to methanol ratios were 45:55, 40:60 and 25:75 for A, B and C, respectively. The flow-rates were 1.0 ml/min for A and B and 1.3 ml/min for C. Chromatographic peaks were monitored at 254 nm for B and C and fluorimetrically for A using an excitation filter of 315 nm and an emission cut-off filter of 425 nm.

Calibration graph

A 1-ml volume of working reagent A, B or C was subjected to reaction 0.0–0.20 μmol of hydrogen peroxide. After 15 min, 0.5 ml of AHP solution (A) or 0.15 ml of nicotinic acid solution (B and C) was added and replicate injections of 5 μl were made for each sample.

Diamine oxidase activity

A 5-μl volume of enzyme solution (1 mg/ml) and 1 μl of 0.1 *M* putrescine in 0.05 *M* phosphate buffer (pH 6.5) were added to 1 ml of working reagent A, B or C. The resulting mixture was incubated at 37°C for different times, then, 0.5 ml of AHP solution (A) or 0.15 ml of nicotinic acid solution (B and C) was added and 5 μl were injected into the chromatograph.

RESULTS AND DISCUSSION

Several mobile phases were investigated in order to develop a single system for all three reagents. Different ratios of 0.01 *M* diammonium hydrogen phosphate buffer (pH 4.9) to methanol gave the best results. The oxidized compounds and the components of each reagent were completely separated within 5 min.

Retention times and detection limits are shown in Table I.

Linear relationships between the peak-area ratios (II: AHP, V: nicotinic acid and VIII: nicotinic acid) and the amount of hydrogen peroxide were found over the range investigated (up to 0.20 μmol) and can be expressed by the following equations:

(A) $y = 10.534\,x + 0.094$; $r = 0.990$

(B) $y = 4.9512\,x + 0.115$; $r = 0.968$

(C) $y = 7.456\,x + 0.376$; $r = 0.978$

TABLE I

RETENTION TIMES AND DETECTION LIMITS

Reagent	*Compound*	*Retention time (min)*	*Detection limit (ng)*
A	I	–**	–**
	II	1.80	2.5
	AHP (I.S.)	2.60	0.1
B	III	4.52	250
	IV	4.27	3.3
	V	3.50	9.8
	Nicotinic acid (I.S.)	2.76	11
C	VI	2.39	18
	VII	2.79	36
	VIII	3.70	48
	Nicotinic acid (I.S.)	2.02	11

* For abbreviations, see Fig. 1 I.S. = internal standard.
** No fluorescence.

where y represents the amount of hydrogen peroxide (μmol) and x is the peak-area ratio.

The activity of diamine oxidase was calculated from the concentration of the enzymatically produced hydrogen peroxide, using the equation

$$\text{activity (u/mg protein)} = \frac{\mu\text{mol } H_2O_2}{\text{time} \cdot \text{mg protein}}$$

In order to ensure favourable kinetics, diamine oxidase was incubated with a large excess of putrescine and the reaction was stopped by injecting the sample into the chromatograph. The results for incubations of 2, 7 and 12 min are shown in Fig. 2A, B and C, respectively.

The reproducibility of the method was determined by replicate analyses of the same samples and the activities were found to be 1.474 u/mg $\pm$ 1.2% for A, 1.7 u/mg $\pm$ 2.1% for B and 1.5 u/mg $\pm$ 2.4% for C.

CONCLUSIONS

The results illustrate the potential of HPLC for the assay of diamine oxidase. The sample preparation is minimal; the analysis time is short (5 min) and the oxidized products are completely separated from the reagents, thereby eliminating any interferences. Reagent A was found to be preferable in that it can be prepared rapidly and allows more accurate and sensitive detection.

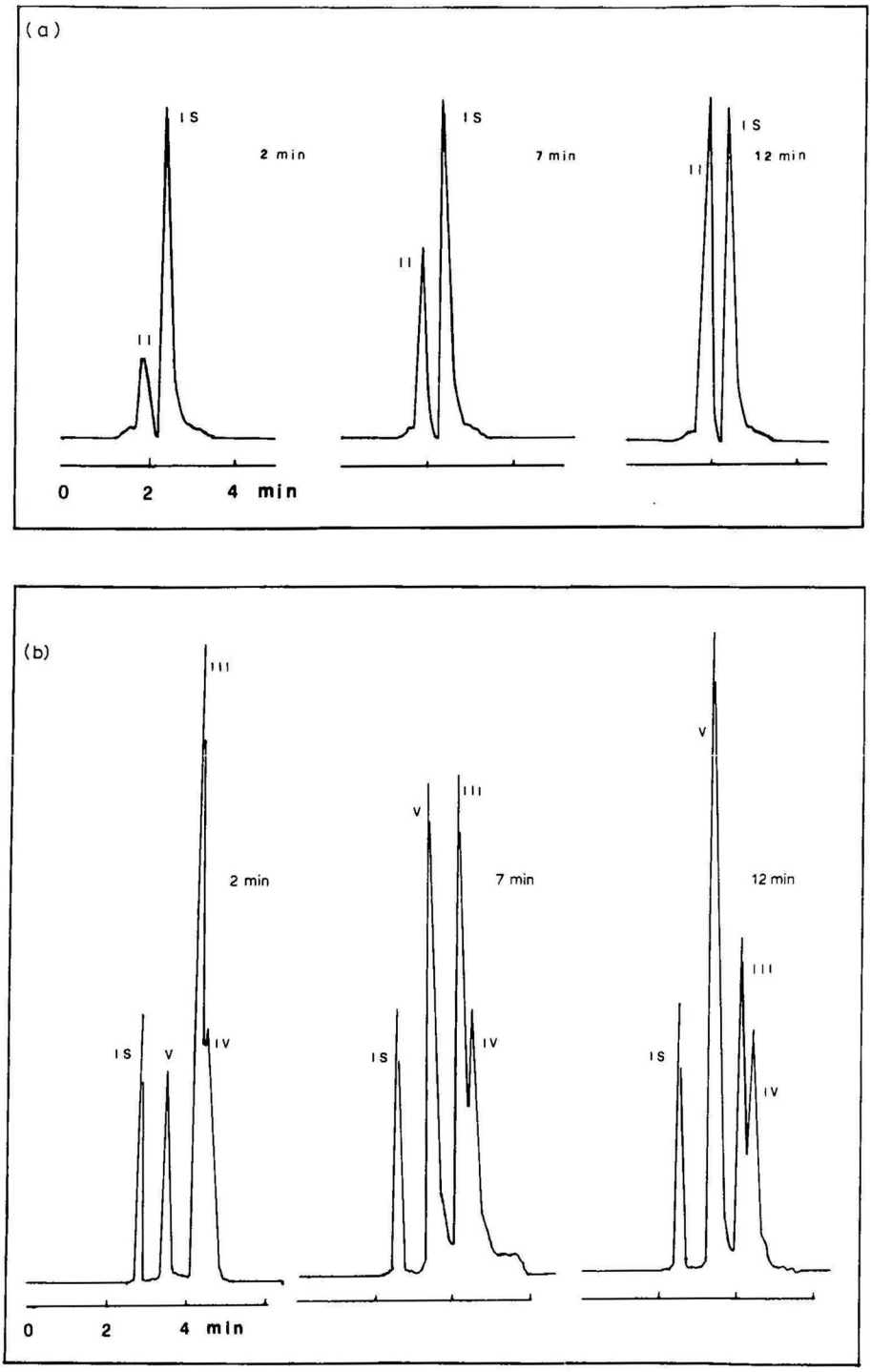


Fig. 2.

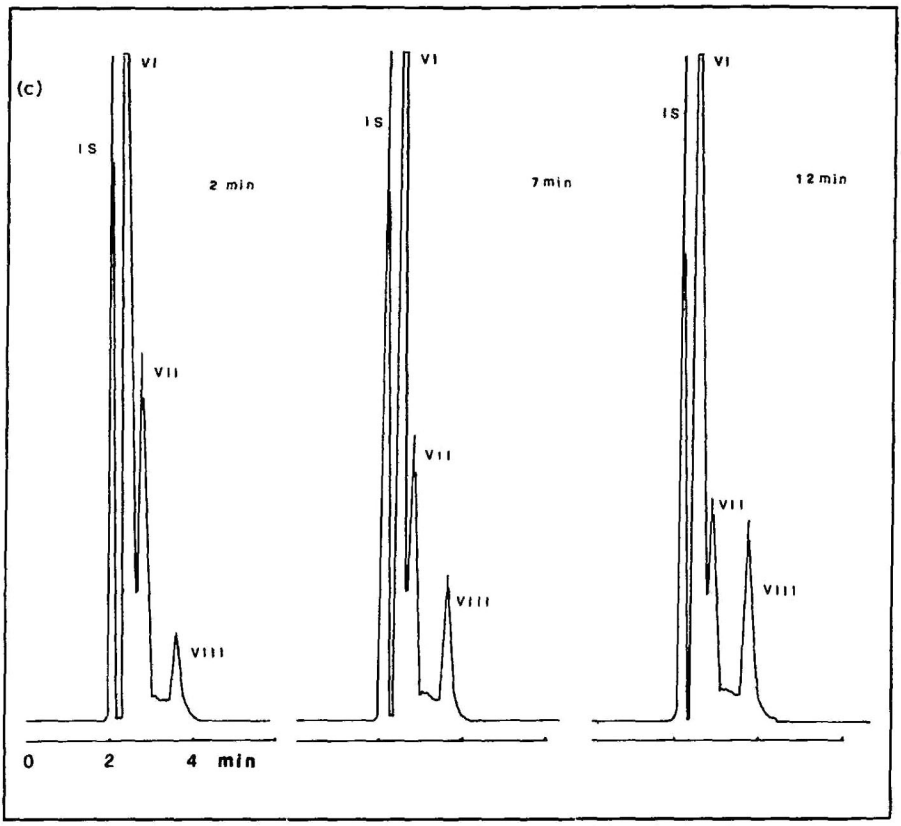


Fig. 2. Chromatograms of diamine oxidase incubated with putrescine for 2, 7 and 12 min. (a) II = 2,2′-Dihydroxy-3,3′-dimethoxybiphenyl-5,5′-acetic acid; I.S. = 2-amino-4-hydroxypteridine. Fluorescence detection (315/425 nm). (b) III = 3,5-Dichloro-2-hydroxybenzenesulphonic acid; IV = 4-aminoantipyrine; V = 4′-aminoantipyril-3-chloro-5-sulphonic acid-1,4-benzoquinoneimine; I.S. = nicotinic acid. Ultraviolet detection (254 nm). (c) VI = Pyridoxine; VII = 4-amino-N,N-diethylaniline; VIII = 4′-amino-N,N-diethylanilyl-3,4-dihydroxymethyl-6-methyldihydropyridine-2,5-dioneimine; I.S. = nicotinic acid. Ultraviolet detection (254 nm).

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THIN-LAYER CHROMATOGRAPHIC DETERMINATION OF BETAMETHASONE DIPROPIONATE IN SEMI-SOLID PHARMACEUTICAL PREPARATIONS

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(First received December 21st, 1981; revised manuscript received February 25th, 1982)

SUMMARY

A novel and specific thin-layer chromatographic method for determination of betamethasone dipropionate in semi-solid pharmaceutical preparations is described. Thin-layer chromatography was carried out on silica gel K6F-plates using cream or ointment extracts obtained with chloroform or ethanol respectively. A direct determination was performed by a chromatogram-scanner in the reflectance detection mode with zigzag scanning. Betamethasone dipropionate was determined at λ_{max} 240 nm. The method described is rapid, enabling a good reproducibility; it is suitable for quality control and stability investigations. The relative standard deviation is 1.3%.

INTRODUCTION

Interest in developing versatile techniques for corticosteroid determination in semi-solid preparations has grown continually over the last 30 years. The production of new pharmaceutical formulations containing new corticosteroids provides a challenge to the development of better analytical techniques than those available. Among many methods devised for corticosteroid determination[1,2] three are included in Pharmacopoeias and followed by manufacturers. These methods are based on the reactions between corticosteroids and (a) tetrazole salts[3], (b) phenylhydrazone[4] and (c) isoniazide[5].

Some problems appear when applying these methods owing to certain ingredients and decomposition products. Therefore, Graham *et al.*[6] recommended a column procedure to avoid or at least decrease the interferences present. Graham *et al.*[7] employed a similar procedure for analysis of betamethasone and its monoesters in pharmaceutical preparations. According to ref. 8, high-performance liquid chromatography should be used for betamethasone dipropionate analysis.

This paper reports a thin-layer chromatographic (TLC) method for determination of betamethasone dipropionate in semi-solid preparations. Betamethasone dipropionate can be determined directly on the TLC plate by a chromatogram-scanner after separation of the compound from interfering components.

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EXPERIMENTAL

Materials

All solvents, tetrazolium blue, triphenyltetrazolium chloride and isoniazide were of analytical reagent grade (E. Merck, Darmstadt, G.F.R.). Commerical silica gel K6F TLC plates, 20 × 20 cm (Whatman, Clifton, NJ, U.S.A.), layer thickness 0.25 mm, were used. Solutions were spotted on to the TLC plates using micropipettes.

Drug and formulations

Betamethasone dipropionate was a gift from Schering Corporation (U.S.A.). Betamethasone dipropionate ointment (Diprogent Ointment; Belupo Pharmaceutical Works, Yugoslavia; licenced by Schering Corporation) and betamethasone dipropionate cream (Beloderm Cream and Diprogent Cream, Belupo, licensed by Schering) were purchased. Ointment and cream contained 0.05% betamethasone in the form of dipropionate. Diprogent Ointment and Diprogent Cream contained 1000 international units per g of gentamicin in the sulphate form.

Apparatus

The following were used: a Shimadzu dual-wavelength TLC scanner, Model CS-910, with a dual-pen recorder (Philips, Model PM 8222); Varian Techtron spectrophotometer, Model 635; M4020 shaker from Köttermann (Hanigsen, G.F.R.); "Superspeed" centrifuge from Sorvall (Newtown, CN, U.S.A.) and a Grant BT3 block thermostat (Cambridge, Great Britain).

Sample preparation

Ointments and creams —Method A. The procedure of Graham *et al.*[6] was followed except that an amount of ointment (or cream) containing the equivalent of 1 mg betamethasone was used instead of 5 mg betamethasone.

Ointments —Method B. The procedure given in ref. 9 for assay preparation was followed, except that: (a) 30 ml of ethanol were used instead 10.0 ml of ethanol and 5.0 ml of internal standard solution, (b) the extraction was repeated once with 15 ml of ethanol and (c) the sample extracts were combined into a 50-ml volumetric flask and diluted to volume with ethanol.

Creams —Method B. An accurately weighed amount of cream, equivalent to about 1 mg betamethasone, was transferred to a separator, 150 ml of 10% hydrochloric acid were added and mixed. The mixture was extracted with four 20-ml portions of chloroform, shaking each portion for about 2 min. The chloroform phase was filtered through about 3 g anhydrous sodium sulphate into a 100-ml volumetric flask and made up to volume with chloroform. A 10.0-ml volume of the solution was transferred to a 20-ml conical test-tube and evaporated to dryness at 50°C in a block thermostat under a nitrogen stream, whereupon 5.0 ml of methanol were added to the residue. The tube was heated for 5 min in a 60°C water-bath, then agitated vigourously for 3 min, adjusted to room temperature and centrifuged for 5 min at 2611 × *g*.

Determinations

Proposed method. Sample solution and standard solution (50 μl or 30 μl of ointment sample obtained by *Method B* respectively) were spotted successively on the

TLC plate. The TLC tank was lined with filter-paper saturated with the solvent mixture chloroform–acetone (70:10) and the system was allowed to equilibrate for 60 min. After placing the TLC plate into the tank the solvent front was allowed to migrate 15 cm from the origin (*ca.* 45 min). The developed plate was dried in a stream of air for 30 min. The spots were located under short-wavelength UV-light.

The TLC plate was scanned at 20 mm/min in a direction perpendicular to the direction of development, using the following operating conditions: photometric mode, dual-wavelength, λ_s = 240 nm, λ_r = 400 nm; detection mode, reflection; measuring mode, absorbance; stage scanning mode, zigzag; working curve linearizer, channel 1. The speed of the recorder was 20 mm/min. The profile and integration curves were recorded for each spot on the TLC plate. The peak heights were measured for the integrated values of spots of sample solution and standard solution. The betamethasone content in the preparation was calculated from[10]

$$S\,(\%) = \frac{Y_p X_s \cdot 100}{Y_s X_p}$$

where S = percentage of betamethasone in preparation, X_s = mass of standard applied (μg), X_p = mass of sample applied (μg) (calculated from the declared quantity in the preparation), Y_p = mean of heights (cm) of the integrated values of the sample spots and Y_s = mean of heights (cm) of the integrated values of the standard spots.

Tetrazole method. The procedure as described previously[11] was followed, except that triphenyltetrazolium chloride was used instead of tetrazolium blue. The same procedure was used with 20.0 ml of standard solution (concentration 10 μg/ml) and 20 ml of ethanol as blank.

This procedure was used for determination of betamethasone dipropionate in ointment and cream after extraction by *Method A*.

Isoniazide method. Five millilitres of a chloroform extract of cream, obtained by *Method B*, was used. The procedure of Umberger[5] was used, and repeated with 5.0 ml of standard solution (concentration 10 μg/ml).

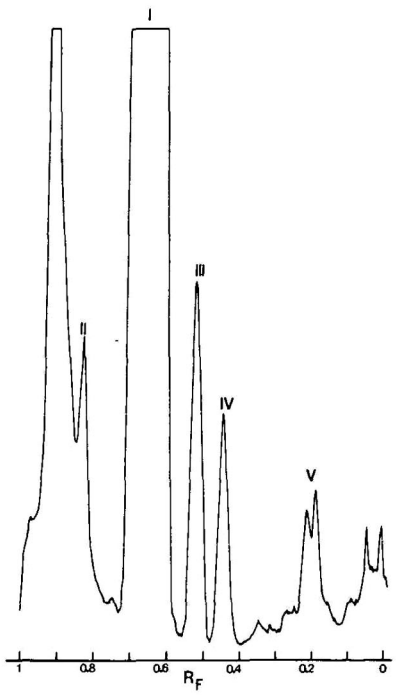
RESULTS AND DISCUSSION

Specificity of the determination

Two solvent systems from other studies[11,12] were tested: (a) chloroform–acetone (70:10) and (b) chloroform–ethyl acetate (50:50); the former was more satisfactory, giving complete resolution of betamethasone dipropionate and accompanying substances (Fig. 1).

Ultraviolet absorption spectra of betamethasone dipropionate and accompanying substances on the TLC plate were constructed by plotting absorbance at different wavelengths (Fig. 2). The maximum absorbance of betamethasone dipropionate was at 240 nm. In selecting the dual-wavelength settings this wavelength was used for the sample side, while 400 nm —a wavelength at which no absorption occurs— was used for the reference side.

When the developed chromatogram was sprayed with tetrazolium blue in alkaline methanol[13] and triphenyltetrazolium chloride in alkaline methanol (prepared as tetrazolium blue in alkaline methanol) positive reactions were obtained with ac-



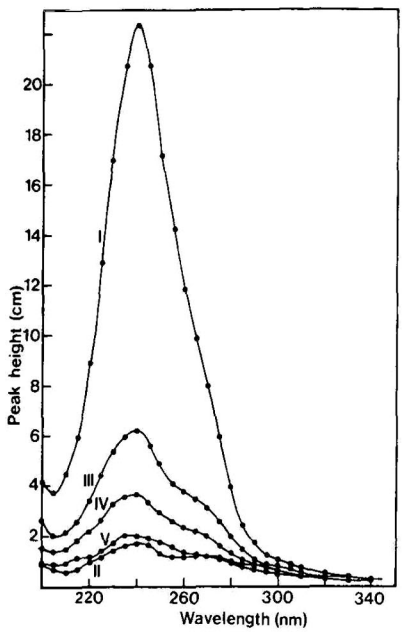


Fig. 1. Chromatogram of betamethasone dipropionate and accompanying substances obtained by linear scanning of the TLC plate. Peaks: I = betamethasone dipropionate; II–V = accompanying substances. 400 μg of betamethasone dipropionate were spotted.

Fig. 2. Ultraviolet absorption spectra of betamethasone dipropionate and accompanying substances obtained by scanning the TLC plate after separation in the solvent mixture chloroform–acetone (70:10). Spectra: I = betamethasone dipropionate, hR_F = 67; II = accompanying substance, hR_F = 85; III = accompanying substance, hR_F = 52; IV = accompanying substance, hR_F = 45; V = accompanying substance, hR_F = 20.

companying substances. Therefore, these substances can interfere in the colorimetric determination of betamethasone dipropionate with tetrazolium blue or triphenyltetrazolium chloride reagents.

The interference of ingredients in cream was investigated. Extracts of cream were spotted on the TLC plates and developed with the solvent mixture chloroform–acetone (70:10). Inspection of the developed chromatograms under short-wavelength UV-light revealed only spots of betamethasone dipropionate. Then the developed chromatograms were sprayed with the previously mentioned reagents or with isoniazide in acidic methanol[5]. Two spots from ingredients (Fig. 3) were found to interfere in colorimetric determinations of betamethasone dipropionate.

Separation of betamethasone dipropionate from accompanying substances and excipient interferences by TLC in the mobile system chloroform–acetone (70:10) offers a specific assay of betamethasone dipropionate on the TLC plate by use of a chromatogram scanner.

Linearity of response

Using the curve linearizer, a linear relation between integrated values and the

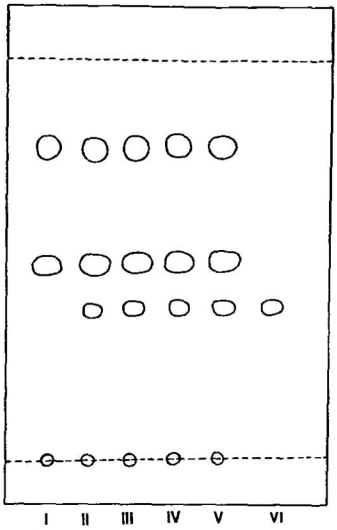


Fig. 3. Separation of betamethasone dipropionate and excipient interferences from cream. Bands: I = extract of cream without betamethasone dipropionate; II and V = extracts of cream obtained by *Method B*; III and IV = extracts of cream obtained by *Method A*; VI = betamethasone dipropionate.

amounts of betamethasone dipropionate spotted was obtained. Since not more than 1.5 μg per spot were placed on the TLC plates, the procedure was found to be satisfactory.

Accuracy and reproducibility of determination

It is known that reproducibility depends on local variations on a individual TLC plate as well as variations between plates. In order to determine which factors are dominant the following tests were carried out. Four TLC plates were spotted with the cream extracts obtained according to *Method B*. Onto each TLC plate three spots of sample solution and three spots of standard solution (concentration 1 μg per spot) were spotted alternatively. The found concentration (Table I) for each spot of sample was calculated by the mean of the integrated values of three spots of standard solution. The mean betamethasone contents, S.D. and R.S.D. were calculated for each plate and these results were used for calculation of the corresponding mean values for four TLC plates. Variations within a plate are found to be 1.03–4.38%, whereas variations between plates were 1.06%. The R.S.D. data clearly show the existence of local variations within a plate and in spotting. In order to avoid these variations three spots of sample solution and three spots of standard solution should be spotted on the TLC plate one after another. The content of betamethasone should be calculated from mean integrated values of standard and sample.

The method proposed was compared with the tetrazole and isoniazide methods. Samples of betamethasone dipropionate cream, prepared in the laboratory, were assayed in quintuplicate (Table II). TLC determinations were carried out with extracts obtained by both *Methods A* and *B*. In addition, the same extracts were analysed by the tetrazole or isoniazide method. The highest recovery was obtained by TLC determination with extracts from *Method B* (99.2%) and R.S.D. was 1.3%.

TABLE I

REPRODUCIBILITY OF TLC DETERMINATIONS ON SAME AND DIFFERENT TLC PLATES

Sample solution and standard solution (concentration 1 μg per spot) were spotted on four TLC plates, one after another.

Plate No.	*Spot No.*	*Found concentration (% of added)*	*Mean*	*S.D.*	*R.S.D. (%)*
1	1	97.9			
	2	99.8	98.3	1.27	1.29
	3	97.4			
2	1	100.4			
	2	96.5	99.8	3.04	3.05
	3	102.5			
3	1	97.0			
	2	95.0	98.5	4.31	4.38
	3	103.4			
4	1	99.3			
	2	101.1	100.5	1.04	1.03
	3	101.1			
		Mean	99.3		
		S.D.	1.05		
		R.S.D. (%)	1.06		

TABLE II

ACCURACY OF DETERMINATIONS BY THE PROPOSED METHOD AND COLORIMETRIC METHODS

0.5 mg of betamethasone in the form of dipropionate were added per 1 g of cream base.

Sample No.	*Found concentration (% of added)*			
	By proposed method		*By tetrazole method*	*By isoniazide method*
	Extract by Method A	*Extract by Method B*		
1	83.9	98.4	82.4	90.1
2	103.1	101.1	91.1	100.5
3	95.6	99.9	90.8	91.5
4	109.4	98.7	104.1	96.9
5	103.1	97.8	107.8	96.5
Mean	99.02	99.18	95.24	95.10
S.D.	9.76	1.32	10.46	4.25
R.S.D. (%)	9.80	1.33	10.98	4.47

TLC determination of betamethasone dipropionate extracted by *Method A* shows high R.S.D. (9.8%); even worse results were obtained employing the tetrazole method (R.S.D. 11.0%). These could be ascribed to variations in the extraction procedure of *Method A*. Additional interferences in tetrazole method arise from excipient interferences and accompanying substances. In the TLC determinations

these interferences have been avoided. The recovery of the isoniazide method is as high as in the tetrazole method, while reproducibility is markedly better (R.S.D. 4.5%).

Results of the analysis of commercial preparations of ointments and creams, containing only betamethasone dipropionate as active component, or in combination with the antibiotic gentamicin sulphate, are given in Table III. The determinations were carried out with several cream and ointment batches as well as with several samples of the same batch. Results for extracts obtained by *Method B* and applying the proposed method were compared with results for the tetrazole and isoniazide methods. The best reproducibility in respect of the declared amounts was achieved by the proposed method, whereas the worst results were obtained by the tetrazole method. Hence, the latter is not reliable for determination of betamethasone dipropionate in creams and ointments if not preceded by sample clean-up.

TABLE III

ANALYSIS OF COMMERCIAL OINTMENTS AND CREAMS CONTAINING BETAMETHASONE DIPROPIONATE

Type of sample	*Batch*	*Sample from tube No.*	*Found concentration (% of declared)*		
			By proposed method	*By tetrazole method*	*By isoniazide method*
Beloderm	A	1	104.6	107.0	110.6
Cream	A	1	101.9	104.3	103.5
	A	1	104.9	84.4	100.0
	B	1	100.8	106.1	100.5
Diprogent	C	1	96.9	96.8	98.1
Cream	C	2	98.4	78.0	98.0
	D	1	101.2	–	–
Diprogent	E	1	99.4	108.0	
Ointment	E	2	100.8	110.0	
	F	1	99.3	100.1	
	F	2	101.5	92.7	
	G	1	99.0	106.1	
	G	1	100.9	110.4	
	G	2	102.3	116.0	
	H	1	102.1	97.8	
	J	1	99.6	73.2	
	J	1	101.4	93.5	

In conclusion, the proposed TLC determination is suitable for the quality control of betamethasone dipropionate ointments and creams. It is rapid and specific, and the precision and accuracy of assay are better than with existing colorimetric methods.

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Note

Optimierung der Aldehyd–Schwefelsäurereagenzien zur Detektion in der Dünnschicht-Chromatographie

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Zur Detektion werden in der Dünnschicht-Chromatographie (DC) häufig Aldehyd–Schwefelsäurereagenzien zur Farbbildung angewandt. Wir konnten kürzlich zeigen, dass hierbei 3 farbbildende Reaktionen ablaufen[1]. Zunächst werden durch die starke Mineralsäure die entsprechenden Substanzen umgesetzt, dabei entstehen neben primär schwach farbigen Produkten wahrscheinlich Cyclopentenylkationen[2,3]. Diese kondensieren dann mit dem entsprechenden Aldehyd zu mehr oder weniger intensiv gefärbten Verbindungen. Bei höherer Säurekonzentration kommt noch unerwünschterweise eine Autokondensation der Aldehyde zu polymeren Farbstoffen hinzu[4]. Deshalb sollten die in den Reagenzien angewendeten Aldehyde eine hohe Carbonylaktivität zeigen, gleichzeitig jedoch nur in geringem Masse zur Autokondensation neigen. Ferner müssen in den Aldehyden geeignete Substituenten vorhanden sein, die bewirken, dass die Kondensationsprodukte mit den umzusetzenden Verbindungen einen hohen Extinktionskoeffizienten im sichtbaren Bereich haben. Die Anforderungen an die Aldehyde sind also gegenläufig. Deshalb kann eine theoretische Abschätzung der Aldehydaktivität[5] eine experimentelle Untersuchung nicht ersetzen. Auch die aus der klassischen Analytik bekannten Reagenzien, wie nach Kägi–Miescher (s. Lit. 6 und 7), Ekkert[8], Komarowsky (s. Lit. 9 und 10) und andere können wegen der andersartigen Reaktionsbedingungen nicht direkt übertragen werden.

EXPERIMENTELLES

Schichtmaterial

Selbst beschichtete Glasplatten (Streichverfahren[14]) des Formats 20 × 20 cm, TLC-Kieselgel 60 HF_{254} mit einer mittleren Korngrösse von 15 μm (Merck, Darmstadt, B.R.D.) unter Zusatz von Acronal 250 D (BASF, Ludwigshafen, B.R.D.). Zusammensetzung der Suspension: 30 g Kieselgel + 98 ml Wasser + 1.8 ml Acronaldispersion; Mengenangabe für 5 DC-Platten, Nassschichtdicke 250 μm.

Detektionsreagenzien

Zu 100 ml eines abgekühlten Gemisches aus Methanol–Eisessig–konz. Schwefelsäure (85:10:5) werden jeweils 0.5 g eines zu untersuchenden Aldehyds (s. Tabelle I) zugesetzt. Davon werden pro 20 × 20 cm-Schichtfläche 20 ml aufgesprüht. Die Erwärmung erfolgte unter Beobachtung auf einer Heizplatte vom Typ Thermoplate (Desaga, Heidelberg, B.R.D.).

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Probesubstanzen

Etherische Öle, wie z.B. von *Mentha piperita, Foeniculum vulgare, Solidago spec., Acorus calamus, Salvia officinalis.*

Es wurden 1% Lösungen hergestellt und 2 µl punktförmig aufgetragen.

Optimierung der Detektionsbedingungen und Vergleich der Aldehyde.

Es wurden Temperaturen zwischen 50 und 150°C erprobt, und die Zonenfärbung zunächst nach 30 sec, dann jeweils nach doppelten Zeitabständen fotografisch dokumentiert. Später wurden die Fotografien visuell nach den Grauwerten ausgewertet.

Bestimmung der Erfassungsgrenzen

Sie erfolgte nach Chromatographie und anschliessender visueller Auswertung der Chromatogramm-Zonen.

ERGEBNISSE UND DISKUSSION

Nach Arbeiten von Katz[5] und Schaltegger[11,12] war zu erwarten, dass insbesondere diejenigen aromatischen Aldehyde, deren Substituenten die Elektronendichte am Kern erhöhen, zu hochempfindlichen Reagenzien führen würden. Deshalb erfolgte auch die Auswahl unter besonderer Berücksichtigung dieser Gruppe. Als Testsubstanzen dienten sowohl etherische Öle als auch häufig vorkommende Terpenderivate und eine grössere Anzahl von Steroiden. In Tabelle I sind sowohl die positiven Kriterien, wie hohe Farbintensität der Substanzzonen als auch die an sich unerwünschte Schichtverfärbung angegeben. Als Vergleich diente jeweils das aldehydfreie Reagenz, das heisst die von ihm hervorgerufenen schwachen Färbungen.

Es ergibt sich, dass von den aliphatischen Aldehyden die Färbungen weder verstärkt noch die Farbtöne nennenswert geändert werden. Die schwachen Schichtverfärbungen sind wohl auf ein beginnendes Verkohlen des verwendeten Bindemittels zurückzuführen. Gering ist ebenfalls der farbverstärkende Einfluss von unsubstituierten aromatischen Aldehyden; er wird von der deutlichen Schichtfärbung kompensiert.

Verwendet man jedoch aromatische Aldehyde mit Substituenten mit positiven induktiven oder positiven mesomeren Effekten, so verändern sich die Detektionsfarben beträchtlich. Statt der vorherrschend gelben, orangen und braunen Färbungen mit nichtsubstituierten Verbindungen erhält man nun meist blaue oder violette Farbtöne. Ausserdem ist die Farbintensität zumeist stark erhöht.

Mit zunehmender Zahl an Substituenten wird dieser Effekt zwar immer ausgeprägter, aber gleichzeitig verstärkt sich die Verfärbung der Schicht. Deshalb sind alle dreifach substituierten Verbindungen in diesem Reagenz unbrauchbar; desgleichen auch die 2,5- und 3,5-disubstituierten aromatischen Aldehyde. Zum Teil ist die Schichtfärbung so intensiv und tritt so rasch auf, dass die eigentlichen Substanzzonen nicht erkennbar sind.

Gute Ergebnisse erhält man dagegen mit *p*-Methoxybenzaldehyd (Anisaldehyd), 2,4-Dimethoxybenzaldehyd und einigen 3,4-disubstituierten aromatischen Aldehyden. Man erzielt mit ihnen bei etwa vergleichbaren Erfassungsgrenzen farblich gut differenzierte Zonen. Am intensivsten erscheinen die Färbungen mit dem 2,4-

TABELLE I

DIE EIGNUNG VERSCHIEDENARTIGER ALDEHYDE ZUR DETEKTION VON TERPENDERIVATEN IN DER DÜNNSCHICHT-CHROMATOGRAPHIE IM VERGLEICH ZUM ALDEHYDFREIEN REAGENZ
− = Schwach; + = deutlich; + + = stark; + + + = sehr stark. +I = electronenschiebende; −I = electronenziehende Substituenten.

Aldehyd	*Charakterisierung des Aldehyds*	*Optimierte Detektionsbedingungen**	*Farbintensität der Zonen*	*Schichtfärbung*	*Intensität der Schichtfärbung*
Aldehydfreies Reagenz	0	125°C;5–10 min	−	Grau	−
Formaldehyd		125°C;5–10	−	Grau	−
Acetaldehyd	Aliphaten	min	−	Grau	−
Propionaldehyd			−	Grau	−
Benzaldehyd	Unsubsti-	100°C;5–10	+ +	Gelb	+
Zimtaldehyd	tuierte	min	+	Gelbgrün	+
Phthaldialdehyd	Aromaten		+	Gelb	+
Anisaldehyd	Aromaten mit	90°C; 10 min	+ + +	Rosa	+
4-Benzyloxybenzaldehyd	einem Sub-	90°C; 10 min	+ + +	Orange	+ + +
4-Dimethylaminobenz-	stituenten	110°C; 5–10 min	+	Braun	+
aldehyd	(+I Effect)				
4-Carboxybenzaldehyd	(−I Effekt)	110°C; 10 min	+	Gelb	+
4-Dimethylamindobenz-			+	Braun	+
aldehyd					
Vanillin		90°C;5–10	+ + +	Gelbgrün	+ +
Isovanillin		min, unter	+ + +	Gelbgrün	+ +
Piperonal		Beobachtung	+ +	Gelbgrün	+ +
3,4-Dihydroxybenzaldehyd	Aromaten		+ + +	Gelbgrün	+ +
3,4-Dimethoxybenzaldehyd	mit		+ + +	Gelbgrün	+ +
4-Benzyloxy-3-meth-	zwei		**	Graubraun	+ + +
oxybenzaldehyd	Substituenten				
2,4-Dimethoxybenzaldehyd	(+I Effekt)		+ + +	Violett	+ +
2,5-Dimethoxybenzaldehyd			**	Orange	+ + +
3,5-Dimethoxybenzaldehyd			**	Dunkelgrün	+ + +
2,4,6-Trihydroxy-	Aromaten	80°C; 5 min	**	Orange	+ + +
benzaldehyd	mit drei				
3,4,5-Trimethoxy-	Substituenten	80°C; 5 min	+ +	Gelbbraun	+ + +
benzaldehyd	(+I Erffekt)				
5-Methylfurfural	Heteroaromat	100°C; 5 min	+ +	Braun	+

* Gültig bei Erwärmung auf der Thermoplate (Desaga, Heidelberg, B.R.D.).
** Durch die starke Schichtfärbung ist die Zonenlage unkenntlich.

Dimethoxybenzaldehyd-Reagenz. Allerdings tritt hier die Schichtverfärbung stärker in Erscheinung als bei Verwendung von einfach substituierten Aldehyden. Nachteilig ist die auf nur wenige Stunden begrenzte Haltbarkeit dieser Reagenzlösung. Ähnliches gilt für das an sich häufig benutzte Vanillin und für die anderen disubstituierten Aldehyde.

Mit Abstand am besten eignet sich das Anisaldehyd–Schwefelsäurereagenz;

TABELLE II

ERFASSUNGSGRENZEN UND FARBEN BEI DETEKTIONEN VON STEROIDEN MIT DEM ANISALDEHYD–SCHWEFELSÄURE-REAGENZ.

Bei Verwendung der Thermoplate wurde unter Beobachtung 10 min auf 120°C erhitzt. Die beobachteten Farben gelten für Substanzkonzentrationen zwischen 1 und 10 μg.

Erfassungsgrenze in μg pro Zone		
0.01–0.1	*0.1–1*	*1–10*
Androsteron (blau-violett)	Cholesterol (violett)	Aldosteron (grau-blau)
Dihydrotestosteron (blau-violett)	Cholsäure (grau-blau)	Cortison (ocker)
Epi-androsteron (blau-violett)	Dehydro-epi-androsteron (rot-violett)	Hydrocortison (grau-braun)
Lanosterol (blau-violett)	Desoxycholsäure (grau-violett)	Prednisolon (grau-grün)
Östradiol (oliv)	3α,7α-Dihydroxy-5β-cholansäure (violett)	Tetrahydrocortison (grau)
Östriol (grün-blau)	Digitonin (grau-blau)	
Pregnantriol (blau)	Digitoxigenin (grün-blau)	
Pregnenolon (blau-violett)	Digitoxin (grau-violett)	
Sitosterol (blau-violett)	Digoxin (violett)	
Stigmasterol (blau-violett)	Ethinylöstradiol (blau-violett)	
Testosteron (chromoxid-grün)	Gitoxin (blau)	
	Östron (grau-grün)	
	Pregnandiol (grau-blau)	
	Progesteron (ocker)	

vorteilhaft ist, dass man es bei gleicher Wirksamkeit im Kühlschrank mehrere Wochen vorrätig halten kann. Hinzu kommt die Preiswürdigkeit und toxikologische Unbedenklichkeit des verwendeten Aldehyds. Die Aldehydkonzentration im Reagenz sollte bei etwa 0.5 % liegen. Es hat sich gezeigt, dass höhere Konzentrationen, etwa von 1 oder 2 %, schnell zu störenden Schichtverfärbungen führen und Konzentrationen unter 0.1 % für eine quantitative Umsetzung nicht mehr ausreichend sind.

Entgegen der oft gegebenen und auch im Europäischen Arzneibuch aufgenommenen Vorschrift ist es sinnvoll, den Aldehyd stets als letzten Bestandteil zu dem *abgekühlten* Gemisch von Säuren und Methanol zuzugeben. Dies senkt die sonst unvermeidliche thermische Belastung des Aldehyds und erhöht so die Haltbarkeit des Reagenzes beträchtlich.

Die Wahl geeigneter Reaktionsbedingungen ist bei Verwendung des Anisaldehyd-Schwefelsäurereagenzes äusserst unkritisch. Sie hängt in erster Linie von der

Säureempfindlichkeit der zu detektierenden Verbindungen ab. Beachtenswert ist jedoch die Tatsache, dass die Nachweisempfindlichkeit sehr stark von der Konstitution der entsprechenden Verbindungen abhängt, eine allgemein gültige Regel kann bei der Vielzahl der ablaufenden Reaktionen nicht gegeben werden. Die Nachweisempfindlichkeit liegt, wie die Tabelle II zeigt, sehr unterschiedlich. Manche Verbindungen lassen sich in Mengen von 10 ng nachweisen, bei anderen liegt die Nachweisempfindlichkeit bei 1–100 μg.

Generell wurde gefunden, dass man bei Verwendung tieferer Erhitzungstemperaturen und kürzerer Detektionszeiten selektivere Anfärbungen erhält. So ergibt sich beispielsweise beim Kalmusöl, dass man bereits bei Zimmertemperatur eine Blaufärbung von Pre-Isocalamendiol und Isocalamendiol erhält[15], und erst bei einer Temperaturerhöhung die Zahl der Farbzonen beträchtlich zunimmt. Die höchste Empfindlichkeit ergibt sich jedoch zumeist erst dann, wenn sich die Schicht bereits schwach verfärbt.

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Note

Analysis of boron–halogen derivatives of bis(1,2-dicarbollyl)cobalt(III) anions by capillary isotachophoresis

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The system nitrobenzene–cobaltocarborane of the type $[(C_2B_9H_{11-n}X_n)_2\text{-}CO]^-H^+$, where n = 1–4 and X = Cl (Br), has excellent extraction properties for mono- and divalent cations (see, *e.g.*, refs. 1–4). Halogen atoms present in the molecule of this extraction agent (in the positions B-8 and B-8′ of the dicarbollyl skeleton) remarkably improve its chemical stability[5]. High stabilities to radiaton, typical of this group of compounds, make them suitable for the isolation of fission products (^{137}Cs and ^{90}Sr) from acidic solutions[3,4].

Mixtures of derivatives are formed on chlorination[6,7] or bromination[8,9] of the substrate $[(C_2B_9H_{11})_2Co]^-H^+$, (Cs^+). Some of them can be obtained in a pure form by gel chromatography (Sephadex LH-20) using methanol as an eluent[5,10]. The identity and purity of the isolated compounds is usually evaluated by ^{11}B NMR spectroscopy[11].

The similar physical and chemical properties of DCC {the abbreviation for $[(C_2B_9H_{11})_2Co]^-$} and its halogen derivatives make difficult their chromatographic separation and consequently their analysis. Recently, an extensive chromatographic investigation of heteroborane anions was published by Plzák *et al.*[12]. Homologous series of halogenated sandwich complexes of the above type (for structural formulae see Fig. 1) were not included in this investigation. However, the retention behaviour of this group of compounds evaluated under similar conditions[10] implies hardly any separations.

The aim of this work is to show that capillary isotachophoresis (ITP) is a powerful technique for the separation of DCC and its halogen derivatives. Because of the low solubilities of cobaltoboranes in water their ITP separations were carried out in water–methanol and water–ethanol mixtures.

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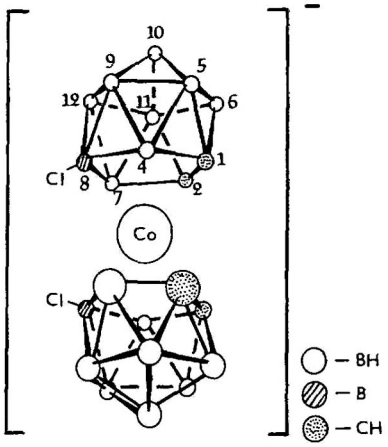


Fig. 1. Structural formula of the dichloro derivative of cobaltocarborane anion (Cl_2DCC). The order of halogenation of DCC is as follows: 1, position 8; 2, 8′; 3, 9; 4, 9′; 5, 12; 6, 12′.

EXPERIMENTAL

Apparatus

An instrument for ITP similar to that developed by Everaerts *et al.*[13] was used. Parts which are in contact with solutions were made of poly(tetrafluoroethylene) (PTFE). A fluorinated ethylene–propylene copolymer (FEP) capillary tube of 0.3 mm I.D. was used. Detection was performed with a conductivity detector[13,14]. Material to be analysed was delivered with the aid of a six-way valve[13] also made of PTFE. Stabilized current was supplied by a unit developed by Havaši[15].

Reagents

Chemicals used were of pro analysi purity and if necessary were purified by conventional methods[16]. Trichloroacetic and benzoic acids were obtained from Lachema (Brno, Czechoslovakia), N-ethylmorpholine from Fluka (Buchs, Switzerland). Water, methanol and ethanol were doubly distilled before use. The caesium salt of DCC was prepared by Dr. J. Plešek (Institute of Inorganic Chemistry, Czechoslovak Academy of Science, Řež near Prague, Czechoslovakia). Halogen derivatives of DCC were prepared in our laboratories.

The pH values of the leading and terminating electrolytes were measured as described in the literature[13,17]. A PHM-64 pH meter provided with a G 202 C glass electrode and a K-401 calomel electrode (all from Radiometer, Copenhagen, Denmark) was used.

RESULTS AND DISCUSSION

As mentioned above, DCC as well as its halogen derivatives are sparingly soluble in water. Therefore, ITP separations in methanol–water mixtures were studied. Small differences in the effective mobilities of the constituents to be separated were typical for this pair of solvents. Optimization of the separation according to p*K*

TABLE I

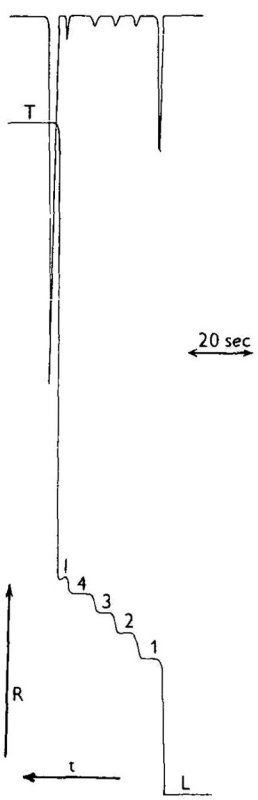
OPERATIONAL SYSTEM SUITABLE FOR THE SEPARATION OF DCC AND ITS HALOGEN DERIVATIVES BY ITP

Cl_3Ac^- = Trichloroacetate; $C_6H_5COO^-$ = benzoate; N-ETM = N-ethylmorpholine; MOW = Mowiol 8-88 (Hoechst, Frankfurt/M, G.F.R.).

	Leading electrolyte	*Terminating electrolyte*
Solvent	Ethanol–water (87:13 v/v)	Ethanol–water (95:5 v/v)
Anion	$Cl_3CH_2COO^-$	$C_6H_5COO^-$
Counter-ion	N-ETM	N-ETM
pH*	6.4	7.0
Additive**	0.01 % MOW	–

* Measured as described in refs. 13 and 17.

** For the role of the additive see ref. 13.



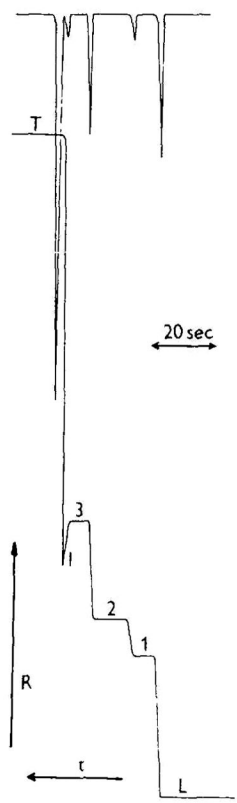


Fig. 2. An isotachophorogram of the separation of chloro derivatives of DCC. For the operating conditions see Table I. Driving current 10 μA. 1 = DCC; 2 = ClDCC; 3 = Cl_2DCC; 4 = Cl_3DCC; L = leading anion; T = terminating anion; I = impurity; R = increasing resistance; t = increasing time.

Fig. 3. An isotachophorogram of the separation of some bromo derivatives of DCC. 1 = BrDCC; 2 = Br_2DCC; 3 = Br_6DCC. Other symbols as in Fig. 2.

values is ineffective in this instance as these compounds are strong electrolytes in non-aqueous solvents[10,18]. Therefore, other solvents were considered.

An unexpected improvement of the separation was obtained when ethanol–water mixtures were employed. Differences in the effective mobilities of DCC and its halogen derivatives in this solvent system were sufficient for full resolution of available members of the homologous series. Several operating systems are available for ITP separations of these compounds if the ethanol content is sufficiently high (more than 80% v/v). The one used throughout this work is given in Table I.

Isotachophorograms of the separation of chloro and bromo derivatives of DCC are given in Figs. 2 and 3, respectively. The order of migration of the derivatives indicates that their different molecular weights are responsible for the separation (strong electrolytes, same charge type, very close structural properties). However, solvation effects probably also play a rôle as insufficient resolution was found in methanol–water mixtures. A detailed investigation of the solvation effects for this group of constituents and for different solvents is the subject of current research in this laboratory.

Possible applications of ITP, *e.g.*, for control of purity and stability to radiation, are clear from the above isotachophorograms. An application to the analytical evaluation of a crude product is given in Fig. 4. We can see that the dichloro deriva-

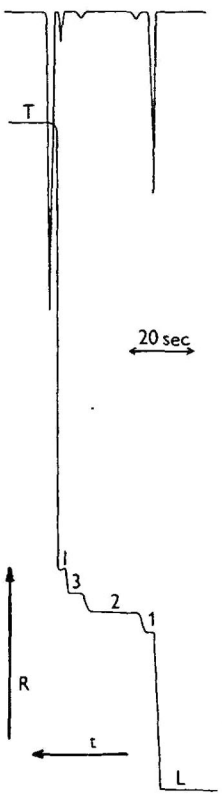


Fig. 4. Analytical evaluation of crude Cl_2DCC. 1 = ClDCC; 2 = Cl_2DCC; 3 = Cl_3DCC. Other symbols as in Fig. 2.

tive of DCC (Cl_2DCC) is accompanied by two by-products ClDCC ($\approx 16\%$) and Cl_3DCC ($\approx 27\%$).

The use of ITP for analysis of metallocarboranes, has several advantages:

(1) The analysis time was approximately 15 min in this investigation. However, modification of the apparatus, *e.g.*, by use of coupled columns[19], can decrease this substantially.

(2) The reproducilibity of determination is very good (1–2% of the amount to be determined is typical).

(3) Only small amounts of material are necessary for a complete analysis, which can be of great importance when radioactive material is involved.

(4) The solvent in which the sample is dissolved was found to be of minor importance in this application[20].

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Note

Gas chromatographic separation of carbohydrate enantiomers as (—)-menthyloxime pertrifluoroacetates on silicone OV-225

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Two methods for the separation of enantiomeric carbohydrates are possible. The carbohydrates can be derivatized with achiral reagents and a chiral stationary phase is utilized. The separation of trifluoroacetyl (TFA) and TFA-methylglycoside derivatives of enantiomeric carbohydrates on a capillary column coated with XE-60–L-valine-*S*-α-phenylethylamide has been reported[1,2]. Each carbohydrate enantiomer produces up to four peaks due to the α- and β-pyranosides and furanosides, respectively.

Alternatively derivatives can be prepared with chiral reagents. Oxidation of aldoses to aldonic acids, esterification with a chiral alcohol and acetylation result in diastereomeric derivatives, which allow partial separation[3,4]. Drawbacks of this method are the difficult preparation of derivatives and its limitation to aldoses. Also, trimethylsilyl (−)-2-butanol glycoside derivatives of carbohydrates have been applied in gas chromatography (GC)[5]. In both instances the chiral alcohols used were not optically pure.

In this paper new diastereomeric derivatives of enantiomeric carbohydrates useful for GC are described. Each carbohydrate enantiomer produces two peaks, the *syn*(*Z*) and *anti*(*E*) alkoximes, derivatization is very easy and GC on OV-225 mostly shows good separations of enantiomers.

EXPERIMENTAL

Apparatus

A Hewlett-Packard 5830A gas chromatograph equipped with a 50-m capillary column wall-coated with OV-225 (WGA, Griesheim, G.F.R.) was used. The split liner was filled to about 2 cm with 3% OV-225 on Chromosorb W HP (80–100 mesh), held on both sides by quartz-wool.

Materials

The carbohydrates and trifluoroacetic anhydride were obtained from Sigma (Munich, G.F.R.) and (−)-menthol, (±)-2-butanol, (−)-2-methyl-1-butanol, sodium acetate and ethyl acetate from Fluka (Buchs, Switzerland). O-(−)-Menthylhydroxylammonium chloride, O-(±)-2-butylhydroxylammonium chloride and O-(−)-2-methyl-1-butylhydroxylammonium chloride were synthesized from the sodium alcoholates and chloramine[6].

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TABLE I

RETENTION TIMES OF THE (±)-2-BUTOXIME PERTRIFLUOROACETYL DERIVATIVES OF CARBOHYDRATES

50-m capillary column; conditions as in Fig. 1. Each *E* and *Z* isomer can give two peaks, the (+)- and the (−)-2-butoxime derivatives.

Carbohydrates	*Retention time (min)*					
	Anti (E) isomer			*Syn (Z) isomer*		
Erythrose		15.11			16.41	
Ribose		17.29		18.76		18.87
Arabinose		17.52			20.03	
Xylose	19.04		19.13		20.59	
Lyxose		18.77		20.21		20.34
Allose		19.02		20.93		21.09
Altrose	19.64		19.76		21.59	
Glucose	20.91		21.01		22.62	
Mannose	20.45		20.57	22.05		22.19
Gulose		20.69		22.55		22.75
Idose	21.47		21.58		23.77	
Galactose		20.79			23.97	
Talose		20.90		22.55		22.68
Fructose		21.21			22.55	
Sorbose		22.23			22.96	
Tagatose		23.64			23.83	
Fucose		15.62			18.25	
Rhamnose	16.41		16.47	17.34		17.47

Derivatization

To about 0.5 mg of a carbohydrate a solution of 4 mg of O-(−)-menthylhydroxylammonium chloride, 2.5 mg of O-(±)-2-butylhydroxylammonium chloride or 2.5 mg of O-(−)-2-methyl-1-butylhydroxylammonium chloride and 3 mg

TABLE II

RETENTION TIMES OF (−)-MENTHYLOXIME PERTRIFLUOROACETYL DERIVATIVES OF ENANTIOMERIC CARBOHYDRATES

50-m capillary column; conditions as in Fig. 2. Each carbohydrate enantiomer gave two peaks, the *E* and the *Z* isomers.

Carbohydrate	*Retention time (min)**			
	D-*Enantiomer*		L-*Enantiomer*	
Glyceraldehyde	11.94	15.24	11.71	15.14
Ribose	18.55	25.01 (A)	17.50	24.84 (A)
Arabinose	18.15	29.53	19.01	29.94
Xylose	22.10 (B)	31.44 (C)	22.18 (B)	31.17 (C)
Lyxose	20.80	30.95	22.03	31.37
Glucose	25.54 (D)	40.30 (E)	25.78 (D)	40.28 (E)
Mannose	25.03	37.05	26.15	37.63
Galactose	27.95	45.60	26.63	44.63
Fucose	15.31	23.36	14.64	22.89

* Pairs of peaks labelled A, B, C, D and E overlap.

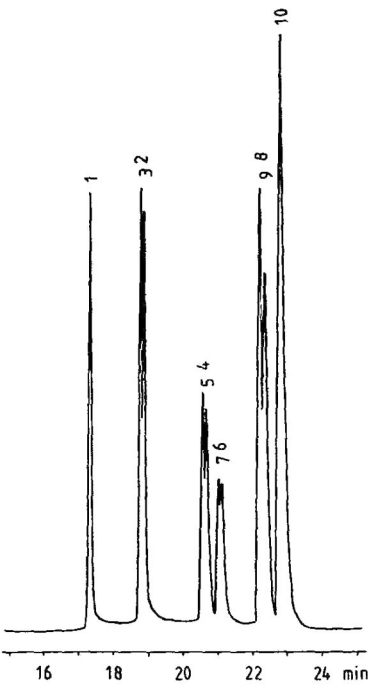

Fig. 1. Gas chromatogram of (±)-2-butoxime pertrifluoroacetyl derivatives of ribose, mannose and glucose. Temperatures: column, 100°C for 2 min then increased from 100 to 180°C at 5°C/min; injection and detector, 250°C. Gas flow-rates: nitrogen carrier gas, 2 ml/min; hydrogen, 20 ml/min; air 200 ml/min. Sample volume: 1 μl. Splitting ratio: 1:12. Peaks: 1,2,3 = ribose; 4,5,8,9 = mannose; 6,7,10 = glucose.

of sodium acetate in 100 μl of water were added. Further preparation was carried out as described previously[7].

RESULTS AND DISCUSSION

First experiments to separate enantiomeric carbohydrates as alkoxime pertrifluoroacetates were made with 2-methyl-1-butoximes and 2-butoximes. The (−)-2-methylbutoxime pertrifluoroacetates of racemic mixtures of carbohydrates were not separated on a 50-m capillary column coated with OV-225, whereas the (±)-2-butoxime pertrifluoroacetates of the investigated aldoses mostly gave three peaks, but hexulose (±)-2-butoxime pertrifluoroacetates showed no splitting (see Table I and Fig. 1). If the aldose had a *threo* configuration at $C_{2,3}$, preferentially the first peak split, whereas an *erythro* configuration at $C_{2,3}$ resulted in splitting of the second peak [except that erythrose, arabinose, galactose and fucose (6-deoxygalactose) gave only two peaks and mannose and rhamnose (6-deoxymannose) gave four peaks].

As the separation of carbohydrate 2-butoxime pertrifluoroacetates was not good and *R*- and *S*-2-butanol are very expensive and moreover not optically pure, experiments with (−)-menthyloxime pertrifluoroacetates were carried out. Of the nine aldoses investigated the enantiomers of two (xylose and glucose, *xylo* configuration at $C_{2,3,4}$) cannot be separated and the others showed separation of the *anti* (*E*)

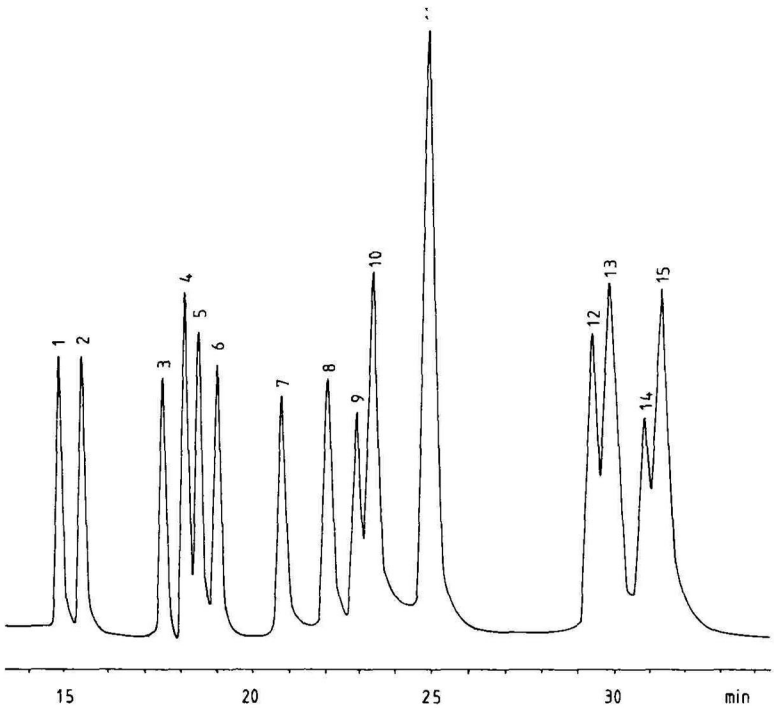

Fig. 2. Gas chromatographic separation of enantiomers of fucose, ribose, arabinose and lyxose as (−)-menthyloxime pertrifluoroacetates. Column temperature: 180°C (isothermal). Other GC conditions as in Fig. 1. Peaks: 1,9 = L-fucose; 2,10 = D-fucose; 3,11* = L-ribose; 4,12 = D-arabinose; 5,11* = D-ribose; 6,13 = L-arabinose; 7,14 = D-lyxose; 8,15 = L-lyxose. * Overlapping peaks.

isomer of the oxime (arabinose) or of the *E* and *Z* isomer (glyceraldehyde, ribose lyxose, mannose, galactose and fucose; see Table II and Fig. 2). It is remarkable that the enantiomer with the hydroxyl function at C_2 on the left-hand side in Fischer's projection always appeared first.

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Note

9-(Chloromethyl)anthracene: a useful derivatizing reagent for enhanced ultraviolet and fluorescence detection of carboxylic acids with liquid chromatography

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Many carboxylic acids, including the fatty and bile acids, are difficult to monitor spectrophotometrically at low concentrations in liquid chromatography (LC) because the acids have relatively weak absorption bands above 200 nm; however, derivatization of the acids with moieties that either enhance ultraviolet absorption or fluorescence can greatly facilitate detectability[1–4]. 9-Anthrylmethyl (9-AM) esters formed from carboxylic acids with 9-anthradiazomethane (ADAM) show both high absorptivity and intense fluorescence, appear to be as readily separated on reversed-phase LC columns as the products from other derivatization reagents[4] and are very stable[5]. This paper describes the use of 9-(chloromethyl)anthracene (9-ClMA) as an alternative reagent for the preparation of 9-AM esters of carboxylic acids prior to LC separation for those analysts who prefer to avoid the inconvenience of generating the diazoreagent. Spectral properties, detection limits, rates of formation and two examples of separations with fatty acid esters and glycine-conjugated cholates are discussed.

EXPERIMENTAL

Reagents and chemicals

Fatty acids, bile acids and glycine-conjugated bile salts were obtained from Sigma (St. Louis, MO, U.S.A.), and methanol, ethanol, acetonitrile, cyclohexane, dimethylformamide (DMF) and tetramethylammonium hydroxide (TMH) (24% in methanol) were obtained from MCB (Norwood, OH, U.S.A.). Solvents were purified according to standard procedures[6]. Dicyclohexyl-18-crown-6 was obtained from DuPont (Wilmington, DE, U.S.A.). 9-Anthraldehyde and purified anthracene (Gold Label Grade) were purchased from Aldrich (Milwaukee, WI, U.S.A.). Preparation of 9-CIMA from 9-(hydroxymethyl)anthracene gave bright yellow needles with m.p. 137–138°C (lit.[7] 137.5–138°C).

9-AM esters of lauric, myristic, palmitic and stearic acid were prepared in millimolar quantities by the general derivatization procedure using TMH and 9-CIMA described below. The esters were purified (>98% by LC analysis) by chromatography over neutral silica gel and recrystallization from ethanol. Structures of the esters were confirmed by infrared, proton magnetic resonance and mass spectroscopic

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TABLE I

UV ABSORPTION, FLUORESCENCE AND MELTING POINT DATA OF FOUR 9-ANTHRYLMETHYL FATTY ACID ESTERS

The esters contained traces of anthracene estimated to be less than 1% by LC analysis.

Fatty acid ester	*Molar absorptivity (1 mol $^{-1}$ cm^{-1})**		*Fluorescence relative quantum yield*** *ex. 365 nm*	*Corrected m.p. (°C)*
	256 nm	*365 nm*		
9-AM Laurate	$1.98 \cdot 10^5$	$1.06 \cdot 10^4$	0.34, 0.34	51–52
9-AM Myristate	$1.95 \cdot 10^5$	$1.04 \cdot 10^4$	0.34, 0.33	60–61
9-AM Palmitate	$1.94 \cdot 10^5$	$1.03 \cdot 10^4$	0.34, 0.1	67–68
9-AM Stearate	$1.92 \cdot 10^5$	$1.01 \cdot 10^4$	0.34, 0.1	73–74

* In methanol solvent.

** Relative to anthracene which has a quantum yield in ethanol of 0.30. First value in methanol, ethanol, acetonitrile and water–methanol (10:90); second value in water–methanol (20:80).

analysis. Melting points of the pale yellow crystalline substances are given in Table I with key molar absorptivities and quantum yields. The esters of oleic, linoleic and linolenic acid were oily substances and special purification techniques were not attempted.

Instrumentation

LC separations shown in Figs. 1 and 2 were obtained with a Waters LC system (Waters Assoc. Milford, MA, U.S.A.) including a Model 6000A pump, 440 Absorbance Detector, U6K Injector and a 30-cm "Fatty Acid" reversed-phase column. Fluorescence detection limits were determined with a Kratos (Schoeffel) (Westwood, NJ, U.S.A.) FS 970 Fluorescence Detector. Molar absorptivities (Table I) were calculated from absorbance data obtained on a Varian (Palo Alto, CA, U.S.A.) Cary 1605 Spectrophotometer, and relative quantum yields (Table I) were calculated from excitation and emission spectra generated with a Varian SF 330 Spectrofluorometer and a Perkin-Elmer (Norwalk, CT, U.S.A.) MPF 44B Spectrofluorometer fitted with a corrected spectra accessory. No significant differences were ascertained in quantum yields determined from either corrected or non-corrected emission spectra.

Derivatization procedure

The general procedure for the derivatization of carboxylic acids with 9-CIMA was similar to Greeley's method[8], and the procedure using sodium salts of carboxylic acids followed the method of Durst *et al.*[9]. Most reactions were performed in 2-ml graduated test-tubes and heated with a "Temp Block" (Labline Instruments, Melrose Park, IL, U.S.A.). Transfers of solutions and injections into the liquid chromatograph were done with Hamilton (Reno, NV, U.S.A.) microsyringes. Stock solutions of reagents and carboxylic acids were prepared at approximately 2 m*M* concentrations.

For carboxylic acids. Fatty or bile acids in methanol were treated with a slight excess of methanolic TMH. The sample was dried by passing a slow stream of nitrogen over the surface of the liquid. Acetonitrile was then added to give acid salt

concentrations ranging from mM to μM. After addition of enough 9-CIMA (in cyclohexane) to give a two- to ten-fold excess depending on the concentration of the original acid, the mixture was heated for the desired time. At mM concentrations of acid a two-fold molar excess of 9-CIMA was sufficient for rapid and quantitative reactions; however, at μM concentrations, a ten-fold molar excess of 9-CIMA was used. After cooling, the volume was adjusted by the addition of acetonitrile and an aliquot injected into the chromatograph for analysis.

For sodium salts of carboxylic acids. Appropriate quantities of methanolic solutions of the sodium salts of fatty acids, bile acids or glycine-conjugated bile acids were dried by a slow stream of nitrogen. An equal molar quantity of dicyclohexyl-18-crown-6 catalyst in acetonitrile was added and then solvent to give the desired concentrations. 9-CIMA was added to give a two- to ten-fold ratio as described above. After heating the sample was cooled and analyzed.

RESULTS AND DISCUSSION

Key spectral data of the four 9-AM esters of lauric, myristic, palmitic and stearic acid, the four common saturated fatty acids, are summarized in Table I. Molar absorptivities at 256 nm ranged from $1.92 \cdot 10^5$ to $1.98 \cdot 10^5$ and from $1.01 \cdot 10^4$ to $1.06 \cdot 10^4$ l mol^{-1} cm^{-1} at 365 nm, the excitation wavelength used for fluorescence analysis, consistent with literature values for anthracene[10] and 9-methylanthracene[11]. Quantum yields of the 9-AM esters relative to anthracene (lit.[12] 0.30) were 0.34 in the helium-purged solvents, methanol, ethanol and acetonitrile. The intense absorption band at 256 nm facilitated the detection of 0.1 pmol of the four 9-AM esters with a 10/1 signal-to-noise ratio at 0.005 a.u.f.s. after LC separation with 5% water–methanol. Approximately 2 fmol of 9-AM esters were monitored with the fluorescence detector (excitation 365 nm, emission 412 nm) after separation under similar conditions. Using a combination of the two detection systems, signals were linearly dependent on concentration over a 10^7-fold range.

Lloyd[13] considered several complications associated with the use of fluorescent derivatives in LC analysis, including the effects of solvent and oxygen on quantum yields and the photolability of derivatives. Although none of these factors was significant in the reversed-phase separations of 9-AM esters in this study, they could become important in procedures with solvents that quench fluorescence in anthracene[14]. Molecular aggregation, as noted with other derivatives of fatty acids[3,13], appeared significant as the concentration of water increased beyond 10%. With 9-AM esters of palmitic and stearic acids, the quantum yields dropped rapidly from 0.34 to 0.1 as the water concentration increased from 10 to 20%. The myristate ester was only slightly affected by the same change. The presence of oxygen in non-purged methanol led to a 10% decrease in quantum yield, a small factor for LC detectability and eliminated by the use of degassed solvents in the separation.

Carboxylic acids can be alkylated with 9-CIMA by a variety of different routes similar to those described in the literature for reactions of acids with alkyl halides[15]; however, either of the two processes, using quaternary ammonium hydroxide to form a quaternary ammonium salt of the carboxylic acid prior to alkylation[8] or using a "crown" catalyst with the sodium salt of the carboxylic acid during alkylation[9], gave excellent yields of derivatives within a reasonable time. Fatty acids in 10 μM concen-

trations were 98% alkylated in 15 min with a ten-fold excess of 9-CIMA in acetonitrile at 75°C. Lower concentrations of acids required longer heating periods or higher temperatures in sealed vials. Although the reaction was slightly faster in DMF solvent and could tolerate higher temperatures in open systems without solvent loss, absorbance signals from the DMF interfered with the signals of the lower-molecular-weight esters at 10 μM ester concentrations. Therefore, acetonitrile, which is transparent at 254 nm, was clearly the solvent of choice for alkylations with low concentrations of carboxylic acids.

For preparing 9-AM esters from carboxylic acids in protic solvents at lower than μM concentrations, ADAM would be the preferred reagent because it reacts more rapidly and selectively in protic solvents than 9-CIMA[4]. ADAM would also be the reagent of choice for base-sensitive carboxylic acids, such as the polyunsaturated fatty acids which may rearrange under the conditions for rapid alkylation with 9-CIMA.

Fig. 1 demonstrates the separation of six fatty acid esters and Fig. 2 shows the separation of three glycocholates and two types of cholates on a reversed-phase column with water–methanol mixtures. Principal by-products of the alkylation procedure, 9-(hydroxymethyl)anthracene and 9-(methoxymethyl)anthracene, eluted rapidly at the start of both separations and did not interfere with signals for the 9-AM

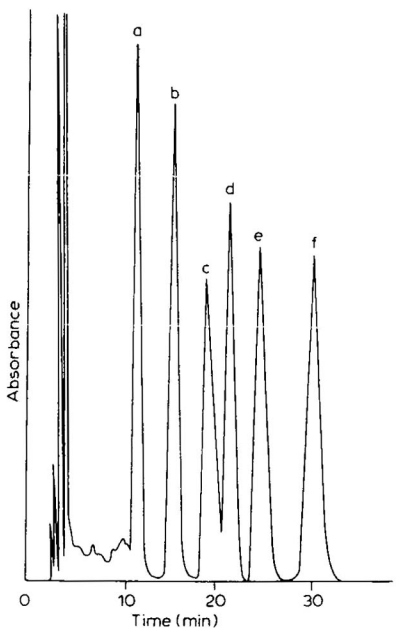

Fig. 1. Chromatogram of six 9-anthrylmethyl fatty acid esters: a = 9-AM laurate; b = 9-AM myristate; c = 9-AM linolenate; d = 9-AM palmitate; e = 9-AM oleate and f = 9-AM stearate. Separation on a Waters 30-cm "Fatty Acid" column with water–methanol (12:88) at 0.75 ml/min.

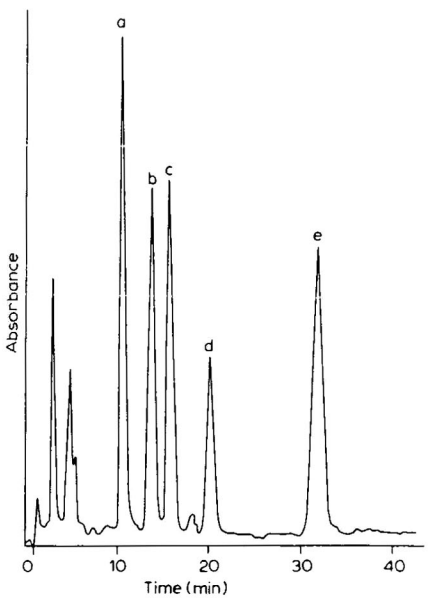

Fig. 2. Chromatogram of three 9-anthrylmethyl glycocholates and three 9-anthrylmethyl cholates; a = 9-AM glycocholate; b = 9-AM glycochenodeoxycholate; c = 9-AM glycodeoxycholate; d = 9-AM cholate; e = a mixture of 9-AM deoxycholate and 9-AM chenodeoxycholate. Separation on a Waters 30-cm "Fatty Acid" column with water–methanol (18:82) at 0.75 ml/min.

esters. With *p*-bromophenacyl bromide as the alkylating agent, glycocholic *p*-bromophenacyl ester was separated from excess of derivatizing reagent with a preliminary separation by thin-layer chromatography[16]. The by-products and excess of 9-CIMA do not interfere with normal-phase separations of 9-AM cholates[17], so that 9-CIMA is a useful reagent for both normal and reversed-phase separations.

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Note

Gelchromatographie von Cellodextrinen an Sephadex LH-20

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Für die Trennung von Oligosacchariden werden in erster Linie chromatographische Verfahren angewendet, wobei für die Isolierung von einzelnen Komponenten wohl nur säulenchromatographische Verfahren in Frage kommen. Die Trennung von Cellodextrinen, das sind Oligomere von β-Glukose, gelingt an Aktivkohle–Celit Säulen[1]. Analog zur Papierchromatographie ist die Trennung an Zellulosesäulen selbst möglich[2,3], doch wegen der Auswaschbarkeit von löslichen Cellodextrinen aus der Säule scheint dieses Material für die Untersuchung von Zelluloseabbauprodukten eher ungeeignet. Die Entwicklung der Gelchromatographie brachte nun die Möglichkeit die Oligomeren nach dem Molekulargewicht zu fraktionieren[4,5]. Bei der Verwendung von Dextrangelen[6–9], die durchaus zufriedenstellende Ergebnisse zeigen, kann es neben einer ebenfalls möglichen Auswaschung von löslichen Kohlehydratbestandteilen aus dem Gel, zu einer Wechselwirkung zwischen den freien OH-Gruppen des Gels und den OH-Gruppen der zu trennenden Kohlehydrate kommen[7]. Polyacrylamidgele zeigen neben besseren physikalischen Eigenschaften auch eine geringere Tendenz zu Wechselwirkungen[10]. So konnten Stärkedextrine bis zu einer Grösse von 13 Glukoseeinheiten getrennt werden[11].

In Verbindung mit der Hochleistungsflüssigchromatographie (HPLC) konnten Trennungen von Oligosacchariden unter grosser Zeitersparnis durchgeführt werden; so konnten auf Säulenfüllmaterialien mit entständigen NH_2-Gruppen oder druckresistenten Gelen rasche Trennungen nach der Molekülgrösse durchgeführt werden[12,13]. Eine weitere Möglichkeit zur Trennung von Zelluloseabbauprodukten ist über die entsprechenden Salpetersäureester an Styragelen mit Tetrahydrofuran als Eluens möglich[14].

In der vorliegenden Arbeit wird versucht die gute Löslichkeit der Kohlehydrate in Dimethylformamid (DMF) dazu auszunützen, eine Fraktionierung von Cellodextrinen an einem hydrophoben Gel wie z.B. Sephadex LH-20, durchzuführen. Durch Vergleich mit zwei bekannten Systemen Sephadex G-25 und Bio-Gel P-2 sollte untersucht werden, inwieweit Wechselwirkungen zwischen dem Gel und den zu trennenden Kohlehydraten an Sephadex LH-20/DMF die Fraktionierung nach dem Molekulargewicht (MG) beeinflussen.

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EXPERIMENTELLER TEIL

Material

Glukose, Cellobiose und Raffinose standen als p.A. Reinsubstanzen zur Verfügung. Das Gemisch der Cellodextrine wurde durch Acetolyse von Cellulosepulver (Merck) nach Vorschrift[1] hergestellt, wobei die freien Zucker mit Natriummethylat aus den Zuckeracetaten freigesetzt wurden. Das Endprodukt wurde dünnschichtchromatographisch auf Hochleistungsdünnschichtchromatographie (HPTLC)-Platten (Merck) analysiert[15]. Die Trennung erfolgte im Laufmittel Äthylacetat–Essigsäure–Methanol–Wasser (60:15:15:10) und gelingt bis zur Cellotetraose; Cellopentaose und Cellohexaose bleiben am Start sitzen. Die Methode hat allerdings den grossen Vorteil sehr rasch Ergebnisse zu liefern (Laufzeit *ca.* 15–20 min) und es sind nur kleine Probenmengen notwendig, da noch 0.1 μg Zucker detektiert werden können. Die Detektion erfolgt durch Sprühen oder Tauchen in Diphenylamin–Anilin–Phosphorsäure-Reagens[16]. Durch *in situ* Messen der Transmission bei 610 nm mit einem Dünnschichtscanner ist auch eine quantitative Auswertung möglich.

Methodik

Sephadex G-25F (Pharmacia) und Bio-Gel P-2 (200–400 mesh, Bio-Rad Labs.) wurden über Nacht in destilliertem Wasser gequollen, Sephadex LH-20 (Pharmacia) in DMF. Die Säulen wurden nach Vorschrift[17] gefüllt und über Nacht mit dem entsprechenden Eluens equilibriert. Die Qualität der Säulenfüllung und das Ausschlussvolumen wurden mit Blue-Dextran (Pharmacia) bestimmt.

Die Säulen, Flow Adaptoren, Schlauchverbindungen und Ventile waren alles Pharmacia Produkte in SR-Ausführung (solvent resistent). Die jeweiligen Säulendimensionen werden bei den einzelnen Versuchen gesondert angegeben. Die Probenaufgabe erfolgte mit einer Probenschleife (Inhalt = 0.72 ml oder 1.2 ml), die mit Hilfe von 2 Vierwegventilen in den Lösungsmittelfluss geschaltet werden konnte. Das Eluens wurde mit einer Dosierpumpe (P 1, Pharmacia) gefördert, über die auch die Durchflussgeschwindigkeit eingestellt wurde. Die Detektion der chromatographierten Substanzen erfolgte kontinuierlich mit einem Differentialrefraktometer (Siemens). Das Eluat wurde noch mit einem Fraktionssammler fraktioniert, um die Fraktionen mit HPTLC noch zusätzlich identifizieren zu können (Apparatur siehe auch Fig. 1).

ERGEBNISSE

Gemisch der Cellodextrine

Das Gemisch der Cellodextrine, das nach Acetolyse von Cellulosepulver[1] hergestellt wurde zeigte folgende relative Zusammensetzung (HPTLC):

Glukose	= 22%
Cellobiose	= 25%
Cellotriose	= 29%
Cellotetraose	= 15%
Cellopentaose	= 7%
Cellohexaose	= 2%

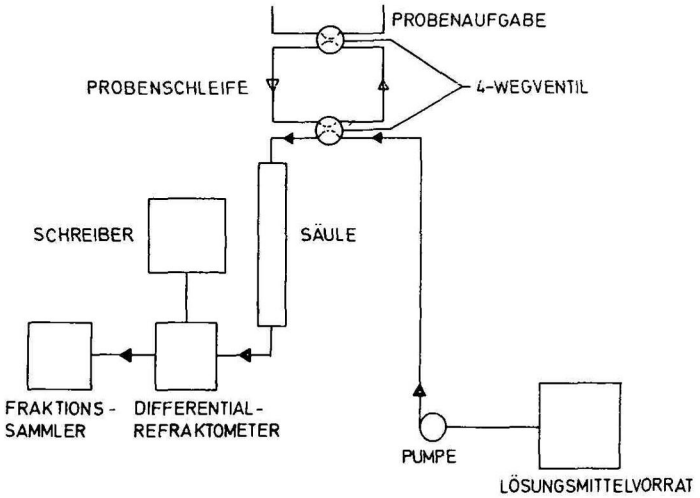

Fig. 1. Fliessschema und Apparatur.

Gelchromatographie

Wenn man die in Tabelle II angeführten Werte für K_d gegen log MG in ein Diagramm einträgt (Fig. 3), so ergibt sich für die Systeme Sephadex G-25 und Bio-Gel P-2 jeweils mit Wasser als Eluens eine Kurve, wobei aber die K_d Werte der Einzelsubstanzen mit den entsprechenden des Cellodextringemischs sehr gut übereinstimmen. Diese Abweichung vom theoretischen linearen Verlauf, die in Übereinstimmung mit früheren Ergebnissen[6,10,11] steht, tritt beim System Sephadex LH-20/DMF nicht sichtbar auf. Die K_d Werte für Raffinose und Saccharose dagegen lassen keinen linearen Zusammenhang mit dem log (MG) erkennen. Bei einem Bettvolumen von 356 ml ist die Trennung der Cellodextrine sehr gut (siehe Fig. 2).

DISKUSSION

Bei der Verwendung von hydrophilen Gelen wie Sephadex G-25 oder Bio-Gel P-2 zur Trennung von Oligosacchariden ist der Zusammenhang zwischen K_d und log MG aufgrund von Wechselwirkungen zwischen Gel und chromatographierter Sub-

TABELLE I

KENNZAHLEN FÜR SÄULEN UND GELPACKUNGEN

	Sephadex G-25	*Bio-Gel P-2*	*Sephadex LH-20*	
Säule (mm)	700 × 16	700 × 16	500 × 10	1000 × 25
Bettvolumen, V_t (ml)	115.6	121.5	36.9	356
Ausschlussvolumen:				
V_o (ml), Blue Dextran	46.0	43.5	18.0	124.0
Durchfluss (ml/h)	17.2	17.2	10.9	80.0
Probevolumen (ml)	0.72	0.72	0.72	1.2
Probekonz., (mg/ml)	*ca.* 5	*ca.* 5	*ca.* 5	*ca.* 15
Eluens	Wasser	Wasser	DMF	DMF

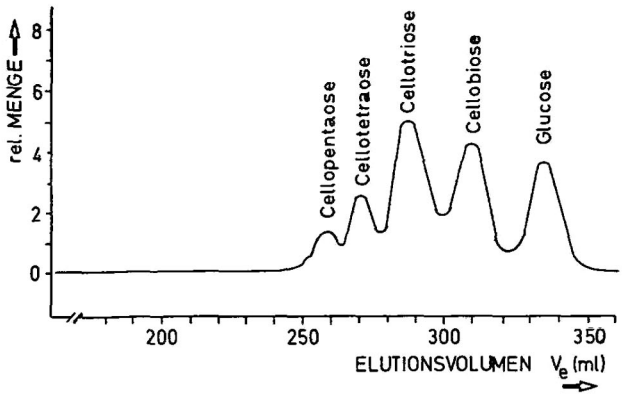

Fig. 2. Trennung der Cellodextrine an Sephadex LH-20 mit DMF als Eluens.

stanz nicht über den gesamten Bereich linear. Polyacrylamidgele (Bio-Gel) liefern neben einer leichteren Handhabung beim Füllen der Säulen, wie schon in früheren Arbeiten[7,10] erwähnt, etwas bessere Ergebnisse. Die Trennung der Cellodextrine gelingt mit dem System Sephadex LH-20/DMF ganz ausgezeichnet; hierbei zeigen die einzelnen Peaks keinerlei wesentliche Verbreiterungen und auch bei einem raschen Durchfluss (16.3 ml h^{-1} cm^{-2}) wird eine klare Trennung der Oligomeren erreicht. Innerhalb der Reihe der Oligosaccharide, die nur aus Glukoseeinheiten aufgebaut sind, ist die Beziehung zwischen log MG und dem K_d-Wert weitgehend linear (siehe Fig. 3); somit also ist die Bestimmung von Molekulargewichten höherer Cellodextrine (oder Stärkedextrine), auch bei einer Eichung des Systems mit Substanzen mit nur kleinem Molekulargewicht, hinreichend genau.

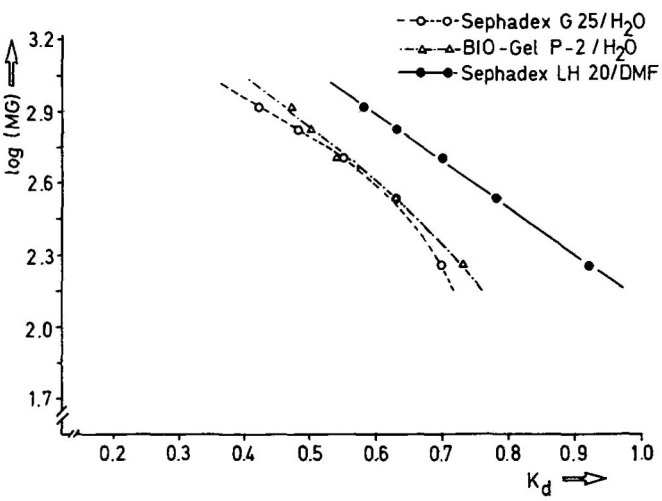

Fig. 3. Beziehung zwischen K_d und log MG. O---O, Sephadex G-25/Wasser; △·····△, Bio-Gel P-2/Wasser; ●——●, Sephadex LH-20/DMF.

TABELLE II

ZUSAMMENSTELLUNG VON V_e (ELUTIONSVOLUMEN), K_d $\left(=\frac{V_e - V_0}{V_t - V_0}\right)$ UND MOLEKULARGEWICHTEN DER IN DEN VERSCHIEDENEN SYSTEMEN UNTERSUCHTEN OLIGOSACCHARIDE

Oligosaccharide (Testsubstanzen)	*MG*	*log MG*	*Sephadex G-25/Wasser (V_t = 115.6 ml)*		*Bio-Gel P-2/Wasser (V_t = 121.5 ml)*		*Sephadex LH-20/DMF (V_t = 36.9 ml)*		*Sephadex LH-20/DMF (V_t = 356.0 ml)*	
			V_e (ml)	K_d	V_e (ml)	K_d	V_e (ml)	K_d	V_e (ml)	K_d
Glukose	180	2.2553	95.0	0.70	100.2	0.73	35.1	0.90	355.0	0.92
Cellobiose (pur)	342	2.5340	89.6	0.63	92.8	0.63	32.6	0.77	309.0	0.78
Raffinose	504	2.7024	84.0	0.54	85.0	0.53	24.7	0.31	198.0	0.32
Saccharose (Cellodextrin-gemisch)	342	2.5340	–	–	–	–	25.6	0.40	218.5	0.42
Glukose	180	2.2553	95.0	0.70	100.2	0.73	–	–	335.0	0.92
Cellobiose	342	2.5340	89.6	0.63	92.8	0.63	–	–	309.0	0.78
Cellotriose	504	2.7024	84.3	0.55	85.3	0.54	–	–	286.0	0.70
Cellotetraose	667	2.8241	79.4	0.48	82.3	0.50	–	–	270.0	0.63
Cellopentaose	829	2.9186	75.2	0.42	79.9	0.47	–	–	258.0	0.58

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Note

Purification of luciferase by affinity elution chromatography on Blue Dextran columns

Comparison of Sepharose and silica as support matrices

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The popularity and significance of the luciferase-catalysed bioluminescence in the firefly is well documented. Its remarkable specificity and sensitivity towards adenosine triphosphate (ATP) has induced us to work out methods for the purification of luciferase. The essence of the reaction is the production of light from luciferin in the presence of ATP, Mg^{2+} and molecular oxygen as shown in eqns. 1 and 2

$$LH_2 + ATP + E \rightarrow E \cdot LH_2 \cdot AMP + PP \quad (1)$$

$$E \cdot LH_2 \cdot AMP + O_2 \rightarrow E + P + CO_2 \quad (2)$$

where LH_2 = luciferin, E = enzyme, $E \cdot LH_2 \cdot AMP$ = the luciferyl adenylate complex, P = the product oxyluciferin and PP = the pyrophosphate.

Several partial and complete luciferase purifications have been reported[1–3]. However, some of them require lengthy chromatographic procedures while others do not yield complete separation. This communication describes an improved separation technique for luciferase.

Recent literature on nucleotide-dependent enzymes deals with their purification by affinity elution chromatography on Blue Dextran Sepharose, a semi-specific high-molecular-weight compound substituted with the mono-chlorotriazinyl dye Cibachron Blue F3GA which can mimic a polynucleotide, presumably due to an attraction of the blue chromophore to a nucleotide-binding site of the enzyme. It has been reported by Thompson *et al.*[4] that Blue Dextran linked to Sepharose may be used as an affinity chromatographic medium for many proteins which bind dinucleotides and ATP.

EXPERIMENTAL

Cyanogen bromide-activated Sepharose 4B was purchased from Pharmacia, firefly lantern, Blue Dextran and ATP from Sigma and DTT from E. Merck. All other chemicals were of reagent grade.

Sample preparation

Extraction of firefly abdomen was carried out according to the method of

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Antonik[5] with slight modifications. A 1-ml volume of dithiothreitol (DTT) (195 mg/ml) was added per 100 mg of firefly abdomen at the end of the centrifugation. This was necessary for the stability of the crude extract. The preparation was dialysed against 0.02 *M* Tris–acetate, pH 7.8 with 1 m*M* EDTA for 24 h with three changes of the buffer.

Luciferase assay

Luciferase activity was estimated by PICO ATP apparatus (Jobin Yvon, France). The reaction mixture containing 50 μl of 10^5 pg/ml ATP in 0.01 *M* MOPS (3-N-morpholinopropanesulphonic acid), pH 7.4 containing 10 m*M* $MgSO_4$, 50 μl of luciferin (0.168 μg/ml) and 50 μl of luciferase sample was introduced in a special cuvette in the PICO ATP apparatus at 18–20°C and the intensity of light in millivolts during the first 2 sec of emission was recorded by a photomultiplier. The unit of activity is the maximum intensity of light recorded in millivolts per picogram ATP per milligram protein at 562 nm.

Chromatography

Blue Dextran was coupled to oxiran silica by the procedure of Chang *et al.*[6]. Blue Dextran Sepharose was prepared by the method of Ryan and Vestling[7]. The efficiency of the coupling reaction was controlled by measuring the differential absorbance in the visible region of the coupled gel suspended in 87% glycerine. A 0.3-ml aliquot of the dialysed extract was injected into the column of Blue Dextran coupled to Sepharose or silica and the elution was started with 0.01 *M* MOPS containing 20 m*M* $MgSO_4$ and 1 m*M* DTT and continued until no protein was detected in the fractions. Then the eluent was changed to 0.5 m*M* ATP in 0.01 *M* MOPS containing 20 m*M* $MgSO_4$ and 1 m*M* DTT to desorb the enzyme. The ballast proteins were stripped by eluting with a 3 *M* KCl solution. After each use, the columns were washed with four volumes of 3 *M* KCl and then exhaustively equilibrated with the starting eluent, 0.01 *M* MOPS, pH 7.4 containing 20 m*M* $MgSO_4$ and 1 m*M* DTT, before the start of the next cycle. Under these conditions no loss of the blue chromophore was observed. The same resin can be successfully used for more than six elutions.

Dialysis of the fractions

The fractions (1.0 ml) were pooled into groups of three and dialysed against 0.02 *M* Tris–acetate, pH 7.8 with 1 m*M* EDTA for 16–19 h. All operations were carried out at 4°C. Each pooled fraction was tested for luciferase activity and protein concentration. The absorbance at 278 nm was corrected against ATP in MOPS.

Sodium dodecyl sulphate (SDS)-gel electrophoresis and isoelectric focusing

The homogeneity of the preparation of purified luciferase was checked by SDS-gel electrophoresis in 7.5% polyacrylamide gel, pH 8.0–9.0. Isoelectric focusing was carried out in the range pH 3.5–10.0 with 8 *M* urea on 360 μm thick polyacrylamide gels freshly prepared on a cellophane support according to Gorg *et al.*[8].

RESULTS

A comparison of chromatography on Blue Dextran coupled to Sepharose and silica is shown in Table I. The elution profiles are shown in Figs. 1 and 2.

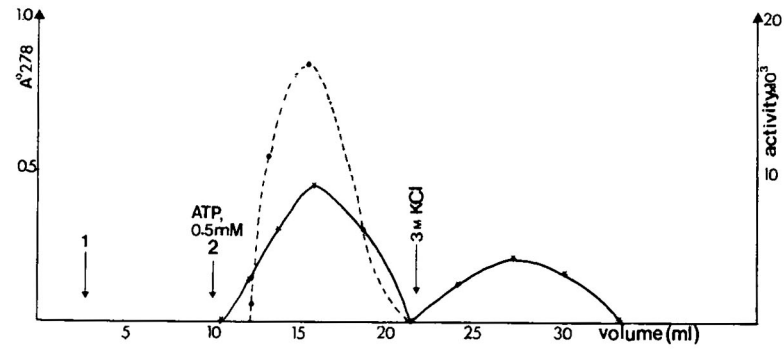

Fig. 1. Elution profile of luciferase on Blue Dextran coupled to Sepharose. A column of Blue Dextran Sepharose equilibrated with 0.01 *M* MOPS containing 20 m*M* $MgSO_4$ and 1 m*M* DTT, pH 7.4 was loaded with 0.3 ml of crude luciferase. The eluent was changed to 0.01 *M* MOPS containing 20 m*M* $MgSO_4$, 1 m*M* DTT and 0.5 m*M* ATP. Column fractions (1 ml) were assayed for enzyme activity and protein concentration (see Experimental). ——, Absorbance at 278 nm; – – –, luciferase activity.

It is evident that Blue Dextran coupled to silica is more favourable for luciferase purification since we were able to recover about 300% of the initial activity compared to about 180% in the case of Sepharose Blue Dextran. The purification in the two cases is 61-fold and 51-fold respectively for silica- and Sepharose-based derivatives (Table I). This method is superior to that of Lundin *et al.*[9], who used $(NH_4)_2SO_4$ precipitation followed by isoelectric focusing but obtained only a 8.1-fold purified enzyme with 65% of the initial activity. We found good evidence for the homogeneity of our purified sample on Blue Dextran Sepharose and silica by SDS-gel electrophoresis. Isoelectric focusing showed the presence of one band at a pH of about 5.0. The purified fractions did not show the presence of other contaminating enzymes present in the extract.

DISCUSSION

Blue Dextran is known to complex with a wide range of proteins because it is specific for a super-secondary structure called the dinucleotide fold. This structure forms the ATP-binding site in phosphoglycerate kinase[10] and NAD-binding sites in lactate dehydrogenase and many other enzymes[11].

In our studies, the nucleotide ATP which has the highest affinity for luciferase

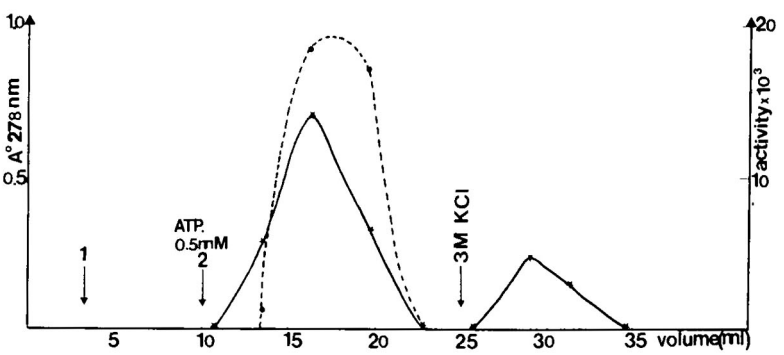

Fig. 2. Chromatogram of luciferase purified on Blue Dextran silica. Conditions and curves as for Blue Dextran Sepharose.

TABLE I

PURIFICATION OF LUCIFERASE ON BLUE DEXTRAN COUPLED TO SEPHAROSE AND SILICA

Purification steps	*Total protein*		*Total activity*		*Specific activity*	*Purification factor*
	Absolute value (mg)	*% Initial*	*Absolute value*	*% Initial*		
1 Crude extract	25.6	100	243,000	100	9346	0
2 Dialysed extract	1.5	5.8	247,300	102	164,900	17.0
3 Purified sample from Blue Dextran sepharose	0.96	3.7	460,100	180	479,300	51.0
4 Purified fraction from Blue Dextran silica	1.3	5.2	742,800	300	571,400	61.0

was most effective in eluting the bound enzyme. The optimum concentration of ATP was 0.5 m*M*. By contrast, NaCl at a concentration at least 200 times that of the specific ligand ATP was required to desorb the enzyme from the column. Thus ligand specificity for luciferase was made use of to distinguish binding to the dinucleotide fold from non-specific ionic binding. In our case, the relative affinity of Blue Dextran and ATP for the same active site of luciferase must have facilitated the selective elution of the enzyme from the presumably large number of proteins and other compounds in the firefly abdomen homogenate which are bound to the affinity column by non-specific interactions.

In a previous comparison of metal–chelate adsorbents based on Sepharose and silica, for the separation of nucleotides, a higher capacity was reported for silica-based adsorbent[12]. We find the same behaviour in this case, as the Blue Dextran silica column has a higher enzyme-binding capacity than the corresponding Sepharose-based adsorbent. The affinity status of these Blue Dextran columns remains controversial. Many authors have recently pointed out that these are in fact pseudo-affinity interactions. However it is too premature to draw any conclusion on the details of the interaction or to state the mode of inhibition of luciferase with respect to Blue Dextran and ATP. In the presence of ATP, the equilibrium between free enzyme and enzyme bound to Blue Dextran could be shifted towards free enzyme. We confidently predict the evidence of a dinucleotide fold in firefly luciferase.

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Note

Columns for simultaneous gas chromatographic determination of ppt* levels of the atmospheric tracers sulphur hexafluoride and bromotrifluoromethane in ambient air samples

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Sulphur hexafluoride has been widely used in tracer experiments for the identification and simulation of air pollution from stacks and diffuse sources. It was selected for such investigations mainly because of its non-toxic character, chemical stability and easy detection at very low concentrations by gas chromatography (GC) using an electron-capture detector.

The separation of SF_6 from air components, such as oxygen, is a major analytical problem. In some earlier investigations a packed silica gel column, followed by an activated charcoal column and alumina oxide columns, were used[1,2]. However, the SF_6 signal then appears after the oxygen signal, causing interference problems and a decreased detection limit. Some rather sophisticated methods have also been described using preconcentration and backflush of the sample to improve the detection limit to a few ppt[1,3]. By introducing specially treated molecular sieve columns[4–6], from which SF_6 is eluted before oxygen, some of the problems mentioned above have been overcome, enabling the direct determination of a few ppt. However, most of the columns described need a reactivation of the molecular sieve after a few days to maintain the separation efficiency. This makes them less suitable for long-term measurements and automatic devices.

The application of two tracer gases is of considerable advantage for the evaluation of more complicated emissions especially when several sources are involved. Bromotrifluoromethane has been used in dual-tracer experiments due to its comparable properties to SF_6. However, there are some difficulties in the GC detection procedure. The response factor of the electron-capture detector for $CBrF_3$ is about two orders of magnitude lower than that for SF_6, and the separation of both tracers from oxygen is difficult. Lamb[6,7] suggested a rather complex procedure for the preparation of a molecular sieve column, which makes possible the separation of both tracers from oxygen. Unfortunately, his columns were difficult to reproduce, and the lifetime was rather short.

The aim of this work was to simplify the activation procedure, to improve the reproducibility of the separation efficiency and to increase the column lifetime. A detailed description of a method is given, which allows the preparation of highly

* Throughout this article, the American billion (10^9) and trillion (10^{12}) are meant.

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stable molecular sieve columns for trace analysis of SF_6 and $CBrF_3$ in ambient air samples. The simultaneous detection of about 5 ppt SF_6 and 100 ppt $CBrF_3$ in a 1-ml air sample is possible.

EXPERIMENTAL

Instruments

A simple home-made gas chromatograph was used for all experiments. The whole system, including the electron-capture detector, was operated isothermally either at room temperature or at 40–60°C. The electron-capture detector was of the pin-cup type equipped with a tritium copper foil of 400 mCi. The detector was operated at a pulse width of 2 μsec, a pulse rate of 250 μsec and a pulse amplitude of −30 V. A two-channel recorder (Model 585; Linear Instrument, Irvine, CA, U.S.A.) was used for the registration of the chromatograms. For quantitative analysis a Model 3390 integrator system (Hewlett-Packard, Palo Alto, CA, U.S.A.) was employed. A manual valve (Valco six-port HP valve; Valco Instruments, Houston, TX, U.S.A.) with a loop of 1 or 5 ml was used for sample introduction. Ambient air samples were collected in 50-ml polyethylene disposable syringes using a home-made automatic sampling device.

Nitrogen of 99.995% purity was used as the carrier gas. A metal bellow pressure reduction valve (Type HBS300; L'Air Liquide, Paris, France) and a stainless-steel molecular sieve trap were used to prevent contamination of the GC system. Calibrations were done by means of an exponential dilution vessel with standard gas mixtures prepared from 99.9% pure SF_6 and $CBrF_3$ (Kali Chemie, Hannover, G.F.R.)

Column preparation

Dual tracer column. A stainless-steel column (2.5 m × 2 mm I.D.) was connected to a 25-ml glass pipette filled with about 12 ml of 5A molecular sieve (80–100 mesh). It is very important to remove any dust from the column material by washing it with 0.1 *M* hydrochloric acid and deionized ultrapure water[8]. After washing, the molecular sieve was dried for 12 h at 300°C. A commercially available, prepurified and acid-washed Type 5A molecular sieve (80–100 mesh) (No. 5605; Alltech, Deerfield, IL, U.S.A.) is also suitable and can be used without pretreatment. The other end of the column was connected to a diaphragm vacuum pump (Type Al 17, Neuberger Inc.) with an empty impinger in between. Nitrogen, at a pressure of 1.3 bar, was applied to the inlet of the glass molecular sieve pipette and the column filled slowly, with slight tapping. The pressure should be increased step by step to about 2 bar at the end of the filling procedure. The packing has to be done with extreme care to prevent the formation of dust from the molecular sieve. After filling, the column was activated at 300°C for 12 h under a nitrogen flow of about 10 ml/min.

Single-tracer column. This column allows only the separation of SF_6 from the oxygen signal. It was prepared as described above. Aluminium oxide (Alumina F-1, 80–100 mesh, No. 2-0284; Supelco, Bellefonte, PA, U.S.A.) was used for packing and the column was activated at 400°C for 12 h.

Measuring procedure

The gas chromatograph, operated at ambient temperature and an inlet pressure of 3 bar, was calibrated with standard gas mixtures from an exponential dilution vessel. Ambient air samples were taken with automatic samplers in 50-ml syringes. After the injection of 1 ml air, SF_6, $CBrF_3$ and oxygen were eluted within 3 min. Because of the presence of other halogenated trace compounds in the samples, one has to wait about 5 min before the next injection. This conditioning time is reduced to about 3 min when the column is operated at 40°C. From the single-tracer column, oxygen and SF_6 were eluted within 1 min at an inlet pressure of 1.1 bar.

RESULTS AND DISCUSSION

First measurements of SF_6 were made with the alumina columns, which were easy to prepare and gave a reasonably low detection limit of about 5 ppt (see Fig. 1). Based on the work of Simmonds *et al.*[4], columns packed with standard molecular sieve were used for some investigations. These were only able to separate SF_6 from the oxygen signal and had to be activated after a short time. Dietz and Cote[5] proposed a nitric oxide-treated molecular sieve column to improve both peak shapes and long-term stability. However, separation of both SF_6 and $CBrF_3$ could not be achieved.

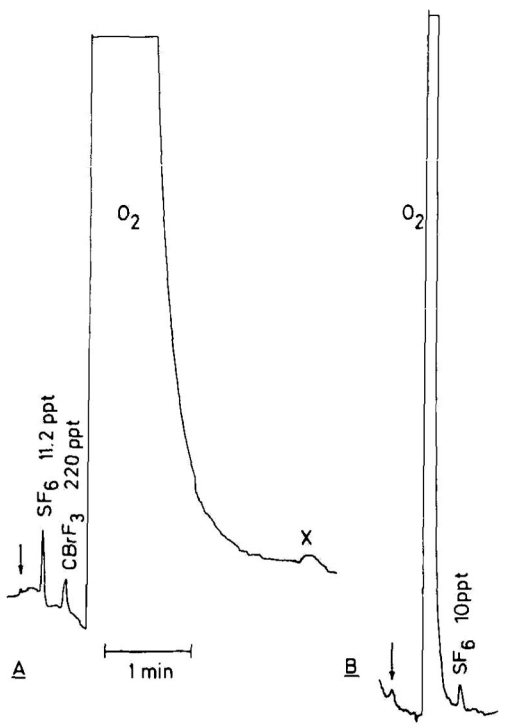

Fig. 1. Gas chromatograms of tracer gases on molecular sieve and aluminium oxide. A, Chromatogram of an ambient air sample separated on molecular sieve. The column was operated at room temperature; X = halogenated compound. B, Standard mixture containing 10 ppt SF_6 separated on aluminium oxide at room temperature.

Further experiments to separate both $CBrF_3$ and SF_6 from oxygen were conducted with the molecular sieve column prepared according to the method of Lamb[6,7], which recommends an overnight activation procedure at 300°C, followed by a partial deactivation with water. Another suggestion was to activate the column at 175°C for 12.5 h. All of these columns, filled with standard quality molecular sieve, however, showed a relatively high pressure drop, and had to be operated at 3.6 bar inlet pressure. For the separation of both tracers from oxygen, the columns were useful only for a few days, and their performance decreased rapidly thereafter.

The use of other stationary phases, such as Carbosphere (80–100 mesh) (Alltech, No. 5682) and a carbonaceous molecular sieve (Carbosieve B, 60–80 mesh, Supelco, No. 1-0250), gave no improvements. Both column packings showed extreme tailing of the oxygen signal and insufficient separation of the SF_6 signal from both oxygen and $CBrF_3$.

The standard quality molecular sieve, used in the first experiments, contained a lot of very fine particles, which made the preparation of low pressure drop columns very difficult. Furthermore, the molecular sieve material is rather brittle and does not tolerate vibration of the column under the filling procedure. The large pressure drop and high content of dust do not allow a reproducible activation of the column. The use of acid-washed, dust-free molecular sieve (see Experimental) and the development of a careful packing procedure eliminated the problems mentioned above. The activation was carried out at 300°C or more to get stable and highly active columns.

The purity of the carrier gas is decisive for the long-term stability. A pressure regulator with metal bellow seals and a stainless-steel filter cartridge (filled with molecular sieve) were used to maintain the purity of the nitrogen. Commercially available purification cartridges, made from acrylic glass, cannot be recommended since they may cause contamination, which disturbs the function of the detector.

The presence of late elution peaks of chlorinated hydrocarbons considerably increases the analysis time. The operation of the separation column at an elevated temperature (about 40–50°C) reduces the retention time for such compounds, without any influence on the separation efficiency for the tracer gases. A sample analysis cycle of 3 min is then possible (see Fig. 1).

The lower detection limit can be improved to 2 ppt and 50 ppt, respectively (signal-to-noise ratio 5:1) when a 5-ml loop is used. The separation efficiency deteriorates slightly, but is still sufficient for the low concentrations.

Applications of the tracer technique

The GC system described has been used for several thousands of samples without any serious problems. Applications of the tracer gas technique have been in the assessment of inert gas ventilation system efficiency in oil tankers, investigations of the transport of pollutants in the primary aluminium industry[9] and the control of the air conditioning and ventilation system efficiency in buildings. Major organic compounds in air, such as aliphatic and aromatic hydrocarbons, did not influence the lifetime of the column. Even under field conditions, where heavily polluted air masses were analysed, no reactivation or bake-out of the column was necessary for at least 3 months.

For indoor measurements, interferences caused by Freons from very small leakages in refrigeration systems can be a severe problem. The presence of ppb

amounts of Freons, which elute just after the tracer compounds, precludes any determination.

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Note

Reversed-phase high-performance liquid chromatographic separation of ribosyl, 2′-deoxyribosyl and arabinosyl nucleosides of adenine and hypoxanthine*

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One area of analysis which has lent itself extremely well to high-performance liquid chromatographic (HPLC) methods is the separation of nucleic acid components. Because of the importance of these compounds in biological systems many investigations have been conducted in order to find rapid and reproducible methods for their separation[1].

In connection with our studies on the metabolism of arabinosyl nucleosides possessing antiviral activity, we required a quantitative separation of the ribosyl, 2′-deoxyribosyl and arabinosyl derivatives of adenine and hypoxanthine. A further requirement was that the separated components be readily collectable, free from interfering materials and easily analyzed by liquid scintillation spectrometry.

Early methods of separating nucleosides were based on experiences acquired with nucleotides, namely ion exchange chromatography. However, recent work has involved the more rapid process of reversed-phase high-performance liquid chromatography (RP-HPLC)[2–7]. We now have examined this process to accomplish the required separation.

Previous work has focused on the separation of ribosyl nucleosides from 2′-deoxyribosyl nucleosides[2,3], pyrimidine ribosyl nucleosides from purine ribosyl nucleosides[4,6] and the arabinosyl derivative of adenine from the arabinosyl derivative of hypoxanthine[5]. Previously reported separations of the three carbohydrate classes by means of thin-layer chromatography (TLC) of nucleotides[8], mixtures of nucleotides, nucleosides and bases[9] and anion exchange HPLC[10] were not suitable for our needs.

We wish to report two methods found suitable for the separation of the title compounds. One method involves an isocratic separation while the second utilizes a gradient system.

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EXPERIMENTAL

Chemicals and reagents

Ammonium dihydrogen phosphate (Fisher), sodium borate (J. T. Baker), boric acid (E. Merck) and sodium periodate (Eimer and Amend) were all of reagent grade and used without further purification. The methanol (Burdick & Jackson) and acetonitrile (Fisher) were HPLC grade. The mixed borate–phosphate buffer used in the final analyses contains 80 parts 0.075 *M* $Na_2B_4O_7$ and 20 parts 0.01 *M* $NH_4H_2PO_4$ with the pH adjusted to 6.03.

Adenosine (Ado) and inosine (Ino) were from Nutritional Biochemicals; 2′-deoxyadenosine (dAdo) and 2′-deoxyinosine (dIno) were from Sigma; 9-β-D-arabinofuranosyladenine (ara-A) and 9-β-D-arabinofuranosylhypoxanthine (ara-H) were obtained through the courtesy of Dr. H. Machamer, Warner-Lambert/Parke-Davis Co., Detroit, MI, U.S.A.

Apparatus

The HPLC system consisted of a dual piston pump and controller (Nester Faust) and an Altex Model 153 fixed UV detector set at 254 nm with instrument attenuation at 0.02 a.u.f.s. The column was either a Partisil PXS 10/25 C_8 (25 × 3 cm) or a Partisil PXS 10/25 ODS-2 (25 × 3 cm) (both from Whatman).

Procedures

The water used throughout was distilled and all solutions were passed through a Millipore filter (0.45 μm) and degassed prior to use. All runs were conducted at ambient temperature at a flow-rate of 1 ml/min and a chart speed of 0.25 cm/min. Adjustments to pH were made by adding crystalline boric acid to the buffered solutions prior to mixing with the organic solvents.

RESULTS AND DISCUSSION

Initial studies were conducted using the ODS-2 column in attempts to separate Ado, ara-A, Ino and ara-H. With this column good separation was achieved for the adenine derivatives. Ara-H and Ino were eluted first, but together, while ara-A and Ado were clearly separated from each other and from the hypoxanthine derivatives. Different ratios of phosphate buffer (0.01–0.10 *M*) to methanol and phosphate buffer (0.01–0.10 *M*) to acetonitrile covering a pH range of 3.07–7.40 were utilized but failed to separate ara-H and Ino.

In order to investigate the conditions necessary to separate Ino and ara-H, we explored chromatography on a reversed-phase (C_8) column. A slight separation of Ino and ara-H was obtained using a phosphate buffer–methanol solvent system at pH 7 but improvement in the degree of separation was desired. The technique of carbohydrate–borate complexation has been explored to effect the separation of ribosyl, 2′-deoxyribosyl and arabinosyl nucleosides on both anion and cation exchange systems[10]. Replacing the phosphate buffer with a borate buffer at pH 7 provided a baseline separation of these two nucleosides. However the baseline separation of ara-H and Ado was lost. In the borate system the elution order was reversed and the ribosyl derivatives were eluted before the corresponding arabinosyl derivatives. The

cis-diol functionality facilitated the formation of stable borate complexes which were readily eluted on the reverse phase medium. Complexing with the *trans*-diol system was less complete resulting in relatively longer retention times for the arabinosyl compounds. By a comparison of the retention times in these two systems it was noted that borate concentration had a greater effect on retention times than either the phosphate concentration or pH.

To obtain a larger difference in retention times of ara-H and Ado the borate buffer was modified by the addition of varying amounts of phosphate buffer. The retention times of ara-A and ara-H were very sensitive to changes in the borate/phosphate ratio. Increases in concentration of borate in these mixtures increased the retention times of ara-A and ara-H while the opposite effect was observed for both Ado and Ino. As a result, we were able to achieve a baseline separation of the four previously discussed nucleosides on the C_8 column using a mixed borate–phosphate (75:25) buffer at pH 7.16 and acetonitrile in a ratio of 98:2.

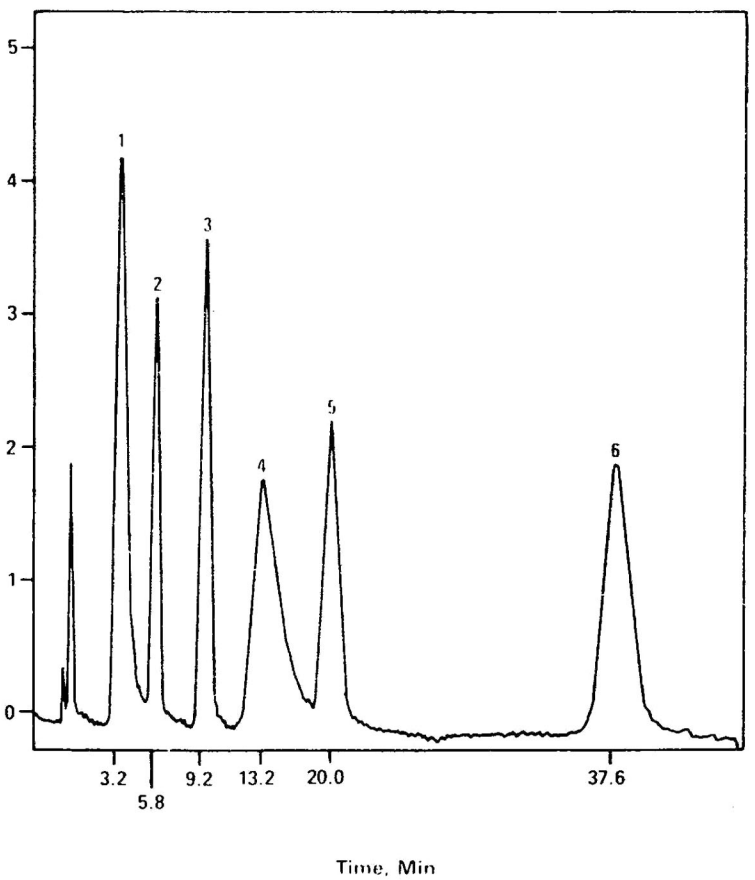

Fig. 1. Isocratic elution of ribosyl, 2′-deoxyribosyl and arabinosyl derivatives of adenine and hypoxanthine. The following conditions were employed: column, Partisil PXS 10/25 C_8; mobile phase, buffer–methanol (95:5); pressure, 900 p.s.i.; detection, UV at 254 nm (0.02 a.u.f.s.). The compounds eluted in the following order: 1 = Ino; 2 = ara-H; 3 = dIno; 4 = Ado; 5 = ara-A; 6 = dAdo.

When we included the deoxynucleosides, however, we were unable to separate dIno and Ado. Through a series of minor adjustments to the buffer system, pH and changing the organic solvent to methanol a complete separation of all six nucleosides was effected (see Fig. 1).

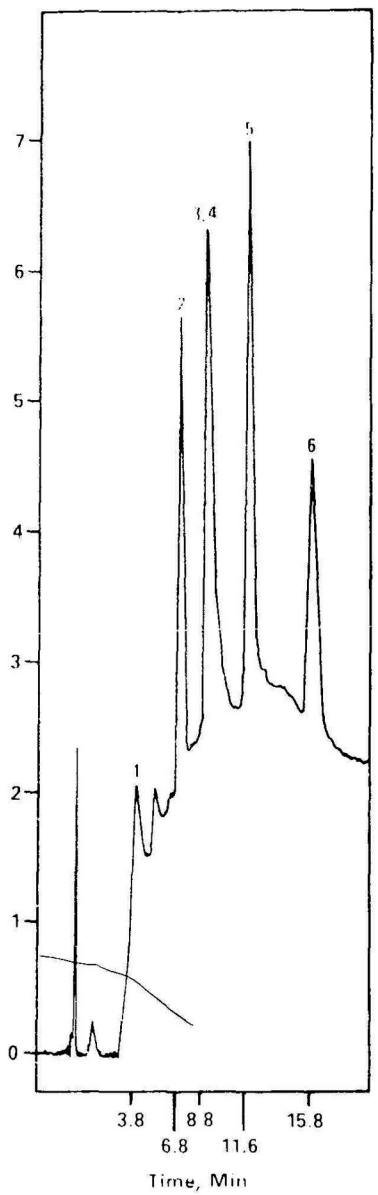

Fig. 2. Gradient elution of ribosyl, 2′-deoxyribosyl and arabinosyl derivatives of adenine and hypoxanthine. The following conditions were employed: column, Partisil PXS 10/25 C_8; mobile phase, linear gradient from buffer–methanol (99:1) to (75:25); pressure, 900 p.s.i.; detection, UV at 254 nm (0.02 a.u.f.s.). Compounds as in Fig. 1.

Even though this separation of the six nucleosides satisfied our immediate needs, the elaborate composition of the solvent system was seen as a potential problem in repetitive analyses. Consequently we undertook an examination of gradient conditions in hopes of achieving a simpler solvent system. Utilization of a mixed borate/phosphate buffer–methanol (from 99:1 to 75:25) failed to separate the most difficult pair, namely dIno and Ado, but was very effective in separating all of the other nucleosides (see Fig. 2). For most metabolic studies this particular difficulty was not seen to be important.

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Note

Determination of fenitrothion, bioresmethrin and piperonyl butoxide in aerosol concentrates by high-performance liquid chromatography

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Formulations consisting of fenitrothion [O,O-dimethyl-O-(4-nitro-*m*-tolyl) phosphorothioate], bioresmethrin [5-benzyl-3-furylmethyl(±)-*trans*-chrysanthemate] and piperonyl butoxide {5-[2-(2-butoxyethoxy)ethoxymethyl]-6-propyl-1,3-benzodioxole} in various ratios have been evaluated and proposed for use as grain protectants[1,2]. Methods published to date for the determination of fenitrothion include infra-red analysis[3], gas–liquid chromatography[4–6] and reversed-phase high-performance liquid chromatography (RP-HPLC)[7]. Bioresmethrin has been determined by gas chromatography–mass spectrometry (GC–MS)[8] and GC[9,10]. Several methods have also been described for the determination of piperonyl butoxide (Pip./But.) including GC–MS[8] and HPLC[11]. Although several methods have been described for the determination of the individual technical materials and their determination at the residual level in various matrices, little has been published on the analysis of formulations containing all three components.

The method described here was developed for the rapid determination of all the above three pesticides in aerosol concentrate formulations. Determination of the three components is achieved by RP-HPLC separation, followed by UV detection at 240 nm.

EXPERIMENTAL

Apparatus

The analyses were performed on a Waters Model 6000A pump, equipped with a U6K injector and Model 450 variable-wavelength UV detector (Waters Assoc., Sydney, Australia). A Brownlee Labs. RP-8 (10 μm), 25 cm × 4.6 mm I.D. reversed-phase column was used (Activon Scientific Services, Granville, Australia). The detector was coupled to a Curken 250-1 recorder (Varian, Sydney, Australia) and injections were made with a Hamilton 25-μl syringe (Waters).

Reagents and standards

Fenitrothion (99.5%), bioresmethrin (93.7%) and piperonyl butoxide (97.0%)

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were obtained from Cooper Australia (N.S.W., Australia), acetonitrile and methanol (HPLC grade, Burdick & Jackson) from Alltech (N.S.W., Australia).

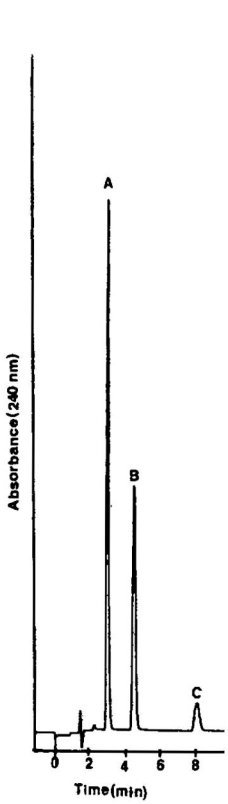
Preparations of standard

A solution of 0.65 g fenitrothion, 0.40 g piperonyl butoxide and 0.10 g bioresmethrin in 100 g methanol was prepared and diluted 1 ml to 10 ml in methanol to

TABLE I

SAMPLES OF AEROSOL CONCENTRATES ANALYSED

Sample	*Component (g/100 g dichloromethane)*		
	Bioresmethrin	*Pip./But.*	*Fenitrothion*
1	0.75	3.50	6.00
2	1.00	4.00	6.50
3	1.25	4.50	7.00



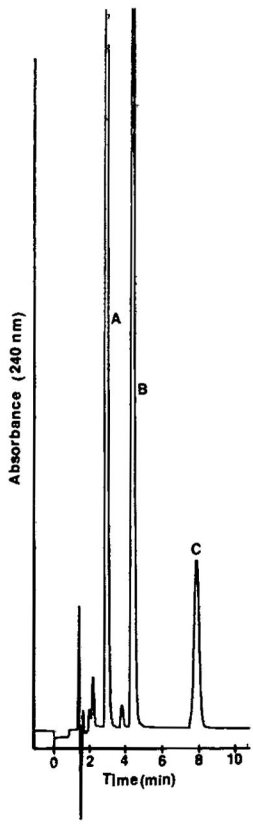


Fig. 1. Chromatogram of a standard solution of fenitrothion (A), piperonyl butoxide (B) and bioresmethrin (C). Retention times are 3.0, 4.4 and 7.8 min respectively. Detector set at 0.2 a.u.f.s. Conditions as in Experimental.

Fig. 2. Chromatogram of standard solution as in Fig. 1 but with detector set at 0.04 a.u.f.s.

TABLE II

RECOVERY OF BIORESMETHRIN, PIPERONYL BUTOXIDE AND FENITROTHION FROM AEROSOL CONCENTRATES

Bioresmethrin			*Pip./But.*			*Fenitrothion*		
Added (%)	*Found (%)*	*Recovery (%)*	*Added (%)*	*Found (%)*	*Recovery (%)*	*Added (%)*	*Found (%)*	*Recovery (%)*
0.75	0.76	101.3	3.5	3.60	102.9	6.0	6.21	103.5
0.75	0.76	101.3	3.5	3.70	105.7	6.0	6.24	104.0
0.75	0.77	102.7	3.5	3.60	102.9	6.0	6.21	103.5
1.0	1.02	102.0	4.0	3.97	99.3	6.5	6.51	100.2
1.0	1.00	100.0	4.0	3.99	99.8	6.5	6.48	99.7
1.0	1.03	103.0	4.0	4.06	101.5	6.5	6.59	101.4
1.25	1.24	99.2	4.5	4.58	101.8	7.0	7.00	100.0
1.25	1.27	101.6	4.5	4.53	100.7	7.0	7.08	101.1
1.25	1.28	102.4	4.5	4.51	100.2	7.0	7.12	101.7
	Mean	101.5		Mean	101.6		Mean	101.7

give an analytical standard consisting of 0.065% fenitrothion, 0.040% piperonyl butoxide and 0.010% bioresmethrin.

Preparation of samples

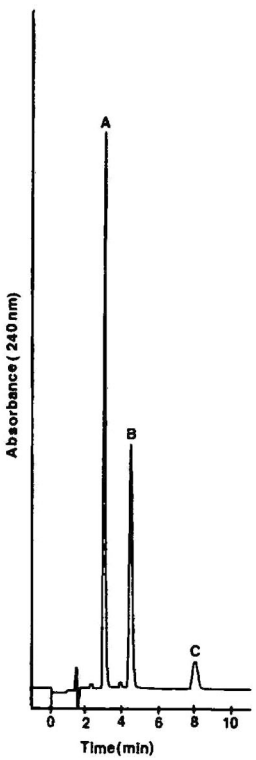
A 1-ml sample of each of the concentrates containing the three components in methylene chloride, as shown in Table I, was separately diluted to 100 ml in methanol.

Preparation of mobile phase

The mobile phase was prepared by adding 700 ml of acetonitrile to 300 ml distilled water and degassing under vacuum.

Chromatographic conditions

Flow-rate: 2.0 ml/min. Detector settings: 240 nm and 0.2 a.u.f.s. for fenitrothion and piperonyl butoxide; 0.04 a.u.f.s. for bioresmethrin. Chart speed: 0.5 cm/min. Injection volume: 20 μl, each in triplicate.



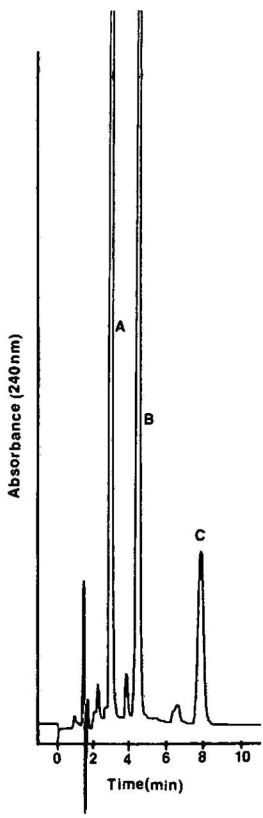


Fig. 3. Chromatogram of aerosol concentrate containing fenitrothion (A), piperonyl butoxide (B) and bioresmethrin (C) with detector set at 0.2 a.u.f.s. Conditions as in Experimental.

Fig. 4. Chromatogram of aerosol concentrate as in Fig. 3 but with detector set at 0.04 a.u.f.s.

RESULTS AND DISCUSSION

Figs. 1 and 2 show typical chromatograms for the standard solution with the detector set at 0.2 a.u.f.s. and 0.04 a.u.f.s. respectively. The retention times under the chromatographic conditions used are 3 min (1.5 cm), 4.4 min (2.2 cm) and 7.8 min (3.9 cm) for fenitrothion, piperonyl butoxide and bioresmethrin respectively. Figs. 3 and 4 show chromatograms of an aerosol concentrate containing a nominal 0.010% bioresmethrin, 0.040% piperonyl butoxide and 0.065% fenitrothion.

As can be seen in Table II, the average recoveries of each component for the three concentration ranges investigated are: bioresmethrin, 101.5% (range 99.2–102.4%); piperonyl butoxide, 101.6% (range 99.3–105.7%) and fenitrothion, 101.7% (range 100.0–104.0%).

Due to the large differences in the concentrations of the three components it is necessary to perform two chromatographic runs for each solution; in one run the detector is set at 0.2 a.u.f.s. to quantitate fenitrothion and piperonyl butoxide, in the second run the detector is set at 0.04 a.u.f.s. to quantitate bioresmethrin. The complete analysis time is approximately 30 min.

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Note

Sensitive and rapid detection of paraoxon by thin-layer chromatography and strips using enzyme inhibition and Ellman's method

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Thin-layer chromatography (TLC) in combination with enzyme inhibition (EI) has been widely used in the detection of organophosphorus, carbamate and organochlorine pesticides as described in review and survey articles by Mendoza[1–3] and also in refs. 4–17. The principle consists in the visualization of the chromatogram by enzymes that are inhibited by the substances to be analysed and subsequently stained by colour reactions. Thus, the colour develops on the background and colourless spots appear as a consequence of the enzyme-inhibiting action of the substances.

For organophosphorus and carbamate pesticides, cholinesterases have been used as enzymes together with various substrates. Artificial substrates which slowly react with cholinesterase, yielding coloured products either directly or after subsequent reactions, show medium to high sensitivity.

In the present investigation butyrylthiocholine was used as an analogue of the natural substrate in combination with Ellman's reagent together with horse serum cholinesterase. Butyryl- and acetylthiocholine are widely used in the determination of cholinesterase activity[18,19]. This system has high sensitivity and reacts rapidly; both features make it suitable to applications under field conditions where rapid detection of dangerous material is necessary. In case the TLC method is not applicable in such situations, a simplified modification has been designed consisting of a simple strip test.

Paraoxon is used in this study as an example of a cholinesterase inhibitor. The presented TLC–EI is run simultaneously with a conventional TLC and a TLC–EI from the literature using indoxyl acetate as a substrate. The latter has been described to be very sensitive (see refs. 1–3 and 5).

MATERIALS

TLC plates (silica gel 60 F_{254}, 20 × 20 cm × 0.5 mm) were obtained from E. Merck (Darmstadt, G.F.R.). Cholinesterase from horse serum (lyophilised, 4 U/mg) was purchased from E. Merck. Butyrylthiocholine iodide was research grade (Serva, Heidelberg, G.F.R.). Ellman's reagent [5,5′-dithiobis-(2-nitrobenzoic acid), pure] was obtained from Serva and indoxyl acetate, methanol, *n*-hexane, diethyl ether and ethanol (all analytical-reagent grade) from E. Merck. $Na_2B_4O_7$, NaOH, NaH_2PO_4,

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Na_2HPO_4 and NaCl were commercial products. Filter paper circles were of diameter 12.5 cm (Nr. 602 h, Schleicher & Schüll, Dassel, G.F.R.).

METHODS

TLC

Portions (each 5 μl) of 10^{-2}–10^{-8} *M* paraoxon solutions in *n*-hexane were applied to the starting line of the TLC plate. The eluent consisted of *n*-hexane–methanol–diethyl ether (3:1:1) and was allowed to migrate nearly to the top of the TLC plate.

Conventional TLC detection

The paraoxon can be visualized by alkaline hydrolysis when it forms the yellow 4-nitrophenolate anion. With aqueous alkali this reaction occurs very slowly because paraoxon has a poor solubility in water. Therefore 10% methanolic NaOH was used as a spray reagent, which led to rapid decomposition at room temperature.

TLC detection with EI

(a) After evaporation of the eluent the TLC plate was sprayed with a solution of 0.5 mg/ml horse serum cholinesterase in 50 m*M* sodium phosphate buffer (pH 7.7) and dried in the air for about 1 h.

(b) Instead of horse serum cholinesterase a 1:10 diluted human pool plasma in the phosphate buffer was used.

(c) The horse serum cholinesterase was dissolved in 10 m*M* sodium tetraborate buffer (pH 9.2).

Colour development with Ellman's reagent and butyrylthiocholine after TLC–EI

The spray reagent consisted of 1.5 m*M* Ellman's reagent and 7 m*M* butyrylthiocholine in the above mentioned phosphate buffer. Ellman's reagent is stable for several weeks in the buffer if kept cool. Butyrylthiocholine has to be stored as a concentrated solution in distilled water (*e.g.* 70 m*M*) and should be diluted with Ellman's reagent to the final 7 m*M* concentration each day when it is needed. White spots on a yellow background, developed at the very latest 5 min after spraying the plates, show the presence of paraoxon. This spray reagent is used with the TLC–EI (a) and (b) above.

Colour development with indoxyl acetate

The spray reagent was prepared immediately before use by dissolving 15 mg of indoxyl acetate in 1 ml of absolute ethanol and subsequent addition of 10 ml of the previously described borate buffer. This method is a modification of the method used by Mendoza[1-3] and Bogusz and Borkowski[5]. The modification seems to work better under the conditions of the present work. White spots on a blue background, appearing up to 30 min after spraying the plates, show the presence of paraoxon. The described spray reagent is used with the TLC–EI (c) above.

Strip test for paraoxon detection

(a) Filter paper strips were impregnated with 1 mg/ml horse serum cholines-

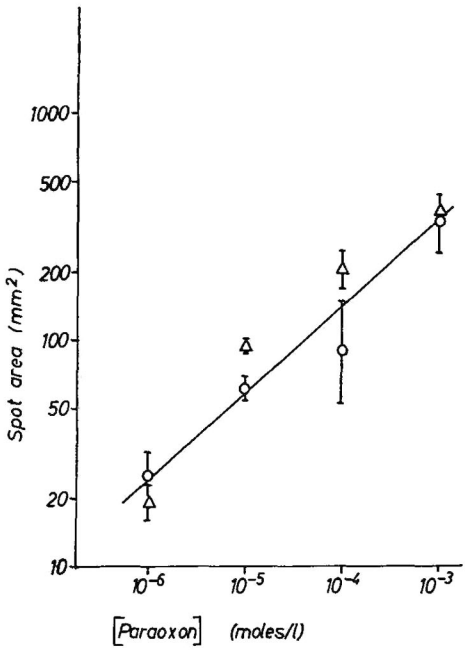

Fig. 1. Double logarithmic plot of spot area *vs.* paraoxon concentration for TLC–EI with indoxyl acetate (△, $n = 3$) and Ellman's reagent (○, $n = 4$). (The area of elliptic spots was calculated after measurement of the long and short axis of the ellipse. Deformed spots at higher paraoxon concentrations were evaluated by counting of squares.)

terase solution in the aforementioned phosphate buffer, dried in the air and kept in a cool dry place. For the test they are slightly wetted with an aqueous paraoxon sample for 5 min. Then they are wetted again with the Ellman's reagent–butyrylthiocholine mixture mentioned above. A blank strip is compared with the test strip, which is treated in the same way with the exception that the aqueous sample did not contain paraoxon nor any other cholinesterase inhibitor. If the blank strip shows a more intense yellow colour than the test strip 5 min after reagent addition, the test is positive.

(b) The filter paper was impregnated with undiluted human pool plasma instead of horse serum cholinesterase. All other procedures are carried out as outlined under (a).

RESULTS

In all cases, TLC showed good reproducibility since the spot magnitude was fairly well correlated with the amount of paraoxon applied (see Fig. 1). The R_F-value was 0.31. The detection limits of the different methods are summarized in Table I.

DISCUSSION

The results clearly show the superiority of the enzymic methods over conventional TLC. Purified horse serum cholinesterase yields (at least for paraoxon) better

sensitivity than human plasma and is claimed to work as well as bovine liver homogenate[5]. Both the new enzymic and the indoxyl acetate reference methods show a high degree of sensitivity. The advantage of the new method is the rapid colour development, which may be useful in emergency cases*. As already mentioned, the time needed for the spot development at the detection limit is *ca.* 5 min for the butyrylthiocholine and 30 min for the indoxyl acetate method. The detection limit is equal for both methods (see Table I). A drawback of the new method as compared to the reference method is the instability of the colour, as the coloured 3-carboxy-4-nitrothiophenolate anion generated during the reaction is slowly oxidized back to Ellman's reagent (see ref. 20). This drawback however is not serious, since the colour does not disappear rapidly and in emergency determinations one would watch the colour development in order to get the information as soon as possible.

TABLE I

DETECTION LIMITS OF DIFFERENT TLC METHODS

Method	*Enzyme*	*Substrate*	*Chromogen*	*Approximate detection limit (ng)*
TLC, no EI (alkaline hydrolysis)			Paraoxon	500
TLC–EI	Human plasma	Butyrylthiocholine	Ellman's reagent	3
TLC–EI	Horse serum cholinesterase	Butyrylthiocholine	Ellman's reagent	0.3
TLC–EI	Horse serum cholinesterase	Indoxyl acetate	Indoxyl acetate	0.3
Strip-test	Human plasma	Butyrylthiocholine	Ellman's reagent	0.5*
Strip-test	Horse serum cholinesterase	Butyrylthiocholine	Ellman's reagent	0.1*

* One drop of sample applied.

The new method, as well as all the other TLC–EI methods, should be suitable to all cholinesterase inhibiting organophosphorus and carbamate compounds. Those organophosphorus compounds that do not inhibit *in vitro* have to be activated by appropriate methods, *e.g.* that described by Ackerman *et al.*[4]. This is necessary in the described strip-test as well, if such compounds occur.

The latter test is a simple but very useful tool for rapidly checking the possible danger from cholinesterase inhibitors directly where they occur. Clearly, with this test a qualitative analysis is impossible, but the cholinesterase inhibiting power of the investigated sample should be correlated to possible dangerous effects in the organism. The new TLC–EI method, on the other hand, is useful for qualitative analysis and is also sufficiently reproducible for semi-quantitative determinations. The latter may be carried out graphically using the regression line shown in Fig. 1.

* In these cases a shorter treatment of the TLC plates with the enzyme, *e.g.* 10 min, is recommended as the loss of sensitivity is small compared with the treatment described under Methods.

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Book Review

Environmental problem solving using gas and liquid chromatography, by R. L. Grob and M. A. Kaiser, Elsevier, Amsterdam, Oxford, New York, 1982, XIII + 240 pp., price Dfl. 127.00, US$ 59.00, ISBN 0-444-42065-7.

The extremely rapid development of the analytical techniques used in environmental analysis and the wide range of recent applications would make a book on the present situation in environmental research very desirable but very difficult to write.

The authors of the present volume feel that to select and present the subject matter in a way that will satisfy all potential readers is not feasible. The reviewers are not fully convinced, however, that a better presentation could not have been found. It is their impression that with some chapters there is little difference between those dealing with analytical techniques and those on applications, nor is due weight given to the relative importance of the various topics.

It is felt that the references should have been selected with emphasis on those papers on which actual environmental research is based, but we found that a large number of papers were cited that are of little help to the environmental analyst, and essential contributions have been omitted.

The reviewers feel that, on the whole, there is some doubt whether this book provides the support to environmental analysts in solving their problems that is promised by the title.

PUBLICATION SCHEDULE FOR 1982

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

MONTH	J	F	M	A	M	J	J	A	S	O	N	D
Journal of Chromatography	234/1 234/2 235/1 235/2	236/1 236/2	237/1 237/2 237/3	238/1 238/2 239	240/1 240/2 241/1	241/2 242/1 242/2	243/1 243/2 244/1 244/2	The publication schedule for further issues will be published later.				
Chromatographic Reviews		251/1		251/2								
Biomedical Applications	227/1	227/2	228	229/1	229/2	230/1	230/2					

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