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NATIONAL JOURNAL ON CHROMATOGRAPHY, ELECTROPHORESIS AND RELATED METHODS

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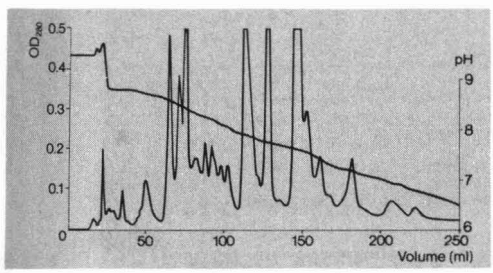

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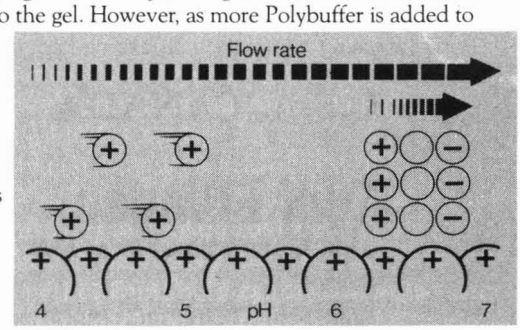

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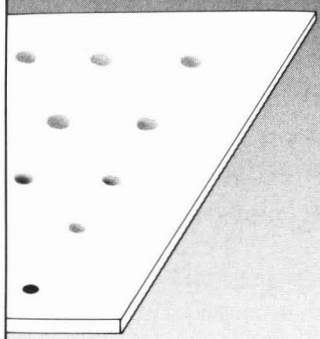

TLC prepared layers coated with hydrophilic silica gel have been in use for a number of years now and the majority of TLC separations can be done on these layers.

However it can be advantageous to make certain separations on lipophilic silica gel i. e. reverse phase chromatography. Due to the rapid development of HPLC, a demand has arisen for TLC plates, which can be used for an orientating pre-separation. In HPLC there is a very definite trend towards to RP separations (e. g. with C_8, C_{18} alkyl silanized silica gels).
After careful consideration it has been decided, that instead of different alkyl silanes (eg. C_2, C_8, C_{18} etc.) only C_{18} silane should be used, but with different percentages of silanization. The reactive groups are totally (100%) or partially (75% and 50%) substituted with alkyl silane. The base material for our RP plates is medium pore silica gel (average pore diameter 60 Å) with mean particle size of 5-10 µm i. e. Nano silica gel. Hereby the different demands of the user are met. Furthermore the use of solvents, which vary from totally organic to strongly aqueous is possible (see following table).
The fluorescent indicator is acid resistant, so that strongly acidic solvents can be used, without any undesirable quenching of the fluorescence. Under short wave UV light the indicator shows a pale blue fluorescence. Substances which absorb UV light appear dark blue to black, on the pale blue background of the plate.

Solvent		Length of separation in mm / 15 min. with Nano RP Plates SIL C 18-100	SIL C 18-75	SIL C 18-50
Methanol/H_2O	= 2:1	45	51	57
	= 1:1	21	32	52
	= 1:2	0	11	50
	= 1:3	0	0	40
	= 0:1	0	0	0
Acetonitril/H_2O	= 2:1	46	51	62
	= 1:1	30	35	52
	= 1:2	27	33	51
	= 1:3	15	25	48
	= 1:9	0	0	20
Chloroform		64	65	68

Excellent results have been achieved with Nano-RP-Plates for the separation of polycylic aromatics, lipides and steroids.

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CHARACTERIZATION OF REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC STATIONARY PHASES BY MEANS OF PYROLYSIS–GAS CHROMATOGRAPHY

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SUMMARY

Pyrolysis–gas chromatography has been shown to be a valuable tool for the characterization and evaluation of chemically bonded phases for reversed-phase high-performance liquid chromatography. The chain length of the hydrocarbon group bonded to the silica surface could be determined and the functionality of the silanizing agent was established for different C_{18} materials. The technique could also be used to investigate whether the materials had been post-silanized with reagents such as hexamethyldisilazane for the capping of residual silanol groups.

INTRODUCTION

The development of chemically bonded stationary phases (CBSP) on microporous silica supports for use in high-performance liquid chromatography (HPLC) is one of the major factors that has increased the importance of this technique. Hydrocarbons bonded to the silica support, *i.e.*, phases for reversed-phase chromatography, enjoy great popularity and several reviews on commercial stationary phases have been published[1,2].

Because of the increasing number of manufacturers, the type of reversed-phase material for a particular separation is often chosen by chance. However, as frequently pointed out, the packing materials can differ considerably according to their packing characteristics[3], their performance[2,4] and especially their separation properties[4–7]. Variations from batch to batch for phases from the same manufacturer have also been noted[3]. Sometimes these differences can be explained in terms of pore size, pore distribution, particle size, particle distribution, surface area, hydrocarbon chain length, hydrocarbon loading, etc., data which are often given by the supplier. However, often the observed differences are not easily explained in these terms[8].

It has been suggested that the number of unreacted silanol groups on the silica surface can play an important role in reversed-phase chromatography[9], especially when chromatographing basic substances such as amines[4,10].

It appears that more information about the surface properties such as mode

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of modification (monolayer, polymer layer), functionality of silanizing reagent, chain length of reagent, degree of silanization and degree of "capping" is necessary before a complete understanding of the chromatographic behaviour is possible and a comparison between different reversed-phase stationary phases can be made[9]. Various chromatographic tests have been employed to evaluate the influence of silanol groups[8,9] and column performance for comparative purposes[11]. Screen tests have been used for the evaluation of the separation ability of different bonded phases[12]. Methods employed for studying bonded phases, in addition to "standard" elemental analysis, have included IR[13–16] and photoacoustic spectroscopy[17], UV absorption[18], thermal degradation and mass spectrometry (MS)[15,19], alkaline hydrolysis followed by silylation and gas chromatography[20] and titration[21,22]. Unger[23] described various methods for the characterization of HPLC materials.

Pyrolysis–gas chromatography (Py–GC) has found widespread use for the study of polymers[24]. As the reversed-phase silica support can be regarded as a siloxane polymer modified with hydrocarbons of varying chain length, Py–GC should be a useful technique for the study of these materials. A study of silica gels esterified with saturated alcohols by means of Py–GC–MS has been reported[25].

In this investigation we used Py–GC for the characterization of reversed-phase silica gels with different chain lengths and structures of the hydrocarbon moiety. We also studied the influence of "capping" on the pyrolysis and chromatographic results.

EXPERIMENTAL

Apparatus

Pyrolyser. The pyrolyser used was a CDS 150 Pyroprobe (Chemical Data Systems, Oxford, PA, U.S.A.). It consisted of a probe of a platinum-coil type, a quartz tube and a sample holder (see Fig. 1). The sample holder was made by folding a stainless-steel mesh disc for HPLC columns. The probe was inserted into an interface, mounted on the injection port of the gas chromatograph. The interface temperature was set at 275°C. The temperature inside the sample holder was measured by means of an iron–constantan thermocouple.

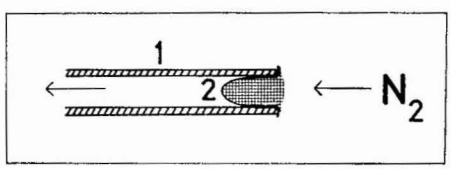

Fig. 1. Diagram showing sample application part placed inside the Pt-coil of the probe. 1 = Quartz tube; 2 = sample holder.

For identification of the pyrolysis products the pyrolyser was connected to the GC part of a Finnigan 4021 mass spectrometer. The Py–GC–MS measurements were carried out using the same conditions as in the Py–GC investigation except for a change from nitrogen to helium as the carrier gas.

Gas chromatography. A Varian 3700 gas chromatograph with a 1.9 mm I.D. stainless-steel column was used. The carrier gas was nitrogen at a flow-rate of 30 ml/

min, and the hydrogen and air flow-rates were 30 and 240 ml/min, respectively. A flame-ionization detector was used at 300°C. The injector temperature was 240°C.

Two GC systems were used: (A) a 1-m Chromosorb 102 column with temperature programming from 50 to 225°C at 20°C/min; and (B) a 2-m column packed with 3% OV-17 on Chromosorb W HP (80–100 mesh) with temperature programming from 70 to 275°C at 20°C/min.

Liquid chromatography. The apparatus, column tubing, fittings and procedure have been described elsewhere[26].

Chemicals and packing materials

Octadecyldimethylchlorosilane and octadecylmethyldichlorosilane were purchased from Magnus Scientific (Sandbach, Great Britain). Octadecyltrichlorosilane, vinylmethyldichlorosilane and trimethylchlorosilane were purchased from E. Merck (Darmstadt, G.F.R.). All solvents (E. Merck) were of analytical-reagent grade and all chemicals were used without further purification.

The packing materials studied were LiChrosorb RP-18, RP-8 and RP-2 (E. Merck), μBondapak C_{18} (Waters Assoc., Milford, MA, U.S.A.), Nucleosil C_{18} (Machery, Nagel & Co, Düren, G.F.R.), Partisil ODS (Whatman, Clifton, NJ, U.S.A.), Spherisorb ODS (Phase Separations Ltd., Queensferry, Clwyd, Great Britain), ODS-Hypersil (Shandon Southern Products, Runcorn, Great Britain) and Zorbax ODS (DuPont, Wilmington, DE, U.S.A.). The silica gels used to prepare the home-made bonded phases were LiChrosorb Si-100 or Si-60 (E. Merck).

Preparation and capping of CBSP

The bonding procedure used for the preparation of home-made CBSP was basically that used by Hemetsberger *et al.*[27] but with variation of the reaction time and the type of original silica gel according to Table I.

TABLE I

MATERIALS AND CONDITIONS USED FOR THE PREPARATION OF HOME-MADE CBSP

Type of modifier	*Designation of product*	*Type of silica*	*Particle diameter (μm)*	*Reaction time (h)*
Trimethylchlorosilane	TMCS-silica	Si-100	5	24
Vinylmethyldichlorosilane	VMDCS	Si-60	10	2
Octadecyltrichlorosilane	OTCS-silica	Si-60	5	1.5
Octadecylmethyldichlorosilane	OMDCS-silica	Si-100	5	24
Octadecyldimethylchlorosilane	ODMCS-silica	Si-100	5	24

The capping of CBSP was carried out in the same way as the preparation of CBSP itself, except that the CBSP was dried at 100°C and 10 mmHg for 1 h and the reaction time was 5 h.

Pyrolysis procedure

The sample (about 60 μg) was placed in the sample holder of the quartz tube, assembled with the probe and put into the interface. The system was conditioned for

about 10 min, during which time any traces of solvents in the sample were evaporated. When a steady baseline had been achieved, the pyrolyser was set at 800°C, whereupon the sample was pyrolysed in 5 sec. Between each run the quartz tube with the sample holder was flushed with a jet of air in order to remove any traces of previous sample.

RESULTS AND DISCUSSION

Gas chromatography

For the purpose of determining the length and type of the hydrocarbon moiety attached to the silica gel, two different GC systems had to be used. Chemically bonded stationary phases with a chain length up to eight carbon atoms were suitably analysed with GC system A. Using this system a good resolution within a reasonable time was obtained. In order to reduce the time of analysis for CBSPs with longer chain lengths, GC system B was employed. This system gave a poor resolution of hydrocarbons below C_8 but was satisfactory for establishing the identity of longer hydrocarbon groups attached to the silica gel.

Pyrolysis conditions

In order to obtain simple pyrograms, *i.e.*, gas chromatograms obtained from pyrolysis products, it would be desirable to be able to choose a pyrolysis temperature at which mainly the C–Si bond was broken while the hydrocarbon chain was left intact (for representative bond energies, see Table II[28]). As the energy of the C–Si bond is dependent on the nature of other groups attached to the silicon atom (see Fig. 2), the splitting pattern will vary with the structure around the silicon atom[16,28,29]. This is in agreement with our experience. Thus, it appears that an exchange of Si–O bonds for $Si–CH_3$ weakens the Si–C bond of, *e.g.*, the octadecyl groups, resulting in an increase in the proportion between the C_{18} and C_{17} peaks on pyrolysis. Although for some compounds it is possible to choose a low pyrolysis temperature at which the C–Si bond is split in preference to the C–C bonds, it appears that for practical work a higher temperature must be used in order to increase the yield of pyrolysis products. At this temperature both C–C and C–Si bonds are split, as shown by the pyrogram in Fig. 3.

TABLE II

BOND ENERGIES

Type of bond	*Bond energy (kcal/mole)*
Si–C	76
Si–O	108
C–C	82.6
C–H	98.7

The choice of pyrolysis conditions must be a compromise because of the conflicting demands of a high yield of products and simplicity of the pyrogram. It was found that the optimal pyrolysis conditions for the purpose of this investigation were a set pyrolysis temperature and pyrolysis time of 800°C and 5 sec, respectively. Measurements with a thermocouple (see *Apparatus*) showed that under these con-

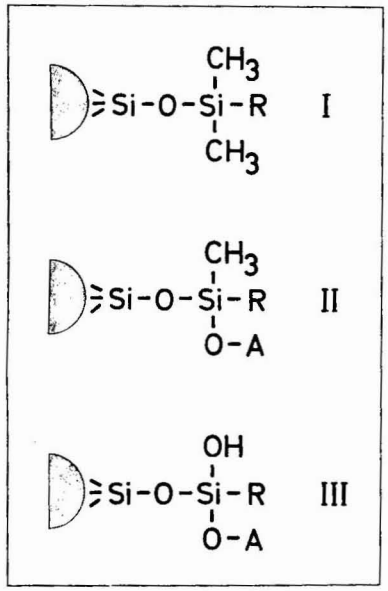

Fig. 2. Schematic formulae of the different structures of CBSPs obtained when mono- (I), di- (II) and tri- (III) functional silanizing agents are reacted with the silica surface. R = Hydrocarbon chain; A = H or Si→.

ditions a temperature of about 495°C was reached in the sample holder. Pyrolysis times longer than 5 sec gave a still increasing temperature. It is thus obvious that the temperature rise time was relatively long and that the pyrolysis of the sample took place during the rise to the set temperature.

Determination of the chain length of the CBSP

Fig. 3 shows the pyrograms of some home-made and commercial CBSPs. Each type of CBSP gave a characteristic pyrogram. The pyrolysis products were a homologous series of predominantly monoalkenes. The formation of alkenes as degradation products from alkanes has been established previously[16,30,31]. Some workers[15,19] claim that the Si–O bond is also cleaved at the temperatures used, giving silicon compounds as pyrolysis products. However, such compounds could not be identified as isolated peaks in the pyrograms.

The determination of the chain length of different CBSPs is based on the determination of the chain length of the latest eluted hydrocarbon in the pyrogram, corresponding solely to breaking of the Si–C bond in the CBSP. The determination was accomplished by comparing the retention times with those from a Py–GC–MS run with a C_{18} CBSP, where the presence and identities of alkenes were confirmed.

Determination of the functionality of the silanizing agent

The fact that the substitution pattern at the silicon atom has a major influence on the bond strength between the silica and the main hydrocarbon chain, as mentioned above, can be utilized to obtain information about the surface structure and the probable functionality (mono-, di or tri-) of the silanizing agent (see Fig. 2). Fig. 4 shows the pyrograms of three different C_{18} materials synthesized in our labora-

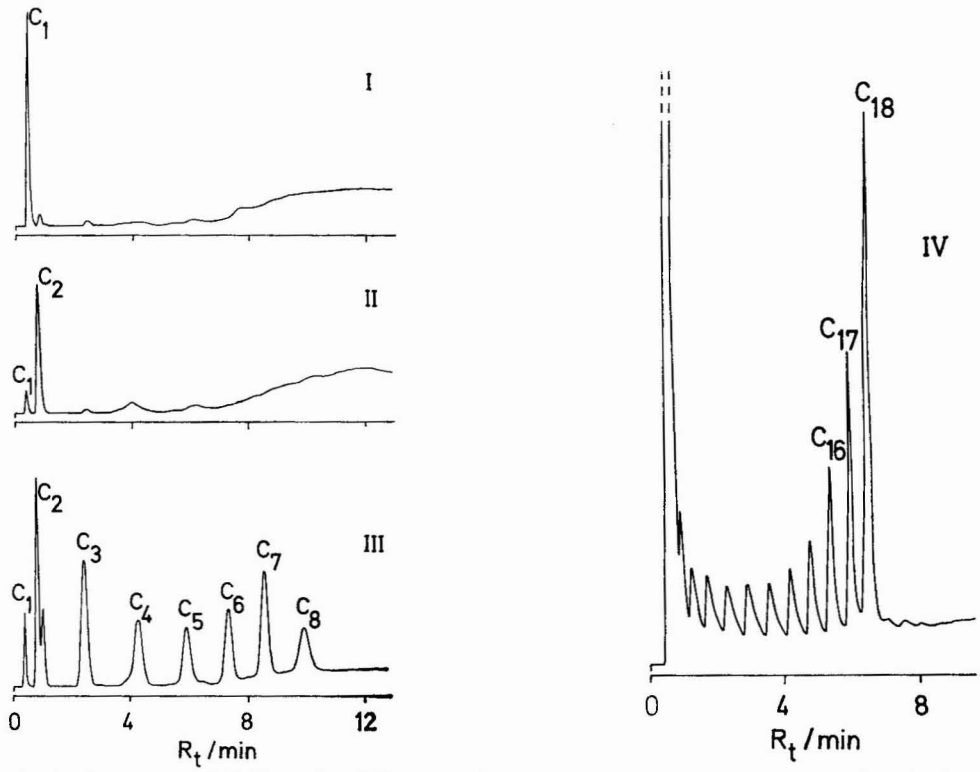

Fig. 3. Pyrograms of CBSPs with different chain length of the hydrocarbon moiety (R). I, R = methyl-, GC system A; II, R = vinyl-, GC system A; III, R = octyl-, GC system A; IV, R = octadecyl-, GC system B. I, II and IV, home-made CBSP; III, LiChrosorb RP-8.

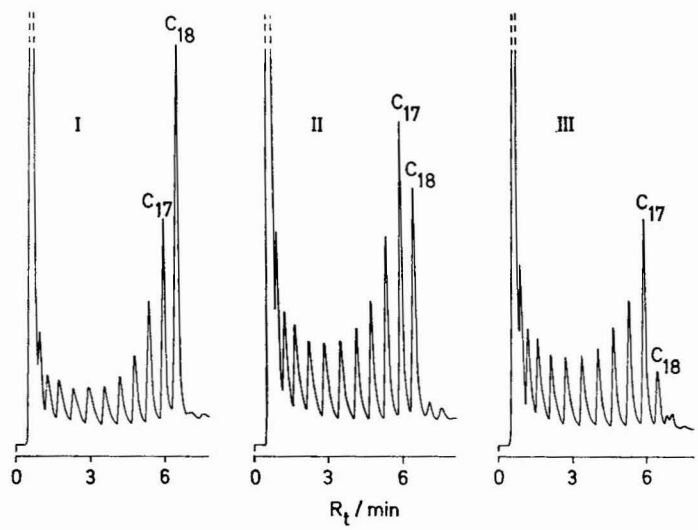

Fig. 4. Pyrograms of three different C_{18} materials. I = ODMCS-silica; II = OMDCS-silica; III = OTCS-silica. GC system B. For explanation see Table I.

tory. As can be seen, the peak height ratios of heptadecene and octadecene vary with the type of silanizing agent.

Table III gives the C_{17}/C_{18} peak height ratios of some C_{18} materials. As demonstrated, the ratio falls into three main groups, which is believed to be due to the different functionalities of the silanizing agents. The deviation of the ratios within the three functionality groups (mono-, di- and tri-) could be attributed to different kinds of original silica and, with di- and trifunctional silanizing agents, to different degrees of post-polymerization. The identity of the silanizing agent was confirmed by the manufacturers in some instances. The precision of the peak-height ratio expressed as relative standard deviation was 6% for measurements during 1 day and 10% for measurements during 3 months.

TABLE III

DETERMINATION OF PROBABLE FUNCTIONALITY OF DIFFERENT C_{18} MATERIALS BY MEANS OF Py–GC

Packing material	*Batch*	*Mean particle diameter (μm)*	*Peak-height ratio C_{17}/C_{18}*	*Probable functionality of silanizing agent*
ODMCS-silica*	Home-made	5	0.54	Mono
OMDCS-silica*	Home-made	5	1.24	Di
OTCS-silica*	Home-made	5	3.5	Tri
Zorbax ODS	18341-66	6	0.76	Mono
μ-Bondapak	58	10	1.18	Di
Lichrosorb	VV58	10	1.30	Di
Partisil	100130	10	3.1	Tri
Spherisorb	MH 15/175	10	3.6	Tri
Hypersil	GA 613	5	4.7	Tri
Nucleosil	8111	5	5.1	Tri
Nucleosil	9101	5	7.5	Tri
Nucleosil	9122	5	6.8	Tri

* For explanation, see Table I.

Verification of capping of CBSP

The necessity for subsequent treatment of CBSPs with TMCS or HMDS for the capping of residual silanol groups has been discussed. The results from elemental analysis of the CBSPs do not give a sufficiently large increase in the carbon content to verify that capping takes place[32]. On the other hand, chromatographic tests indicated that capping does occur and improves efficiency[33,34].

Experience from work with CBSPs at this laboratory pointed to a considerable difference in performance between different batches of the same phase, especially when chromatographing basic substances such as amines and amides. This is demonstrated in Fig. 5, which shows the results for a mixture of some urea derivatives run on three different batches of Nucleosil C_{18} under identical conditions. It can be seen that not only the retentions but also the order of retention are different. It is believed that this behaviour is due to differences in capping of residual silanol groups.

In order to investigate the value of Py–GC for the verification of capping of CBSPs, two kinds of home-made C_{18} phases, one treated with trimethylchlorosilane (TMCS) and one untreated, were subjected to Py–GC (see Fig. 6). It can be seen that

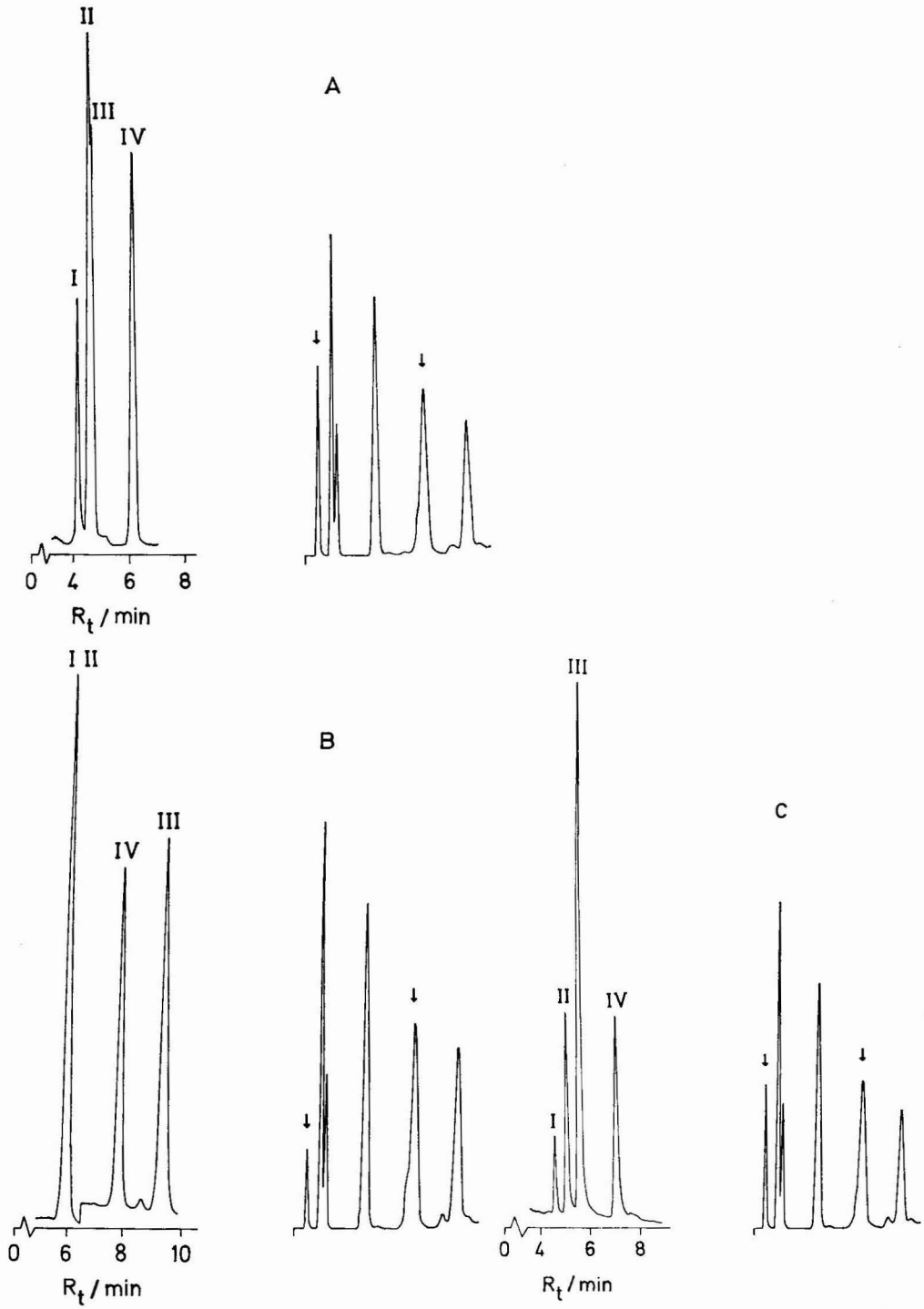

Fig. 5. Liquid chromatogram (left) and corresponding pyrogram (right) of three different batches of Nucleosil C_{18} (5 μm). A, Batch 8111; B, batch 9101; C, batch 0061. Batch 8111 and 0061 are capped and batch 9101 is not capped. Liquid chromatograms: urea derivatives of 9-(N-methyl-aminoethyl)anthracene reagent and (I) toluene 2,6-diisocyanate, (II) toluene 2,4-diisocyanate, (III) hexamethylene diisocyanate and (IV) 4,4′-diphenylmethane diisocyanate; eluent, acetonitrile–water (70:30), the aqueous phase containing 3% of triethylamine, pH 3.0; flow-rate, 2 ml/min; column, 200 × 5 mm Nucleosil C_{18} (5 μm). Pyrograms: GC system A; the arrows mark the C_1 and C_4 peaks.

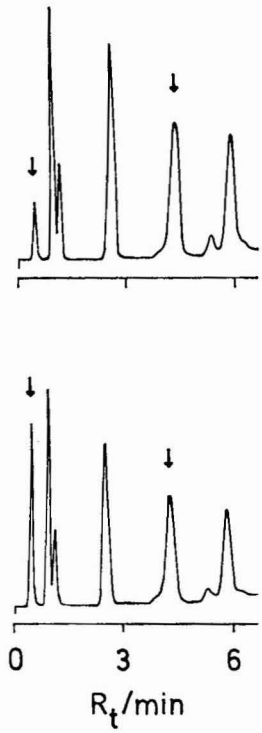

Fig. 6. Pyrograms of OTCS-silica. Top, uncapped material; bottom, capped material. The arrows mark the C_1 and C_4 peaks used for the calculation of the C_1/C_4 peak-height ratios in Table IV. GC system A.

the pyrograms are almost identical, with the exception of the relative height of the methane peak. Thus, it is greater for the capped material. The ratio of the peak heights of methane and propene was taken as a measure of the capping efficiency. Table IV gives the peak-height ratios of the methane/propene peaks for trifunctional capped and uncapped material. Of course, this ratio will vary with the functionality of the silanizing agent. However, as capping is of minor importance for the mono- and difunctional silanizing agents, our investigation has been mainly concerned with the trifunctional one. The precision of this peak height ratio, expressed as relative standard deviation, was 11%.

The three batches of Nucleosil C_{18} mentioned above were pyrolysed (see Fig. 5) and the methane/propene peak-height ratio was determined. The previous investigation (see Table III) indicates that the bonded phase of Nucleosil C_{18} is of the trifunctional type. From Table IV it can be seen that the methane/propene peak-height ratio from batches 9101 and 9122 fits well with an uncapped C_{18} phase of the trifunctional type. Batch 8111 seems to be a capped C_{18} phase of the same type and the third batch (0061) seems also to be capped. These observations were later confirmed by the manufacturer.

In Table IV are also tabulated the carbon contents of some of the pyrolysed phases, determined by elemental analysis. It can be seen that the carbon content

TABLE IV

INVESTIGATION OF CAPPING OF SOME TRIFUNCTIONAL C_{18} MATERIALS BY MEANS OF Py–GC

Packing material	*Batch*	*Mean particle diameter (μm)*	*Peak-height ratio, C_1/C_4*	*Elemental analysis, C (%)*
OTCS-silica*	Home-made	5	0.40**	17.2
Capped OTCS-silica	Home-made	5	1.60***	18.0
Nucleosil	8111	5	1.22§	14.9
Nucleosil	9101	5	0.49**	13.3
Nucleosil	9122	5	0.42**	13.5
Nucleosil	0061	5	0.99§	13.6
Partisil	100130	10	1.82§§	
Spherisorb	MH 15/175	10	0.97§§	
Hypersil	GA 613	5	1.36§§	

* See table I.
** Not capped.
*** Treated with TMCS.
§ Capped according to the manufacturer.
§§ Probably capped.

varies considerably between the different materials investigated. However, this fact does not reveal whether the material is capped or not. In order to try to verify if a packing material is capped on the basis of carbon elemental analysis, a comparison between the carbon contents of a capped and the same uncapped batch must be made.

In Table IV such a comparison was made for the first two packing materials and a slight increase in carbon content was obtained for the capped material. However, it is questionable if this can be taken as a proof of capping, because of the small difference and the fact that it has been stated that the carbon content does not necessarily increase after capping[32].

CONCLUSIONS

Py–GC offers a useful method for the characterization of CBSPs on silica gel. The method is rapid and reasonably reliable and can be used without any pretreatment of the samples. The method can be employed for studying both the nature of the hydrocarbonaceous phase and the functionality of the silanizing agent. It is also useful for establishing if the phase has been capped or not and the capping efficiency. It is believed that further quantitative investigations could give a more detailed picture of the structure of CBSPs and such investigations are planned for the near future.

ACKNOWLEDGEMENTS

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CHARACTERISTICS OF IMMOBILIZED TANNIN FOR PROTEIN ADSORPTION

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SUMMARY

The adsorption of proteins on immobilized tannin has been found to fall into one of three classes: in the first the proteins are well adsorbed at neutral to weak acidic pH values, and the adsorption is not affected by salt concentration; in the second the proteins are well adsorbed at acidic or alkaline pH values, but the adsorption is affected by salt concentration; in the third class the proteins are only slightly adsorbed at all pH regions. On the basis of these results, a mixture of three different proteins has been separated using an immobilized tannin column.

INTRODUCTION

Previously, we have immobilized tannin by covalent binding to aminohexyl cellulose using cyanogen bromide or epichlorohydrin[1], and have also reported the characteristics of immobilized tannin prepared by the cyanogen bromide method[2]. Furthermore, we have reported the application of tannin for immobilization of enzymes[3,4] and continuous fining of saké[5,6]. Subsequently it was found[7] that the immobilized tannin prepared by the epichlorohydrin method was more suitable for continuous fining of saké than that obtained by the cyanogen bromide method.

In order further to develop this technique for separation, recovery and removal of proteins, we have now investigated the characteristics of immobilized tannin prepared by the epichlorohydrin method from the viewpoint of protein adsorption.

MATERIALS AND METHODS

Compounds

Filter pulp (No. 4) was purchased from Toyo Roshi (Tokyo, Japan), epichlorohydrin and Chinese gallotannin from Katayama Chemical Industries (Osaka, Japan). Crystalline α-amylase from *Bacillus subtilis* and alkaline protease from *Streptomyces griseus* were obtained from Sigma (St. Louis, MO, U.S.A.), crystalline lysozyme from egg and glucoamylase from *Rhizopus niveus* from Seikagaku Kogyo (Tokyo, Japan), crystalline ovalbumin and crystalline pepsin from porcine stomach from Worthington

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Biochemical (Freehold, NJ, U.S.A.) and bovine serum albumin (Fraction V) and crystalline trypsin from beef pancreas from Miles Labs. (Elkhart, IN, U.S.A.). Gelatin powder was the product of E. Merck (Darmstadt, G.F.R.). All other chemicals were of reagent grade.

Preparation of immobilized tannin

To 3 l of distilled water were added 200 g of filter pulp (No. 4) and the suspension was cooled to 4°C. Three litres of 6 *N* sodium hydroxide were added, and the mixture was gently stirred at 4°C for 3 min, then allowed to stand for 30 min at 4°C. To the alkali-treated cellulose, were added 12 l of distilled water and the suspension was stirred for 30 min at 60°C. Two litres of epichlorohydrin were added, and the mixture was stirred for 30 min at 60°C. The activated cellulose was collected by filtration, and washed with 10 l of distilled water. It was then suspended in 8 l of 0.625% hexamethylenediamine solution, and the mixture was stirred at 60°C for 120 min. After the reaction, the aminohexyl cellulose was collected and washed with 10 l of water. It then was suspended in 10 l of 0.25 *N* sodium hydroxide, 1 l of epichlorohydrin was added and the mixture was stirred for 30 min at 60°C. The activated aminohexyl cellulose was washed with 10 l of water, and then suspended in 8 l of 3% Chinese gallotannin aqueous solution adjusted to pH 7.0 with sodium hydroxide. After addition of 4.8 g of sodium borohydride, nitrogen gas was bubbled into the suspension with stirring for 150 min at 45°C. After the reaction, the immobilized tannin was collected and washed with 10 l of water. It was then suspended in 4.5 l of 30% aqueous acetone, and the pH of the mixture was adjusted to 2.0 with 3 *N* hydrochloric acid. The resulted suspension was stirred for 10 min at 25°C. Immobilized tannin was collected by filtration, washed with 10 l of water, three times with aqueous acetone and finally with 20 l of water. The resulting immobilized tannin preparation was suspended in 5 l of sodium citrate–hydrochloric acid buffer (pH 4.0). The immobilized tannin was then filtered off and stored at 4–10°C in a sealed vessel. The preparation was 1290 g in wet weight (*ca.* 4 l in volume), and contained 81% of water and 4.75% Chinese gallotannin.

Adsorption and desorption of proteins

The adsorption of proteins on immobilized tannin was carried out by shaking the adsorbent with protein dissolved in buffer solution for 30 min at 5°C. The mixture was then filtered, and the concentration of protein in the filtrate was determined.

The desorption of proteins from immobilized tannin was carried out by shaking the immobilized tannin–protein complex with selected desorbents for 15 min at 25°C. After filtration of the mixture, the concentration of protein in the resulting filtrate was determined.

Adsorption capacity of immobilized tannin for proteins

The adsorption capacity of immobilized tannin for proteins was measured by the column method as follows. One gram (wet weight) of immobilized tannin was packed into a column (45 × 15 mm, bed volume 8 ml), and the column was then equilibrated with a buffer solution. A 0.5% protein solution in the same buffer was applied to the column at a flow-rate of 13.8 ml/h at 10°C, until the protein concentration in the effluent became equal to that in the charged solution. Two millilitres of

each fraction were collected, and the concentration of protein in the fractions was determined. The adsorption capacity of immobilized tannin for a protein was calculated as the difference between the amount of protein in the effluent and that in the charged solution.

Chromatography

One gram (wet weight) of immobilized tannin was packed into a column (45 × 15 mm, bed volume 8 ml). After washing with water, the column was equilibrated with 0.05 *M* phosphate buffer, pH 7.5. Proteins dissolved in the same buffer solution were applied to the column at 10°C at a flow-rate of 13.8 ml/h. After washing the column with the same buffer, proteins adsorbed on the column were eluted with 0.05 *M* carbonate buffer, pH 9, and subsequently with 0.05 *M* acetate buffer, pH 4. Two millilitres of each fraction were collected, and the protein concentration and enzyme activity in the fraction were assayed.

Determination of protein

Protein was determined by the method of Lowry *et al.*[8], or by measuring the absorbance at 280 nm.

Determination of enzyme activity

Enzyme activities of trypsin and lysozyme were determined by the methods of Schwert and Takenaka[9] and Shugar[10], respectively.

Determination of ovalbumin

The concentration of ovalbumin was determined by measuring the content of carbohydrate with the phenol–sulphuric acid method.

RESULTS

Titration of immobilized tannin

The immobilized tannins prepared by the epichlorohydrin method and native tannin were titrated with hydrochloric acid and sodium hydroxide, and the resulting potentiometric titration profiles were compared. As shown in Fig. 1, immobilized tannin had two different titratable groups: one is acidic having a pK value (pK_1) of 5.0, the same as that of native tannin; the other is basic having a pK value (pK_2) of 8.5. As the titres for both groups were almost equal, *i.e.*, *ca.* 20 μmoles per gram adsorbent (wet weight), the isoelectric point of immobilized tannin is near neutral pH.

Adsorption of proteins on immobilized tannin

Effect of pH. Various proteins were shaken with immobilized tannin in 0.01 *M* buffer at pH 2–12. All the proteins used were adsorbed to various extents on the adsorbent as shown in Fig. 2a and b. However, the ratios of the extents of adsorption of proteins on immobilized tannin were affected by the pH of the protein solution, the optimum pH for adsorption depending on the protein used. Furthermore, some proteins such as trypsin and gelatin were only slightly adsorbed even at optimum pH.

Effect of salt concentration. In order to study the nature of the binding between proteins and immobilized tannin, the effect of salt concentration on the adsorption

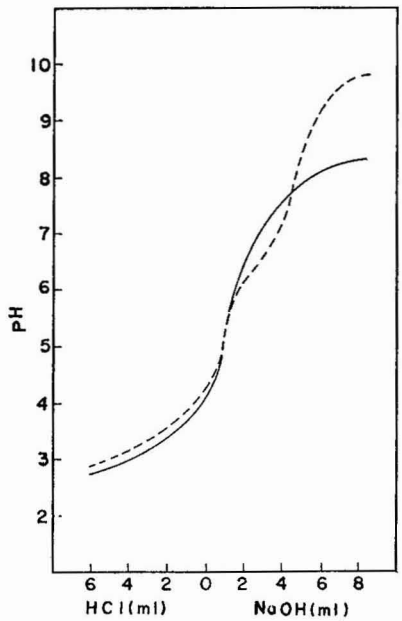

Fig. 1. Titration curve of immobilized tannin. One gram of wet immobilized tannin (--------) or 50 mg of native tannin (———) in 25 ml of water was titrated with 0.01 *N* NaOH or 0.01 *N* HCl using a potentiograph E 336 Metrohm.

of proteins was investigated at optimum pH for adsorption. As shown in Fig. 3, in the cases of ovalbumin and serum albumin having optimum pH at near neutral pH, the salt concentration had little effect on the adsorption. On the other hand, in the cases of pepsin and lysozyme having optimum pH at acidic or alkaline pH, the adsorption decreased with increasing salt concentration. Furthermore, the adsorption of gelatin increased with increasing salt concentration.

Recovery of ovalbumin

The results in Fig. 3 suggested that the binding between ovalbumin and immobilized tannin at the optimum pH for adsorption may not be ionic. In order to study the mode of binding in this case, the desorption of ovalbumin from immobilized tannin was carried out with different eluents. As shown in Table I, the recovery of protein was low when sodium chloride, sucrose, acetone or ethylene glycol was used as eluent. However, the recovery was high when a mixture of sodium chloride and other eluents was used. The desorptions were also carried out with some buffer solutions, and it was seen that buffer solutions of acidic or alkaline pH could also desorb ovalbumin from immobilized tannin.

Adsorption capacity of immobilized tannin for proteins

For practical use of immobilized tannin it is necessary to know its adsorption capacity for proteins. Therefore, pepsin, lysozyme, ovalbumin and trypsin were applied to the column at the respective optimum pH values for adsorption until the protein concentration in the effluent became equal to that in the charged solution;

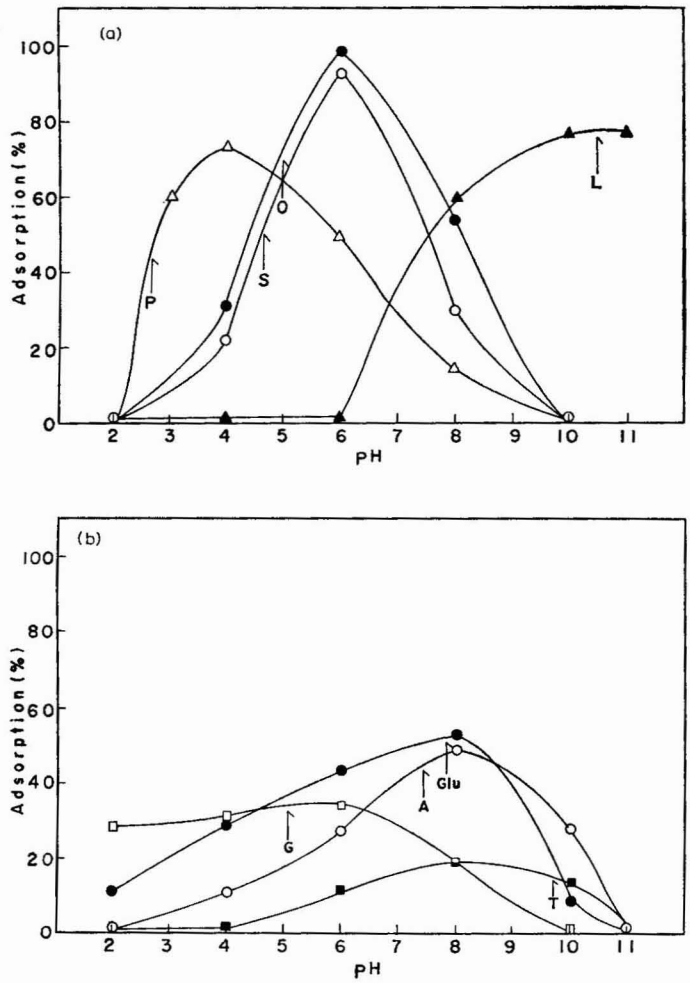

Fig. 2. Effect of pH on adsorption ratio for proteins. Adsorption was carried out by shaking 200 mg (wet weight) of immobilized tannin with 10 ml of 0.1% protein dissolved in the following buffer solutions: pH 2, 0.01 *N* HCl; pH 3–5, 0.01 *M* acetate buffer; pH 5–8, 0.01 *M* phosphate buffer; pH 9–11, 0.01 *M* carbonate buffer. The adsorption ratios were calculated from:

$$\text{Adsorption ratio (\%)} = \frac{\text{(protein used)} - \text{(protein not adsorbed)}}{\text{(protein used)}} \times 100$$

Other conditions are given in the text. Proteins: a, △, pepsin (P); ●, ovalbumin (O); ○, serum albumin (S); ▲, lysozyme (L); b, □, gelatin (G); ○, alkaline protease (A); ●, glucoamylase (Glu); ■, trypsin (T). Arrows indicate isoelectric points.

the adsorption capacities for these proteins were then determined. As shown in Fig. 4, pepsin, lysozyme and ovalbumin were well adsorbed, but trypsin was only slightly adsorbed. From the figure, the adsorption capacity per gram of immobilized tannin (dry weight) was calculated to be 950 mg for pepsin, 600 mg for lysozyme, 540 mg for ovalbumin and 68 mg for trypsin.

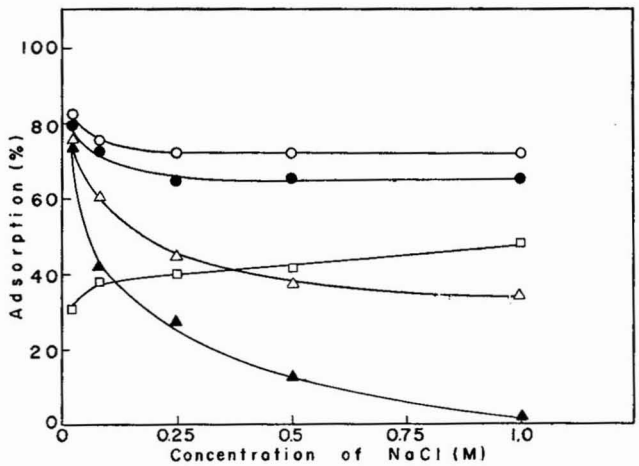

Fig. 3. Effect of salt concentration on the adsorption ratio for proteins. Adsorption was carried out by the method in Fig. 2 except that the pH and salt concentration of the protein solution was varied. Proteins: ●, ovalbumin; ○, serum albumin; □, gelatin; △, pepsin; ▲, lysozyme.

TABLE I

RECOVERY OF OVALBUMIN ADSORBED ON IMMOBILIZED TANNIN

Adsorption was carried out by shaking 35 g (wet weight) of immobilized tannin with 200 ml of 0.5% ovalbumin dissolved in 0.05 *M* acetate buffer solution, pH 6. Desorption was carried out by shaking 1 g (wet weight) with 10 ml of a buffer solution or a 0.05 *M* acetate buffer, pH 6, containing some desorbent. Other conditions are given in the text.

Solvent for desorption	*Recovery of protein (%)*
0.05 *M* Acetate buffer, pH 4	48.8
0.05 *M* Acetate buffer, pH 6	9.6
0.05 *M* Phosphate buffer, pH 8	73.7
0.05 *M* Carbonate buffer, pH 10	81.6
1 *M* Sodium chloride in acetate buffer, pH 6	17.6
50% Sucrose in acetate buffer, pH 6	17.6
30% Acetone in acetate buffer, pH 6	14.7
50% Ethylene glycol in acetate buffer, pH 6	17.3
1 *M* Sodium chloride and 50% sucrose in acetate buffer, pH 6	58.7
1 *M* Sodium chloride and 30% acetone in acetate buffer, pH 6	47.2
1 *M* Sodium chloride and 50% ethylene glycol in acetate buffer, pH 6	80.9

Separation of trypsin, ovalbumin and lysozyme on immobilized tannin

The separation of a mixture of these three pure proteins was tested using an immobilized tannin column. As shown in Fig. 5, trypsin was not adsorbed on the column at neutral pH, whereas the other two proteins were adsorbed and could be separately eluted with alkaline buffer and then acidic buffer without significant loss of protein.

DISCUSSION

In order to develop the separation, recovery and removal of proteins, we have investigated the characteristics of immobilized tannin for protein adsorption.

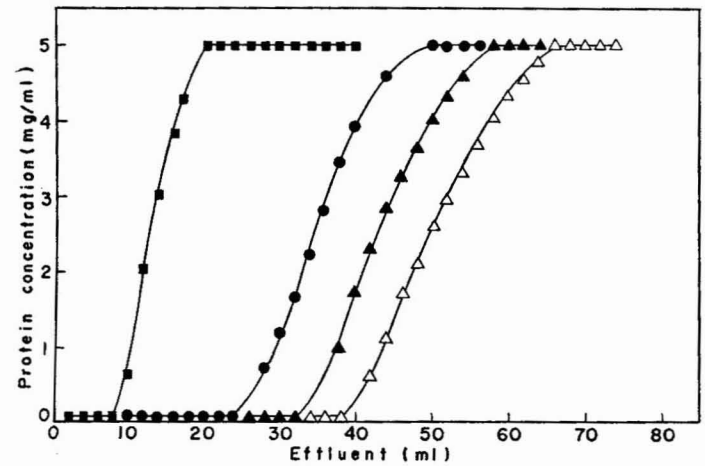

Fig. 4. Adsorption capacity of immobilized tannin for proteins. Buffer solutions for equilibration of the column were 0.05 *M* acetate buffer, pH 4, for pepsin; 0.05 *M* acetate buffer, pH 6, for ovalbumin; 0.05 *M* phosphate buffer, pH 8, for trypsin; and 0.05 *M* carbonate buffer, pH 10, for lysozyme. Other conditions are given in the text. Proteins: ■, trypsin; ●, ovalbumin; ▲, lysozyme; △, pepsin.

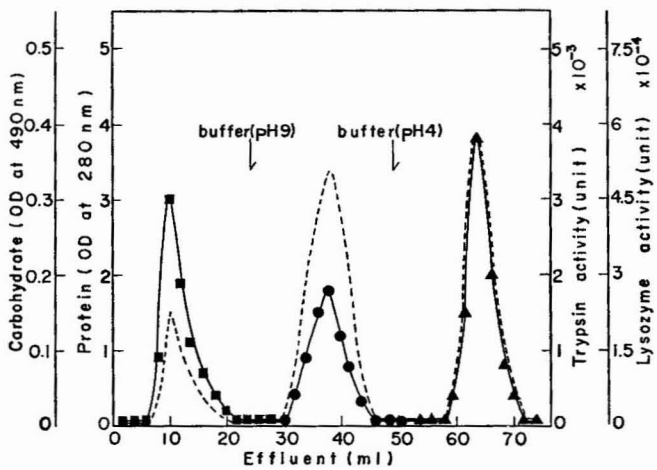

Fig. 5. Separation of a mixture of trypsin, lysozyme and ovalbumin on immobilized tannin. Two millilitres of protein solution (pH 7.5) containing 10 mg of trypsin, 10 mg of ovalbumin and 10 mg of lysozyme were applied to the column, and the proteins adsorbed were eluted with the specified buffer solutions. Other conditions are given in the text. ■—■, Trypsin activity; ●—●, carbohydrate; ▲—▲, lysozyme activity; – – –, protein.

It is well known that native tannin interacts with proteins mainly through hydrogen bonding[11]. However, as can be seen from Fig. 1, immobilized tannin has both acidic and basic groups. Therefore, it is suggested that ionic forces may also take part in the interaction between proteins and the adsorbent.

As the isoelectric point of immobilized tannin is at near neutral pH, the adsorption of proteins to immobilized tannin at neutral pH must be primarily due to hydrogen bonding, whereas ionic forces only assist the binding. This is borne out by the fact that ovalbumin adsorbed on immobilized tannin could not effectively be eluted by an eluent containing 1 *M* sodium chloride at neutral pH (Table I), and the

adsorption ratio of ovalbumin and serum albumin was not affected by salt concentration (Fig. 3).

On the other hand, immobilized tannin has positive and negative charges at acidic and alkaline pH values, respectively. Thus, in the pH region between the isoelectric point of a protein and that of immobilized tannin, ionic binding forces work in addition to hydrogen bonding and increase the adsorption ratio of proteins as observed in the case of pepsin and lysozyme. In other pH regions, where the charge of the protein is the same as that of immobilized tannin, ionic repulsion forces occur, and the adsorption decreases with increasing repulsion (Fig. 2 a and b). As ionic forces operate at acidic and alkaline pH values, the salt concentration also affects the adsorption of proteins such as pepsin and lysozyme, whose isoelectric points are far from that of immobilized tannin (Fig. 3).

Some proteins such as trypsin and gelatin were only slightly adsorbed on immobilized tannin at all pH regions. It is suggested that these proteins may have a structure which hinders the formation of hydrogen and ionic bonds with the immobilized tannin.

From the above results, proteins can be classified into three types on the basis of their interactions with immobilized tannin: first, proteins having low affinities for immobilized tannin, such as trypsin and gelatin; secondly, proteins having affinities for immobilized tannin through mainly ionic bonding, such as lysozyme and pepsin whose isoelectric points are far from that of immobilized tannin; thirdly, proteins having affinities for immobilized tannin through mainly hydrogen bonding, such as ovalbumin and serum albumin whose isoelectric points are near to that of immobilized tannin.

In order to confirm that the immobilized tannin can be utilized for the separation of proteins, a model experiment for chromatographic separation of different kinds of pure proteins was carried out. The proteins were well separated (Fig. 5). These results suggest that separation, recovery and removal of proteins using immobilized tannin may be carried out by selecting suitable conditions for adsorption and desorption.

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CHEMOTAXONOMIC STUDY OF NEUTRAL COUMARINS IN ROOTS OF *CITRUS* AND *PONCIRUS* BY THIN-LAYER, GAS–LIQUID AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSES

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SUMMARY

Eight neutral coumarins were isolated from the roots of 11 *Citrus* and *Poncirus* cultivars by thin-layer chromatography (TLC). These coumarins included seselin, xanthyletin, poncitrin, osthol, suberosin and xanthoxyletin. The two others were only tentatively identified. The eight were completely resolved by gas–liquid chromatography (GLC) with two different liquid phases. With limited studies these eight were not however, completely resolved by TLC or high-performance liquid chromatography (HPLC). Values from HPLC supported the quantitative values obtained by GLC. Profiles of these eight coumarins were intrinsic to *Citrus* and *Poncirus* species, with hybrids having profiles characteristic of both genera.

INTRODUCTION

Chemotaxonomic marker compounds are being used increasingly in citrus horticulture to better define the parentage of some important cultivars and to help predict the characteristics of new hybrids prior to fruit development[1]. Diseases, cold hardiness and fruit yields of *Citrus* are often associated with specific rootstocks; hence knowledge of compounds found in certain rootstocks and not in others would be useful. In previous publications of our laboratory chemotaxonomy was used in delineating closely related species within the genus *Citrus*[2,3]. Coumarins are among the compounds which have served as chemotaxonomic markers for other workers[4]. In an earlier study on lipids associated with mycorrhizal fungus and citrus roots[5], we found about 60% of the extractable root material to be coumarins. Thus, these extracts seemed to be excellent sources of material for a chemotaxonomic study on *Citrus* rootstocks.

Seselin[6] and xanthyletin[7] are the only coumarins reported to be constituents of *Citrus* roots. Another coumarin, poncitrin, was isolated from the roots of *Poncirus*

trifoliata and appeared to be the major coumarin[8]. Use of gas–liquid chromatography (GLC) for analyzing coumarins has been limited[9–12] due primarily to the need to derivatize coumarins that contain hydroxyls or other polar groups and to the fact that many coumarins are heat and/or acid sensitive. Use of high-performance liquid chromatography (HPLC) on coumarins has been even more limited[13,14].

The current study reports the quantitation of 8 neutral coumarins from *Citrus* and *Poncirus* root extracts. One purpose of this study was to devise a GLC system that would allow all 8 coumarins to be quantified and to verify this quantification by HPLC. The second purpose was to use this GLC method to analyze the coumarins present in the roots of 6 *Citrus* rootstock varieties, *Poncirus trifoliata* and 4 *Citrus* × *Poncirus* hybrids for chemotaxonomic purposes.

METHODS*

Isolation of coumarins and structure determinations

Two- to six-month-old seedlings of 6 rootstocks were obtained from the nursery at the U.S. Horticultural Field Station, Orlando, FL, U.S.A. Two-year-old seedlings and roots from a 9-year-old seedling grapefruit tree were from the budwood liner plot and variety grove respectively, at Whitmore Foundation Farm, Leesburg, FL, U.S.A. The roots were excised, air dried and extracted for lipids with Folch reagent[5]. Individual coumarins were isolated by sequential preparative thin-layer chromatography (TLC) with chloroform[5] and cyclohexane–ethyl acetate (4:1)[6]. Purity of all fractions was determined by GLC. Portions of the 8 coumarins were hydrogenated for 1 h at room temperature and 60 p.s.i., with chloroform as solvent and 1% Pd/C as catalyst. Hydrogenated products were purified by TLC with cyclohexane–ethyl acetate (4:1). The UV spectra of natural and hydrogenated coumarins were determined in ethanol. Mass spectra were determined by gas chromatography–mass spectrometry (GC–MS) of a mixture of the 8 coumarins and a mixture of their hydrogenated derivatives. The mixtures were injected onto a 1.83 m × 2 mm, 1% SP-1000 glass column coupled with a VG Micromass 7070F mass spectrometer operated under 70 eV at 200°C.

Chromatographic analyses

TLC. Migration distances of the isolated root and reference coumarins were determined on 250-μm, 20 × 20 cm, nonactivated silica gel G plates developed with chloroform and with cyclohexane–ethyl acetate (4:1).

GLC. An HP Model 7610A chromatograph, with a flame-ionization detector (FID) and injection heaters at 250°C and helium at 60 ml/min, was equipped with glass columns (1.83 m × 2 mm I.D.). These were packed with 100–120-mesh Gas-Chrom Q that had been coated with the following liquid phases and were operated isothermally at the indicated temperatures: (1) 3% SE-30 at 170°C, (2) 3% Dexsil 300 at 205°C, (3) 1% Dexsil at 180°C, (4) 1% OV-210 at 164°C, (5) 1% Carbowax 20M–terephthalic acid at 200°C, (6) 1% SP-1000 at 205°C and (7) 0.75% Silar 10C at 190°C.

* Mention of a trademark of proprietary product is for identification only and does not recommend its approval by the U.S. Department of Agriculture to the exclusion of others which may also be suitable.

HPLC. All analyses were performed on a Varian 5000 with a Vari-Chrom UV–VIS detector and a MicroPak MCH-10 (reversed phase, 10-μm bonded C_{18} at 14% load, 30 cm × 4 mm I.D.) column. The detector was operated at an absorbance range of 0.5, a band width of 16 and an optimum wavelength of 330 nm. The optimum elution programs were: (1) methanol–water (4:1), flow-rate held constant at 0.5 ml/min for 12 min, increased at 0.15 ml/min to 2.0 ml/min over a 10-min period; (2) acetonitrile–water (3:2), flow-rate held at 0.8 ml/min for 10 min, increased at 0.12 ml/min to 2.0 ml/min over a 10-min period; (3) hexane–tetrahydrofuran, constant flow of 0.5 ml/min; time (min)–% hexane program was as follows: 0–85%, 10–75%, 15–75%, 17–70%, 20–70%, 25–85%, 30–85%.

Quantitation. All rootstocks were analyzed for their neutral coumarin profiles by GLC with the 1% SP-1000 column. From 5 to 30 μg of sample in 2–3 μl chloroform were injected on-column at 205°C. Relative percentages were calculated with the aid of an Autolab Systems IV computing integrator. The means of 2 to 12 replicates of each rootstock analyzed in duplicate are reported. The GLC-determined coumarin profiles of the various rootstocks were compared with profiles obtained by HPLC at various wavelengths.

RESULTS AND DISCUSSION

Structures

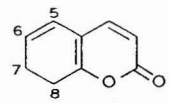
Two major fluorescent bands with R_F values of 0.43 and 0.33 were observed when the root lipid extracts were separated by TLC with chloroform. The major component of the 0.43 band was the angular seselin (I) (Table I) while the major component of the 0.33 band was the linear xanthyletin (II), as indicated by their UV spectra[6,7]. The mass spectra of these two compounds were nearly identical with *m/e* at 228 (molecular ion), 213 (base ion) and fragmentation ions at 185, 125 and 70; typical of non-substituted pyranocoumarins[15,16]. Upon hydrogenation of the compounds the 280–345 nm UV absorption maxima were lost. Molecular ions in the mass spectra of the hydrogenated coumarins were both at 232 while the base ions were at 217, in accordance with the characteristics of dihydropyranocoumarins[17].

Osthol (III) and suberosin (IV) isolated in this study are the biological precursors of the angular seselin and the linear xanthyletin, respectively[17,18]. Both III and IV gave very similar UV and mass spectra and were the same as spectra on a sample of osthol obtained from grapefruit peel oil[19] and the mass spectrum reported for suberosin[18]. These two prenyl-methoxy coumarins upon hydrogenation lost their 320-nm absorption bands. Retention times of isolated osthol were the same as for authentic osthol in all TLC, GLC and HPLC systems. Upon hydrogenation both isolated and authentic osthol gave the same retention times.

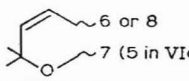
Xanthoxyletin (V) gave the same mass spectrum as reported[15,20] for monomethoxylated pyranocoumarins. Its UV spectrum was identical to that of an authentic sample and agreed with its reported spectrum[20]. Upon hydrogenation the coumarin showed enhanced UV absorption in the 230–238-nm area, a characteristic of hydrogenated methoxypyranocoumarins[8]. Retention times and volumes of isolated and authentic xanthoxyletin were the same on all TLC, GLC and HPLC systems used in this study. Their dihydro derivatives likewise gave the same chromatographic times and volumes. Coumarin VI had a mass spectrum nearly identical to that of

TABLE I

FOUND AND PROPOSED STRUCTURES OF NEUTRAL COUMARINS IN *CITRUS* AND *PONCIRUS* ROOTS



Coumarin
Substituents



Dimethylpyrano

$-CH_2-CH=C(CH_3)_2$ $-OCH_2CH=C(CH_3)CH_2CH_2CH=C(CH_3)_2$

$-C_5H_9$ $-OC_{10}H_{17}$ (geranyloxy-)

Code	*Common name*	*Position of substituents on coumarin ring*			
		5	*6*	*7*	*8*
I	Seselin*			Dimethylpyrano	
II	Xanthyletin*		Dimethylpyrano		
III	Osthol*			$-OCH_3$	$-C_5H_9$
IV	Suberosin*		$-C_5H_9$	$-OCH_3$	
V	Xanthoxyletin*	$-OCH_3$	Dimethylpyrano		
VIa	Braylin**		$-OCH_3$	Dimethylpyrano	
VIb	Luvangetin**		Dimethylpyrano		$-OCH_3$
VIc	Alloxanthoxyletin**	Dimethylpyrano		$-OCH_3$	
VII	Poncitrin*	$-OCH_3$	Dimethylpyrano		$-C_5H_9$
VIII	Geranyloxypyranocoumarin***		Dimethylpyrano		$-OC_{10}H_{17}$

* Found structure.
** Structure of VI is thought to be one of these three compounds.
*** Proposed structure.

xanthoxyletin. Three monomethoxylated pyranocoumarins besides xanthoxyletin (V) are reported to be present in *Rutaceae*[17] braylin (VIa), luvangetin (VIb) and alloxanthoxyletin (VIc). Until a standard is obtained for chromatographic comparison with VI, its structure remains uncertain.

Poncitrin (VII) was isolated as a minor component of the "xanthyletin" TLC band. Its UV and mass spectra were identical to reported values for poncitrin, and its hydrogenated derivative's UV spectrum was the same as reported for tetrahydroponcitrin[8].

The mass spectrum of the 8th coumarin (VIII) gave only a 4% ion at *m/e* 365. The presence of this weak anion may be a reflection of the facile fragmentation of an allylic aliphatic side chain [15,19] since the next ions were at 242 (92%), 227 (25%), 211 (100%) and 183 (49%). The molecule failed to produce an isolatable compound when subjected to hydrogenation under conditions used for the other 7 coumarins. Likewise, two geranyloxycoumarins found in grapefruit peel oil[19] —7-geranyloxycoumarin and bergamottin— failed to produce an isolatable compound under these hydrogenation–TLC conditions. The UV spectrum of VIII had a maximum at 301 nm. Structures which support this data are 5-methoxy-8-geranyloxy psoralen[21] and a

geranyloxy pyranocoumarin. The first structure was ruled out since an authentic sample of this compound gave a HPLC RRV (RRV = retention volume of coumarin/retention volume of seselin) of 1.08 while the corresponding RRV for VIII was 1.80 (Table II). Pyranocoumarins with geranyloxy substituents have not been reported in the literature. Thus, further studies need to be carried out on coumarin VIII before its structure can be established.

TABLE II

MIGRATION RATIOS OF NEUTRAL *CITRUS* AND *PONCIRUS* ROOT COUMARINS RELATIVE TO SESELIN ON TLC, GLC AND HPLC

Chromatographic system	R_F *or ret. vol. of seselin*	*Migration ratios*						
		II	*III*	*IV*	*V*	*VI*	*VII*	*VIII*
TLC								
Chloroform	R_F 0.43	0.83	0.98	0.88	0.86	0.95	0.83	0.95
Cyclohexane–ethyl acetate (4:1)	R_F 0.47	0.77	0.77	1.00	0.81	1.11	1.09	0.51
GLC								
1% Dexsil 300	180 ml	1.48	1.60	2.07	2.64	2.39	5.33	2.95
1% SP-1000	297 ml	1.48	1.33	1.81	1.99	2.15	2.37	2.65
HPLC								
Methanol–water	5.6 ml	1.00	1.00	1.29	1.10	1.25	1.67	1.35
Acetonitrile–water	5.6 ml	0.97	1.33	1.55	1.08	1.28	3.02	1.27
Hexane–tetrahydrofuran	4.5 ml	1.25	1.31	0.94	1.06	0.92	0.86	1.80

TLC. Various methods have been used to compare migration patterns of similar compounds in chromatography. In this study we found the simplest and most meaningful was a comparison of all migrations to that of seselin (I). This coumarin had the lowest molecular weight and was the most mobile of the eight neutral coumarins. By this method we were able to compare the structural influences of the eight coumarins on their migrations in all three chromatographic systems.

The relative retention times (RRT = $R_{F\text{coumarin}}/R_{F\text{seselin}}$) of the eight neutral coumarins on plates developed with chloroform fell into two ranges, 0.83–0.88 and 0.95–1.00 (Table II). For coumarins I to V and VII these ranges corresponded to pyranocoumarins and their biological precursers with the linear and angular structures respectively. With the cyclohexane–ethyl acetate system the addition of a methoxy or prenyl group onto the basic pyranocoumarin structures increased their RRT values from 0.04 to 0.33 RRT units. With cyclohexane–ethyl acetate coumarin VIII had an RRT of 0.51 while the RRT values of standards were: imperatorin, 0.64; phellopterin, 0.42; 5-methoxy-8-geranyloxy-psoralen, 0.65 and bergamottin, 1.26. The closeness of VIII's RRT to the RRT values of the first three standards indicate that the geranyloxy substituent of VIII was on carbon 8. The slight but distinct differences in the eight root coumarins' RRT values in the two TLC systems allowed them to be isolated by preparative TLC.

GLC. Previously we found that under temperature-programmed conditions with a 3% SP-1000 column, the eight coumarins were eluted after the fatty acid methyl esters[5]. The last coumarin (VIII), however, was unstable giving us erratic quantitative results. Chromatographic studies were conducted to maximize resolution

but minimize the time needed for the coumarins to be on the column. The eight coumarins were analyzed isothermally on a series of low-load columns of increasing polarity and at temperatures needed to elute the last coumarin within 20 min. Best resolutions were obtained on 1% SP-1000 and 1% Dexsil-300 columns.

Isoprenoid ethers such as imperatorin (8-prenyloxy-psoralen) are not stable under GLC conditions, undergoing rearrangement as well as degradation with heat[9]. Steck and Bailey[11] were able to elute 7-prenyloxy-coumarin and phellopterin (5-methoxy-8-prenyloxy-psoralen) but not imperatorin on a 5% SE-30 column. Under our improved conditions such as glass columns, low-load phases, on column injection and silanized supports, we were able to gas chromatograph such isoprenoid ethers as 7-geranyloxy-coumarin and imperatorin on 3% SE-30, 1% Dexsil-300 and 1% SP-1000 as symmetrical peaks in a reasonable length of time. We were, however, able to elute bergamottin (5-geranyloxy-psoralen) as a sharp, non-tailing peak only on the 1% Dexsil-300 column. There thus seems to be a difference in heat stability of 5- and 8-isoprenoid coumarin ethers on various GLC liquid phases. Our ability to elute VIII from both polar and non-polar phases lends support to the proposed 8-geranyloxy-pyranocoumarin structure for VIII.

HPLC. To the best of our knowledge there is no literature on HPLC of pyranocoumarins. The general recommended procedure with C_{18} columns (non-polar, partition chromatography) is to elute with various mixtures of 2 polar solvents, *e.g.*, water–methanol or water–acetonitrile. Resolution of angular seselin from linear xanthyletin (Table I) was the major objective in these HPLC experiments. This objective was not achieved with either of these two solvent systems, although other coumarins were separated (Table II). Baseline separation was obtained, however, for seselin (I) and xanthyletin (II) by using the non-conventional C_{18} column solvents hexane–tetrahydrofuran; the linear isomer was eluted after the angular isomer, as observed in GLC. Contrary to the sequence observed on GLC however, osthol (III) was eluted after its isomer suberosin (IV), the III/IV ratio of RRV being 1.39. The sequence of elution for the linear pyranocoumarins was VII > V > II, the reverse of what one would expect if the column separated these coumarins by adsorption chromatography as indicated by the separation of seselin and xanthyletin.

Chemotaxonomy

Table III shows the neutral coumarin profiles of six *Citrus* taxa, *Poncirus trifoliata* and four *Citrus* × *Poncirus* hybrids, all used as rootstocks. As a group the *Citrus* rootstocks were essentially free of poncitrin (VII), had very little xanthoxyletin (II), from 1 to 2% osthol (III) and various amounts of the other five coumarins. Sweet and sour oranges differed noticeably in their (VI) and (VIII) percentages. Cleopatra mandarin differed from two oranges in relative abundance of (VI) and xanthyletin (II). Rough lemon and Rangpur lime differed from the above three citrus rootstocks by having lower percentages of seselin (I) and high values of both xanthyletin (II) and suberosin (IV). These rootstocks differed from each other in their percentages of these two coumarins. Grapefruit, like Cleopatra mandarin, had 13% (VI) and 1.9% osthol (III). This rootstock however, differed from all of the other five *Citrus* taxa by containing considerably higher percentages of xanthoxyletin (V) and VIII. The profile of *Poncirus trifoliata* contained 78.9% seselin (I), only 11% poncitrin (VII) and less than 4% each of the 6 other coumarins. Its low percentage of poncitrin was un-

TABLE III

RELATIVE PERCENTAGE OF NEUTRAL COUMARINS IN ROOTS OF *CITRUS*, *PONCIRUS* AND *CITRUS* × *PONCIRUS* BY GLC

No.	*Rootstock*	*Relative % in coumarin*							
		I	*II*	*III*	*IV*	*V*	*VI*	*VII*	*VIII*
1	Sweet orange (*Citrus sinensis* [L.] Osb.)	67.9	13.8	1.1	4.4	—*	1.8	—	11.0
2	Sour orange (*C. aurantium* L.)	71.3	13.3	1.0	2.6	0.4	6.1	—	5.3
3	Cleopatra mandarin (*C. reticulata* Blanco)	68.9	7.0	1.9	4.0	0.5	13.5	—	4.2
4	Rough lemon (*C. limon* [L.] Burm. F.)	34.1	37.6	0.5	24.4	—	0.6	—	2.8
5	Rangpur lime (*C. reticulata* var. *austera* Swing)	37.1	49.1	0.6	10.1	0.5	0.6	0.1	1.9
6	Seedling grapefruit (*C. paradisi* Macf.)	41.9	20.7	1.9	—	4.8	13.2	—	17.5
7	Carrizo citrange (*Poncirus trifoliata* × *C. sinensis*)	54.7	22.8	0.3	1.5	5.0	1.0	7.4	7.3
8	Troyer citrange (*P. trifoliata* × *C. sinensis*)	57.5	19.6	0.3	1.2	4.6	0.3	7.7	8.8
9	Rusk citrange (*C. sinensis* × *P. trifoliata*)	43.9	27.7	0.2	0.9	7.0	0.1	11.5	8.7
10	Swingle citromelo (*C. paradisi* × *P. trifoliata*)	48.7	20.7	1.4	1.5	7.1	10.9	8.3	1.4
11	Trifoliata orange (*Poncirus trifoliata* [L.] Raf.)	78.9	3.3	0.1	1.1	4.0	—	11.0	1.6

* If present below 0.1 relative %.

expected in the light of a previous study[8]. The two citranges Carrizo and Troyer cannot readily be distinguished from each other in the grove; nor could we distinguish them, whether by a chemotaxonomic study of their leaf hydrocarbons[2] or of their root coumarins. Rusk, the third citrange, differed from the other two by having higher percentages of all three of the linear pyranocoumarins. Like the citranges, the citrumelo, "Swingle", showed its *Poncirus* parentage by the presence of 8 % poncitrin (VII). The citrumelo reflected its grapefruit parentage by its 10.9 % VI and 1.4 % osthol (III). In general *Citrus* had higher amounts of the angular pyranocoumarin and its biological precurser osthol (III) while *Citrus* × *Poncirus* contained more linear pyranocoumarins. In agreement with previous studies[6,7], furanocoumarins, which are widely found in aerial parts of citrus, were not found in the roots.

In previous *Citrus* scion studies many cultivars were available[3,4]; however, in our study the number of rootstocks available was limited. With the exception of Carrizo-Troyer, these 11 rootstocks each had characteristic root neutral coumarin profiles. These coumarins appear to be confined to the roots of these trees. Two exceptions to this are the reports of osthol in the peel of fruit[17] and xanthyletin in the stem of souorange[6]. All eight of the coumarins were readily quantitated by GLC using a low phase-load and relatively low oven temperatures. Under these conditions the unstable VIII was eluted without decomposition. Our limited trials with HPLC could not resolve all eight coumarins on a single column. However we were able to resolve "critical pairs" of coumarins on a reversed-phase C_{18} column, using mixtures of two non-polar solvents. Placing coumarin VIII in a non-destructive chromatographic environment (HPLC) clearly showed that it was present in the roots and was not an artifact formed under GLC conditions.

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THIN-LAYER CHROMATOGRAPHY OF AROMATIC AMINES ON AMMONIUM MOLYBDOPHOSPHATE AND TUNGSTOPHOSPHATE

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SUMMARY

The use of ammonium tungstophosphate in the thin-layer chromatography of 35 primary aromatic amines was investigated, with respect to the influences of the exchanger concentration in the layer, the acidity and salt concentration in the eluent and the kind of the organic solvent. Many interesting separations among the amines have been carried out. On ammonium molybdophosphate layers the amines are oxidized, giving rise to blue spots, and sample amounts ten times as large as on ammonium tungstophosphate must be employed.

INTRODUCTION

Recently, two synthetic inorganic exchangers have been used in the thin-layer chromatography (TLC) of organic compounds. Thus, ammonium molybdophosphate (AMP) was employed for the separation of sulphonamides[1], while ammonium tungstophosphate (AWP) showed great advantages with respect to AMP in the separation of amino acids[2] owing to its lower redox potential and lack of colour. In comparison with organic exchangers[3] and with silanized silica gel impregnated with anionic and cationic detergents[4], on ammonium tungstophosphate a different affinity sequence of the amino acids and a higher sensitivity was found. Layers of silica gel impregnated with small amounts of pyridinium tungstoarsenate have also allowed an improvement in the separation of some nitrogenous organic compounds with respect to silica gel alone[5,6].

We have now examined the behaviour of 35 primary aromatic amines on AWP and AMP layers. This class of compounds is very suitable for the study of the retention mechanism on these exchangers and for comparison with the results achieved on layers of organic exchangers[7–9] and with the soap TLC[10,11].

EXPERIMENTAL

Standard solutions of amines were prepared by dissolving the compounds in methanol– 1 *M* hydrochloric acid (1:1) and stored in dark bottles. Fresh solutions were used for those amines which decompose easily, *i.e.*, phenylenediamines. The amines were detected with a solution of 5% N,N-dimethyl-*p*-aminobenzaldehyde (*p*-DAB) in ethanol–glacial acetic acid (5:1, v/v).

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Ammonium tungstophosphate was prepared by dissolving 50 g of phosphotungstic acid (E. Merck, Darmstadt, G.F.R.) in 200 ml of 2 *M* nitric acid; 200 ml of 2 *M* ammonium nitrate were added with stirring. The suspension was filtered and the solid was rinsed with water and air-dried. The layers (thickness 300 μm) were prepared with a Chemetron automatic apparatus by mixing the desired amount of AMP (Bio-Rad Labs., Richmond, CA, U.S.A.) or AWP (0.5–8 g) in 50 ml of water. To the suspension, shaken with a magnetic stirrer, were added 2 g of calcium sulphate hemihydrate (first passed through a 200-mesh sieve). After 10 min the aqueous slurry was sprayed on the plates.

The measurements were carried out at 25°C. The migration distance was 10 cm unless otherwise stated.

RESULTS AND DISCUSSION

Layers of ammonium tungstophosphate

Influence of the AWP concentration. In order to examine the influence of the exchanger concentration on the retention of the aromatic amines, the R_F values of 35 compounds were determined on layers of calcium sulphate hemihydrate mixed with increasing amounts of AWP, by eluting with water.

The development time increases from 30 to 70 min with increasing proportion of AWP in the layer. On layers of calcium sulphate hemihydrate alone most amines run with the solvent front giving rise, in some cases, to elongated spots. In the presence of small amounts of AWP, *i.e.*, AWP:$CaSO_4 \cdot \frac{1}{2}H_2O$ ratio = 0.5:2, the retention of the amines, particularly of those without acid groups, is increased; under these conditions, the spots are diffuse even when spherical, and for this reason some amines cannot be detected at the amounts reported in Table I. As the AWP concentration in the layer is increased a sharp increase in the retention of all compounds and more compact spots are observed. For most amines a limiting R_F value is not reached even at high percentages of exchanger, as observed in the case of amino acids[2].

The sequence of the R_F values for the aromatic amines does not change with increasing AWP concentration in the layer, except for the data in the first column in Table I where the chromatographic behaviour of the compounds is only slightly affected by the exchanger owing to its small concentration in the layer.

The influence of the substituent groups on the chromatographic behaviour of the amines can be discussed in relation to aniline. The introduction of a $-CH_3$, $-Br$, $-Cl$ group and of a second $-NH_2$ group in the ring involves an increase in the affinity towards the stationary phase. The opposite behaviour is observed in the presence of a sulphonic group or (except for the *meta* isomers) of carboxylic and nitro groups in the molecule. The last result is peculiar to these exchangers since neither organic exchangers[7–9] nor silanized silica gel impregnated with detergents[10,11] exhibited this behaviour. The presence of a second aromatic nucleus in the molecule, *e.g.* α-naphthylamine, results a sharp decrease in the R_F value. As regards the influence of the substituent group position on the chromatographic characteristics, the *para* isomers are more strongly retained in the case of toluidines and of arsanilic acids, and the *meta* isomers in the case of bromo-, chloro- and nitroanilines and amino-benzoic acids. Such behaviour could be useful in the separation of isomers.

Another peculiar characteristic is the high sensitivity in the detection of

TABLE I

R_F VALUES OF PRIMARY AROMATIC AMINES ON THIN LAYERS OF CALCIUM SULPHATE HEMIHYDRATE MIXED WITH INCREASING AMOUNTS OF AWP

Eluent: water. n.d. = Not determined; e.s. = elongated spot.

Amine	*AWP:$CaSO_4 \cdot \frac{1}{2}H_2O$ ratio*				*Amount (μg)*
	0.5:2	*2:2*	*4:2*	*8:2*	
Aniline	0.50	0.21	0.15	0.12	0.02
o-Toluidine	0.45	0.20	0.14	0.11	0.02
m-Toluidine	0.43	0.19	0.12	0.09	0.02
p-Toluidine	0.37	0.19	0.10	0.08	0.02
o-Bromoaniline	n.d.	0.18	0.11	0.09	0.02
m-Bromoaniline	0.29	0.11	0.07	0.07	0.02
p-Bromoaniline	0.33	0.13	0.08	0.07	0.02
o-Chloroaniline	n.d.	0.18	0.11	0.09	0.03
m-Chloroaniline	0.40	0.17	0.10	0.08	0.02
p-Chloroaniline	0.45	0.20	0.12	0.09	0.03
2,4-Dichloroaniline	n.d.	0.18	0.11	0.08	0.4
o-Nitroaniline	0.82	0.62	0.52	0.41	0.3
m-Nitroaniline	0.50	0.20	0.14	0.08	0.02
p-Nitroaniline	0.78	0.55	0.45	0.35	0.03
o-Aminobenzoic acid	0.68	0.45	0.35	0.20	0.04
m-Aminobenzoic acid	0.46	0.23	0.15	0.10	0.02
p-Aminobenzoic acid	0.72	0.48	0.36	0.23	0.04
4-Amino-3,5-dimethylbenzoic acid	0.68	0.48	0.37	0.24	0.05
3,4-Diaminobenzoic acid	0.37	0.22	0.15	0.11	0.3
3,5-Diaminobenzoic acid	0.14	0.07	0.04	0.02	0.05
o-Aminophenylarsonic acid	0.80	0.70	0.65	0.56	0.2
p-Aminophenylarsonic acid	0.75	0.65	0.59	0.50	0.1
o-Aminophenylsulphonic acid	0.90	0.77	0.73	0.60	0.1
m-Aminophenylsulphonic acid	0.90	0.78	0.74	0.61	0.1
p-Aminophenylsulphonic acid	0.91	0.85	0.84	0.75	0.1
1,3,6-Xylidene-4-sulphonic acid	0.90	0.77	0.75	0.62	0.2
2-Aminotoluene-5-sulphonic acid	0.90	0.82	0.80	0.69	0.2
4-Aminotoluene-3-sulphonic acid	0.90	0.73	0.68	0.55	0.2
6-Amino-4-chloro-3-toluenesulphonic acid	0.90	0.74	0.70	0.58	0.2
o-Phenylenediamine	0.02	0.01	0.01	0.00	0.5
m-Phenylenediamine	0.02	0.01	0.01	0.00	0.03
p-Phenylenediamine	0.01	0.01	0.01	0.00	0.01
α-Naphthylamine	e.s.	0.04	0.03	0.03	0.05
1-Naphthylamino-7-sulphonic acid	0.85	0.69	0.60	0.45	0.1
1-Naphthylamino-4-sulphonic acid	0.88	0.78	0.70	0.55	0.1

aromatic amines. The sensitivity on AWP layers is 3–100 times higher than on cellulose-based organic exchangers[7–9] and on silanized silica gel impregnated with detergents[10,11].

Besides water, 1 *M* nitric acid was also employed as eluent. This resulted in a greater compactness of the spots even at low AWP concentrations in the layer so that all the amines could be detected. Aminophenylarsonic acids and α-naphthylamine, however, give rise to elongated spots on the layer with 50% AWP. On the basis of the results achieved on eluting with water, or 1 *M* nitric acid, a layer with a 4:2 ratio of AWP:$CaSO_4 \cdot \frac{1}{2}H_2O$ was chosen for further investigations.

Influence of salt concentration and eluent acidity. Table II lists the R_F values of the aromatic amines obtained on layers of AWP:$CaSO_4 \cdot \frac{1}{2}H_2O$ in the ratio 4:2 with changing acidity and salt concentration in the eluent. On eluting with 1 *M* nitric

TABLE II

R_F VALUES OF PRIMARY AROMATIC AMINES ON LAYERS OF AWP:$CaSO_4 \cdot \frac{1}{2}H_2O$ IN THE RATIO 4:2 (w/w)

n.d. = Not determined.

Amine	*Eluent*					*1 M* NH_4NO_3*
	1 M HNO_3	*1 M* HNO_3 + *0.25 M* NH_4NO_3	*1 M* HNO_3 + *0.5 M* NH_4NO_3	*1 M* HNO_3 + *1 M* NH_4NO_3	*1 M* HNO_3 + *2 M* NH_4NO_3	
Aniline	0.35	0.47	0.51	0.61	0.67	0.63
p-Toluidine	0.33	0.44	0.49	0.59	0.66	0.61
o-Toluidine	0.29	0.41	0.47	0.57	0.64	0.59
m-Toluidine	0.25	0.39	0.45	0.55	0.63	0.57
p-Bromoaniline	0.20	0.31	0.38	0.44	0.54	0.46
o-Bromoaniline	0.18	0.30	0.38	0.46	0.56	0.48
m-Bromoaniline	0.15	0.27	0.35	0.43	0.53	0.45
p-Chloroaniline	0.32	0.43	0.49	0.56	0.63	0.58
o-Chloroaniline	0.22	0.37	0.44	0.51	0.62	0.54
m-Chloroaniline	0.21	0.34	0.41	0.49	0.59	0.52
2,4-Dichloroaniline	0.20	0.30	0.38	0.47	0.56	n.d.
p-Nitroaniline	0.30	0.37	0.42	0.47	0.55	0.53
o-Nitroaniline	0.30	0.36	0.39	0.43	0.51	0.52
m-Nitroaniline	0.19	0.26	0.31	0.36	0.48	0.39
p-Aminobenzoic acid	0.48	0.50	0.56	0.63	0.70	0.66
o-Aminobenzoic acid	0.32	0.38	0.46	0.54	0.64	0.60
m-Aminobenzoic acid	0.30	0.36	0.44	0.51	0.62	0.55
4-Amino-3,5-dimethylbenzoic acid	0.31	0.34	0.42	0.50	0.58	0.55
3,4-Diaminobenzoic acid	0.35	0.38	0.46	0.55	0.63	0.55
3,5-Diaminobenzoic acid	0.05	0.13	0.20	0.30	0.44	0.33
p-Aminophenylarsonic acid	0.44	0.52	0.56	0.66	0.73	0.75
o-Aminophenylarsonic acid	0.36	0.40	0.45	0.56	0.64	0.75
p-Aminophenylsulphonic acid	0.81	0.81	0.82	0.85	0.86	0.82
o-Aminophenylsulphonic acid	0.68	0.66	0.67	0.72	0.73	0.72
m-Aminophenylsulphonic acid	0.72	0.70	0.71	0.77	0.77	0.75
1,3,6-Xylidene-4-sulphonic acid	0.71	0.70	0.71	0.75	0.76	0.75
2-Aminotoluene-5-sulphonic acid	0.76	0.75	0.78	0.81	0.83	0.79
4-Aminotoluene-3-sulphonic acid	0.63	0.60	0.64	0.68	0.70	0.68
6-Amino-4-chloro-3-toluene-sulphonic acid	0.63	0.60	0.64	0.64	0.64	0.66
p-Phenylenediamine	0.02	0.10	0.20	0.34	0.51	0.35
o-Phenylenediamine	0.02	0.13	0.23	0.36	0.52	0.37
m-Phenylenediamine	0.02	0.12	0.22	0.36	0.52	0.37
α-Naphthylamine	0.08	0.13	0.19	0.26	0.36	0.28
1-Naphthylamino-4-sulphonic acid	0.70	0.65	0.67	0.70	0.72	0.68
1-Naphthylamino-7-sulphonic acid	0.62	0.59	0.60	0.62	0.63	0.61

* R_F value of the first solvent front = 0.75.

acid the compounds with sulphonic or arsonic groups and some of those with nitro or carboxylic groups were similarly or more retained than with water. This behaviour indicates that the presence of a positive charge in the molecule, related to the protonation of the $-NH_2$ group, results in a greater affinity of the compounds towards the exchanger as is the case with a decrease in their anionic characteristics[2]. In the case of the remaining amines, which are more weakly retained than with water and which are in the cationic form at the pH of the eluent, the lower retention with respect to the non-ionized form can be ascribed to the influence of the hydrogen ion as counter ion rather than to a lower affinity towards the exchanger of the protonated form with respect to the free-base form. In fact, as the nitric acid concentration in the eluent is increased, *i.e.*, to 2 *M*, the R_F values of these amines increase remarkably.

Plots of the R_M values as a function of the nitric acid activity in the 0.5–2 *M* concentration range were linear and almost parallel to the abscissa for most sulphonated amines and *o*-nitroaniline, with slopes between 0.3 and 0.5 for the other monoamines and between 0.9 and 1.1 for the diamines. This shows that most sulphonated amines and *o*-nitroaniline are not affected by the nitric acid concentration in the eluent, and that for the other monoamines the occurrence in the retention mechanism of an ion-exchange process must be considered. Such a process, however, is not predominant since the values of the slopes are smaller than those predicted theoretically[12]. In the case of the diamines, the ion-exchange process seems more important.

The replacement of 1 *M* nitric acid with the same concentration of ammonium nitrate as eluent results in a remarkable increase in the R_F values of the amines, except for the sulphonated ones. With this eluent the appearance on the layer of two fronts, the first of which has $R_F = 0.75$, is observed. On eluting with solutions of constant nitric acid content (1 *M*) but increasing concentration of ammonium nitrate, it is observed that nitric acid affects the retention of the amines less than does ammonium nitrate. The chromatographic behaviour resembles more that obtained on eluting with 1 *M* ammonium nitrate than that with 1 *M* nitric acid.

From the above results and keeping in mind that, on eluting with 1 *M* ammonium nitrate, many amines (nitroanilines, 2,4-dichloroaniline, etc.) are in the free-base form, it seems that the protonation of the amines does not determine their retention by the layer and that the effect of the ammonium ions as counter ions is surprisingly different from that of hydrogen ions. This can be explained by assuming that ammonium nitrate is more strongly adsorbed by the exchanger than nitric acid, thus decreasing the adsorption of the amines both in the protonated and in the free-base form. This "desorption" of the amines seems, therefore, to be the determining factor in the chromatographic behaviour of most amines, at least in the presence of significant amounts of ammonium nitrate in the eluent.

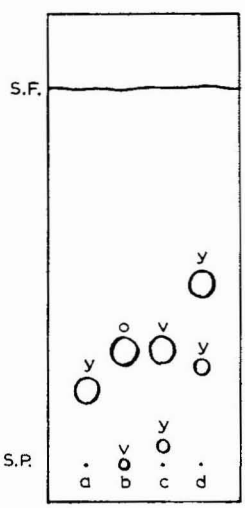
The different affinity of many isomers towards the exchanger on eluting with 1 *M* nitric acid compared with elution with water is very interesting from an analytical point of view. The reversal of the sequence of the two aminophenylarsonic acids is of note in this context. Such characteristics can be used for the separation of groups of isomers with two-dimensional development.

Aqueous–organic eluents. Elution with aqueous–organic mixtures (water–methanol, water–ethanol, water–acetic acid and 1 *M* nitric acid in water–methanol) resulted in the following differences with respect to elution with water or with aqueous

salt solutions: (1) a longer elution time; (2) a greater compactness of the spots. The elution time increased with increasing percentage of the organic solvent in the eluent and it was about 2 h (for a migration distance of 10 cm) when eluting with a 7:3 (v/v) mixture of water–methanol or water–acetic acid. The elution time also increased when methanol or acetic acid was replaced with ethanol.

The percentage of organic solvent in the eluent must not be higher than 50% in order to avoid both high elution time and diffuse spots. The affinity sequence was almost the same when eluting with water–methanol, water–ethanol or water–acetic acid (7:3, v/v). Aniline, α-naphthylamine, amines containing $-CH_3$, –Br and –Cl groups, *m*-nitroaniline, *m*-aminobenzoic acid, 3,4- and 3,5-diaminobenzoic acids, which are strongly retained with the above eluents ($R_F \leqslant 0.10$), can be separated from all the other amines. The addition of methanol to the nitric acid solutions generally yields an improvement in the separation of the amines.

Decomposition products. The high sensitivity of the AWP layers towards aromatic amines can be used to detect the presence of decomposition products in the standard amine solutions. Some aromatic amines, whose solutions are generally considered stable over long periods, give rise, after few days, to a secondary spot other than the main one obtained with a fresh solution. We deemed it useful, therefore, to report in Fig. 1 the chromatographic behaviour of standard solutions of nitroanilines and *p*-aminobenzoic acid, 10 days after their preparation, on layers of AWP:$CaSO_4 \cdot \frac{1}{2}H_2O$ in the ratio 4:2 eluted with 1 *M* nitric acid. The presence of the two spots is observed also when eluting with water.



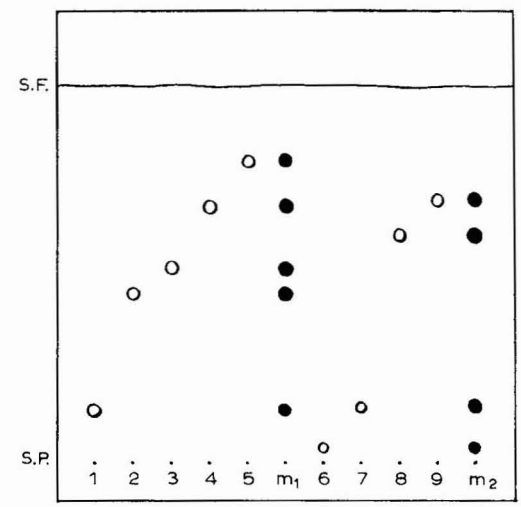


Fig. 1. Thin-layer chromatogram of standard solutions of amines, 10 days after their preparation, on AWP:$CaSO_4 \cdot \frac{1}{2}H_2O$ in the ratio 4:2. Eluent: 1 *M* nitric acid. Amines (1 μg): a = *m*-nitroaniline; b = *p*-nitroaniline; c = *o*-nitroaniline; d = *p*-aminobenzoic acid. Colours after spraying with *p*-DAB: y = yellow; o = orange; v = violet. S.P. = Starting point; S.F. = solvent front.

Fig. 2. Thin-layer chromatogram of some isomers on AWP:$CaSO_4 \cdot \frac{1}{2}H_2O$ in the ratio 4:2. Eluent: water. Amines: 1 = *m*-nitroaniline; 2 = *p*-nitroaniline; 3 = *o*-nitroaniline; 4 = 4-aminotoluene-3-sulphonic acid; 5 = 2-aminotoluene-5-sulphonic acid; m_1 = mixture of 1–5; 6 = 3,5-diaminobenzoic acid; 7 = 3,5-diaminobenzoic acid; 8 = 1-naphthylamino-7-sulphonic acid; 9 = 1-naphthylamino-4-sulphonic acid; m_2 = mixture of 6–9. Other details as in Fig. 1.

From these data, the stability of *m*-nitroaniline, with respect to the other isomers, can be determined. *p*-Aminobenzoic acid was found to be the compound decomposing most quickly, since the second spot appeared 2 days after the preparation of the standard solution. A chromatogram of these compounds 3 weeks after preparation of the standard solutions is similar to that in Fig. 1.

It should be noted that the stability of the solutions of these amines depends on the kind of solvent used. For instance, solutions of nitroanilines in methanol are more stable than those in a 1 *M* hydrochloric acid–methanol (1:1, v/v) (to which Fig. 1 refers).

Layers of ammonium molybdophosphate

Ammonium molybdophosphate exhibits more marked oxidizing properties than ammonium tungstophosphate and for this reason most amines are oxidized when deposited on the layer, giving blue spots. The amines which are more easily oxidized, *i.e.*, phenylenediamines, aniline, toluidines, etc., give rise to blue elongated spots which start from the application point, showing that their oxidation by the exchanger is not complete at the starting point but continues during their migration. An amine which is not yet oxidized reacts with *p*-DAB to give an orange colour.

The oxidation process is dependent on the pH of the eluent and its extent may be decreased with strong acid eluents. Amines containing $-NO_2$ or sulphonic groups are less readily oxidized and do not give rise to blue elongated spots.

The chromatographic behaviour of the amines on layers of ammonium molybdophosphate is similar to that obtained on ammonium tungstophosphate under the same elution conditions, apart from the lower sensitivity of detection.

Analytical applications

On the basis of the R_F values of Tables I and II and of those obtained on eluting with aqueous–organic mixtures, many separations among the different amines and, particularly, among isomers can be effected. In Fig. 2 are illustrated the separations of nitroanilines, of aminotoluenesulphonic, diaminobenzoic and naphthylaminosuphonic acids on layers of AWP:$CaSO_4 \cdot \frac{1}{2}H_2O$ in the ratio 4:2 eluted with water. The three nitroanilines were separated also with 2 *M* nitric acid on layers of AWP:$CaSO_4 \cdot \frac{1}{2}H_2O$ in the ratios 4:2 and 8:2. On both these layers, with this eluent, the affinity sequence (*meta* > *ortho* > *para*) obtained was different from that obtained on eluting with water (*meta* > *para* > *ortho*) or with 1 *M* nitric acid (*meta* > *para* = *ortho*). For instance, on layers with a exchanger:binder ratio of 4:2, the R_F values of nitroanilines are: 0.25 (*meta*), 0.32 (*ortho*) and 0.39 (*para*). The use of 2 *M* nitric acid as eluent allows the separation of the three aminobenzenesulphonic acids on the same layers since the *ortho* and *meta* isomers are well separated from them.

With aqueous–organic eluents, the separation of the three nitroanilines and of nine compounds in total on AWP:$CaSO_4 \cdot \frac{1}{2}H_2O$ in the ratio 4:2 has been achieved (see Fig. 3). This separation involves the three isomers of aminobenzoic acid and demonstrates the different behaviour among amines with a carboxylic, arsonic or sulphonic group. The other compounds containing a sulphonic group exhibit R_F values between those of 4-aminotoluene-3-sulphonic and *p*-aminobenzenesulphonic acids, which are shown in Fig. 3.

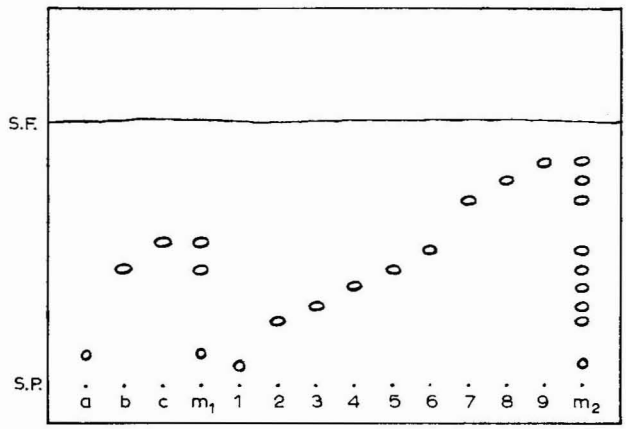


Fig. 3. Thin-layer chromatogram of aromatic amines on AWP:$CaSO_4 \cdot \frac{1}{2}H_2O$ in the ratio 4:2. Eluent: water–ethanol (7:3, v/v). Migration distance: 7 cm. Amines: a = *m*-nitroaniline; b = *p*-nitroaniline; c = *o*-nitroaniline; m_1 = mixture of nitroanilines; 1 = *m*-aminobenzoic acid; 2 = *o*-aminobenzoic acid; 3 = *p*-aminobenzoic acid; 4 = 4-amino-3,5-dimethylbenzoic acid; 5 = *p*-aminophenylarsonic acid; 6 = *o*-aminophenylarsonic acid; 7 = 4-aminotoluene-3-sulphonic acid; 8 = *o*-aminophenylsulphonic acid; 9 = *p*-aminophenylsulphonic acid; m_2 = mixture of 1–9. Other details as in Fig. 1.

The peculiar compactness of the spots and their flat shape allow the separation of compounds that differ only by 0.04 retention units, notwithstanding the short migration distance (7 cm), in an elution time of about 2 h. The best separations among the amines containing $-CH_3$, –Cl and –Br groups have been achieved by eluting with 1 *M* nitric acid in 30% methanol. Aniline ($R_F = 0.30$) is separated from the three toluidines, *meta* (0.28), *ortho* (0.33), *para* (0.33) and from the bromoanilines, *meta* (0.17), *ortho* (0.21), *para* (0.26). The behaviour of the bromoanilines allows the separation of the *para* isomer from the other two isomers. The difference in retention is more marked in the case of chloroanilines where the *para* isomer ($R_F = 0.38$) is much less strongly retained than the *meta* ($R_F = 0.26$) and *ortho* ($R_F = 0.29$) isomers.

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TRACE ANALYSIS OF FREE AMINES BY GAS–LIQUID CHROMATOGRAPHY

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SUMMARY

Amines with chain lengths between one and fifteen carbon atoms were determined accurately at sub ppm levels by direct sample injection into a gas chromatograph. The amines were dissolved in different solvents including water. The prerequisite for success was a suitable column (28% Pennwalt 223 with 4% KOH on Gas-Chrom R, 60–230°C), the use of a nitrogen-sensitive detector (Varian TSD) and the addition of ammonia to all solutions prior to analysis. The ability of ammonia to suppress adsorption of amines on glass surfaces and on the column packing was demonstrated. The influence of solvent, column temperature and sample size on the chromatograms was studied. The decomposition of the packing when aqueous samples were injected was shown to cause interference at some column temperatures.

INTRODUCTION

Interest in the determination of amines has grown rapidly during the last few years, owing to their importance, for example, in the transformation into nitrosamines[1], in allergic reactions[2] and in the determination of isocyanates at low concentrations[3].

The most important limitation in using gas chromatography (GC) for analysis of free amines has been their adsorption on the column, resulting in severe tailing and low reproducibility. The literature on the subject was reviewed by Casselman and Bannard[4]. Most of the investigations were made at high concentrations. Amine separation by capillary GC has also been limited to the high concentration range. Schomburg *et al.*[5] recently reported considerable improvements in the analysis of amines on fused silica columns.

There have been very few papers on trace analysis of free amines. Dunn *et al.*[6] developed a method for separating dimethylamine from trimethylamine in biological samples at the ppm level. Di Corcia *et al.*[7] determined low-boiling aliphatic amines in aqueous solutions at a level of 3 ppm on a modified Carbopack B column. In most other cases, *e.g.*, in refs. 8–10, it was found to be necessary to resort to derivatization prior to analysis.

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EXPERIMENTAL

A Varian 3700 gas chromatograph was equipped with an automatic flow controller for the carrier gas, a flame ionization detector (FID) and a thermionic specific detector (TSD) for nitrogen and phosphorus. A small capillary was inserted in the hydrogen line leading to the TSD. The resulting pressure drop was measured by a precision manometer. A calibration was made so that the hydrogen flow-rate could be obtained from the readings on the manometer.

The TSD response was optimized with a mixture of 2 ppm octylamine and 2000 ppm *n*-nonane in *n*-hexane (sample size 2 μl). The hydrogen flow-rate was varied between 3.3 and 5.9 ml/min and the bias voltage between −4 and −10 V. The bead current was kept to 2.95 scale divisions as recommended in the manual. The selectivity for octylamine over *n*-nonane was 4000 at a hydrogen flow-rate of 4.6 ml/min and a bias voltage of −10 V. The signal-to-noise ratio for octylamine was then 70. The hydrogen flow-rate was approximately the same as that recommended in the manual, but the bias voltage was considerably lower than recommended (−4 V). The air flow-rate to the detector was 175 ml/min.

Some other experiments were made on a Finnegan 4021 gas chromatograph–mass spectrometer.

The column packing was 28% Pennwalt 223 and 4% KOH on Gas-Chrom R (80–100 mesh) (Alltech, Arlington Heights, IL, U.S.A.). The Pennwalt 223 stationary phase was extracted from the packing by methylene chloride and investigated by IR spectroscopy and mass spectrometry (MS). About 10 g of the packing was ffilled into a glass column (190 cm × 3 mm I.D.). The carrier gas (nitrogen) flow-rate of 20 ml/min was near the HETP minimum. The temperature was varied between 60 and 230°C.

Various aliphatic and aromatic amines were used as solutes (see Table I) in different organic solvents as well as in water. Ammonia was added to all solutions to prevent salt formation of the amines and to minimize adsorption on the glass surfaces. Stock solutions of gaseous amines were prepared by dissolving 10 ml of gas in the solvent containing ammonia. The concentration of the amines in the samples was varied between 0.03 ppm and 5 ppm and the sample sizes were 1–7 μl. The sample was injected with a syringe having a non-interchangeable needle.

Special precautions were undertaken to obtain reproducible conditions during sample storage and injection. Samples were stored in glass test-tubes closed with Mininert Valves (Supelco), modified with PTFE caps. The samples were in contact with the membrane only when the valve was opened to insert the syringe. The membranes were grey GC septa. Some membrane types gave disturbing peaks when in contact with alkaline amine solutions. The red type of vial septa could not be used with volatile amines. Memory effects in syringes were reduced by washing first with 25% ammonia in water, then distilled water and finally with the solvent. The syringe was rinsed with ethanol before using a solvent immiscible with water.

The use of a new very stable nitrogen-sensitive detector and the addition of ammonia to all solutions prior to analysis are important improvements in the experimental conditions compared with earlier investigations.

TABLE I

AMINES USED AS SOLUTES IN THIS INVESTIGATION

Solute	*Typical column temperature (°C)*
Dimethylamine	80
Ethylamine	
Isopropylamine	
Methylamine	
Trimethylamine	
Isopentylamine	120
n-Pentylamine	
Triethylamine	
Aniline	190
Benzylamine	
Cyclohexylamine	
Heptylamine	
Hexylamine	
N,N-Dimethylaniline	
Octylamine	
Piperidine	
Tripropylamine	
o-Toluidine	210
Piperazine	
Tributylamine	
Triisopentylamine	
Tripentylamine	
Isophorondiamine	230

RESULTS AND DISCUSSION

Fig. 1 shows the separation of some amines at a level of 1 ppm on a Pennwalt column with the Varian TSD. The baseline noise is low at high temperatures and the peaks are symmetrical. The importance of different parameters for the analysis will now be considered.

The packing

Pennwalt 223, Triton X-100 and Dowfax 9N9 stationary phases are often recommended for amine analysis, although Pennwalt is considered to be the best[11]. The other two stationary phases are polyoxyethers. The mass spectrum of Pennwalt 223 indicates that it is a mixture of two components. The main component consists of a polyether chain terminated by two phenyl rings, with a hydrocarbon chain on each ring. The minor component contains a methyl group instead of one of the phenyl rings. The molecular peaks occurred at *m/e* 554 and *m/e* 366. The IR spectrum of Pennwalt 223 is very similar to that of Triton X-100, except that Pennwalt 223 seems to lack free hydroxyl groups. This is favourable with respect to peak symmetry for hydrogen-bonding solutes. Elemental analysis confirms the absence of nitrogen in the molecule. Thus, column bleeding will only give compounds containing carbon, hydrogen and oxygen. The detector response to the bleeding will be low due to the high detector selectivity. In using a FID at 200°C a sensitivity of 10^{-9} A.f.s.

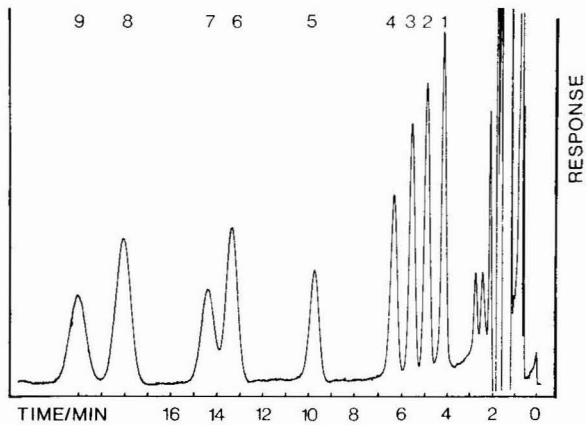

Fig. 1. Chromatogram of a mixture of amines in hexane with 500 ppm ammonia added. Injection 2 μl. Solutes (each 0.5 ppm): 1 = hexylamine; 2 = tripropylamine; 3 = cyclohexylamine; 4 = heptylamine; 5 = octylamine; 6 = aniline; 7 = tributylamine; 8 = N,N-dimethylaniline; 9 = *o*-toluidine. Column: 28% Pennwalt 223 and 4% KOH on Gas-Chrom R (80–100 mesh). Carrier gas: nitrogen, flow-rate 20 ml/min. Detector: TSD with a bias voltage of −10 V, a bead current of 2.95 divisions, a hydrogen flow-rate of 4.6 ml/min and an air flow-rate of 175 ml/min. Temperature: 190°C. Attenuation $4 \cdot 10^{-12}$ A.f.s.

was reported[11]. This is low compared with the sensitivity found usable with TSD in this work, $4 \cdot 10^{-12}$ A.f.s.

A high liquid loading (28%) was selected in order to obtain a high column capacity, which is favourable for trace analysis. The properties of the packing were stable, as demonstrated by the fact that the relative retention of benzylamine compared with heptylamine changed less than 1% during 6 months. The decomposition of the Pennwalt 223 led to a decrease in retention times of about 6% during this time. Thus, the separation efficiency was virtually unchanged during these experiments.

The solvent

Organic solvents. *n*-Hexane, *n*-nonane, diethyl ether and ethanol, as well as mixtures of alkanes and ethanol, were used as solvents. All are suitable for trace analysis of volatile amines with the Varian TSD detector, if the amines are eluted later than the solvent. However, they cannot be used for trace analysis with the FID due to the tailing of the solvents. Even for such a highly volatile solvent as diethyl ether there are difficulties in analysing concentrations of 50 ppm octylamine with the FID. On the other hand, 0.1 ppm octylamine can easily be determined using the TSD. The only way of increasing the sensitivity in organic solvents, when using the FID and Pennwalt packing, seems to be to use solvents which are eluted after the sample components. For this purpose we tested the solvents squalane, diisobutyl phthalate, diisooctyl phthalate, 1,10-dodecanedinitrile and 3,3′-oxydipropionitrile. Diisobutyl phthalate was found to be the best, permitting determinations of a mixture of octylamine and benzylamine at a level of about 10 ppm.

One of the long chain solvents, 3,3′-oxydipropionitrile (ODPN), decomposed on the column at the temperatures investigated (above 160°C). This was confirmed by MS. Direct inlet MS showed the molecular peak of ODPN, while injection of 2 μl

ODPN on the column gave fragments characteristic of propenenitrile. ODPN has a good thermal stability as a GLC stationary phase. It has been analysed as a solute without degradation on high temperature stationary phases such as SF 96[12]. The basic character of the packing might be responsible for the decomposition. A water molecule and two propenenitrile molecules appear as reaction products. We note that another solvent, 1,10-dodecanedinitrile, similar to ODPN, was eluted after about 2 h at 180°C.

It can be concluded that amines having between 5 and 15 carbon atoms can be analysed at a level of about 0.1 ppm with the TSD by using a solvent such as *n*-hexane or ethanol which are both eluted before the sample components (Fig. 2). For volatile amines it is preferable to use an alkane or an alcohol which is eluted after the sample components. The sensitivity for volatile amines is then better than 0.03 ppm (Fig. 3). By making full use of the column capacity, concentrations even less than 0.01 ppm can be determined with a sample size of 5 μl. Ethylamine and trimethylamine were only partially separated on this column. An optimization of the resolution was outside the scope of this paper.

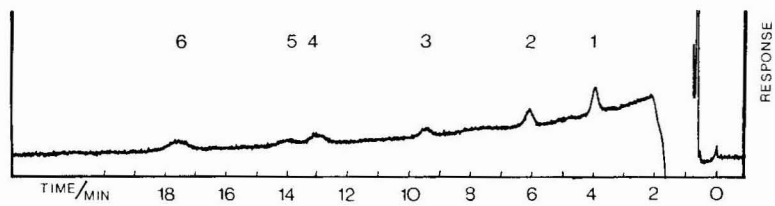

Fig. 2. Chromatogram of a mixture of amines in ethanol with 500 ppm ammonia added. Injection 3 μl. Solutes (each 0.1 ppm): 1 = hexylamine; 2 = heptylamine; 3 = octylamine; 4 = benzylamine; 5 = tributylamine; 6 = N,N-dimethylaniline. Attenuation: $2 \cdot 10^{-12}$ A.f.s. Other conditions as in Fig. 1.

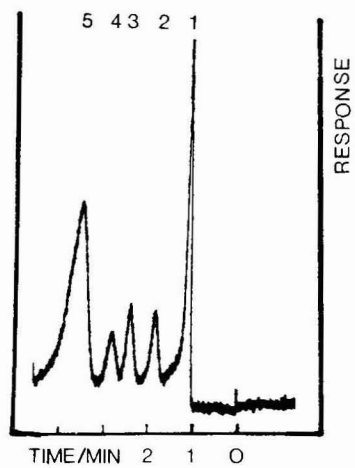

Fig. 3. Chromatogram of volatile amines in ethanol with 500 ppm ammonia added. Solute concentrations: 1–3, about 0.03 ppm; 4, about 0.15 ppm. Sample size 1.8 μl. Solutes: 1 = ammonia; 2 = methyl amine; 3 = dimethylamine; 4 = ethylamine; 5 = isopropylamine. Temperature: 5 min at 80°C, programmed from 80 to 120°C at 20°C/min, then 5 min at 120°C. Attenuation: $1 \cdot 10^{-12}$ A.f.s. Other conditions as in Fig. 1.

Water as solvent. Water was injected (2 μl), when the detector was operated at high sensitivity ($1 \cdot 10^{-12}$ A.f.s. with the TSD and $4 \cdot 10^{-11}$ A.f.s. with the FID). Typical chromatograms are shown in Figs. 4 and 5. From these chromatograms it can be seen that either water or some degradation products are eluted from the column and interfere with the detection. Chromatograms obtained with the FID show that determinations above 100°C would be irreproducible. With the TSD, the ghost peak from the water injection prevents determinations of amines in part of the chromatogram, if the temperature is above 80°C. The baseline is restored much faster than with FID.

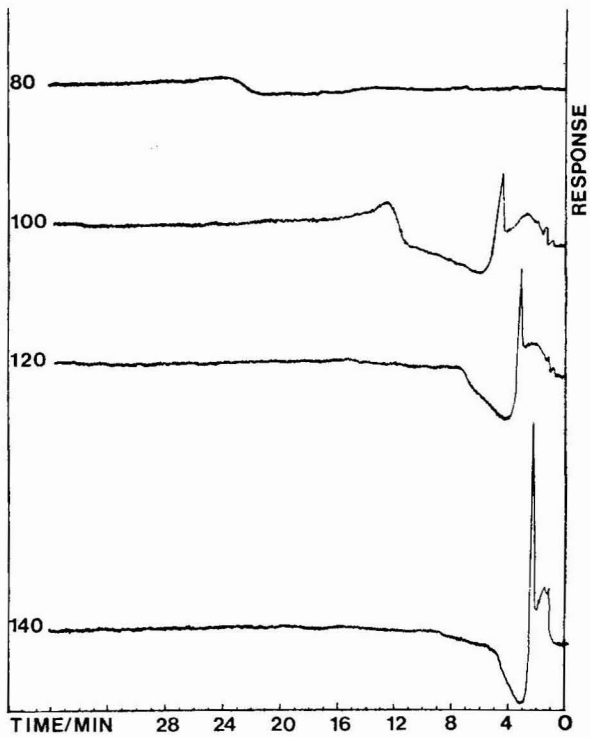

Fig. 4. Chromatograms from injections of 2 μl of water at various temperatures (80–140°C) with TSD. Attenuation: $1 \cdot 10^{-12}$ A.f.s. Other conditions as in Fig. 1.

The problem of interfering peaks when injecting aqueous solutions of amines is well known. Ghost peaks have been obtained on different column packings[13,14]. Onuska[15] reported that the Pennwalt packing is stable at 134°C. It is also stated in a technical bulletin[11] that one of the merits of the Pennwalt 223 packing is that it adsorbs water completely up to 160°C. The earlier investigations were performed at much lower detector sensitivity than used here. The effects shown in Fig. 5 were probably too small to be observed in the previous studies.

The influence of water on the Pennwalt 223 packing was also studied by MS. The baseline spectrum was subtracted from spectra taken during elution of the ghost

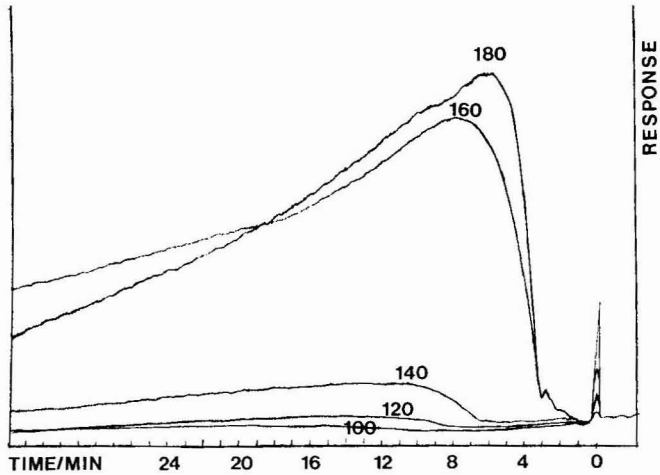

Fig. 5. Chromatogram from injections of 2 μl of water at various temperatures (100–180°C) with FID. Attenuation: $4 \cdot 10^{-11}$ A.f.s. Other conditions as in Fig. 1.

peak. The results showed that the peak was completely or almost completely due to water. On the other hand, it is unlikely that the FID detector would give such a large peak for water. Therefore it seems more probable that small amounts of decomposition products are eluted simultaneously with water. This is also consistent with the results in Fig. 4. The chromatograms, obtained with the TSD, show one sharp positive peak followed by a dip in the baseline. The conditions were chosen so that hydrocarbons gave a small but positive signal. The peak is probably due to hydrolysis products and the dip is caused by a decrease in sensitivity when the ceramic bead is cooled by the eluting water.

For practical analysis of volatile amines with TSD the negative influence of water can easily be avoided by choosing a low column temperature. Fig. 6 shows an analysis at 60°C. The performance of the column at higher temperature can be

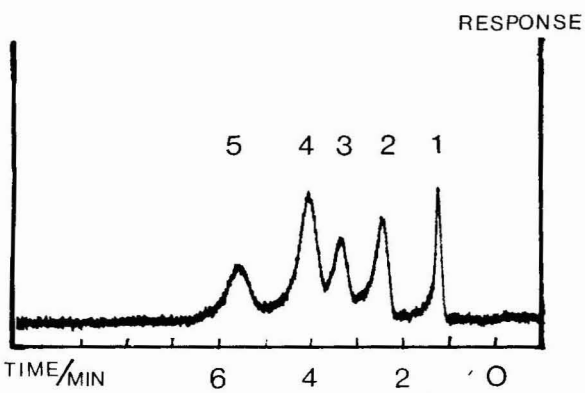

Fig. 6. Chromatogram from injection of 3 μl of a mixture of volatile amines in water. Solute concentration 0.1 ppm. Solutes: 1 = ammonia; 2 = methylamine; 3 = dimethylamine; 4 = ethylamine; 5 = isopropylamine. Temperature: 60°C. Attenuation: $1 \cdot 10^{-12}$ A.f.s. Other conditions as in Fig. 1.

improved substantially if the sample size is decreased. Aqueous amine samples (0.1 ppm, size $\leqslant 1$ μl) can be determined using column temperatures in the range of 100–200°C. One obvious drawback is that the high capacity of the column cannot be utilized, which increases the determination limit for the amines.

Alkali-treated (NaOH or KOH) column packings have been used in those investigations where spurious water peaks and the negative influence of large samples have been noted. Di Corcia *et al.*[7] showed the favourable effect of a decrease of the loading of KOH from 0.8% to 0.3% w/w on Carbopack B. The same effect was achieved by decreasing the amount of water. Thus both these factors must be controlled to optimize the analysis.

The ammonia concentration

Injection of the five lowest boiling amines at 0.03 ppm gave increasing peak areas with ammonia concentrations up to 5000 ppm where they levelled off. The peak areas were corrected for a small amount of volatile amines present in the ammonia reagent. The increase of peak area with ammonia concentration was important only at amine concentrations below 1 ppm. However, some ammonia must be added to the test-tube in which the sample is stored. Results for methylamine and dimethylamine are shown in Fig. 7. The percentage adsorption was calculated from the change in peak areas, taking the area at 5000 ppm ammonia as a reference. For heptylamine and benzylamine (at 1 ppm) the peak area increased by only about 10% and 3% respectively when the ammonia concentration increased from 100 to 1000 ppm. The smaller increase compared with the short chain amines can be explained by their lower polarity. The difference between heptylamine and dibenzylamine is obviously due to the aromatic character of the latter.

The adsoption in test-tubes and syringes was investigated by filling the test-tube with methylamine (0.1 ppm) and ammonia (0–5000 ppm) in ethanol. The tube was emptied and filled with a solution of 5000 ppm ammonia in ethanol. A sample

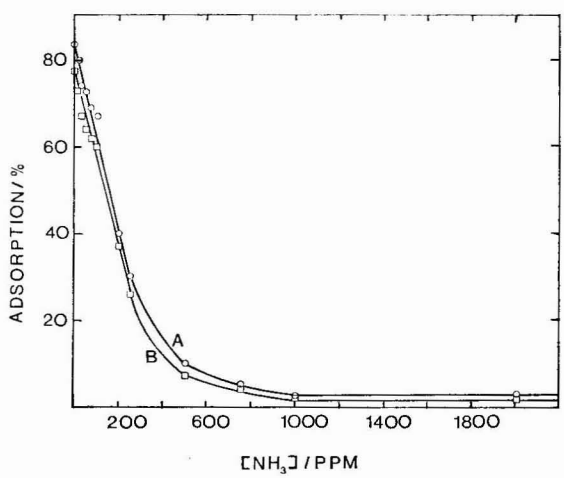

Fig. 7. Percentage adsorption of methylamine (A) and dimethylamine (B) at various concentrations of ammonia and with ethanol as solvent. Solute concentration 0.03 ppm. Column temperature 80°C. Attenuation: $1 \cdot 10^{-12}$ A.f.s. Other conditions as in Fig. 1.

was injected into the gas chromatograph. For this sample, the change in peak area due to adsorption on the columns can be neglected (see Fig. 7). The results are shown in Fig. 8. The percentage adsorption was calculated by taking the peak area from the original test-tube solution, containing 5000 ppm ammonia, as reference. The adsorption decreases rapidly with increasing ammonia concentration, and becomes negligible at 100 ppm. Hexylamine, heptylamine, octylamine and N,N-dimethylaniline were also investigated in the same way. An ammonia concentration of 100 ppm prevented adsorption on glass surfaces even at amine concentrations of 1 ppm.

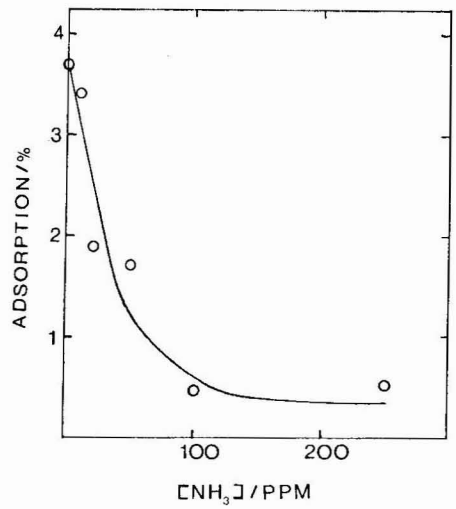

Fig. 8. Adsorption of methylamine on glass surfaces from an ethanolic solution containing various concentrations of ammonia. Concentration of methylamine 0.1 ppm. Temperature: 22°C.

The adsorption in test-tubes and syringes is small compared with the adsorption effects on the column (see Figs. 7 and 8). An ammonia concentration of about 500 ppm is sufficient to obtain a good reproducibility. At this concentration the losses due to adsorption level off, making unimportant small variations in ammonia concentration. The peak shapes of amines at low concentrations, especially for methylamine, are improved after addition of ammonia. The tailing is reduced and the peaks become higher, but the retention times are virtually unchanged. The same behaviour was observed by Dunn *et al.*[6], who used traces of ammonia in the carrier gas stream.

It should be mentioned that, although a nitrogen-selective detector is used, the detector response ratio of ammonia to amines is only $1:10^4$, *i.e.*, large amounts of ammonia can be present with little effect on the baseline.

Reproducibility and linearity

The reproducibility and linearity were tested by using amine samples containing 500 ppm ammonia. The samples were stored in closed vials. Repetitive injection of 2-μl samples gave a standard deviation of 2–4% (five injections) in different solvents (ethanol, *n*-hexane and water). The concentration of volatile amines (chain length up to three carbon atoms) was 0.2 ppm and 0.4 ppm for the others. The reproducibility

is equal to that expected when considering the high detector sensitivity and that the peak area was determined by triangulation. The calibration curves were linear in the range 0.1–5 ppm for the solutes given in Table I. Higher concentrations were not investigated.

The variation of peak area with sample size was investigated up to 7 μl and found to be linear. The high liquid loading (28 %) gives high column capacity.

CONCLUSIONS

With knowledge of the characteristics of the Pennwalt packing, the solvent effects and the influence of ammonia, it is possible to obtain reproducible analyses of amines down to 0.03 ppm for short chain amines and to 0.1 ppm for other amines. The column performance was satisfactory. With organic solvents it was possible to inject samples larger than 7 μl thus decreasing the determination level. Volatile amines can also be determined at low concentrations in water by keeping the column temperature below 80°C. The sensitivity can be increased by another factor of 5–10 by increasing the temperature of the ceramic bead. However, this reduces the lifetime of the bead and decreases the selectivity towards hydrocarbons. The stability of the column packing and the detector mean that the system is well suited for routine analysis of trace amounts of amines. Many of the precautions reported in earlier investigations have not been necessary here, probably because of the addition of ammonia and the use of a selective detector.

ACKNOWLEDGEMENT

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DETERMINATION OF CYANIDES AND THIOCYANATES IN WATER BY HEADSPACE GAS CHROMATOGRAPHY WITH A NITROGEN–PHOSPHORUS DETECTOR

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SUMMARY

A simple method for the determination of trace amounts of CN^- and/or SCN^- in water is suggested. The method is based on the prior transformation of CN^- or SCN^- into HCN; the HCN is separated from the aqueous sample by the headspace technique, analysed by gas–solid chromatography and selectively detected with a nitrogen–phosphorus detector. Cyanides and/or thiocyanates at concentrations between 0.01 and 100 ppm can be detected with a linear response. The procedure has been used for the determination of CN^- in coke-oven water and in coke-oven waste effluent.

INTRODUCTION

In 1973 we proposed a gas chromatographic procedure for the determination of cyanide and thiocyanate[1] that was much simpler, more sensitive and freer from interferences than previous commonly used methods[2–4]. The availability of the headspace technique and of the selective nitrogen–phosphorus detector prompted us to develop a selective and sensitive procedure for the determination of cyanide and/or thiocyanate in water. The procedure for the determination of CN^- is based on three stages; first, transformation into HCN by acidification; second, removal of HCN by the headspace technique; and third, gas chromatographic separation of HCN and selective detection with a nitrogen–phosphorus detector. A similar procedure is adopted for the determination of thiocyanate, the only difference being the quantitative transformation of thiocyanate into HCN according to the reactions:

$$SCN^- + Br_2 + 4H_2O \rightarrow BrCN + SO_4^{2-} + 7Br^- + 8H^+$$
$$BrCN + Red \rightarrow HCN + Br^- + Ox$$

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(where Red = SO_2 or I^- and Ox = SO_4^{2-} or I_3^-). If cyanide is present prior to the oxidation step, it must be transformed into unreactive cyanohydrin by an excess of formaldehyde or removed by boiling the solution previously acidified to pH 2.

EXPERIMENTAL

Reagents

KCN, KSCN, 85% H_3PO_4, 40% HCHO, Na_2SO_3, KI and phenol were pure products obtained from Carlo Erba (Milan, Italy). Bromine water was prepared as a saturated solution of bromine (Carlo Erba) in distilled water.

Gas chromatograph

An F42 gas chromatograph (Perkin-Elmer) equipped with a headspace device and a nitrogen–phosphorus detector was used. The column (2 m × 0.3 cm I.D.) was made of borosilicate glass and packed with Porapak Q (80–100 mesh), supplied by Waters Assoc. (Milford, MA, U.S.A.). Nitrogen was used as the carrier gas at a flow-rate of 40 ml/min; the flow-rates of hydrogen and air were 4 and 150 ml/min, respectively. The injector and detector temperatures were 120 and 150°C, respectively, and the oven temperature was 100°C. The detector wire was heated as recommended by the manufacturer (Perkin-Elmer).

The headspace conditions were as follows: needle temperature, 100°C; flushing of sampling capillary, 15 sec; pressurization of sample vial, 60 sec; sample withdrawal from headspace, 5 sec; 20-ml vials thermostated at 80°C for 10 min were used. Under these conditions the retention time of HCN was 5 min.

Analysis of CN^-

The procedure described below can be adopted regardless of the presence of SCN^- in the sample. A 5.0-ml volume of aqueous sample containing between 0.01 and 100 ppm of CN^- and 2 drops of 85% H_3PO_4 (to give a pH of 2) are introduced into a 20-ml vial. The sample is then analysed under the conditions given above. Any oxidizing agents present in the sample must be reduced with Na_2SO_3 prior to heating of the vial.

Analysis of SCN^- in the absence of CN^-

A 5.0-ml volume of aqueous sample containing between 0.02 and 200 ppm of SCN^- and 2–3 drops of 85% H_3PO_4 (to give a pH of 2) are introduced into a 10-ml volumetric flask. Bromine water is added dropwise until a persistent yellow colour is obtained, and the excess of bromine is removed after 5 min by the addition of 0.5 ml of 5% phenol solution. The quantitative removal of the excess of bromine is indicated by the attainment of a colourless solution.

BrCN is reduced by means of 0.5 ml of 0.1 *M* Na_2SO_3 solution or a few crystals of KI. Distilled water is added to the 10-ml mark. A 5.0-ml volume of this solution is introduced into the headspace vial and analysed under the conditions given above.

Analysis of SCN^- in the presence of CN^-

Two procedures are suggested for eliminating the interference of CN^-.

Removal of CN^- by heating. A 50.0-ml volume of the sample, to which 1 ml of 85% H_3PO_4 has been added is boiled for 20 min in a beaker. After cooling, the sample is treated as described in the previous section.

Treatment of the sample with formaldehyde. To 4.00 ml of sample in a 10-ml flask, 0.2 ml of phosphate buffer (pH = 7) and 0.1 ml of 4.0% (w/v) HCHO are added. After 5 min, 0.5 ml of 85% H_3PO_4 and 3–4 drops of bromine water are added. The solution is allowed to stand 15 min (it must still be yellow), then the excess of bromine is removed by the addition of 0.5 ml of 5% phenol solution. The BrCN formed is reduced by adding 0.5 ml of 0.1 *M* Na_2SO_3 solution or some crystals of KI. The solution is diluted to 10 ml with distilled water and 5.0 ml are introduced into a 20-ml headspace vial and analysed as described above.

Calibration graphs

Calibration graphs for both CN^- and SCN^- can be obtained by diluting solutions containing known concentrations of CN^- according to the method described above and plotting peak height against concentration.

RESULTS AND DISCUSSION

The chromatogram in Fig. 1 shows that HCN gives a symmetrical peak, so the peak height can be conveniently used instead of peak area in the calculation. The peak height is proportional to the concentration of HCN or SCN^-, at least in the concentration range between 0.01 and 100 ppm. More concentrated solutions have not yet been tested. The plot in Fig. 2, obtained by using a solution containing known concentrations of HCN and SCN^- (transformed into HCN by the previously described procedure) shows the linearity of the response and the quantitativeness of the transformation of SCN^- into CN^-. Table I gives some results for a series of measure-

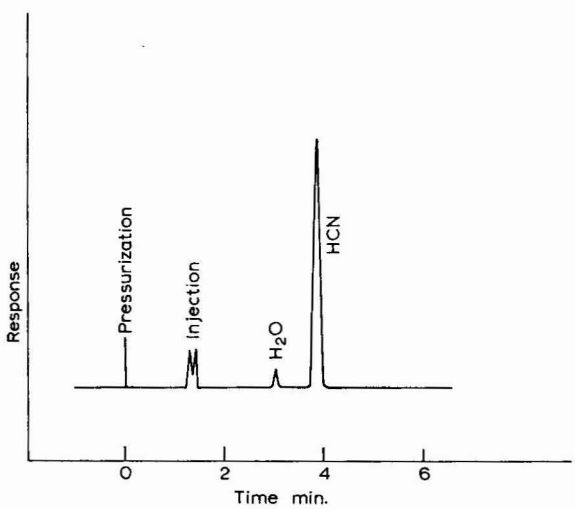

Fig. 1. Gas chromatogram obtained from a 0.10 ppm aqueous solution of cyanide.

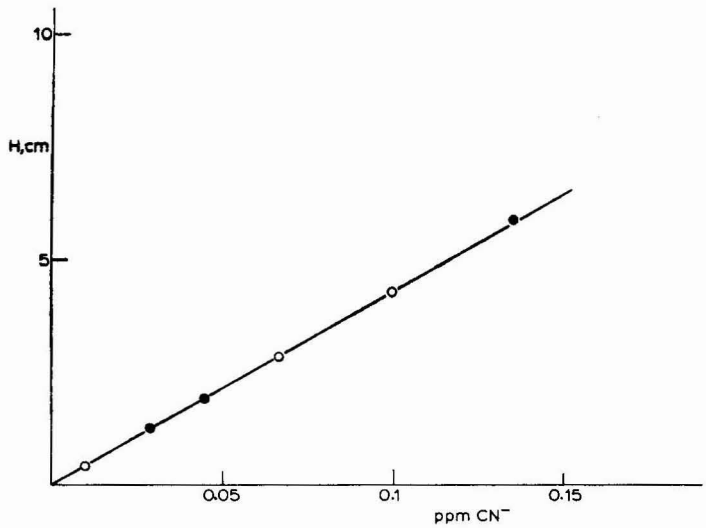

Fig. 2. Calibration graph obtained from standard aqueous solutions of (○) cyanide and (●) thiocyanate as cyanide.

ments on CN^-. The precision and accuracy of the method are indicated in the last two columns in Table I.

The determination of CN^- under the conditions described is not affected by the presence of SCN^-. No response was shown by solutions containing up to 100 ppm of SCN^-.

TABLE I

RESULTS OF SOME TEST MEASUREMENTS

Amount of CN^- (ppm)	*No. of measurements*	*Average peak height (cm)*	*Standard deviation (cm)*	*Relative standard deviation (%)*
50.0	4	7.6	0.12	1.6
3.00	5	10.7	0.10	0.93
0.05	4	8.2	0.12	1.5

For the determination of SCN^- in the presence of CN^- it is sufficient to boil the sample for 20 min, after acidifying at pH 2, in order to remove completely CN^-. No loss of SCN^- was observed because of the heating. Similar results were obtained by adopting the procedure that involves the use of formaldehyde. Tables II and III gives the results of a series of measurements carried out to demonstrate the quantitative recovery of SCN^- after both thermal and formaldehyde treatment for the removal of HCN. The response for HCN does not vary appreciably when volumes of solutions between 3 and 10 ml are used in the headspace vial.

Table IV shows the effect of the salt concentration on the response for HCN. The matrix effect can be avoided by making use of the method of standard additions, as illustrated by the results in Table V and the plot in Fig. 3 for a solution containing 2 ppm of CN^-.

TABLE II

COMPARISON BETWEEN BOILED AND UNBOILED SCN^- SOLUTIONS

Unboiled samples		*Boiled samples*	
CN^- (ppm)	*Peak height (cm)*	*CN^- (ppm)*	*Peak height (cm)*
100	11.1	100	11.1
10	14.1	10	14.2
1	7.2	1	7.1

TABLE III

RECOVERY OF SCN^- IN SAMPLES TREATED WITH FORMALDEHYDE AFTER OXIDATION BY BROMINE

Samples containing HCHO			*Peak height of 2 ppm SCN sample without HCHO (cm)*
SCN^- (ppm)	*Reaction time (min)*	*Peak height (cm)*	
2	5	10.6	11.0
2	10	10.8	
2	15	11.1	
2	20	11.0	

TABLE IV

INFLUENCE OF SODIUM CHLORIDE CONCENTRATION ON THE HCN RESPONSE

Amount of CN^- (ppm)	*NaCl added (%)*	*Peak height (cm)*	*Increase (%)*
1.00	0	7.4	—
1.00	10	8.2	11
1.00	20	8.8	19
1.00	30	9.4	27

TABLE V

STANDARD ADDITIONS METHOD APPLIED TO A 2 ppm CN^- SAMPLE

Aliquot No.	*Amount of CN^- present (μg)*	*Sample volume (ml)*	*CN^- added (μg)*	*Peak height (cm)*
1	10	5.00	0.00	5.5
2	10	5.00	1.00	6.1
3	10	5.00	2.00	6.5
4	10	5.00	4.00	7.7
5	10	5.00	5.00	8.3

The presence of Fe^{2+}, Fe^{3+} and Cu^{2+} in the sample decreases the response for HCN. The results in Table VI indicate that the greatest effect is caused by Cu^{2+}.

Reducing agents do not interfere in the analysis of CN^-, but oxidizing substances have to be reduced prior to heating of the sample. Oxidants and reducing agents do not interfere in the determination of SCN^-.

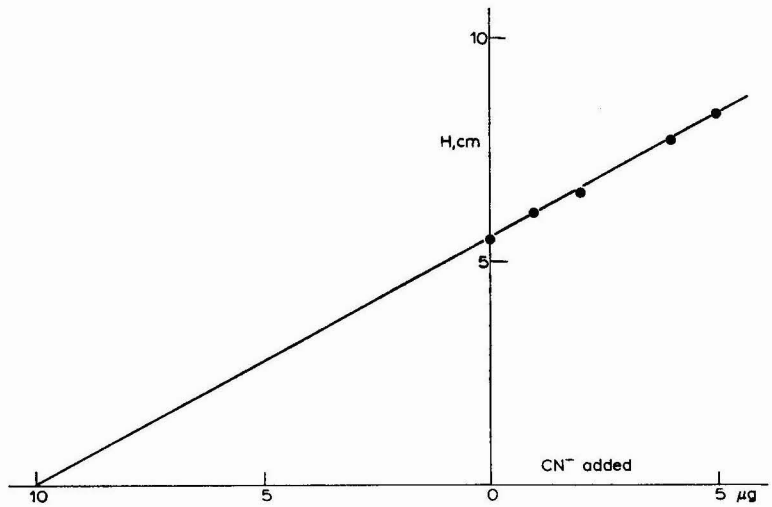

Fig. 3. Standard additions plot for the results in Table V.

TABLE VI

INFLUENCE OF SOME IONS ON THE RESPONSE OF HCN

Amount of CN^- (ppm)	*Amount of Fe^{2+} (ppm)*	*Peak height (cm)*	*Amount of Fe^{3+} (ppm)*	*Peak height (cm)*	*Amount of Cu^{2+} (ppm)*	*Peak height (cm)*
1	0	12.8	0	12.8	0	12.8
1	10	11.7	10	11.0	10	8.4
1	50	11.3	50	9.9	50	8.2
1	100	11.1	100	8.8	100	8.0

For comparable amounts of CN^- and SCN^-, the time of analysis can be considerably decreased by determining CN^- and the sum of CN^- and SCN^- on two different aliquots of the sample, SCN^- being obtained by difference.

The use of the headspace technique is likely to make the proposed method extremely selective (*i.e.*, free from interferences).

The procedure described, indicated as the "HS method", has been used for the determination of CN^- in coke-oven water and in coke-oven waste effluent for disposal in the sea. The same samples were also analysed by the previous procedure[1,5], indicated as "BrCN method".

Analysis of CN^- in coke-oven water and waste effluent

Analysis of CN^- in coke-oven water. A 1.00-ml volume of aqueous sample containing between 25 and 5000 µg of CN^- and 5 ml of 20% H_3PO_4 (to give a pH of 2) are introduced into a 50-ml flask and the solution is made up to 50 ml with distilled water. A 5.00-ml volume of the solution is introduced into a 20-ml vial and analysed under the conditions given under Experimental. Any oxidizing agents present in the sample must be reduced with Na_2SO_3 prior to analysis.

The calibration graph for CN^- can be obtained by diluting solutions containing known concentrations of CN^- according to the method described above. The peak heights are plotted against amount of CN^-.

Analysis of CN^- in coke-oven waste effluent for disposal in the sea: method of standard additions. Into four 10-ml flasks, are introduced 5.0 ml of aqueous sample containing between 0.1 and 1000 μg of CN^-, 1.0 ml of 20% H_3PO_4 and 0.0, 1.0, 3.0 and 5.00 μg of CN^-. The solutions are made up to 10 ml with distilled water and 5.00 ml of each solution are introduced into four 20-ml vials and analysed under the conditions given under Experimental. The peak heights are plotted against amount of CN^-. The points give a straight line and the intercept with the abscissa represents the amount of CN^- present in a particular sample. Any oxidizing agents present in the sample must be reduced with Na_2SO_3 prior to analysis.

As the HCN content in coke-oven water is of the order of 50–100 ppm, it is convenient to dilute the sample 50-fold in order to avoid matrix effects without any decrease in accuracy. In such a case it is possible to use the calibration graph directly. In the samples of coke-oven waste effluent for disposal in the sea, the HCN concentration is between 0.2 and 0.5 ppm so that it is not possible to avoid matrix effects by dilution. For these samples the results obtained by a calibration graph method have poor accuracy; matrix effects are avoided by using the standard additions method and the results are accurate.

Typical results for the analysis of CN^- in some coke-oven waste waters and coke-oven waste effluents for disposal in the sea are reported in Table VII.

The results for samples A–D analysed by the HS method and the BrCN method are in good agreement. Samples E–H show good agreement when analysed by the HS method and the standard additions method; the results obtained by using a calibration graph are appreciably higher owing to the different matrix of the standards and the samples.

The accuracy of the procedure was tested by spiking samples A–H with a known amount of CN^-; the recoveries, reported in the last two columns in Table VII, are excellent.

TABLE VII

COMPARISON WITH BrCN METHOD AND ACCURACY OF PROPOSED PROCEDURE

Sample	*CN^- determined (ppm)*			*CN^- added (ppm)*	*CN^- recovered by HS method (ppm)*	
	BrCN method	*HS method*				
		By calibration graph	*By standard additions method*		*By calibration graph*	*By standard additions method*
A	53	52	–	25.0	25.1	–
B	65	65	–	20.0	20.0	–
C	42	43	–	20.0	25.2	–
D	58	57	–	5.00	5.04	–
E	0.20	0.23	0.21	0.150	–	0.150
F	0.37	0.41	0.38	0.150	–	0.150
G	0.25	0.28	0.25	0.150	–	0.149
H	0.30	0.34	0.30	0.020	–	0.021

In contrast to the BrCN method, the procedure suggested here allows CN^- to be determined regardless of the presence of SCN^-, which is always present in the samples studied; in addition, the linearity range is wider and the procedure is easily automated.

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SEPARATION OF α- AND β-TRYPSIN BY HYDROPHOBIC INTERACTION CHROMATOGRAPHY

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SUMMARY

The separation of α- and β-trypsin by means of hydrophobic chromatography on Spheron P 300 was investigated with respect to the separation conditions, *i.e.*, salt concentration, pH, temperature, sample loading, flow-rate and support particle size. The optimal conditions have been selected at low pH (3.0) where the autodigestion of trypsin is suppressed. This method based on different exposures of hydrophobic amino acid residues of α- and β-trypsin is rapid, simple and effective for both analytical- and preparative-scale separations.

INTRODUCTION

The structure of the trypsin molecule and the mechanism of its action have been studied in considerable detail. At present trypsin is one of the best known proteins. Therefore it provides an important and widely used model system for kinetic as well as physical studies. Such studies require a well defined form of trypsin, preferably the uncleaved, single chain β-trypsin which retains full enzymatic activity. Studies in which trypsin is used for protein fragmentation require enzyme of the same quality[1]. Most commercial trypsin preparation contain some inactive material and ψ-trypsin of low activity[2], in addition to both the active forms, β-trypsin and the double chain α-trypsin. The content of the individual constituents is often unknown, however.

A number of methods for the isolation of α- and β-trypsin has been developed based on ion-exchange[2] and affinity[3–5] chromatography. The method of Schroeder and Shaw[2] using chromatography on SE-Sephadex at neutral pH with *p*-aminobenzamidine in the elution buffer has proved to be the most successful and is frequently employed.

In this paper we introduce a procedure for effective separation of α- and β-trypsin based on differences in the accessibility of the hydrophobic amino acid residues in

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the neighbourhood of the active site[6]. The fractionation of both active forms of trypsin was studied with respect to the type of salt, salt concentration, pH, temperature, sample loading and flow-rate. Optimal conditions for preparative as well as analytical scale separations have been determined.

MATERIALS AND METHODS

Trypsin from bovine pancreas (lyophilized, TRL L AA, 207 U/mg) was obtained from Worthington (Freehold, NJ, U.S.A.), trypsinogen from bovine pancreas (3000 NF E/mg) from Fluka (Buchs, Switzerland) and *p*-aminobenzamidine from Koch-Light (Colnbrook, Great Britain). Spheron P 300 (20–40 μm) was supplied by Lachema (Brno, Czechoslovakia), Spheron P 300 (14.7 $\pm$ 1.6 μm) from Spolek (Ústí n. Labem, Czechoslovakia). Other chemicals (analytical grade) were from Lachema. The sorbent extraction and other characteristics have been reported previously[7].

Equipment

The chromatographic apparatus consisted of a Type 68005 minipump connected to a 2-ml pre-column, a 75-μl sample loop, an injection septum head, a precision bore glass column (8.00 mm I.D.) with adjustable ends and a thermostated water jacket. The absorbance was recorded at 254 or 280 nm on a Type UVM-4 spectromonitor. All these instruments were made by the Instrument Development Workshops of the Czechoslovak Academy of Sciences. Polyacrylamide gel electrophoresis of reduced protein samples in sodium dodecyl sulphate were described previously[6]. The pH value were determined on a digital MV 87 pH meter (Prätcitronic, G.D.R.), using a GK 2301 C electrode (Radiometer, Copenhagen, Denmark). They were not corrected for high salt concentrations. Determination of tryptic activity was done in the usual manner[6].

RESULTS AND DISCUSSION

In previous papers[7,8] Spheron was shown to provide a rigid and highly cross-linked glycolmethacrylate matrix and non-polar binding sites suitable for hydrophobic interaction chromatography of large peptide fragments and particularly of proteins. In contrast to the frequently used Sepharose derivatives which interact also with the hydrophobic surface amino acid residues of proteins in crevices ("pockets"), Spheron displays different mechanical as well as binding properties. It was found to interact entirely with the surface exposed groups of proteins.

Salt concentration dependence of α- and β-trypsin separation

Trypsin is one of the proteins with both types of non-polar surface side chains mentioned above. However, the separation of α- and β-trypsin can be accomplished more easily on Spheron. β-Trypsin appears to exhibit a larger exposed non-polar surface area than α-trypsin, localized in the proximity of the active site[6]. Consequently, β-trypsin was found to be more retained on the Spheron column than α-trypsin under all the conditions examined. Fig. 1 illustrates the elution pattern of a commercial trypsin sample obtained with a Spheron P 300 column in the course of isocratic elution with various concentrations of $(NH_4)_2SO_4$ in the elution buffer. The elution volumes of both active forms at 20°C and 30°C are indicated. Pseudotrypsin (or

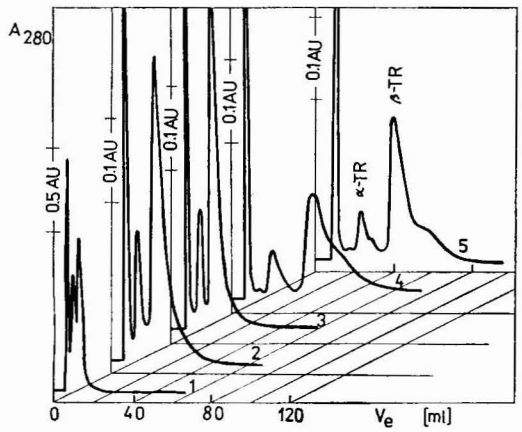

Fig. 1. Elution profiles of a commercial trypsin sample on HIC at pH 3.0 using various concentrations of ammonium sulphate. 1, 0.3 *M* $(NH_4)_2SO_4$, 20°C, 6 mg of trypsin; 2, 0.5 *M* $(NH_4)_2SO_4$, 20°C, 5 mg of trypsin; 3, 0.5 *M* $(NH_4)_2SO_4$, 30°C, 4 mg of trypsin; 4, 0.7 *M* $(NH_4)_2SO_4$, 20°C, 4 mg of trypsin; 5, 0.7 *M* $(NH_4)_2SO_4$, 30°C, 4 mg of trypsin. Trypsin was dissolved in 100 μl of the elution buffer [0.05 *M* formic acid with a given concentration of $(NH_4)_2SO_4$, pH adjusted to 3.0 with 2 *M* NaOH] and applied to a Spheron P 300 (14.7 ± 1.6 μm) column (250 × 8.0 mm I.D.), flow-rate 30 ml/h. A_{280} = Absorbance at 280 nm; V_e = elution volume in ml; α-TR = α-trypsin; β-TR = β-trypsin; AU = absorbance unit.

ψ-trypsin) and products of trypsin degradation as well as some impurities are present in the first peak, α-trypsin is in the second peak and β-trypsin is found in the third one. ψ-Trypsin as well as the majority of impurities present in the sample displays only very low retention even at high ammonium sulphate concentrations. On the other hand, both α- and β-trypsin are markedly retained if the salt concentration is increased; this effect is typical of hydrophobic interaction chromatography (HIC). If the concentration of $(NH_4)_2SO_4$ is raised from 0.3 *M* to 0.7 *M* at pH 3.0 the elution volume of β-trypsin is increased three-fold. An increase in the salt concentration followed by higher retention gives rise to an improvement in the peak resolution. Accordingly, the peak width is reduced at higher temperatures (30°C) because of the higher diffusion velocities. This brings about a remarkable restriction of tailing (Fig. 1). Likewise, a better separation of the small peak following the major peak of β-trypsin seems to contribute to the reduced tailing.

A significant decrease in the retention of β-trypsin can be accomplished by the addition of a low-molecular-weight trypsin inhibitor, *p*-aminobenzamidine to the elution buffer at a concentration of *ca.* $1 \cdot 10^{-3}$ *M*. The HIC pattern of natural trypsin inhibitors and their complexes with trypsin, as well as the above phenomenon, suggest that the hydrophobic amino acids adjacent to the trypsin active site are implicated in the trypsin retention on Spheron[6].

pH dependence

Fig. 2 illustrates the elution pattern of a commercial trypsin sample in 0.5 *M* $(NH_4)_2SO_4$ at various pH of the elution buffer. A pH decrease from 8.4 to 5.5 results in a decrease in the retention of both α- and β-trypsin. In contrast, a further decrease in pH to 4.0 results in a small increase in the retention, whereas a decrease in the

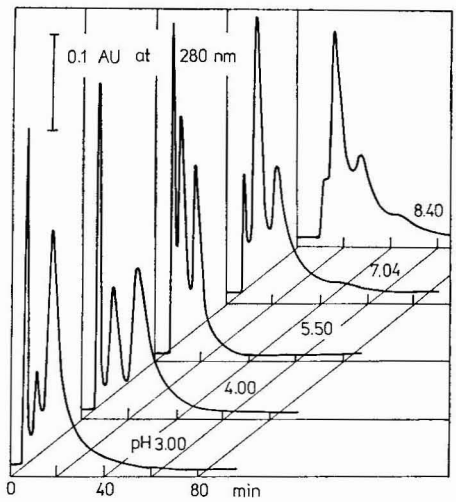

Fig. 2. Elution profiles of a commercial trypsin sample on HIC in 0.3 *M* $(NH_4)_2SO_4$ at different pH values. The elution buffer at pH 3.0 and pH 4.0 was the same as in Fig. 1.1; for pH 5.5, 7.04 and 8.4, 0.03 *M* phosphate buffer containing 0.3 *M* $(NH_4)_2SO_4$ was used. The elution profiles at pH 4.0 and 5.5 were obtained with 0.05 *M* acetate buffer as well. Flow-rate: 60 ml/h at 20°C. Sample: 0.77 mg of trypsin in 70 μl of the elution buffer. For other conditions and symbols see Fig. 1.

retention was again observed when the elution buffer was adjusted to pH 3.0 (Fig. 2). A different pattern of pH-dependence was found when a higher concentration of NaCl in the elution buffers was used to enhance the interaction between trypsin and the support. The relatively flat dependence of the retention upon pH was found in the range pH 8.0–5.5 in 0.5 *M*, 1 *M* and 1.5 *M* NaCl solutions. When the pH was less than 5.5 the retention increased sharply with increasing acidity[6].

The nature of the hydrophobic interaction between a protein and a chromatographic support appears to be complex. Both the salts examined, NaCl and $(NH_4)_2SO_4$, exhibit "salting out" characteristics and are known to enhance hydrophobic interactions[9–11]. The pH dependence, however, is essentially different with the two salts as a result of the character of the anion. The HIC data available for a variety of proteins in buffers containing either $(NH_4)_2SO_4$ or Na_2SO_4 and KCl or NaCl are in agreement with the dominant rôle of the anion.

An apparent increase in the content of α-trypsin as compared to β-trypsin at pH 4.0 is illustrated in Fig. 2. The α-trypsin peak is four times larger than that at pH 3.0, and small changes during sample preparation and chromatography are observed even at pH values as low as 3.5 (not shown in Fig. 2).

For pH $\geqslant$ 5.5, the first peak containing ψ-trypsin and the other inactive impurities is reduced. This is explained by the fact that material responsible for this peak is cleaved by active trypsin into small peptides. Moreover the peptides of higher absorbance at 280 nm, containing aromatic amino acids, show a higher retention as compared to the unaffected material and to the peptides of lower hydrophobicity.

Considering the autolysis of trypsin, the separation procedure at pH 3.0 appears to be optimal for isolation of β-trypsin as well as for a rapid analysis of trypsin samples.

Temperature dependence

The temperature dependence of the trypsin retention in 0.3 *M* $(NH_4)_2SO_4$ at pH 3.0 was investigated in the range 15–50°C. The increase in temperature does not influence the peak size of the main components, *i.e.*, of α- and β-trypsin, but the first peak of inactive constituents is reduced. This peak is influenced even by temperatures as low as 25°C. When the temperature is raised to 35°C, a decrease in the second peak of α-trypsin occurs (Fig. 3). Further increase in temperature brings about a decrease in total recovery and at 50°C only a negligible portion of the material applied was recovered. Within the temperature range 20–45°C the retention of α- and β-trypsin also decreases with increasing temperature. In contrast, the trypsinogen retention in the same elution system [0.3 *M* or 0.5 *M* $(NH_4)_2SO_4$, pH 3.0] is increased as the temperature is raised. Trypsinogen is eluted between the α- and β-trypsin peaks at 20°C (Fig. 4), whereas at the same salt concentration at a higher temperature (30°C) the elution volume of trypsinogen equals that of β-trypsin.

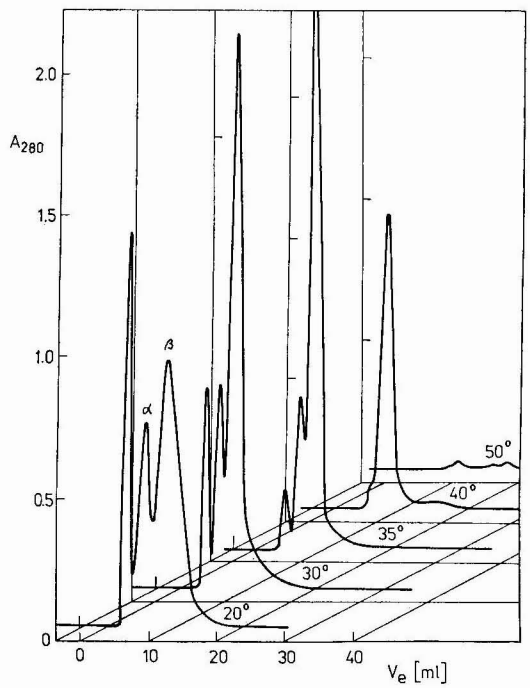

Fig. 3. Elution profiles of a commercial trypsin sample on HIC at different temperatures. The conditions are as in Fig. 1.1, except for temperature.

For the analysis of the activation products of trypsinogen the optimal temperature is 20°C, in 0.5 *M* $(NH_4)_2SO_4$, pH 3.0. Better resolution can be achieved at higher temperatures but is obviously limited by protein stability. A temperature of 30°C was found to be optimal for the rapid separation of α- and β-trypsin and for the analysis of their content in commercial samples.

Flow-rate and particle size dependence

The diffusion constants of proteins are about one order of magnitude lower

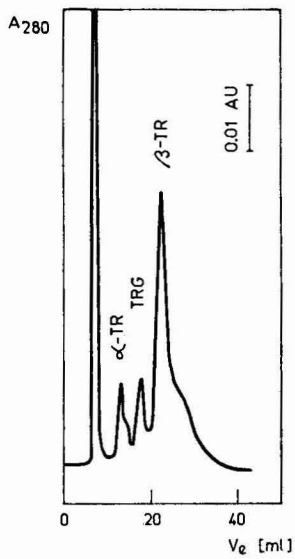

Fig. 4. Separation of components of crude trypsin and trypsinogen. Sample: 1.2 mg of commercial trypsin and 0.15 mg of trypsinogen in 70 μl of the elution buffer. Flow-rate: 7 ml/h at 22°C. Other conditions as in Fig. 1.2. TRG = Trypsinogen.

than those of low-molecular-weight solutes. Accordingly, with respect to the particle size, much higher flow-rates than that at the minimum plate height are often used[12]. The elution profiles shown in Figs. 1–4 were obtained on a column packed with

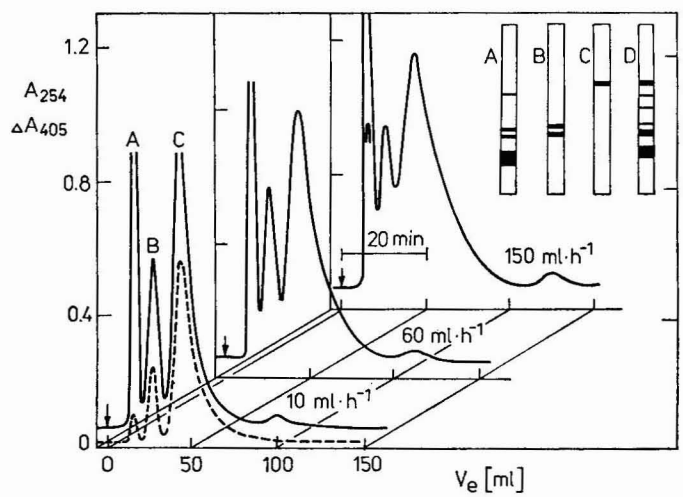

Fig. 5. Elution profiles of α- and β-trypsin at different flow-rates. A 70-mg sample of crude commercial trypsin in 300 μl of the elution buffer was applied to a Spheron P 300 (20–40 μm) column (505 × 8.0 mm I.D.) and eluted with 0.05 M formate buffer containing 0.5 M $(NH_4)_2SO_4$, pH 3.0, 22°C, at the flow-rates indicated. A_{254} = Absorbance at 254 nm (———); A_{405} = absorbance at 405 nm (– – – –) indicating the trypsin activity. The peak fractions A, B and C were used for sodium dodecyl sulphate electrophoresis in polyacrylamide gel, as shown in the insert. A = ψ-Trypsin and inactive impurities; B = α-trypsin; C = β-trypsin; D = commercial trypsin sample.

particles of mean size 14.7 μm, whereas Figs. 5 and 6 illustrate the effect for the particles of size 29 μm. Fig. 5 shows the elution profile and resolution corresponding to a 15-fold increase in flow-rate (from 10 ml h^{-1} to 150 ml h^{-1}, *i.e.*, a linear flow-rate of 2.8 mm sec^{-1}).

Influence of sample loading

The influence of the sample loading upon separation of α- and β-trypsin is illustrated in Fig. 6. A low sample loading (*ca.* 0.9 mg/ml) results in a high retention of the individual trypsin forms. With higher loadings, the resolution is poorer but the separation of β-trypsin remains satisfactory, even when the sample loading is as high as 9 mg/ml. Therefore the small column used in these experiments (27 ml) can be used quite effectively for the preparation of β-trypsin.

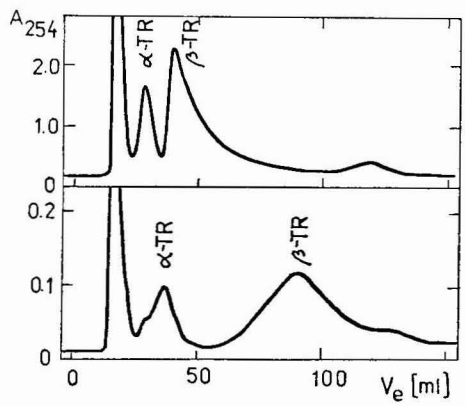

Fig. 6. Dependence of α- and β-trypsin separation on sample loading. Upper curve: 240 mg of crude trypsin in 600 μl of the elution buffer. Lower curve: 24 mg of trypsin in 200 μl of the elution buffer. Flow-rate: 10 ml/h. For other conditions see Fig. 5.

CONCLUSIONS

Separation of α- and β-trypsin by HIC offers many advantages: the simple isocratic elution system prevents autodigestion of trypsin, no inhibitor (*e.g.*, *p*-aminobenzamidine) is needed and the separation can be performed at room temperature. The rigid chromatographic support permits the use of particles of small diameter at high pressures and flow-rates.

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LIGAND-EXCHANGE CHROMATOGRAPHY OF RACEMATES

XIII. MICROPREPARATIVE RESOLUTION OF L,D-LEUCINE

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SUMMARY

Data are presented on the enantioselectivity and efficiency of ligand-exchange chromatographic resolution of racemic α-amino acids using a polystyrene resin with L-hydroxyproline groups and saturated with Cu^{2+}. Micropreparative resolution of L,D-leucine is discussed with respect to the degree of sorbent saturation with Cu^{2+}, elution rate, eluent concentration, operating temperature, column loading and column parameters.

INTRODUCTION

Ligand-exchange chromatographic (LEC) resolution of racemates is based on the interaction between the enantiomers and an optically active metal complex fixed in the sorbent phase. This method appears to be highly efficient for the resolution of amino racemates, hydroxy acids, amino acids and their derivatives, and has recently been reviewed[1].

Rogozhin and Davankov[2] suggested that polystyrene sorbents containing L-α-amino acid group should be used as sorbents for LEC. Sorbents of this kind saturated by Cu^{2+}, Ni^{2+} and Zn^{2+} show a high enantioselectivity towards racemic α-amino acids.

Of special interest are sorbents containing cyclic amino acid groups[3]. In such sorbents L- and D-isomers of many amino acids have sorption energies differing by 200–1000 cal/mol. Such a high sorption enantioselectivity is a prerequisite for the qualitative chromatographic resolution of racemates.

Possible reasons for the high enantioselectivity of LEC using these sorbents have been studied with the help of data for corresponding model compounds[4]. The explanation evidently lies in the conformational and solvational effects of the diastereomeric sorption complexes.

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It was found in earlier studies of LEG using asymmetric sorbents that a high enantioselectivity could be combined with a low efficiency of chromatography, resulting in most cases in only a partial racemate resolution requiring several tens of hours.

LEC of such sorbents is described by intradiffusion kinetics[5,6]. The efficiency of chromatography rises markedly with an increase in sorbent capacity, swelling capacity and decrease in particle size. Racemate resolution time can be much shortened by appropriate selection of the degree of sorbent saturation by Cu^{2+} and of the ammonia concentration in the eluent[5].

A sorbent containing proline groups on a polystyrene matrix saturated by Cu^{2+} has been used for preparative resolution of proline racemates[7]. Data were presented showing the effect of elution rate and ammonia concentration on the degree of racemate resolution.

It should be noted that the attainable degree of racemate resolution depends on both the enantioselectivity and the LEC efficiency. Therefore we have made a systematic study of the efficiency and selectivity of LEC using L,D-leucine as the sample.

EXPERIMENTAL

An asymmetric sorbent with L-hydroxyproline groups was obtained by amination of the chloromethylated macronet polystyrene matrix with methyl L-hydroxyprolinate hydrochloride as described in ref. 8. The sorbent capacity was 3.86 mmol/g, its swelling capacity in water was 250% and the particle size was *ca.* 50 μm. A styrene copolymer with 0.7% divinylbenzene containing 5.3% of additional cross-links of the diphenylmethylene type[6] was used as a matrix.

Sorbent saturated to the required degree with Cu^{2+} (ref. 5) was suspended in 0.1 *N* NH_4OH and transferred into a column. Samples of L-leucine and L,D-leucine in the form of 5% solutions were introduced into the column by a microsyringe and eluted by 0.1–1.0 *N* NH_4OH at a flow-rate of 5–50 ml/h. A liquid-flow spectrophotometer was used as detector. Chromatographic studies were conducted with the aid of the LKB Vario Perpex peristaltic pump.

RESULTS AND DISCUSSION

The retention volumes of amino acid L- and D-isomers vary over a wide range with changes in copper saturation and eluent concentration. Fig. 1 shows the relationship between retention volumes (V) of L- and D-leucine isomers expressed in units of the column free volume and the degree of sorbent saturation with metal ions. A sorbent fully saturated with Cu^{2+} has all fixed amino acid groups in the form of bischelate copper complexes. It exhibits the highest affinity for enantiomers of the resolving amino acid. In the case of bidentate amino acids, the strongest sorption complexes are those containing the D-enantiomer. The enantioselectivity of the sorption, α, is characterized by the ratio of the retention volumes of the D- and L-enantiomers. A decrease in the degree of copper saturation from 100 to 59% causes a 4.5-fold reduction of V_L and V_D while α remains unchanged.

The retention parameters of L- and D-amino acids can be varied by changing the ammonia concentration in the eluent; an increase in ammonia content results in

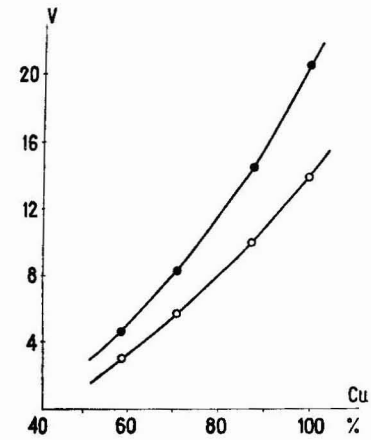
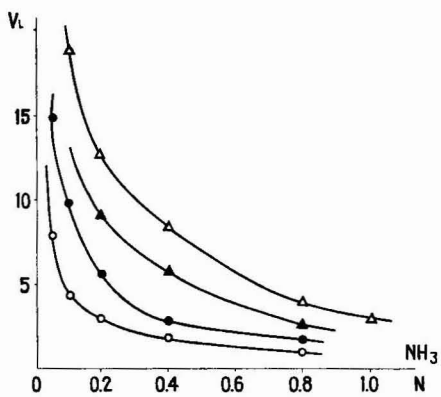


Fig. 1. Retention volumes of leucine enantiomers as a function of the degree of sorbent saturation by Cu^{2+}. ●—●, V_D; ○—○, V_L, Column: 140 × 9 mm. Eluent: 0.2 *N* NH_4OH; flow-rate 50 ml/h.

a reduction of V_L and V_D (Fig. 2). The dependence on ammonia concentration has the same features for sorbents with different degrees of saturation by Cu^{2+}.

Similar values of L-isomer retention for sorbents with different degrees of copper saturation may be obtained by using eluents of different ammonia content. However, the retention of the D-isomer will be different and decrease with increasing eluent concentration. Thus, the enantioselectivity of LEC of amino acid racemates decreases with increasing ammonia content in the eluent (Fig. 3) and is independent of the retention of the resolving amino acid and the degree of sorbent saturation by metal ions. With an increase in eluent concentration, ammonia replaces water in the axial positions of the amino acid sorption complexes, which apparently causes the reduction of α in the case of bidentate α-amino acids. To improve the selectivity of chromatography, eluents with a lower ammonia content should be used.



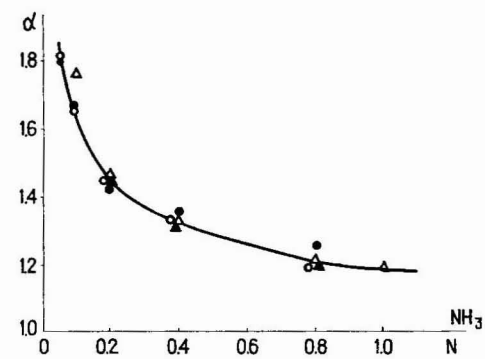


Fig. 2. Retention volumes of L-leucine as a function of ammonia concentration in the eluent. Degree of saturation of the L-hydroxyproline resin by Cu^{2+}: △, 100%; ▲, 88%; ●, 70%; ○, 59%. Sample: 1 mg of L,D-Leu. Chromatographic conditions as in Fig. 1.

Fig. 3. Dependence of enantioselectivity of sorption, α, for L,D-Leu upon ammonia concentration in in the eluent. Degree of sorbent saturation by Cu^{2+}: △, 100%; ▲, 88%; ●, 70%; ○, 59%.

The sorption rate on complex-forming sorbents tends to rise with increasing temperature for processes which can be described by both diffuse and chemical kinetics[9]. A significant increase in the efficiency of LEC of amino acids was observed with increasing temperature[10]. It was therefore of interest to study the effect of temperature on the efficiency of LEC of polystyrene sorbents containing fixed L-amino-acid groups. Fig. 4 shows the temperature dependence of chromatographic efficiency, selectivity and the retention volume of leucine enantiomers. The efficiency of chromatography is almost independent of temperature over a wide temperature range. Also, an increase in temperature has only slight affects on enantioselectivity and amino acid retention volume. Therefore an increase in LEC temperature cannot be used to improve the degree of resolution of racemic leucine.

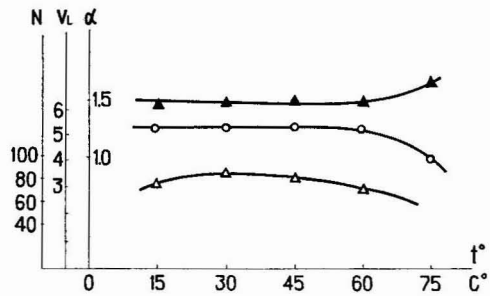


Fig. 4. Chromatography of 1.2 mg L,D-Leu showing temperature dependence of the number of effective plates, N (△) enantioselectivity, α (▲) and the retention volume of L-Leu, V_L (○). Column: 140 × 9 mm. Eluent: 0.2 N NH_4OH; flow-rate 35 ml/h.

The time required for LEC was investigated by studying the effects of elution rate and sample volume upon the efficiency of chromatography. In 140 × 9 mm columns the LEC efficiency was not affected by sample volume over the range 5–200 μl. However, an increase in the elution rate from 30 to 100 ml/h resulted in a slow reduction of efficiency (Fig. 5). The degree of racemate resolution and the chromatography efficiency can be raised without changing the chromatography time by simultaneous increase of the column length and elution rate.

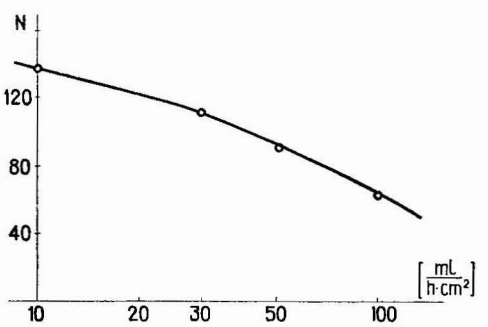


Fig. 5. Chromatography of 2.0 mg L-Leu showing the number of effective plates, N, as a function of elution rate. Column: 140 × 9 mm.

There is a pronounced dependence of the LEC efficiency upon the column loading (Fig. 6). A high efficiency for resolving small quantities of racemate can be achieved on a sorbent with *ca.* 50-μm particles. However, the efficiency is drastically reduced and the chromatographic zone croded in short (14 cm) columns under high-load conditions. On changing to longer (34 cm) columns with high loadings only the upper part of the column is overloaded while the larger part is active at low amino acid concentrations in a zone with efficiency approaching that of a low-load process.

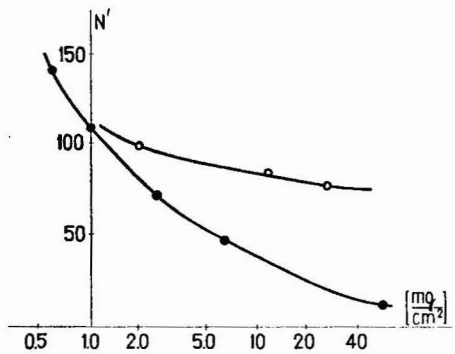


Fig. 6. Chromatography of L-leucine showing the number of effective plates per 10 cm of column length, N', as a function of the amount of L-Leu per cm² of the column cross-section. ○—○, Column 340 × 10 mm; eluent 0.2 N NH_4OH, flow-rate 60 ml/h. ●—●, Column 140 × 9 mm, flow-rate 50 ml/h.

The number of effective chromatographic plates, N, increases with the column length. At low loads this increase is proportional to the increase in length, while on the micropreparative scale it is several times higher. Increase of the column length appears to be more effective in micropreparative LEC resolution than reduction of the load at the expense of increase in cross-section. One can readily resolve 20 mg of L,D-leucine on a 34-cm column (Fig. 7).

The amount of racemate separated can be raised by further increase of the column length.

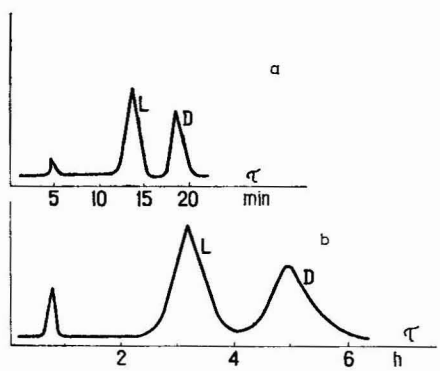


Fig. 7. Chromatography of L,D-leucine on a sorbent with L-hydroxyproline groups, satureted by Cu^{2+}. a, Particle size 10 μm, column 100 × 2 mm, sample 0.01 mg L,D-leucine, eluent 0.2 N NH_4OH, flow-rate 5 ml/h. b, Particle size 50 μm, column 340 × 10 mm, sample 20 mg L,D-leucine, eluent 0.1 N NH_4OH, flow-rate 60 ml/h.

CONCLUSION

Six hours were required to effect a quantitative resolution of 20 mg of L,D-leucine using L-hydroxyproline as sorbent (particle size *ca.* 50 μm), 70% saturated by Cu^{2+}, with 0.1 *N* NH_4OH as an eluent on a 340 × 10 mm column. The enantioselectivity of this process, α, is 1.6, whereas in the resolution of proline on L-proline resin[7] it is as high as 4 (ref. 3).

However, ways exist for further improving the efficiency and reducing the time of chromatography. Thus, the use of a sorbent with particle size of 10 μm on a 10-cm column enables qualitative resolution of leucine in minutes.

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MIXED RETENTION MECHANISMS IN GAS–LIQUID CHROMATOGRAPHY

IV. COMPARISONS OF SILANIZED AND CARBOWAX-MODIFIED SUPPORTS*

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SUMMARY

The relative contributions of partition and adsorption to the retention volume were determined for polar solutes on a non-polar stationary phase (*n*-octadecane) coated on differently treated diatomaceous supports: acid-washed; cleaned with gaseous HCl at high temperatures; silanized; and polymer-coated with Carbowax 20M. Supports which were only cleaned with gaseous HCl showed high adsorption. The silanized support had intermediate adsorption and wettability properties. The extent of wetting was dependent on the procedure for cleaning the initial support. The Carbowax-modified support was very inert, the adsorption effects were negligible and the wettability by the stationary phase very poor.

Thus Carbowax-modified supports, owing to low adsorptivity and poor wetting (which in this case minimizes gas–liquid interface adsorption), are well suited to the analysis of polar compounds. Additionally, such supports should be of great value for physico-chemical measurements. The impairment of efficiency due to poor wetting seems to be of minor importance.

INTRODUCTION

Among several known methods for deactivation of diatomaceous supports for gas–liquid chromatography (GLC), silanization has been the most widely used. The common methods of silanization are well known and the literature concerning this problem is very rich. It has been found that, for steric reasons, the full blocking of silanol groups on a clean silica surface is not possible[1]. The silica skeletons of diatomites are the main components of diatomaceous supports, but they also contain aluminium silicates and some impurities, mainly metal oxides, which form electron donor–acceptor active sites not easily blocked by silanization[2]. To improve the silanization is still of vital importance.

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The method by Welsch *et al.*[3], used for glass capillary deactivation, seems to be the most effective. It is based on treatment with silane vapour at 300°C. Grob *et al.*[4] have adapted this method for their general procedure for glass capillary column preparation, which included exhaustive cleaning of the surface and a temperature of 400°C. Similar procedures could give a more effective silanization than common, commercial methods, also on diatomaceous supports. This has, to our knowledge, not yet been tried.

The siloxane layers formed by silanization are low-energy surfaces, which are poorly wetted by most liquids used as stationary phases in GLC. It has been argued that silanized supports are not wetted even by *n*-hydrocarbons, which have the lowest surface tensions of stationary phase liquids used[5].

So there seems to exist a conflict between the aims of getting a surface which is as inert as possible and a surface which is well wetted by the stationary phase. It is desirable that the latter should form a homogenous thin layer. The geometrical (porous) and crystallographic structure of the support can also be of a great importance to the ability of the liquid to cover the whole surface[6].

Aue and co-workers[7,8] developed a new method of deactivation of diatomaceous supports by Carbowax 20M, which was used to form a highly stable, non-extractable thin layer on the support surface. Such supports are now commercially available. Glass capillary columns are also deactivated by similar procedures[9,10]. The chromatographic properties of such supports are very good, but there is a lack of knowledge about the physicochemical bases for their unusual characteristics, owing to the lack of sufficiently precise methods for characterizing such thin polymer layers. It is assumed[7] that at least a part of the Carbowax molecule lies on the support surface, attached to it at many points by non-covalent forces. The layer is also assumed to be of about monolayer thickness.

If we accept this picture, the covering of solid surfaces with such layers should be more effective than silanization, thereby giving a higher degree of inertness. This is also in agreement with chromatographic experience.

To compare the properties of silanized supports and Carbowax-modified supports, a recently presented [11] procedure for the separation of different retention mechanisms has been used.

EXPERIMENTAL

Techniques

Retention volumes were measured with the automatic equipment described elsewhere[12] which enables direct measurement of retention volume, without involving the concept of retention time. Vapour samples of widely varying concentrations were injected under computer control. All calculated results[13] were stored on flexible discs for further processing. A semiempirical equation[11] was fitted to the retention data (retention volume *vs.* sample size). By this technique, he relative magnitudes of the different retention mechanisms, adsorption and partition, could be calculated.

Materials

Supports of Polish production were used in different modifications as follows. B1: Polsorb B, as received from factory (Permedia, Lublin, Poland). B2: B1 cleaned

with gaseous HCl at 850°C according to the procedure of Aue *et al.*[14]. B3: B2 modified with Carbowax 20M according to the procedure of Daniewski and Aue[8]. Elemental analysis of this material, stabilized in vacuum at 150°C for 4 h, yielded 0.07–0.08% carbon, approximately corresponding to monolayer coverage, in the agreement with Aue *et al.*[7]. The quality of the support was checked by the chromatographic method recommended by Aue *et al.*[14] B4: B2 silanized by vapours of dimethylchlorosilane at 80°C (10 g of support was packed in a glass column, mounted in a GC-oven kept at 80°C and the silanizing agent was injected in 15 portions, each of *ca.* 50 μg[15]. B5: B1, acid-washed and silanized as described above. All supports were standardized before use by heating to 200°C overnight in a slow stream of oxygen-free nitrogen. The particle size was 0.125–0.25 mm. The specific surface areas are listed in Table I. The support material Polsorb B is very similar to Chromosorb G in mode of production and general characteristics[16].

TABLE I

SPECIFIC SURFACE AREAS OF THE SUPPORTS

Support type	*S* (m^2/g)
B1	0.46
B2	0.96
B3	1.05
B4	0.91
B5	0.77

n-Octadecane was used as stationary phase (≥99%, F 28–30°C, Fluka, Buchs, Switzerland).

Samples were of p.a. grade (Merck, Darmstadt, G.F.R.).

Columns

Glass columns, V-shaped (800 × 2.5 mm I.D.) were used. The support was coated with liquid according to the method suggested by Conder and Young[17] and filled into the columns in small portions, between which the tube was carefully tapped.

All columns are listed in Table II. The column name indicates the support used and the approximate loading of liquid phase. The dead volume of all columns was *ca.* 2 ml and the weight of the packings *ca.* 1.5 g. The amount of stationary phase was determined from weighings during column preparation. Owing to bleeding of the liquid phase, this value decreased with the use of the column. This has been compensated for[18].

Experimental conditions

All runs were made at 60.0°C. The carrier gas was hydrogen and the flow-rate was *ca.* 20 ml/min. Dead volumes were measured by the injection of methane.

Surface area measurements

The surface areas of the different supports were measured with the nitrogen thermal-desorption method[19] first described by Nelsen and Eggertsen[20]. Absolute values of standards (one non-modified, one silanized, and one Carbowax-modified support) have been calculated from three points on the BET isotherm.

TABLE II

EXPERIMENTAL RESULTS

Column	*Hexane* V_N *(ml)*	*Diisopropyl ether*		*Methanol*	
		V_N^P *(ml)*	V_N^A *(ml)*	V_N^P *(ml)*	V_N^A *(ml)*
B2-0		22	12,000		
0.3	0.37	16	655		
3	5.15	14	332		
8	12.23	14	321		
12	20.77	24	218		
B3-0				0.39	0.16
1	1.84	1.49	0	0.33	0.26
3	5.29	4.03	0	0.50	0.21
5	8.50	6.68	0	0.58	0.19
7	12.06	9.28	0	0.60	0.20
10	16.99	13.60	0	0.82	0.29
B4-0		0.003	6.20		
1		1.53	3.56		
3		3.35	1.94		
5		6.74	1.24		
7		8.75	1.91		
8		10.97	1.42		
10		13.44	1.51		
B5-0		−0.22	4.71		
1		0.75	3.26		
3		4.95	2.73		
5		6.16	4.23		
7		8.78	4.80		
10		12.93	2.44		
15		20.05	1.28	1.11	28.01

RESULTS AND DISCUSSION

The technique described in ref. 21, *i.e.* measuring net retention volume, V_N, as a function of sample size, n, varying over at least four decades, on columns with different loadings (0–*ca.* 15%, w/w) of *n*-octadecane, was carried out for all support types B2–B5. Samples were hexane, diisopropyl ether and, in some cases, methanol. A non-polar stationary phase and polar solutes are chosen, as in such systems a considerable gas–liquid surface adsorption appears, by which the size of the gas–liquid surface areas can be estimated. Also liquids such as *n*-octadecane are, owing to surface tension considerations, the only ones which have a chance to wet a surface ideally deactivated by silanization.

The contribution to V_N from partition, V_N^P, and adsorption, V_N^A, for diisopropyl ether and methanol, calculated by the procedure giv n in ref. 11 (where V_N^P and V_N^A are denoted A and C, respectively) are collected in Table II. All values are normalized to 1 g of column filling. When a zero appears in the table for V_N^A, no fitting was performed as the V_N values did not vary with n. In such cases, V_N^P is simply the mean of the values of V_N. For hexane, only the mean V_N values are given, for the same reason. The validity of the assumption that V_N^P is the contribution to V_N from partition is discussed below.

Adsorption and wetting on silanized supports

Plots of V_N^A *versus* liquid loading, λ', for diisopropyl ether on the silanized supports B4 and B5 (Fig. 1) are similar to those observed earlier on another type of silanized support, Supasorb AW DMCS (Fig. 4b in ref. 21); however, the magnitude

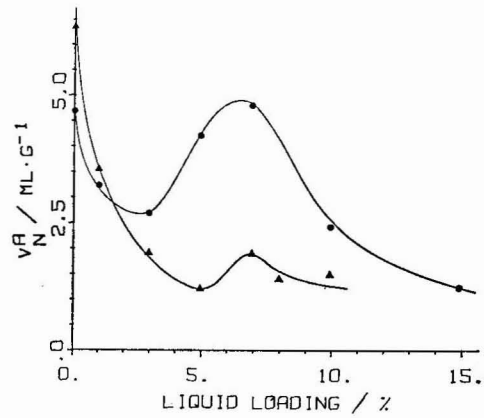

Fig. 1. Adsorption contribution to retention volume of diisopropyl ether *versus* liquid loading of *n*-octadecane for the supports B4 and B5 (HCl-gas treated, silanized and acid-washed, silanized) respectively. ▲, B4; ●, B5.

of the maximum is considerably smaller for B5 and nearly insignificant for B4. This maximum must, as was discussed earlier[21], be attributed to adsorption on the liquid–gas interface (LGI). The adsorption on covered supports will additionally be the sum of the contributions from the gas–solid interface (GSI) and the liquid–solid interface (LSI). In the present context, the GSI adsorption (on uncovered parts of the support) is assumed to decrease continuously as λ' increases and the LSI adsorption is assumed to be negligibly small. To estimate the area of the LGI, the value of the adsorption coefficient, K_I, of diisopropyl ether on a *n*-octadecane surface is needed. This was measured[22] to $55 \cdot 10^{-6}$ m. If an upper estimate of the LGI adsorption is taken as the maximum value at λ' *ca.* 7%, and the lower estimate is found by subtracting the minimum value at λ' *ca.* 4%, the LGI areas will be 0.013–0.034 m^2/g for B4 and 0.038–0.087 m^2/g for B5, which means 1.4–3.7% and 5.0–11.3% of the support area for B4 and B5, respectively. Similar estimates for Supasorb (for which the specific surface area is 0.55 m^2/g, measured as described above) yield 0.27–0.31 m^2/g or 49–55% of the support area. Thus the silanized supports seem to be not completely wetted.

The adsorption coefficient K_A for GSI adsorption was calculated from the BET areas and V_N^A at zero coverage. The result is $6.8 \cdot 10^{-6}$ m and $6.1 \cdot 10^{-6}$ m for B4 and B5, respectively, and $10.8 \cdot 10^{-6}$ m for Supasorb AW DMS[21]. The more thorough cleaning and hydroxylation of the B4 support prior to silanization, which is assumed to result in a more effective silanization, does not seem to have appreciably influenced the adsorbing properties (probably more uncovered silanol groups exist on B4) of the uncoated support, but the liquid wetting properties are apparently different.

Adsorption and wetting on bare support

For the non-silanized support B2, the adsorption (Fig. 2) is initially very high but levels off to a fairly stable value. The GSI adsorption coefficients, K_A, can be calculated to about 10^{-2} m, but the very high value of V_N^A at $\lambda' = 0$ is for experimental reasons very uncertain. On this support, complete coverage is expected for values of λ' greater than 2% and the adsorption is accordingly much smaller for even low liquid loadings. If the area of the GLI is assumed to be the same as the BET area, which is true only at low liquid loadings, the contribution to V_N^A from GLI adsorption would be only *ca.* 50 ml. The LSI adsorption is assumed to be approximately constant, so the decrease in total adsorption for coverages greater than 2% must be attributed to some residual GSI adsorption, perhaps at pointed edges or other places which are difficult to cover.

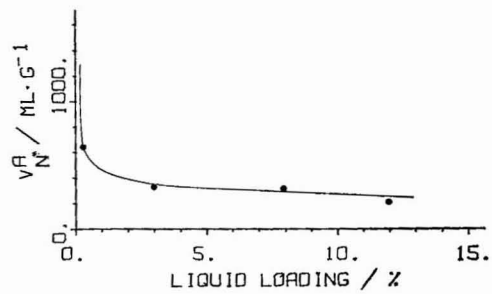

Fig. 2. Adsorption contribution to retention volume of diisopropyl ether *versus* liquid loading of *n*-octadecane for the support B2 (HCl-gas treated).

Adsorption and wetting on Carbowax-modified support

On B3 the adsorption of diisopropyl ether was not detectable. The corresponding results for methanol (Fig. 3) show a completely different picture from the previous ones in that the lowest adsorption is observed on the uncovered support and that the adsorption slowly increases as the liquid loading is increased. However, the small magnitude of these values makes this trend hardly reliable. K_A for methanol on uncovered B3 support is calculated to $0.16 \cdot 10^{-6}$ m. As the adsorption of methanol on another column (B5-15, see Table II) is 22 times greater than of diisopropyl ether, the K_A for the latter substance can be estimated to $7 \cdot 10^{-9}$ m, *i.e.* roughly 10^6 times less than on untreated B2 support and 10^3 times less than on silanized support.

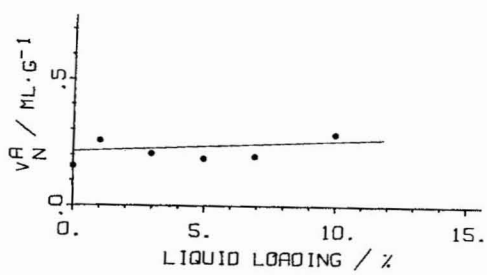

Fig. 3. Adsorption contribution to retention volume of methanol *versus* liquid loading of *n*-octadecane for support B3 (Carbowax-modified).

Furthermore, the area of the GLI is for these columns very small and difficult to estimate. The maximum probable magnitude at 10% *n*-octadecane loading can be calculated from the V_N^A value of methanol for the B3-10 column, the conversion factor between methanol and diisopropyl ether, and K_I for diisopropyl ether. The result is $2.4 \cdot 10^{-4}$ m²/g or *ca.* 0.02% of the support area.

Apparently, the B3 support is very poorly wetted by *n*-octadecane which can be inferred from its very weak adsorption properties.

The very low adsorption effects on B3 are in agreement with the results found by Aue and Younker[23], who measured distribution ratio as a function of peak height for various solutes. They found a deviation from linearity only for very large sample sizes, (Fig. 5 in ref. 23), which supports the view that the retention mechanism on Carbowax-modified supports is similar to partition.

General discussion about wetting

The wetting properties of a solid are governed by the critical surface tension, γ_c, of the solid[24]. γ_c has been estimated to 22 dynes/cm for silanized supports by Serpinet[5], which is a lower value than the surface tensions of most liquids which consequently should not wet. To estimate a value of γ_c for a Carbowax-modified surface we can compare the γ_c for polyethylene (27–36 dynes/cm) and for poly(oxyethylene) (43 dynes/cm)[25]. Thus it does not seem probable that γ_c for the Carbowax-modified supports can be less than that for the silanized surface and, consequently, it does not seem to be a reason for less wetting on Carbowax-modified supports. A more probable reason would be less influence of non-bonded silanol groups and impurities which are more effectively covered by the Carbowax layer than by the silylation. The value of γ_c of the silanized layer was estimated for an ideally silanized surface, without consideration of non-bonded silanol groups and impurities. A true, average value for a real surface is certainly higher, and so are the possibilities of wetting. From the comparison between B4 and B5, with B4 being more thoroughly cleaned prior to silanization, this view can be substantiated, with the additional implication that mainly impurities will be responsible for the higher wetting of B5. As was seen above, B4 seems to have more uncovered silanol groups than B5, owing to more effective hydroxylation. The Supasorb support[21] is, in all the aspects studied here, inferior to both B4 and B5. It can also be remarked that although a completely silanized surface should have similar properties to the Carbowax-modified surface, with respect to critical surface tension and inertness, the common silanization of diatomaceous supports does not effectively cover the surface. The silanization techniques, however, might still be justified as they permit a higher working temperature of the chromatographic column than Carbowax modification, and thus could have a potential for development.

The highest efficiency of a chromatographic column is achieved if the thickness of the liquid phase is small, *i.e.* the support is well wetted. On the other hand, considerable reduction of the liquid surface area in the case of deactivated supports causes a reduction of the LGI adsorption, together with a reduction of adsorption by the support itself. Also, taking into account that even on a wetted surface, according to the model of Giddings[26], the liquid is not spread out in a homogeneous layer, but mainly situated in pores, the disadvantage of bad wetting for highly deactivated supports seems to be of minor importance.

The partition contribution to V_N

The V_N values for hexane on B2 and B3 columns form straight lines when plotted against liquid volume, V_L (corrected for bleeding). The regression parameters are given in Table III and the results confirm the validity of the procedures for column preparation and measurement of retention volume. The slope of such a line is the partition coefficient K_L. The significant positive intercept for the B3 columns is expected, as the polymer layer of this modified support is assumed to contribute to partition retention[23].

For diisopropyl ether, the V_N^P values were plotted in the same way and regression lines were calculated (Table III). For B3, B4 and B5 supports, the slopes coincide well with each other and with previous similar measurements[11] and with static determinations[27] of the partition coefficient. The negative intercepts for B4 and B5 are not statistically significant. For B3 a positive, significant intercept approximately equal to that for hexane is observed. For the B2 support, no meaningful regression line can be calculated. The V_N^P values are in this case calculated in the presence of overwhelming adsorption effects and are not accurate; however, they are of the correct order of magnitude, especially for greater loadings.

TABLE III

REGRESSION PARAMETERS OF PARTITION RETENTION VOLUME *VERSUS* LIQUID LOADINGS

Solute	*Column type*	*Intercept*	*Slope*
Hexane	B2	−0.02	128.2
	B3	0.32	127.2
Diisopropyl ether	B3	0.28	99.8
	B4	−0.14	101.2
	B5	−0.32	101.4

The conclusions from these studies are the same as before[11]: V_N^P equals $V_L \cdot K_L$, the partition contribution to V_N, and thus the parameter V_N^A equals the adsorption contribution at infinite dilution.

CONCLUSIONS

Carbowax-modified supports with their great, near to ideal inertness seem to be promising materials for chromatographic analysis, especially of polar compounds. The possibility of using the support itself, which gives a highly efficient column with linear behaviour for most sample types, might be of great importance in the future. Also, such materials will be of a great interest for physico-chemical applications of gas chromatography, for example in the field of solution chemistry, where all adsorption effects have to be minimized.

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SEMIEMPIRICAL PREDICTION OF RETENTION OF SOME AMPHOLYTES ON A CARBON ADSORBENT*

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SUMMARY

Experimental data were analyzed for several model ampholytes, and parameters were obtained enabling the prediction of the retention behaviour of these substances in dependence on pH and the ionic strength. The possibility of the semi-empirical optimization of the separation conditions is demonstrated.

The experiments were carried out with a JaDo carbon (prepared by reduction of poly(tetrafluoroethylene)), packed in a relatively short (6 cm) column.

INTRODUCTION

The chromatographic process is frequently discussed in terms of numerical treatment and expression. Many authors have dealt with the behaviour of weak electrolytes in reversed-phase high-performance liquid chromatography (theoretically[1], for ODS[2–5], Amberlite XAD copolymers[6–9], a carbonaceous adsorbent[10]). In most cases the effects of pH, ionic strength (I), the organic phase and sometimes temperature were studied separately. The approach described here is one of semi-empirical procedures, which, however, describes the dependence of the retention behaviour of ampholytes on both pH and I. On the basis of experimental data it enables inter- and extrapolation of these dependences over a selected real range of the experimental conditions. The following treatment, which involves the construction of a map in pH and I coordinates depicting the overlap of the solutes studied, was chosen to obtain a complete picture of the possibilities of solving the separation problem with a given column, and replaces the tedious trial and error method.

THEORETICAL

The retention behaviour of weak electrolytes is described by a phenomenological equation for acid–base equilibrium. The dependence of the capacity ratio on the eluent pH at a constant I is given by

$$\kappa = (\kappa_0 + \sum_{i=1}^{n} (\kappa_i \prod_{j=1}^{i} K_j/a_{H+}^{i}))/(1 + \sum_{i=1}^{n} (\prod_{j=1}^{i} K_j/a_{H+}^{i})) \quad (1)$$

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where κ is the ampholyte capacity ratio at the given I;

κ_i for $i \in \langle 0;n \rangle$ are the limiting capacity ratios of the individual ionization forms at the given I;

K_j for $j \in \langle 1;n \rangle$ are the apparent dissociation constants of the acids ordered according to decreasing acidity at the given I;

a_{H^+} is the proton activity;

n is the number of groups that are ionized in the given range of experimental conditions.

To describe the effect of the ionic strength, its effect on the apparent dissociation constant and an indirect effect on the limiting capacity ratios must be expressed. The former dependence was expressed in terms of the relationship derived by Davies[11] as modified Debye–Hückel equation:

$$K_j = K_j^0 \cdot 10^{((2j-1)\cdot A \cdot I^{1/2}/(1+I^{1/2}) - (2j-1)\cdot 0.1 \cdot I)} \tag{2}$$

where K_j^0 is the thermodynamic dissociation constant;

A is an empirical constant (estimated as 0.5093 $mol^{-1/2}\, l^{1/2}$);

I is the ionic strength, $mol\, l^{-1}$.

The effect of the surface tension of the mobile phase on the limiting capacity ratios is known from the literature[2]. The linear dependence of $\ln \kappa_i$ on the electrolyte concentration can, under certain conditions, be replaced by a linear dependence on I:

$$\ln \kappa_i = a_i I + b_i \tag{3}$$

where a_i and b_i are constants characterizing the i-th ionization form of the ampholyte. To decrease the error stemming from the above simplification, the components of the buffers used must have similar character (*e.g.* uni-bivalent with a bulky anion) leading to similar changes in the surface tension with changing concentration.

Further, it can be assumed that eqn. 3 gives parallel lines for various ionization forms of a single substance. The number of parameters required for characterization of the chromatographic behaviour of an ampholyte is thus decreased. On substitution from eqns. 2 and 3 into eqn. 1 the capacity ratio is obtained as a function of two variables, pH and I. If experimental points are fitted to a plane by regression analysis, dissociation constants K_j^0 are obtained (or tabulated values can be used), as well as the limiting capacity ratios, which together with slope a, enable the capacity ratio value to be calculated.

EXPERIMENTAL AND CALCULATIONS

A Pye Unicam LC-XP liquid chromatograph was used with a 20-μl Rheodyne sampling loop and a Pye Unicam LC-UV variable wavelength detector. The detection was carried out around 200 nm. A stainless steel column, 6 × 0.43 cm I.D., packed with a JaDo 1141/3 adsorbent (sample No. II in ref. 10) was employed at laboratory temperature. The column efficiency used in calculations was $N = 120$ at $L = 6$ cm with linear flow-rate $u = 1.5$ mm sec^{-1}. The buffers were prepared from p.a. dihydrogen phosphate, hydrogen phosphate and phosphoric acid. The ionic strength was adjusted with Na_2SO_4. The amino acid solute samples, ornithine, lysine, arginine

TABLE I

DISSOCIATION CONSTANTS OF THE MODEL AMPHOLYTES[12]

Amino acid	pK_1	pK_2
Orn	1.705	8.69
Lys	2.18	8.95
Arg	1.822	8.99
His	1.77	6.1

and histidine, were obtained from Lachema (Brno, Czechoslovakia). The dissociation constant values used in calculations are summarized in Table I.

If, for the sake of simplicity, the column efficiency is considered constant for various capacity ratios and the peaks are assumed to be Gaussian, a theoretical intercept of two peaks can be calculated from the relationship

$$t_k = 2/(1/t_n + 1/t_m) \tag{4}$$

The mean value of the overlap was expressed for symmetrical peaks with a unit height,

$$U = 10 \cdot \Big(\sum_{k=1}^{p} \sum_{i=1}^{p} \exp(-(1 - t_k/t_i)^2 N) \Big)/p \tag{5}$$

where p is the number of peaks.

The Gauss–Newton method was used for the regression analysis and the ionic strength was computed by an iteration method[13] on a TI 59 calculator. For graphical dependences, a HP 9830A desk-top calculator was used, and for pseudographical dependences an ICL 4-72* computer was used.

RESULTS AND DISCUSSION

The dependence of the behaviour of four model ampholytes on the eluent pH and I was investigated. The treatment was based on the capacity ratios measured over a pH range from 2 to 6 and $I \in \langle 0.05;0.20 \rangle$. Figs. 1(a–d) depict sections through the $\kappa = f(\mathrm{pH},I)$ plane at constant I. In the measuring range given, this function increased monotonically with increasing I. Table II summarizes the parameters characterizing the individual ampholytes.

A map (Fig. 2) was constructed from these data, in which peak overlap is represented by the isobases. The height scale is non-linear; the overlap of two, three or four peaks is given by heights 5, 15 or 30, respectively. For example, transition of height 5 represents a change in the elution order of two peaks. Abbreviations composed of the initial letters of the solutes express the elution order and are written in the appropriate valleys.

In this way it can be found how the elution order of the amino acids changes on transition from one valley to another. The dot and dash lines denote the regions

* The program written in FORTRAN is available on request.

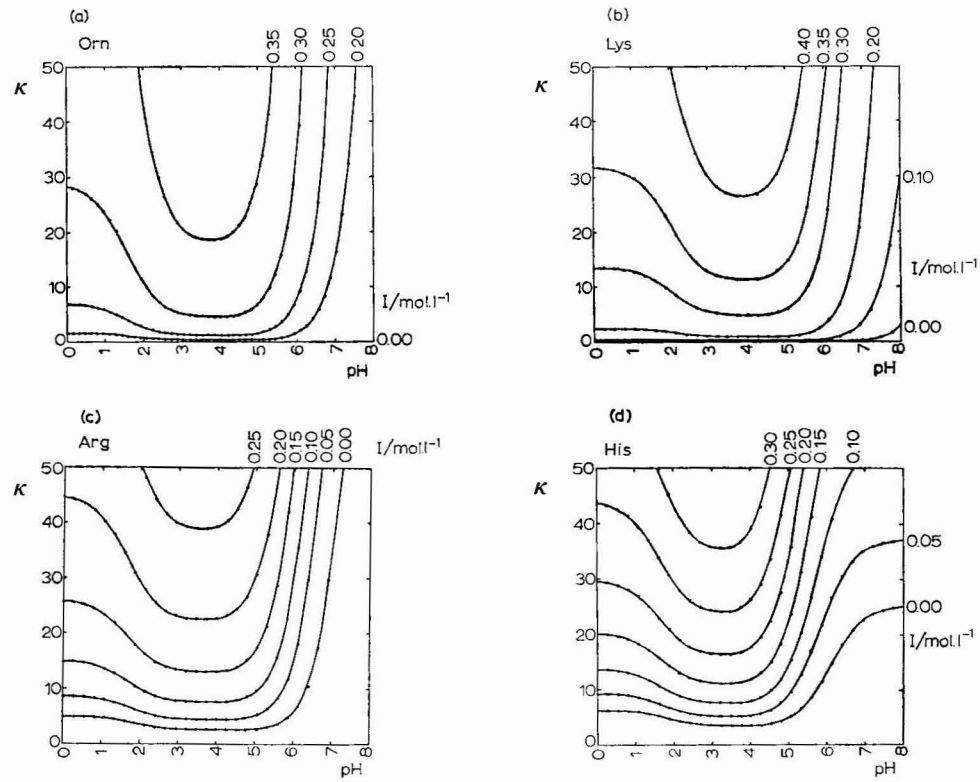

Fig. 1. The effect of the eluent pH on the capacity ratios of different ampholytes at the constant ionic strengths. (a) Ornithine; (b) lysine; (c) arginine; (d) histidine.

TABLE II

SLOPE, a, AND NATURAL LOGARITHM OF THE LIMITING CAPACITY RATIOS, b_i, AT $I = 0$ (EQN. 3)

Amino acid	a	b_1	b_2	b_3
Orn	28.399	−5.155	−7.084	0.0178
Lys	17.061	−2.511	−3.599	3.298
Arg	10.949	1.616	0.896	8.05
His	7.773	1.843	1.191	3.228

in which U is less than 0.1. In these regions, considering the total analysis time, are found the conditions for separation. The retention time of the last peak in the mixture is denoted by a dashed line. The validity of this map was verified on the chromatogram of a mixture of the four substances (Fig. 3).

On increasing the column efficiency the valleys become wider and the ridges narrower. The overall character of the map changes not only in places where there are barely perceptible high plateaux, but new valleys are also formed at higher efficiencies (at *ca.* 10 times higher efficiency a valley is formed in the top left corner of the map; the total analysis time would be here 4–5 min).

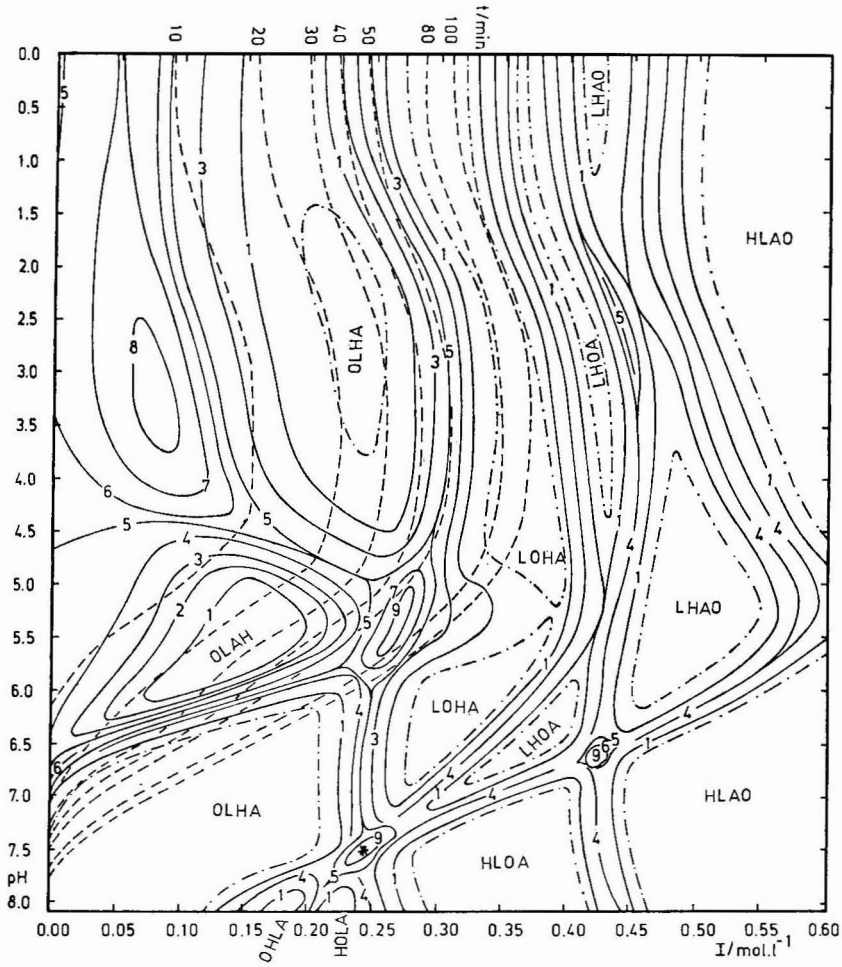

Fig. 2. Map of the influence of the eluent pH and ionic strength on the mean value of the overlap of four studied ampholytes. A = Arginine; H = histidine; L = lysine; O = ornithine.

In view of the many problems with gradient elution it is often advantageous to find isocratic separation conditions. It is possible to introduce further variables (efficiency, temperature, mixed media, etc.) and to assemble a collection of maps from which either suitable conditions can be directly found, or at least an initial point can be obtained for further computer optimization.

CONCLUSION

For practical use of this procedure, a library of data would have to be assembled for a greater number of weak electrolytes, using an adsorbent that is sufficiently widely used and is readily available. As the retention is determined by the size of the non-polar part of the molecule, the content of various elements, the polarization of some bonds, the steric conditions, etc., further work should be directed toward estimation of the absolute values of the limiting capacity ratios.

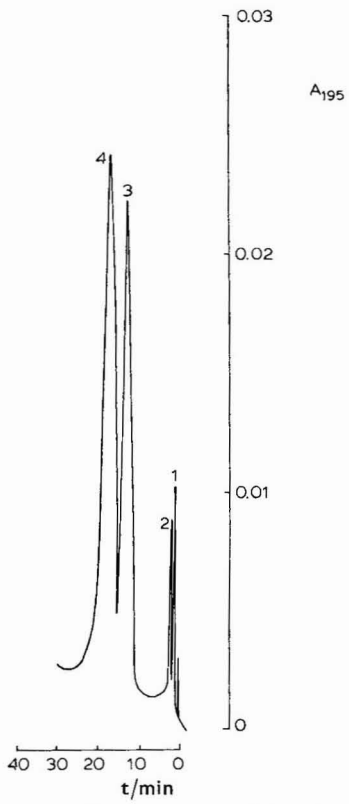

Fig. 3. Chromatogram of four basic amino acids. Operating conditions: eluent, 0.11 *M* phosphate buffer; pH 2.0; $I = 0.2$ mol l^{-1}; flow-rate; 1 ml min^{-1}; detector, UV. Peaks: 1 = Orn; 2 = Lys; 3 = His; 4 = Arg.

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REVERSED-PHASE CHROMATOGRAPHY OF KETO AND HYDROXY DERIVATIVES OF ADAMANTANE AND DIAMANTANE*

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SUMMARY

Chromatographic elution data for adamantane and diamantane derivatives, mostly diketones, hydroxyketones and dihydroxy derivatives, have been measured at 30, 50 and 70°C. Octadecyl-silica was used as the stationary phase, and methanol–water and acetonitrile–water as the mobile phases. For each mobile phase, three or four different concentration ratios of the relevant components were used.

From the viewpoint of the chromatographic behaviour of the studied compounds, the number of functional groups has the largest effect. When this number increases, the retention time of the compound in question decreases.

Interactions of difunctional compounds with octadecyl-silica are strongest when the two groups are adjacent. Retention time increases when the two groups get closer to each other.

Generally, retention time increases as the number of carbon atoms in the molecule increases. This is also valid for ring compounds, as well as for increasing number of rings in the molecule. Selectivity is influenced by changes of the mobile phase composition if acetonitrile or methanol are present. On the contrary, changes of the mobile phase concentrations influence the selectivity only a little. The dependence of log k' on the logarithm of the methanol or acetonitrile concentration in the mobile phase is linear for the most of the compounds studied. Temperature changes have little effect on the selectivity; the dependence of log k' on temperature is linear.

INTRODUCTION

High-performance liquid chromatographic (HPLC) data for monocyclic and polycyclic alcohols and ketones of the adamantane type on silica gel have been reported in previous papers[1,2]. In adsorption liquid chromatography, the combination of silica gel and relatively non-polar mobile phases proved to be suitable for separating adamantane and diamantane derivatives containing one polar functional group. As the number of hydroxy and keto groups in the molecule increases, however, the corresponding retention times increase and the solubility of these compounds in the

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mobile phase decreases, making their detection by means of an RI detector rather difficult. This problem cannot be solved in a satisfactory manner even by using a mobile phase with a higher elution strength value.

In the present paper, the chromatographic behaviour of keto and hydroxy derivatives of adamantane and diamantane is described for octadecyl-silica as stationary phase and methanol–water and acetonitrile–water mixtures as mobile phases.

EXPERIMENTAL

Apparatus

A Varian 8500 liquid chromatograph with a syringe pump was used, connected to an RI detector and an A25 dual-channel strip-chart recorder (Varian, Palo Alto, CA, U.S.A.). Sample injection using the stop-flow technique was performed; a 10-μl syringe (Hamilton, Bonaduz, Switzerland) was used. The column used was a MicroPak CH-10 (Varian; 25 cm × 2.1 mm I.D.), packed with silica gel LiChrosorb Si-60 with chemically bonded octadecyl.

Retention data were calculated on an HP 9830 A calculator connected to an HP 9866 A thermal printer (Hewlett-Packard, Avondale, PA, U.S.A.). Graphic processing of data was carried out on the same calculator equipped with an HP 9862 A plotter.

Reagents

Nearly all the standard compounds used for measurements were prepared in our laboratory. Diamantane-3,5-diol, diamantane-3,6-diol and diamantane-1,3-diol were kindly provided by Prof. M. A. McKervey from the University College, Cork, Ireland. Monocyclic ketones were kindly provided by the Department of Organic Technology, Prague Institute of Chemical Technology. Methanol, analytical grade (Lachema, Brno, Czechoslovakia) was used without further treatment. Acetonitrile (VEB Jenapharm Laborchemie, Apolda, G.D.R.) was distilled before use.

Mobile phase

The mobile phases were prepared from weighed degassed components. The compositions are listed in Table I.

TABLE I

MOBILE PHASE COMPOSITIONS

No. 1	*CH_3OH (%)*	*H_2O (%)*	*No. 2*	*CH_3CN (%)*	*H_2O (%)*
a	30	70	a	20	80
b	40	60	b	30	70
c	50	50	c	40	60
d	70	30			

Procedure

The column was thermostated and retention data were measured at three temperatures (30, 50 and 70°C). The flow-rate of the mobile phase was 60 ml/h. The

column was conditioned by washing with fresh mobile phase for 2–4 h (the total volume of mobile phase passed through the column before measurement was 250 ml). The dead volume of the column was determined by measuring the retention time of water.

Retention data were measured on chromatograms obtained by injecting solutions of compounds in methanol or acetonitrile. The retention data are listed in Tables II–VII.

TABLE II

RETENTION DATA

Mobile phase, methanol–water (30:70)

Compounds	*Temperature (°C)*						*log k'/ 10°C*
	30		*50*		*70*		
	t_R *(sec)*	k'	t_R *(sec)*	k'	t_R *(sec)*	k'	
Adamantanone	171	3.07	143	2.41	127	2.01	0.0048
Adamantane-2,4-dione	75	0.78	77	0.82	64	0.51	0.0045
Adamantane-2,6-dione	66	0.57	63	0.49	59	0.40	0.0040
2-Thiaadamantane-4,8-dione	66	0.57	61	0.45	57	0.35	0.0055
Adamantane-1-ol-4-one	60	0.43	58	0.37	54	0.29	0.0040
Adamantane-4-ol-2-one	76	0.81	73	0.73	68	0.63	0.0028
Cyclohexanol	99	1.35	88	1.09	82	0.95	0.0038
Adamantan-1-ol	222	4.29	186	3.43	152	2.63	0.0053
Adamantan-2-ol	335	6.98	252	5.00	202	3.80	0.0065
Adamantane-1,2-diol	112	1.66	100	1.38	89	1.13	0.0043
Adamantane-1,3-diol	68	0.63	63	0.49	59	0.41	0.0045
Adamantane-2,6-diol	66	0.57	58	0.38	51	0.21	0.0108
Diamantane-1,4-diol	164	2.90	141	2.36	119	1.82	0.0050
Diamantane-1,6-diol	88	1.09	81	0.93	75	0.79	0.0035
Diamantane-1,7-diol	127	2.01	115	1.73	104	1.46	0.0033
Diamantane-1,9-diol	173	3.11	152	2.61	136	2.24	0.0035
Diamantane-4,9-diol	80	0.89	74	0.75	67	0.60	0.0043
4-Oxahomoadamantan-5-one	105	1.50	95	1.27	87	1.07	0.0038
Adamantane-2^a,4^a-diol	123	1.94	108	1.58	97	1.31	0.0043
Adamantane-2^a,4^e-diol	89	1.13	80	0.91	74	0.77	0.0040
Adamantane-2^e,4^e-diol	77	0.82	70	0.67			

RESULTS AND DISCUSSION

The effect of the number of hydroxy and keto groups in the molecule

When comparing chromatographic behaviour of monocyclic and polycyclic alcohols and ketones of the adamantane type in the case of reversed phases (using methanol–water and acetonitrile–water mixtures as mobile phases) with their behaviour on silica gel[1,2], alumina, or other stationary phases[3] (using relatively non-polar mobile phases), the expected reversal in chromatographic behaviour is not observed in all cases. When using silica gel, the retention time (t_R) increases with increasing number of polar functional groups, t_R for alcohols being higher than t_R for ketones. In the case of reversed-phase chromatography, t_R decreases with increasing number

TABLE III

RETENTION DATA

Mobile phase, methanol–water (40:60)

Compounds	*Temperature (°C)*						*log k'/ 10°C*
	30		*50*		*70*		
	t_R *(sec)*	k'	t_R *(sec)*	k'	t_R *(sec)*	k'	
Cyclohexanone	95	1.27					
Adamantanone	150	2.57	123	1.94	108	1.56	0.0055
Adamantane-2,4-dione	68	0.62	63	0.50	59	0.41	0.0045
Adamantane-2,6-dione	62	0.48	60	0.43	56	0.34	0.0035
2-Thiaadamantane-4,8-dione	60	0.43	56	0.34	54	0.29	0.0043
Adamantan-1-ol-4-one	59	0.41	70	0.66	54	0.29	0.0035
Adamantan-4-ol-2-one	70	0.66	65	0.56	63	0.50	0.0030
Cyclohexanol	86	1.06	77	0.83	72	0.71	0.0043
Adamantan-1-ol	181	3.31	143	2.41	131	2.11	0.0050
Adamantan-2-ol	246	4.86	197	3.70	158	2.77	0.0063
Adamantane-1,2-diol	97	1.31	87	1.07	77	0.84	0.0050
Adamantane-1,3-diol	65	0.54	60	0.43	57	0.35	0.0048
Diamantane-1,4-diol	140	2.34	117	1.74	100	1.38	0.0058
Diamantane-1,6-diol	77	0.83	73	0.74	70	0.66	0.0025
Diamantane-1,7-diol	107	1.54	100	1.37	86	1.06	0.0033
Diamantane-1,9-diol	138	2.29	119	1.83	104	1.46	0.0048
Diamantane-3,6-diol	105	1.50	92	1.18	84	0.99	0.0045
Diamantane-4,9-diol	75	0.78	68	0.63	63	0.50	0.0048
4-Oxahomoadamantan-5-one	90	1.14	81	0.94	75	0.79	0.0040
Adamantane-2^a,4^a-diol	102	1.43	95	1.27	85	1.02	0.0025
Adamantane-2^a,4^e-diol	77	0.82	73	0.73	66	0.58	0.0038
Adamantane-2^e,4^e-diol	68	0.61	65	0.56	61	0.46	0.0030

of functional groups. Generally, retention times of adamantanoid substances substituted with hydroxy or keto groups depend mainly on the position of the group and on the mobile phase used, rather than on the character of the group.

Functional group arrangement

Generally speaking, retention times of difunctional compounds are greater if the groups are nearer each other. In case of substitution on secondary carbon atoms, the retention time of adamantane-2,4-dione is greater than that of adamantane-2,6-dione; similarly, all adamantane-2,4-diol stereoisomers are eluted after adamantane-2,6-diol. The effect of the mutual arrangement of OH groups in the molecule is significant enough to enable even a separation of all three adamantane-2,4-diol stereoisomers using both methanol (Fig. 1) and acetonitrile in the mobile phase. All adamantanediols with OH groups on secondary carbon atoms are eluted in the following order:

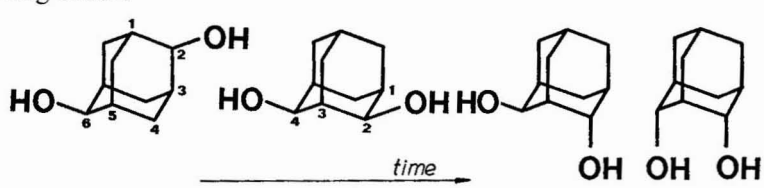


TABLE IV

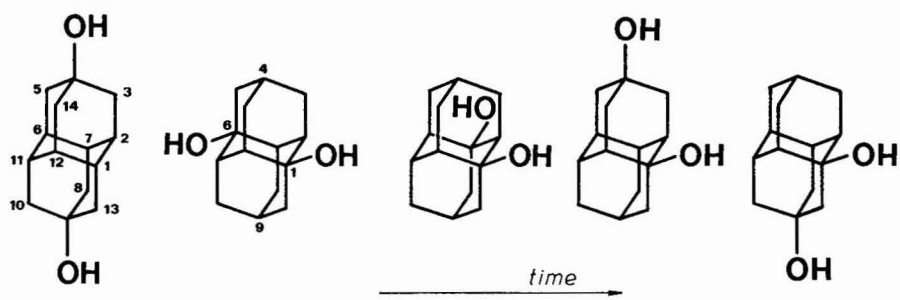
RETENTION DATA

Mobile phase, methanol–water (50:50)

Compounds	*Temperature (°C)*						*log k'/ 10°C*
	30		*50*		*70*		
	t_R *(sec)*	*k'*	t_R *(sec)*	*k'*	t_R *(sec)*	*k'*	
Adamantanone	92	1.20	83	0.98	75	0.79	0.0045
Bicyclo[3,3,1]nonane-2,6-dione	74	0.75	70	0.66	49	0.16	0.0028
Adamantane-2,4-dione	57	0.36	54	0.29	52	0.24	0.0043
Adamantane-2,6-dione	53	0.27	51	0.22	49	0.17	0.0050
2-Thiaadamantane-4,8-dione	51	0.21	49	0.16	47	0.13	0.0058
Adamantan-1-ol-4-one	51	0.22	50	0.18	48	0.14	0.0050
Adamantan-4-ol-2-one	57	0.35	56	0.32	54	0.29	0.0020
Cyclohexanol	73	0.74	59	0.41	57	0.35	0.0040
Adamantan-1-ol	110	1.63	96	1.29	84	1.01	0.0053
Adamantan-2-ol	138	2.29	115	1.73	95	1.27	0.0065
Adamantane-1,2-diol	71	0.68	65	0.55	60	0.44	0.0048
Adamantane-1,3-diol	54	0.29	52	0.24	50	0.20	0.0040
Adamantane-2,6-diol	53	0.25	51	0.21	49	0.16	0.0050
Diamantane-1,4-diol	88	1.10	79	0.89	72	0.71	0.0048
Diamantane-1,6-diol	62	0.46	59	0.41	88	0.37	0.0025
Diamantane-1,7-diol	77	0.82	71	0.70	68	0.62	0.0030
Diamantane-1,9-diol	88	1.09	81	0.93	75	0.79	0.0035
Diamantane-3,6-diol	74	0.76	69	0.64	62	0.48	0.0050
Diamantane-4,9-diol	59	0.41	57	0.36	54	0.29	0.0040
4-Oxahomoadamantan-5-one	66	0.57	65	0.54	61	0.45	0.0040
Adamantane-2^a,4^a-diol	75	0.78	70	0.66	67	0.59	0.0030
Adamantane-2^a,4^e-diol	60	0.43	58	0.37	56	0.32	0.0040

The two adamantane-1,4-diol stereoisomers may be separated in an analogous manner (Fig. 2). The relevent retention times are not listed in the tables because of small amounts of the samples used.

Dihydroxydiamantane derivatives with OH groups on tertiary carbon atoms are eluted in the following order:



Diamantane-3,6-diol, which has one group on a secondary carbon atom, is eluted between diamantane-1,6-diol and diamantane-1,7-diol. Diamantane-3,5-diol, which

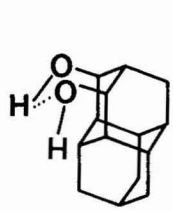
TABLE V

RETENTION DATA

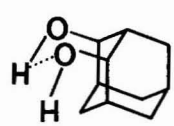
Mobile phase, acetonitrile–water (20:80)

Compounds	*Temperature (°C)*						*log k'/ 10°C*
	30		*50*		*70*		
	t_R *(sec)*	*k'*	t_R *(sec)*	*k'*	t_R *(sec)*	*k'*	
Cyclohexanone	430	2.77	377	2.31	329	1.89	0.0040
Adamantanone	2006	16.60	1440	11.63	1056	8.26	0.0075
Protoadamantan-4-one	1924	15.88	1272	10.16	947	7.31	0.0085
Bicyclo[3,3,1]nonane-2,6-dione	185	0.62	178	0.56	172	0.51	0.0020
Adamantane-2,4-dione	289	1.54	270	1.37	251	1.20	0.0028
Adamantane-2,6-dione	217	0.90	196	0.72	183	0.61	0.0043
2-Thiaadamantane-4,8-dione	278	1.44	245	1.15	218	0.91	0.0050
Adamantan-1-ol-2-one	327	1.87	299	1.62	278	1.44	0.0028
Adamantan-1-ol-4-one	174	0.53	167	0.47	160	0.41	0.0028
Adamantan-2-ol-6-one	178	0.56	169	0.48	161	0.42	0.0033
Adamantan-4-ol-2-one	246	1.16	218	0.91	201	0.76	0.0045
Diamantan-1-ol-3-one	795	5.97	649	4.69	540	3.74	0.0053
Tricyclo[4,4,0,$0^{2,9}$]decan-9-ol-5-one	200	0.76	203	0.78	188	0.65	0.0018
Cyclohexanol	450	2.95	391	2.43	343	2.01	0.0043
Adamantan-1-ol	1836	15.11	1371	11.03	1004	7.81	0.0073
Adamantan-2-ol	2910	24.53	2115	17.55	1498	12.14	0.0078
Adamantane-1,2-diol	347	2.04	318	1.78	292	1.56	0.0030
Adamantane-2^a,4^a-diol	533	3.68	449	2.94	373	2.27	0.0053
Adamantane-2^a,4^e-diol	251	1.20	228	1.00	206	0.81	0.0043
Adamantane-2^e,4^e-diol	195	0.71	187	0.64			
Diamantane-1,4-diol	547	3.80	483	3.24	421	2.69	0.0038
Diamantane-1,7-diol	432	2.79	388	2.41	354	2.11	0.0033
Diamantane-1,9-diol	528	3.63	458	3.02	400	2.51	0.0040
Diamantane-3,5-diol	2496	20.89	1762	14.45	1280	10.23	0.0078
Adamantanonoxim	1178	9.33	1074	8.42	797	5.99	0.0048
4-Oxahomoadamantan-5-one	687	4.95	576	4.05	455	2.99	0.0053
Cyclopentanone	258	1.26	236	1.07	220	0.93	0.0033
Cyclooctanone	1307	10.47	902	6.92	758	5.65	0.0043

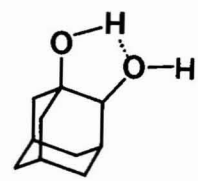
has both functional groups on secondary carbon atoms on one side of the molecule, has a retention time several times greater than those of other dihydroxydiamantane derivatives in acetonitrile–water, being eluted between adamantan-1-ol and adamantan-2-ol. This fact can possibly be accounted for by the formation of an intramolecular hydrogen bond, resulting in another six-membered ring[4] and a decreased interaction with the mobile phase in case of the diaxial 3,5-stereoisomer (I).



I



II



III

TABLE VI

RETENTION DATA

Mobile phase, acetonitrile–water (30:70)

Compounds	*Temperature (°C)*						*log k'/ 10°C*
	30		*50*		*70*		
	t_R *(sec)*	k'	t_R *(sec)*	k'	t_R *(sec)*	k'	
Cyclohexanone	274	1.68	256	1.51	233	1.29	0.0030
Adamantanone	876	7.59	689	5.75	546	4.35	0.0060
Protoadamantan-4-one	840	7.24	641	5.29	511	4.01	0.0065
Bicyclo[3,3,1]nonane-2,6-dione	150	0.47	144	0.41	138	0.35	0.0030
Adamantane-2,4-dione	202	0.98	186	0.82	174	0.71	0.0035
Adamantane-2,6-dione	166	0.62	154	0.51	146	0.43	0.0040
2-Thia-adamantane-4,8-dione	191	0.87	173	0.69	159	0.56	0.0048
Adamantan-1-ol-2-one	217	1.13	202	0.98	197	0.93	0.0020
Adamantan-1-ol-4-one	139	0.36	134	0.32	130	0.28	0.0030
Adamantan-2-ol-6-one	142	0.39	137	0.34	131	0.29	0.0033
Adamantan-4-ol-2-one	168	0.65	160	0.56	151	0.48	0.0033
Diamantan-1-ol-3-one	389	2.82	335	2.29	292	1.86	0.0045
Tricyclo[4,4,0,$0^{2,9}$]decan-9-ol-5-one	155	0.52	152	0.49	149	0.46	0.0013
Cyclohexanol	279	1.74	255	1.50	233	1.28	0.0033
Adamantan-1-ol	791	6.76	637	5.25	509	3.99	0.0058
Adamantan-2-ol	1195	10.72	894	7.76	672	5.59	0.0070
Adamantane-1,2-diol	220	1.18	210	1.06	198	0.94	0.0025
Adamantane-2^a,4^a-diol	301	1.95	269	1.64	243	1.38	0.0038
Adamantane-2^a,4^e-diol	174	0.71	161	0.58	153	0.50	0.0038
Diamantane-1,4-diol	275	1.69	257	1.52	236	1.31	0.0028
Diamantane-1,6-diol	267	1.62	247	1.42	229	1.25	0.0028
Diamantane-1,7-diol	240	1.35	224	1.20	210	1.06	0.0028
Diamantane-1,9-diol	292	1.86	264	1.59	240	1.35	0.0035
Diamantane-3,5-diol	1056	9.35	792	6.76	595	4.83	0.0072
Adamantanonoxim	576	4.65	496	3.86	406	2.98	0.0050
4-Oxahomoadamantan-5-one	364	2.57	317	2.11	275	1.70	0.0045
Cyclopentanone	202	0.98	189	0.85	179	0.75	0.0028
Cyclooctanone	614	5.02	521	4.11	422	3.14	0.0050

The same hydrogen bond may be formed by adamantane-2^a,4^a-diol (II), with a retention time also rather different from those of the other stereoisomers. A retention time only slightly lower than for (II) is observed for adamantane-1,2-diol (III), where the formation of a strained five-membered ring might be possible.

Effect of the basic skeleton size

Retention times increase with increasing number of carbon atoms in the molecule. The observed influence appears strongly for an increasing number of rings *i.e.* cyclohexanone, adamantanone and diamantanone, or cyclohexanol, adamantanol and diamantanol, and especially for an increasing number of carbon atoms in the ring, *i.e.* cyclopentanone, cyclohexanone and cyclooctanone. (The orientation measurement of monofunctional derivatives of diamantane showed the bad solubility and long retention times of these compounds. Therefore, more detailed studies were not performed.)

TABLE VII

RETENTION DATA

Mobile phase, acetonitrile–water (40:60)

Compounds	*Temperature (°C)*						*log k'/ 10°C*
	30		*50*		*70*		
	t_R *(sec)*	k'	t_R *(sec)*	k'	t_R *(sec)*	k'	
Cyclohexanone	214	1.10	200	0.96	189	0.85	0.0028
Adamantanone	460	3.51	401	2.93	332	2.25	0.0050
Protoadamantan-4-one	447	3.38	384	2.76	317	2.11	0.0053
Bicyclo[3,3,1]nonane-2,6-dione	139	0.36	132	0.29	127	0.24	0.0045
Adamantane-2,4-dione	163	0.59	151	0.48	142	0.39	0.0043
Adamantane-2,6-dione	142	0.39	138	0.35	134	0.31	0.0028
2-Thiaadamantane-4,8-dione	155	0.52	146	0.43	138	0.35	0.0043
Adamantan-1-ol-2-one	174	0.71	170	0.67	163	0.59	0.0020
Adamantan-1-ol-4-one	128	0.26	125	0.22	122	0.19	0.0030
Adamantan-2-ol-6-one	130	0.28	126	0.24	122	0.19	0.0038
Adamantan-4-ol-2-one	143	0.40	139	0.36	133	0.31	0.0028
Diamantan-1-ol-3-one	238	1.33	216	1.12	198	0.94	0.0113
Tricyclo[4,4,0,$0^{2,9}$]decan-9-ol-5-one	137	0.35	136	0.33	134	0.32	0.0010
Cyclohexanol	210	1.06	194	0.91	184	0.81	0.0028
Adamantan-1-ol	429	3.21	366	2.59	305	1.99	0.0053
Adamantan-2-ol	560	4.49	462	3.53	372	2.65	0.0058
Adamantane-1,2-diol	177	0.74	168	0.65	160	0.57	0.0028
Adamantane-2^a,4^a-diol	217	1.12	199	0.95	185	0.82	0.0035
Adamantane-2^a,4^e-diol	143	0.40	137	0.35	132	0.29	0.0033
Adamantane-2^e,4^e-diol	131	0.28	126	0.24	123	0.21	0.0035
Adamantane-2,6-diol	119	0.16	119	0.16	117	0.15	0.0013
Diamantane-1,4-diol	217	1.12	183	0.79	172	0.69	0.0028
Diamantane-1,6-diol	187	0.83	180	0.76	171	0.68	0.0023
Diamantane-1,7-diol	174	0.71	163	0.60	155	0.52	0.0033
Diamantane-1,9-diol	206	1.02	187	0.83	172	0.69	0.0043
Diamantane-3,5-diol	550	4.39	430	3.22	342	2.35	0.0068
Adamantanonoxim	349	2.42	300	1.94	252	1.47	0.0053
4-Oxahomoadamantan-5-one	245	1.40	222	1.18	202	0.98	0.0040
Cyclopentanone	181	0.77	168	0.65	161	0.58	0.0033
Cyclooctanone	417	3.09	352	2.45	299	1.93	0.0050

During the measurements on silica the retention times of polycyclic alcohols substituted in a formally identical manner (*e.g.* adamantan-1-ol and diamantan-4-ol or adamantan-2-ol and diamantan-3-ol) were very close[1]. On the reversed phases the retention times of the compounds formally equally substituted are rather different because of the different number of carbon atoms. So, the retention time of the diamantane-3,5-diol mentioned above is essentially longer than t_R of the adamantane-2,4-diols; also, diamantan-4-ol and diamantan-3-ol eluted substantially later than adamantan-1-ol and adamantan-2-ol, respectively.

Even 2-thiaadamantane-4,8-dione has nearly the same retention time as adamantane-2,6-dione in the mobile phase containing methanol. In the mobile phase containing acetonitrile, this heterocyclic derivative is eluted later. The difference in retention times caused by the different structure of protoadamantanone (IV) and

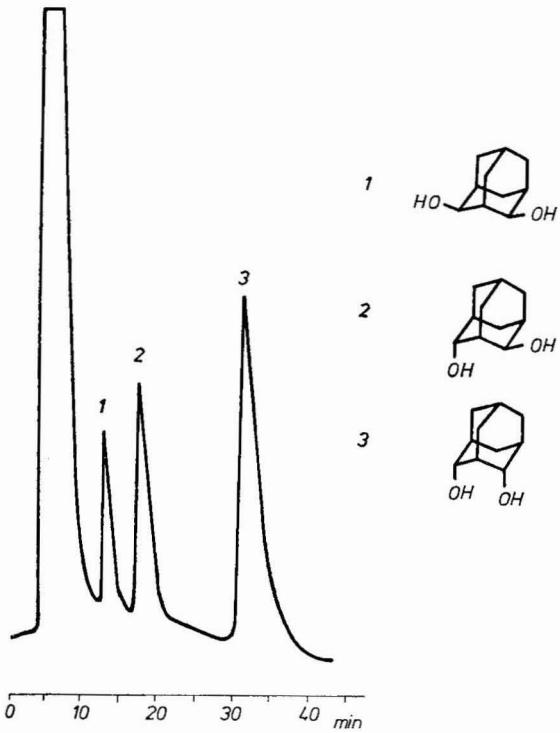


Fig. 1. Separation of the three adamantane-2,4-diol stereoisomers. Column, MicroPak CH-10; mobile phase, methanol-water (20:80); flow-rate, 10 ml/h; temperature, 30°C.

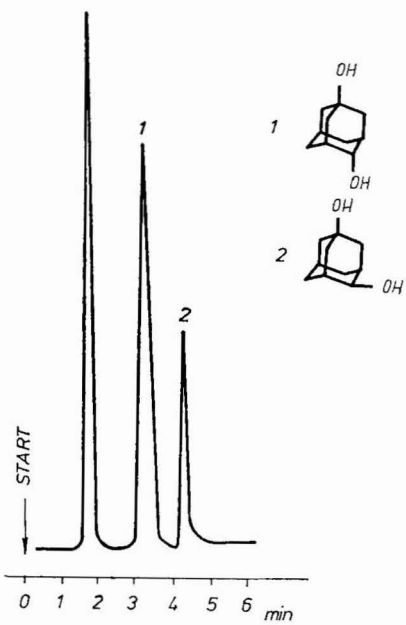
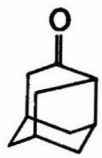


Fig. 2. Separation of the two adamantane-1,4-diol stereoisomers. Column, MicroPak CH-10; mobile phase, methanol-water (30:70); flow-rate, 10 ml/h; temperature, 30°C.

adamantanone (the same number of carbon atoms) appears greatest in the mobile phase with the lowest concentration of acetonitrile at the highest temperature.



IV

The effect of mobile phase composition

An equation derived by Jandera and Churáček[5] describes the dependence of the capacity factor k' on the concentration of acetonitrile in the mobile phase:

$$\log k' = A - n \cdot \log c \qquad (1)$$

where c is the concentration of acetonitrile in the mobile phase, and n and A are constants (Figs. 3 and 4).

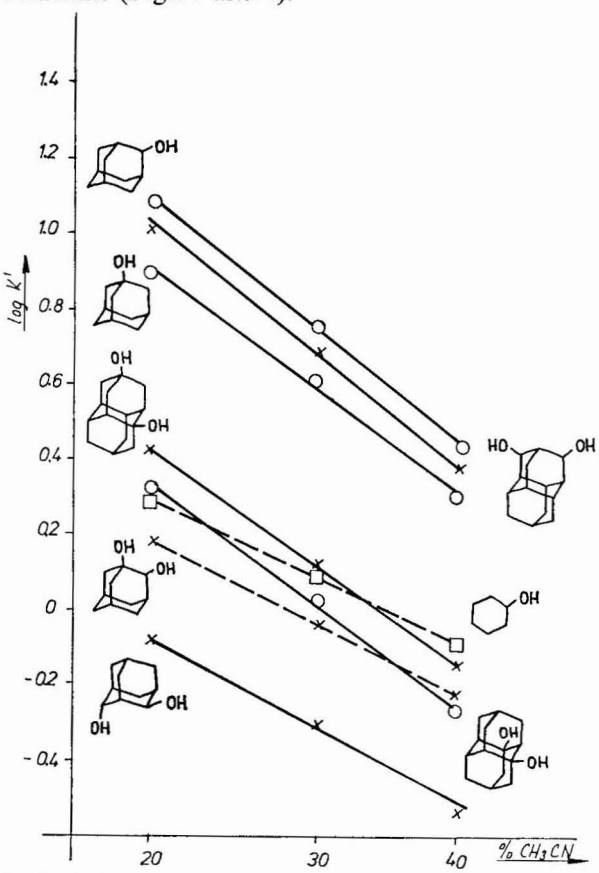


Fig. 3. Variation of the log k' with the concentration of acetonitrile in the mobile phase (water–acetonitrile).

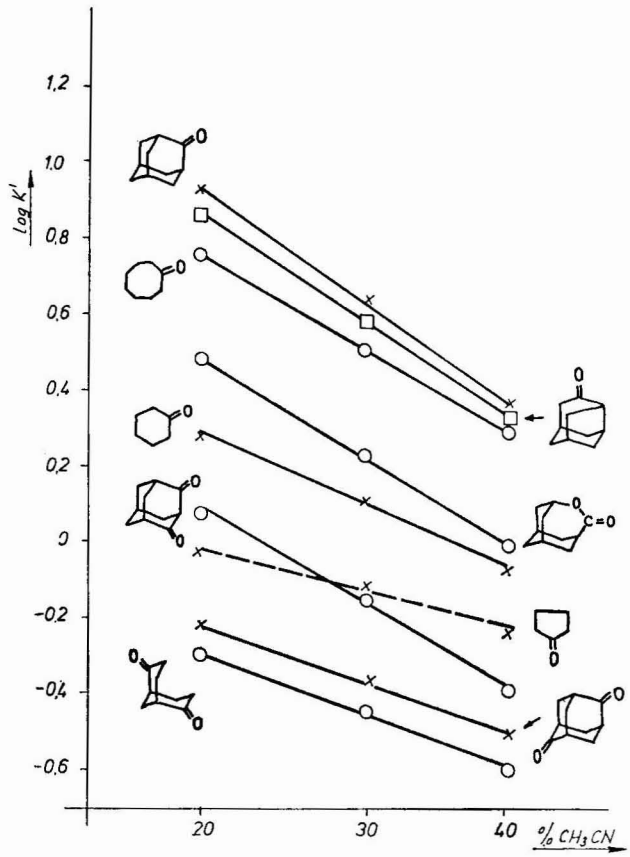


Fig. 4. Variation log k' with the concentration of acetonitrile in the mobile phase (water–acetonitrile).

Fig. 5 shows the dependence of log k' of some compounds on methanol concentration in the mobile phase. In the 40–70% range of methanol content, this dependence is linear and eqn. 1 holds true. The behaviour of the compounds investigated begins to change at 40% methanol, the change being approximately the same for all compounds. However, the selectivity of the separation of individual types of compound, as well as of isomeric derivatives, is not much influenced by changes in methanol concentration.

The dependence of retention times on water concentration is more pronounced for the acetonitrile mobile phase than for the methanol phase. A 10% change in water content in the mobile phase results in a more substantial change in log k' values of the compounds measured when using the acetonitrile–water mixture. The most significant differences were found for adamantanone, protoadamantanone, both adamantanols, and diamantane-3,5-diol, *i.e.* compounds with the highest retention times in the given system.

The mobile phases used are compared in Fig. 6. In both cases, the mobile phase contains 40% of the organic solvent (*i.e.* acetonitrile or methanol) in water. Straight lines representing the selected systems are used for plotting the capacity

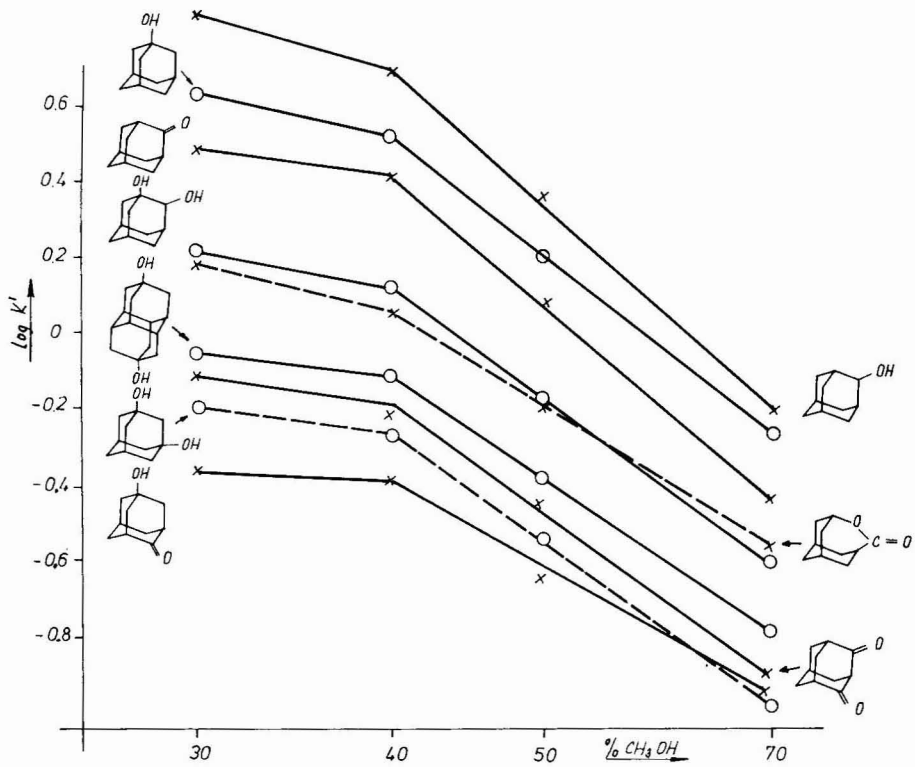


Fig. 5. Variation of the log k' with the concentration of methanol in the mobile phase (water–methanol).

factor values for the compounds chosen. The following conclusions can be drawn:

(1) When methanol replaces acetonitrile, retention times of alcohols increase; this increase is more substantial for molecules with more OH groups and for bigger molecules.

(2) When methanol replaces acetonitrile, retention times of ketones decrease. The elution order adamantan-1-ol, adamantanone, and adamantan-2-ol is changed to adamantanone, adamantan-1-ol and adamantan-2-ol in methanol.

(3) The selectivity of separation of the adamantan-1-ol–adamantan-2-ol pair is *ca.* 1.3–1.4, and is independent of mobile phase composition and temperature.

(4) Retention time differences for compounds substituted on identical secondary carbon atoms by various combinations of keto and hydroxy groups decrease. Thus compounds with nearly the same t_R values in methanol at 30°C are eluted in the order adamantane-2,6-diol, adamantane-2-ol-6-one, and finally adamantane-2,6-dione when acetonitrile is used.

(5) The use of the acetonitrile mobile phase is advisable for separations of ditopic adamantane derivatives.

(6) The selectivity of diamantanediol separations is either the same (1,4/1,9 dihydroxy derivatives) or better (1,6/1,4 and 1,6/1,9 dihydroxy derivatives) in methanol than in acetonitrile.

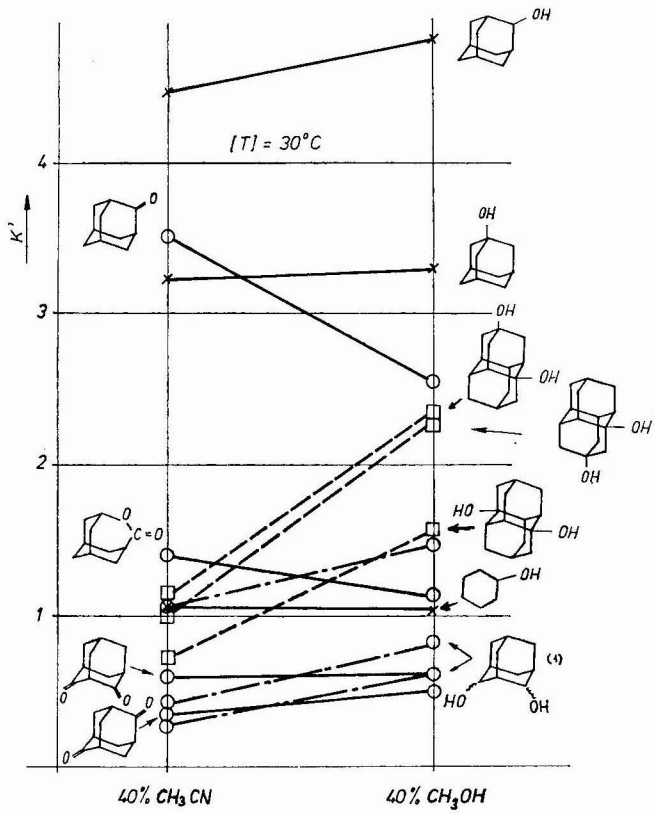


Fig. 6. Comparison of the capacity factors of some compounds, measured in two different mobile phases.

Temperature effects

All measurements in both systems investigated were performed at three temperatures, *viz.* 30, 50, and 70°C. The change of k' or log k' with temperature was studied. Values calculated from retention data, related to a 10°C temperature change, are summarized in Tables II–VII for the corresponding concentrations and compositions of mobile phases.

Capacity factors of the compounds studied are rather independent of temperature. In acetonitrile–water the temperature does not affect the selectivity or the separation efficiency. In case of the methanol mobile phase, there are some small changes improving the separation of several compounds (mostly of low solubility), the solubility being increased at higher temperatures (separation of diamantane-1,4-diol and diamantane-1,9-diol).

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PECTIC ENZYMES*

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SUMMARY

Technical pectic multienzyme preparations, Pectinex Ultra and Rohament P, were chromatographed on an analytical scale using medium pressure liquid chromatography on a glycol methacrylate rigid macroreticular gel, Spheron 1000 and its ion-exchange derivatives. A combination of isocratic and linear-gradient elution (with gradients in ionic strength or pH) was employed and fractions were monitored by measurements of absorbance (A_{285} and A_{254}), conductivity, pH and enzyme activity. Conditions for rapid separations of pectic enzymes are elaborated.

The results indicate the possibilities of separating, the technically undesirable pectin-esterase activity from the other enzyme activities, and of a more detailed biochemical investigation of these enzymes, important for the food industry.

INTRODUCTION

Microorganisms grown in a medium containing pectin produce a mixture of enzymes which catalyze its degradation, via the de-esterification of units of D-galactopyranuric acid and the hydrolysis or β-elimination of glycosidic α-1,4-bonds in D-galacturonan. The composition of the enzyme mixture and the content of individual enzymes are determined by the type of microorganism and the conditions of cultivation. High yields of pectic enzymes are obtained from microscopic fungi, which are therefore often used as sources of commercial and technical preparations of pectinases.

Various methods have been used[1] for the separation of microbial pectic enzymes from cultivation media or commercial preparations, such as ion-exchange chromatography on CM-cellulose[2], DEAE-cellulose[3,4], cellulose phosphate[5], affinity chromatography on pectic acid cross-linked with epichlorohydrin[6] and on its amino

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derivative[7] and gel chromatography on Bio-Gel[8]. These methods generally lead to the separation of one enzyme from a mixture of other enzymes, and all enzymes present in the starting material can be separated only by chromatography on DEAE-cellulose[3,4].

Modern development of high-performance liquid chromatography (HPLC) of biopolymers[9–11] has permitted the application of this rapid method to the separation of pectic enzymes. In earlier papers[12–15] we have demonstrated the possibility of using the matrix of the hydrophilic polymer Spheron® for the preparation of macroreticular ion exchangers. These are suitable for rapid chromatography of enzymes[16] and other proteins[12], simple sugars[17], oligosaccharides[18], nucleoside phosphates[19] and oligonucleotides[20]. We also demonstrated (ref. 16, Fig. 5) the possibility of chromatographing the Czechoslovak technical pectic enzyme Leozym on DEAE-Spheron 300 prepared in the laboratory.

The aim of this paper is to examine the possibilities of HPLC using various commercial derivatives of Spheron 1000 for rapid separation of mixtures of pectic enzymes in preparations of microbial origin, on an analytical scale. The work is oriented towards commercial preparations used in the foodstuffs industry for canning, which contain the pectic enzymes *endo*-D-galacturonanase and *exo*-D-galacturonanase [poly(1–4)-α-D-galactosiduronate glycanohydrolase, E.C. 3.2.1.15 and E.C. 3.2.1.67], pectin-lyase [poly(methyl-D-galactosiduronate) lyase, E.C. 4.2.2.10] and pectin-esterase (pectin pectyl-hydrolase, E.C. 3.1.1.11).

EXPERIMENTAL

Materials

Enzymes. Pectinex Ultra (Ferment, Basel, Switzerland) and two batches of the preparation Rohament P (Rohm and Haas, Darmstadt, G.F.R.) were employed.

Substrates. Citrus pectin, degree of esterification (DE) 65.1%, was purified from Genu Pektin Type B, Slow Set (Københavns Pektinfabrik, Copenhagen, Denmark) by washing with 60% ethanol containing 5% hydrochloric acid and then with 60% and 96% ethanol. Pectic acid was prepared from citrus pectin by repeated alkaline de-esterification with 0.1 *M* sodium hydroxide and subsequent precipitation at pH 2.5. The highly esterified pectin (DE 93.8%) was prepared by esterification of pectic acid with 1 *M* sulphuric acid solution in methanol[21]. Digalacturonic acid was isolated from the partial acid hydrolysate of pectic acid by gel chromatography on Sephadex G-25 (Fine)[22].

Chromatographic materials. Spheron 1000, particle size 25–40 μm, was obtained from Lachema (Brno, Czechoslovakia). Its characteristics are given in ref. 10 (Table I) and in ref. 23 (Table 6.3A); *cf.*, also ref. 13 (Tables I and II). The ion exchangers employed, based on the matrix Spheron 1000, were also from Lachema and their characterization is given in the monograph[23] (Table 5.6B). Nominal capacities: weakly acidic carboxylic cation exchanger Spheron C-1000 (25–40 μm), 1.85 mequiv./g; medium acidic cation exchanger Spheron Phosphate 1000 (40–63 μm), 3.1 mequiv./g; strongly acidic sulphonated cation exchanger Spheron S-1000 (25–40 μm), 1.72 mequiv./g; weakly basic anion exchanger Spheron DEAE-1000 (25–40 μm), 1.5 mequiv./g; strongly basic quaternized anion exchanger Spheron TEAE-1000 (25–40 μm), 1.4 mequiv./g. Other pure chemicals were obtained from Lachema.

Chromatographic methods

The preliminary treatment of Spheron ion exchangers, the chromatographic methods and the equipment used were described in detail in refs. 10, 15 and 16. In this paper a combination of isocratic elution and a linear gradient was employed. The concentrations of the buffers mentioned relate to the counter ions. The chromatographic column from the amino acid analyzer (8 mm I.D.) was packed with 20 cm of the ion exchanger using the slurry method, with occasional pressure pulses of 15–20 atm. The ion exchanger was equilibrated with the first elution buffer. The sample was dissolved in 0.3 ml of the first elution buffer. The eluent (flow-rate *ca.* 3 ml/min) flowed through a tandem system of two flow-through photocells (absorbance recorded at 254 nm and 285 nm) into a fraction collector, the fractions being changed at 90-sec intervals. The pressure in individual experiments was between 4 and 15 atm. The chromatography was carried out at room temperature (20–25°C). The conductivity, pH and enzyme activity of each fraction were measured. The fractions were kept in closed test-tubes, with 2–3 drops of toluene, in an ice-box at 4°C until required for the activity measurements.

Testing methods

The activity of *endo*-D-galacturonanase and *exo*-D-galacturonanase was followed using the method of Somogyi[24] by determining the increase in the number of reducing groups in the reaction mixture composed of 0.8 ml of substrate and 0.2 ml of the enzyme. A 0.5% solution of pectic acid in 0.1 *M* acetate buffer, pH 4.2, was used as substrate for the determination of the activity of D-galacturonanases. For *exo*-D-galacturonanase, a 1 m*M* solution of digalacturonic acid in 0.1 *M* acetate buffer, pH 4.5, was employed.

The activity of pectin-esterase was determined by titration (0.1 *M* sodium hydroxide) of the carboxyl groups set free during reaction for 60 min. A 0.5% pectin solution (DE 65.1%) in 0.1 *M* acetate buffer, pH 4.4, was used as substrate. The reaction mixture contained 5 ml of substrate and 1 ml of enzyme solution.

The activity of pectin-lyase was determined from the increase in absorbance at 235 nm (ref. 25), using a 0.5% solution of highly esterified pectin (DE 93.8%) in 0.1 *M* acetate buffer, pH 5.6, as substrate. The reaction mixture contained 2.5 ml of the substrate and 0.5 ml of the enzyme solution.

RESULTS

The possibility of using HPLC on Spheron derivatives for the separation of technical pectic enzymes was tested with two commercial preparations differing in the content of individual pectic enzymes. Pectinex Ultra is a multienzyme preparation from a culture of *Aspergillus niger*, having a high content of *endo*-D-galacturonanase (N), pectin-esterase (S) and pectin-lyase (L). Chromatography on cellulose phosphate indicated the presence of two *endo*-D-galacturonanases[5]. The activity of *exo*-D-galacturonanase (X), which generally occurs as a minor component of microbial materials, is substantially lower in this material. Rohament P has a high content of *endo*-D-galacturonanase (N), while the activity of other pectic enzymes (S, L, X) in it is low. Therefore some idea of the separation possibilities of pectic enzymes on Spheron derivatives can be obtained on the basis of the results with Pectinex Ultra.

Most of the chromatography on unmodified Spheron 1000 and all its ion-exchanging derivatives was carried out with this preparation, which was also used to determine suitable conditions (buffer composition, pH, gradient) for separation of the enzymes. Rohament P was used for comparison and, in the case of the chromatography on a carboxylated cation exchanger, also for investigating the effect of column loading on the quality of the separation.

Technical pectic enzymes were chromatographed mainly on ion-exchange derivatives. However, in order to elucidate the effect of the matrix on the separation process, chromatography on Spheron 1000 alone, which was not ionogenically modified, was also performed. With Pectinex Ultra, experiments were carried out on Spheron 1000 and weakly, medium and strongly acidic cation exchangers and weakly and strongly basic anion exchangers. With Rohament P, separation experiments on non-modified Spheron 1000 were carried out and the possibility of eluting the column not only with aqueous solutions but also with a solution of *tert.*-butanol was also tested. Chromatography on a weakly and medium acidic cation exchanger was also carried out with two batches of Rohament P.

The chromatography of Pectinex on non-modified Spheron 1000 is illustrated in Fig. 1. Since this macroporous material has an exclusion limit of 1,000,000 daltons and the maximum of the pore-size distribution curve is at diameter 370 Å (ref. 13), we do not consider that the separation is a consequence of gel permeation but of hydrophobic chromatography on the matrix of this macroreticular polymer (*cf.*, ref. 26). However, we emphasize that the conditions used are not favourable for hydrophobic separation and were employed only for comparative purposes with ion-exchange chromatography. While all the esterase activity, S, remains in the hold-up volume and the peak of the pectin-lyase activity, L, is only slightly retarded, the *endo*-polygalacturonanase activity, N, appears in two peaks: one moves with the hold-up volume while the second is more retarded. Since two forms of this enzyme have already been described[5], their separation could have taken place here (in this case the material of the second peak, N, has more hydrophobic properties). However, without further characterization of the fractions the result may be interpreted assuming that the capacity of the column was insufficient for the retention of this most abundant enzyme and that a part of the material N was eluted with the void volume.

Pectinex Ultra gave two peaks of *endo*-polygalacturonanase activity, N, even on a weakly acidic cation exchanger (Fig. 2) at pH 3.5. The first peak is little and the second considerably retarded. However, pectin-lyase is strongly bound on the cation exchanger, while the asymmetry of the peaks may also indicate multiple forms of this enzyme. Replacement of formic acid by acetic acid in buffers of pH 3.5 was not appropriate since the required high concentration disturbed the enzymatic tests. Chromatography at pH 8 (concentration gradient of sodium acetate, not shown) led to the separation of pectin-lyase from the mixture of other enzymes.

The use of a medium acidic cation exchanger having a bivalent functional group for the chromatography of Pectinex Ultra is illustrated in Fig. 3. Although this type of ion exchanger has been found to be very suitable for the chromatography of proteins[12], under the given experimental conditions the separation was not satisfactory. We believe that a further systematic study of a larger number of milder gradients could result in an improvement of the separation even on this ion exchanger.

The chromatography of Pectinex Ultra on a strongly acidic cation exchanger

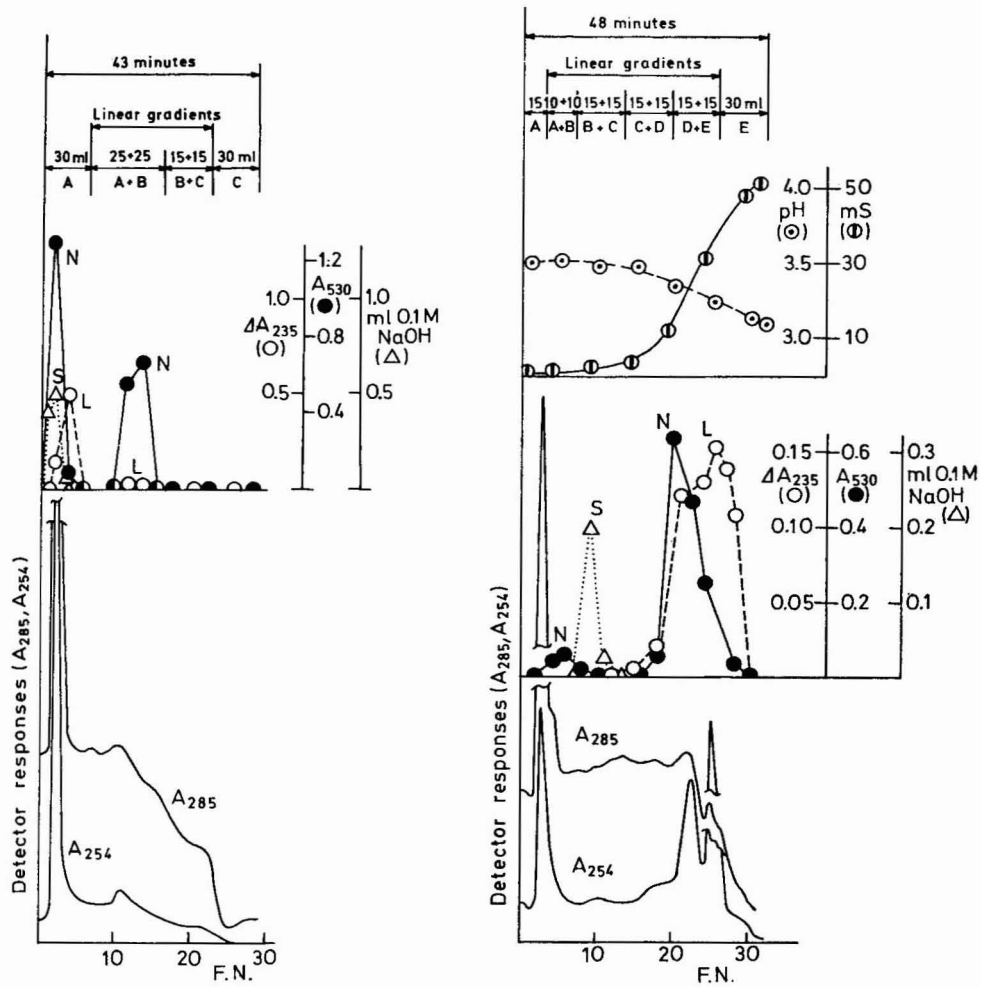

Fig. 1. Chromatography of 25 mg of Pectinex Ultra on unmodified Spheron 1000. Fractions 4.8 ml per 90 sec; pressure 0.4 MPa. Eluents: A, 0.05 *M* sodium hydroxide + acetic acid, pH 3.5. B, 1 *M* sodium chloride. C, 2 *M* sodium chloride. Enzymatic activities: L = pectin-lyase; N = *endo*-D-galacturonanase; S = pectin-esterase. For methods of determination and calculation of results see text. F.N. = fraction number.

Fig. 2. Chromatography of 25 mg of Pectinex Ultra on carboxylated cation exchanger Spheron C-1000. Fractions and pressure as in Fig. 1. The buffers were prepared from sodium hydroxide solutions of the indicated concentration, adjusted with formic acid to pH 3.5: A, 0.05 *M*; B, 0.1 *M*;C, 0.2 *M*; D, buffer C 1 *M* in sodium chloride; E, 2 *M* sodium chloride. Enzymatic activities as in Fig. 1. The pH values were determined using a glass electrode. mS = Electrical conductivity of the effluent in milliSiemens, as an illustration of the ionic strength value. F.N. = fraction number.

gave a satisfactory separation (Fig. 4). The process of separation of pectin-lyase activity, L, and esterase activity, S, into two peaks can be evaluated the same way as the two peaks in Fig. 1. However, this explanation cannot be used for the separation of *endo*-polygalacturonanases, N, because both peaks were retarded to different

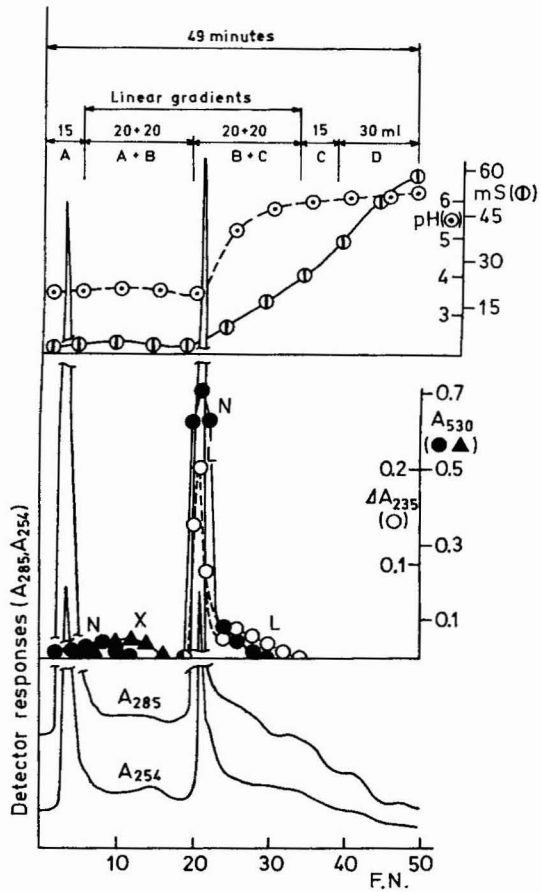

Fig. 3. Chromatography of 25 mg of Pectinex Ultra on Spheron Phosphate 1000. Fractions 2.8 ml per 1 min; pressure 0.3 MPa. The buffers were prepared from ammonium hydroxide of the indicated concentration, which was adjusted to the given pH value with formic acid in the case of buffer A, and with acetic acid in the case of the other buffers: A, 0.05 *M* (pH 3.5); B, 0.3 *M* (pH 6); C, 1 *M* (pH 8), 0.5 *M* in sodium chloride. Enzymatic activities as in Fig. 1, except X = *exo*-D-galacturonanase. F.N. = fraction number.

extents; the largest peak N was the most strongly bound on the strongly acidic cation exchanger.

A promising separation was achieved on a weakly basic anion exchanger (Fig. 5). Here the *endo*-polygalacturonanase activity, N, occurs in three peaks of which the first small peak (in the hold-up volume) may be due to exceeding the column capacity. The change from pH 7 to 5 (which would favour higher stability of *endo*-D-galacturonanase[4]) and the adjustment of gradients (Fig. 6) led to increased retardation of *endo*-polygalacturonanase, N, while the esterase, S, peak moved into the hold-up volume; this may be of great importance — see the Discussion. However, at this pH value of the elution buffer, the pH of the effluent varied. At a still lower pH (4.2–4.5, not illustrated) and by using acetate buffers, all the enzymes were eluted in a single fraction.

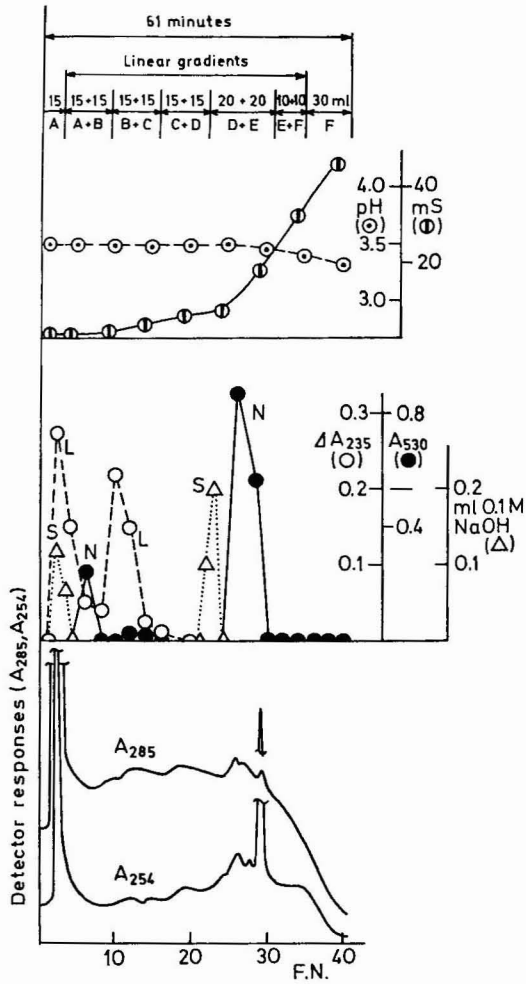

Fig. 4. Chromatography of 25 mg Pectinex Ultra on sulphonated cation exchanger Spheron S-1000. Fractions 4.7 ml per 90 sec; pressure 0.7 MPa. The buffers were prepared from sodium hydroxide of the indicated concentration, adjusted to pH 3.5 with formic acid: A, 0.05 *M*; B, 0.1 *M*; C, 0.2 *M*; D, 0.3 *M*; E, 0.5 *M*, 1 *M* in sodium chloride; F, 2 *M* sodium chloride. Enzymatic activities as in Fig. 1. F.N. = fraction number.

The importance of the adjustment of the gradient in ion-exchange chromatography, using buffers having the same pH value, is evident from the comparison of Fig. 7 with Fig. 5. In this experiment (Fig. 7) the separation of the activities of *endo*-polygalacturonanase, N (two peaks, both retarded), esterase, S, and pectin-lyase, L, was achieved. None of the analysed enzymes occurred in the hold-up volume, so that under these conditions the capacity of the column was not exceeded.

On a strongly basic anion exchanger (Fig. 8) the elution order was similar to that on the weakly basic anion exchanger (Fig. 7), but the separation was not improved. This may be due not only to the stronger basicity of the quaternized functional

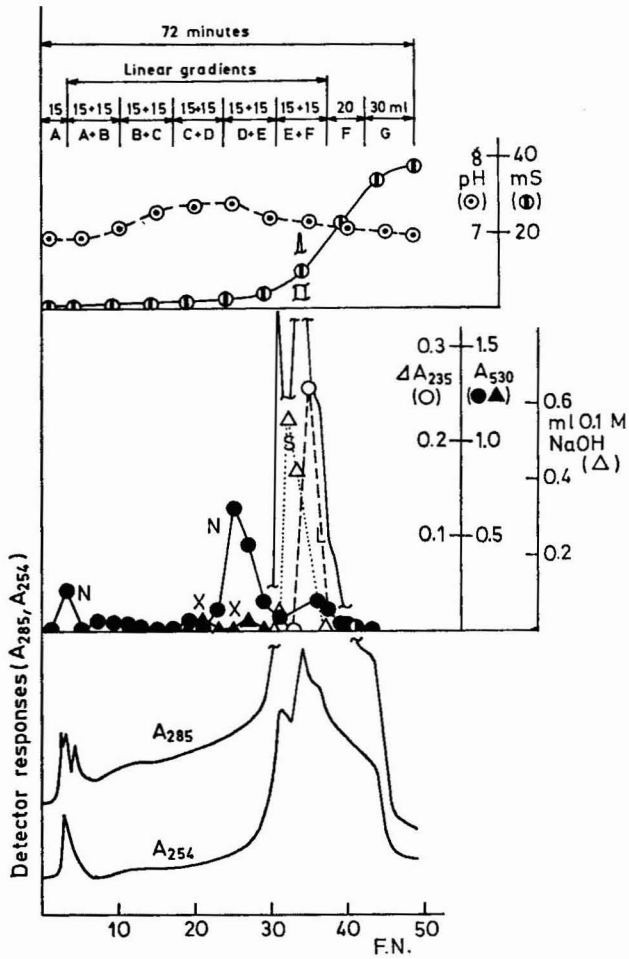

Fig. 5. Chromatography of 25 mg of Pectinex Ultra on the anion exchanger Spheron DEAE-1000, eluting with buffers of pH 7. Fractions 4.4 ml per 90 sec; pressure 0.4 MPa. The buffers were prepared from hydrochloric acid of the given concentration, which was adjusted with Tris to pH 7: A, 0.005 *M*; B, 0.05 *M*; C, 0.1 *M*; D, 0.2 *M*; E, 0.4 *M*; F, buffer E with 1 *M* sodium chloride; G, 0.2 *M* sodium chloride. Enzymatic activities as in Figs. 1 and 3. F.N. = fraction number.

groups, but also to the imperfect nature of the ion exchanger, which was developed as the last of the series of ion exchangers with classical functional groups.

Rohament P was chromatographed on non-modified Spheron 1000 (Fig. 9), first under conditions similar to those employed in the chromatography of Pectinex Ultra (Fig. 1). Whereas in the experiment in Fig. 1 all esterase activity, S, was eluted within the hold-up volume, in Fig. 9 it is considerably retarded. In both experiments (Figs. 1 and 9) the lyase activity, L, is retarded slightly more than the first peak of *endo*-polygalacturonanase activity, N, which is eluted within the hold-up volume. The lyase activity, L, does not belong to a single form of enzyme either in Rohament P (Fig. 9) or in Pectinex Ultra (Fig. 1). The experiment in Fig. 9 was also used to investigate the

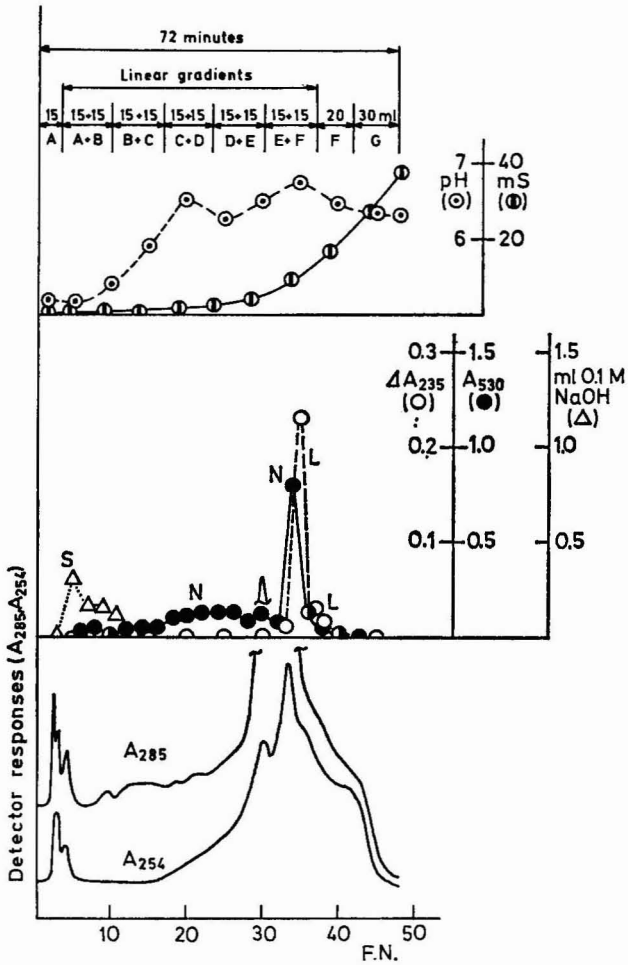

Fig. 6. Chromatography of 25 mg of Pectinex Ultra on Spheron DEAE-1000, on elution with buffers of pH 5. The buffers were prepared as in Fig. 5, but were adjusted to pH 5. Enzymatic activities as in Fig. 1. F.N. = fraction number.

elution of the column with organic solvents, with the aim of removing non-protein or coloured substances that are often present in technical enzyme preparations. Spheron and its ion-exchange derivatives are fairly resistant to organic solvents, but because of the photometric detection they must first be extracted with such solvents (prior to use) in order to eliminate contaminants. No enzyme activity was found in the peaks eluted with eluents containing *tert.*-butanol, even though it was shown in other experiments that some enzymes are stable for short periods in these dilute solutions of *tert.*-butanol. Aqueous solutions of *tert.*-butanol eluted only inactive components of the original preparations, which contained strong chromophores. This indicates the possibility of regenerating the packings by elimination of coloured material.

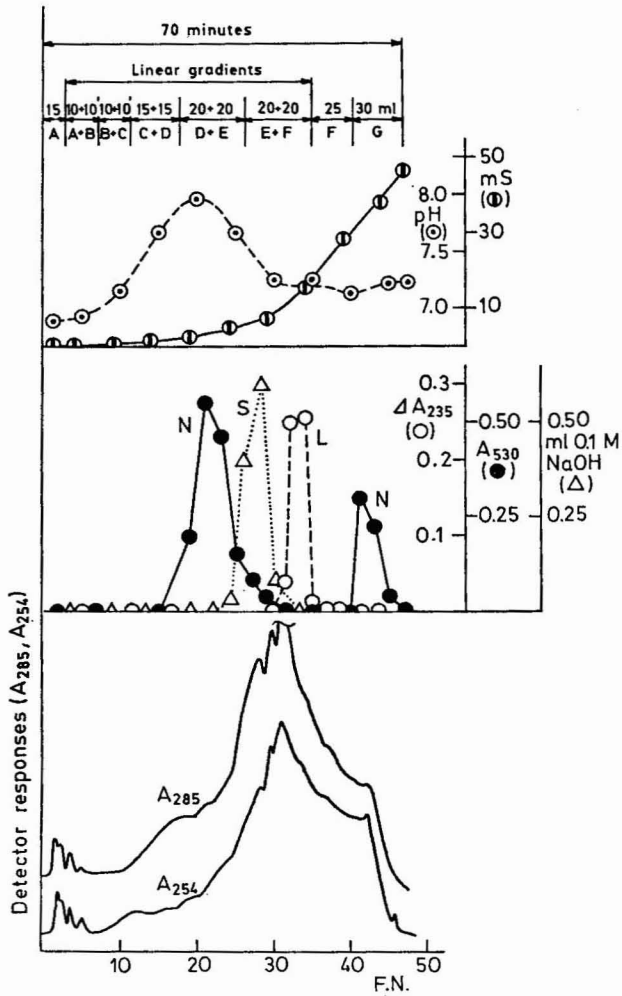

Fig. 7. Chromatography of 25 mg of Pectinex Ultra on Spheron DEAE-1000 with an adjusted gradient elution with buffers of pH 7. The buffers were prepared as in Fig. 5 and the labelling of enzymatic activities is also the same. Fractions 5.1 ml per 90 sec; pressure 0.6 MPa. F.N. = fraction number.

The different characteristics of some enzymes in the analysed preparations were demonstrated by the chromatography of Pectinex Ultra (Fig. 2) and Rohament P (Fig. 10) on a weakly acidic cation exchanger under the same conditions. For example, while pectin-lyase activity, L, is eluted last in Fig. 2, it is eluted first in Fig. 10. In contrast, the esterase activity, S, is eluted more easily from Pectinex Ultra (Fig. 2). A five-fold decrease in the column load did not lead to an improvement in the separation (Fig. 11) under identical conditions, from which it may be concluded that even a loading of 25 mg of pectic enzyme did not limit the separation ability of the column.

The chromatography of Rohament P on a medium acidic cation exchanger (Fig. 12) can be compared with the chromatography of Pectinex Ultra (Fig. 3). The

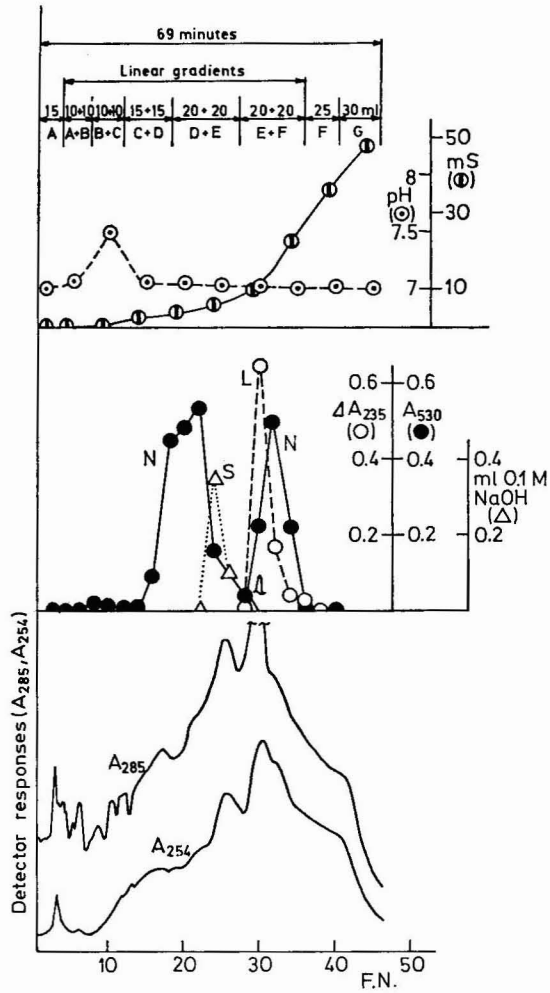

Fig. 8. Chromatography of 25 mg of Pectinex Ultra on strongly basic anion exchanger Spheron TEAE-1000. Fractions 4.6 ml per 90 sec; pressure 0.6 MPa. The buffers of pH 7 were identical to those in Fig. 5, and the labelling of enzymatic activities is also the same. F.N. = fraction number.

results achieved were similar, *i.e.*, both preparations contained small amounts of *exo*-polygalacturonanase, X. Similar comments to those on Fig. 3 can be made on the suitability of the phosphate ion exchangers for the separation.

DISCUSSION

The method described represents an advance upon previous methods of analysis of mixed commercial and technical preparations of pectic enzymes. Up to now the determination of the activity of individual enzymes in these preparations has been considerably complicated by the simultaneous effect of several enzymes on the same substrate, as a consequence of which the effect of the determined enzyme is

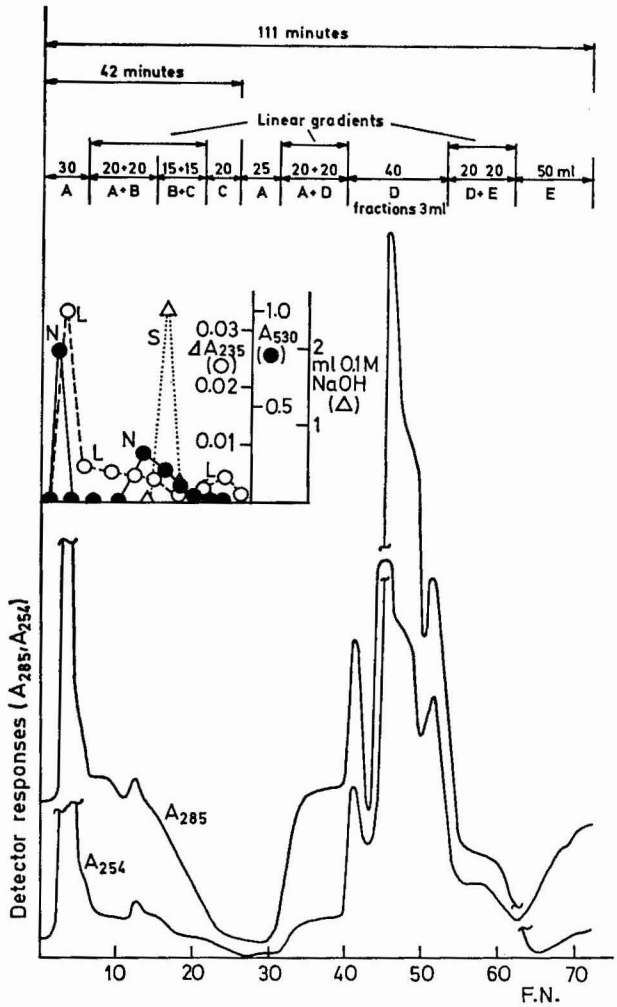

Fig. 9. Chromatography of 25 mg of Rohament P on unmodified Spheron 1000. Fractions 4.5 ml per 90 sec; pressure 0.5 MPa until the elution stage A+D. During the elution D microscopic bubbles appeared in the effluent, the fraction volume dropped to 3 ml and the pressure increased to 1 MPa. During the elution D+E the fraction volume and the pressure returned to normal. Eluents: A, 0.05 *M* sodium hydroxide adjusted to pH 3.5 with acetic acid; B, 1 *M* sodium chloride; C, 2 *M* sodium chloride; D, 50% *tert.*-butanol in buffer A; E, pure water. Enzymatic activities as in Fig. 1. F.N. = fraction number.

partly suppressed. This is true of all pectic enzymes with the exception of *exo*-D-galacturonanase, X, the activity of which can be determined by means of a specific substrate. A further obstacle to the accurate determination of the enzymatic activity consisted in the high content of coloured and reducing contaminants, which considerably decrease the accuracy of the spectrophotometric determination of the activity of pectin-lyase and D-galacturonanases. As a consequence the measured values of the activities were usually subject to an error the magnitude of which was dependent

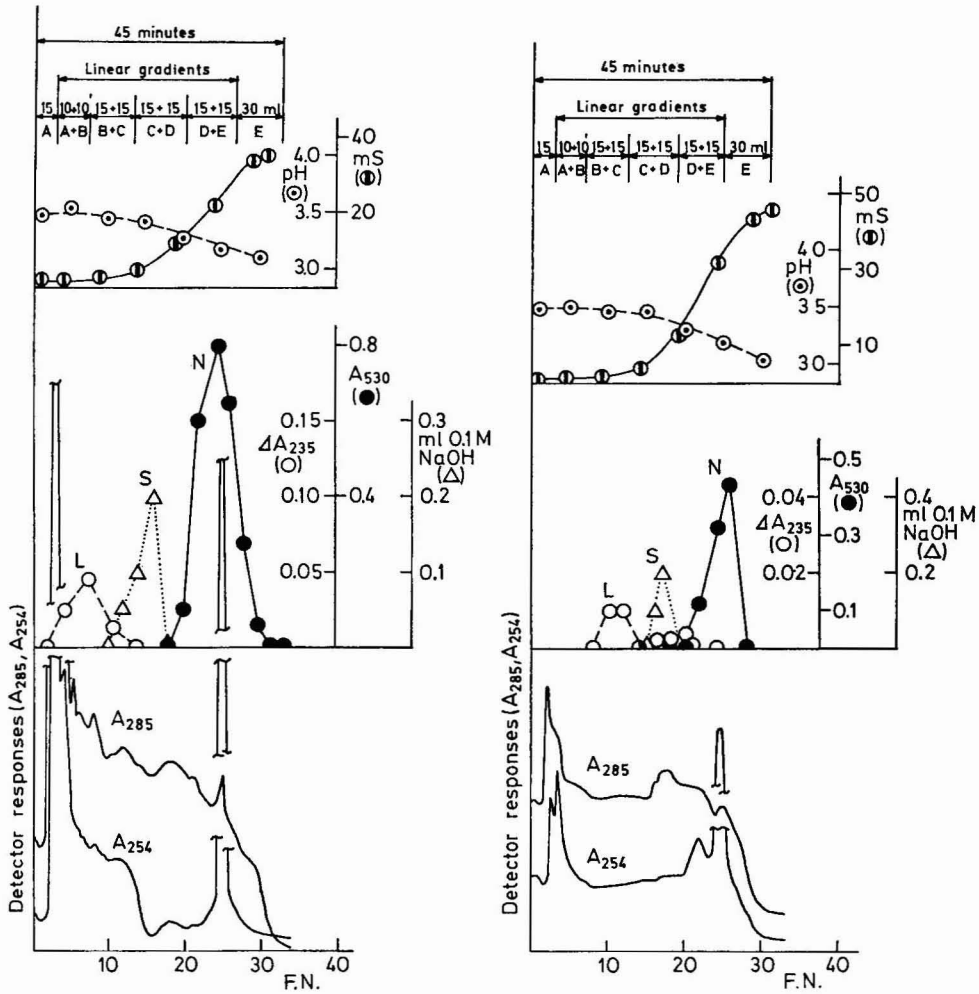

Fig. 10. Chromatography of 25 mg of Rohament P on carboxylated cation exchanger Spheron C-1000. Fractions 4.8 ml per 90 sec; pressure 0.4 MPa. The buffers were identical to those in Fig. 2. Enzymatic activities as in Fig. 1. F.N. = fraction number.

Fig. 11. Chromatography of 5 mg of Rohament P on Spheron C-1000. Fractions 4.7 ml per 90 sec. All other parameters as in Fig. 10. F.N. = fraction number.

on the relative proportions of the enzymes and the contaminants. HPLC on Spheron ion exchangers on an analytical scale, permitting a rapid separation of enzymes, enables the determination of the activity of isolated enzymes freed from contaminants and thus eliminates these drawbacks. The non-ionic reducing substances are often eluted within the hold-up volume. A further advantage of this method is the possibility of a rapid determination of the multiple forms of individual enzymes.

Experience gained from almost all the experiments carried out indicates that the evaluation of the effluent by both UV detectors is not sufficient even for a preliminary choice of separation conditions. Only a complete determination of all enzyme

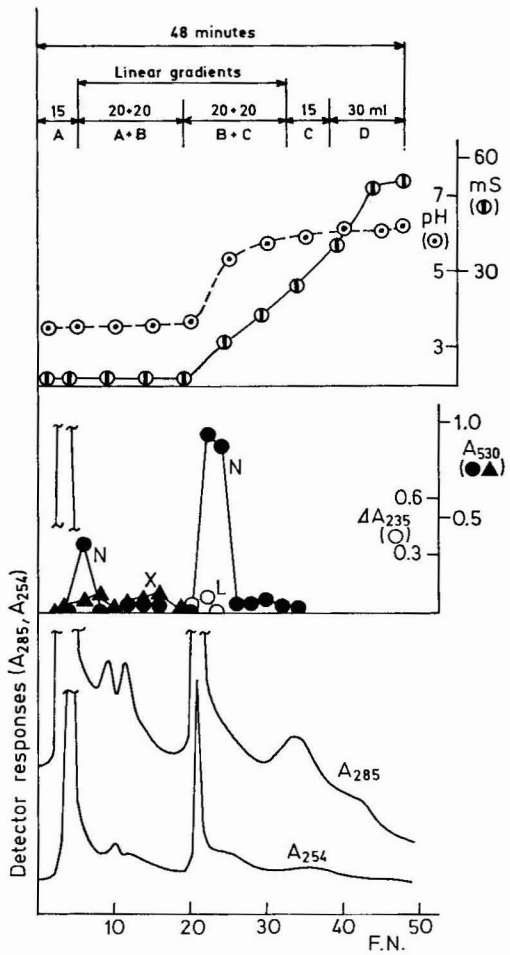

Fig. 12. Chromatography of 25 mg of Rohament P on Spheron Phosphate 1000. Fractions 2.9 ml per 1 min; pressure 0.4 MPa. Buffers A–D as in Fig. 3. Enzymatic activities as in Figs. 1 and 3. F.N. = fraction number.

activities in individual fractions afforded sufficient information for the adjustment of the gradients. Sometimes the peaks of activity occurred in regions where the UV-detectors recorded only a low absorbance, or peaks of high UV-absorbance were devoid of activity. The zones of the proteins are also evidently overlapped by the zones of other coloured contaminants.

The best separation of pectic enzymes was achieved on the anion exchanger Spheron DEAE-1000 with a concentration gradient of HCl–Tris buffer, pH 7.0 (Fig. 7), where both forms of *endo*-D-galacturonanase, N, were separated from Pectinex Ultra in addition to other individual pectic activities. The pectic enzymes from *Aspergillus niger* are acidic proteins with isoelectric points between p*I* 4.8 and 5.4 (ref. 27). At pH 7 they were retained on Spheron DEAE in 0.005 *M* buffer and were gradually eluted at higher concentrations of the buffer, beginning with 0.2 *M*,

in order of increasing acidity, *i.e.*, *endo*-D-galacturonanase I (N), pectin-esterase (S), pectin-lyase (L) and *endo*-D-galacturonanase II (N). The simple gradient which was used in ref. 16 (Fig. 5) for the chromatography of the technical pectic enzyme Leozym does not suffice for a good separation of all required enzymes.

The enzymes of the preparation Rohament behaved similarly on DEAE-Spheron (not illustrated). For their separation also the cation exchanger Spheron C-1000 (Fig. 10) was very suitable; the enzymes were eluted with sufficiently different elution volumes when the concentration gradient of the formate buffer, pH 3.5, was used, in the order pectin-lyase (L), pectin-esterase (S) and *endo*-D-galacturonanase (N). The results of the separation of two different batches of Rohament P on Spheron C-1000 also indicated the differences in the composition and the proportion of the enzymes of various production batches, two fractions with pectin-lyase activity (not illustrated) being found in one batch.

In the chromatography of Pectinex Ultra on the cation exchanger Spheron C-1000 in the formate buffer, pH 3.5 (concentration gradient 0.05–0.2 *M*), pectin-esterase, S, was very well separated from the mixture of enzymes, and was eluted before the other enzymes, with a small admixture of *endo*-polygalacturonanase, N (Fig. 2). On a preparative scale such a method can help to remove pectin-esterase from pectic multi-enzyme preparations. Enzyme of this specificity splits methanol from pectins and therefore it is undesirable in the foodstuff industry branches producing fruit juices and similar products. A similar separation was also achieved on the anion exchanger Spheron DEAE-1000, if the pH of the elution buffer was decreased to 5 (Fig. 6). On the strongly acidic cation exchanger Spheron S-1000 a good separation of the enzymes in the formate buffer, pH 3.5, was obtained (Fig. 4). Individual enzymes were eluted gradually from the cation exchanger, in the order of increasing basicity: *endo*-D-galacturonanase II (N), pectin-lyase (L), pectin-esterase (S) and *endo*-D-galacturonanase I (N).

The polymorphism of the pectic enzymes present in technical pectic preparations is clearly not limited to the two forms of *endo*-D-galacturonanase (N), I, II (Figs. 1–9 and 12). A more detailed inspection of Figs. 1–4 and 9 demonstrates (with the restrictions given in Results) the existence of several forms of lyases, L, separable on ion exchangers. The results of the experiments illustrated in Figs. 1 and 9 indicate that the esterases, S, present in Pectinex Ultra and Rohament P are different. Even the minor component, *exo*-polygalacturonanase, X, of the preparation Rohament P seems to be a composite, when chromatographed on Spheron Phosphate 1000 (Fig. 12). Various reasons for this polymorphism are: existence of genetically conditioned isoenzymes; effect of the limited proteolysis in the cultivation medium; topographic changes in conformers with deviations in the isoelectric points, etc. Chromatography on Spheron 1000 and its ion-exchange derivatives allows the possibility of studying these questions in greater detail. The aim of this paper was to study the chromatographic details not the biochemistry, however it has demonstrated that this method could be employed for such a biochemical study.

CONCLUSIONS

The experiments described in this paper have demonstrated the ability of Spheron ion exchangers in rapid chromatographic separation of pectic enzymes on

an analytical scale. The chemical stability of Spheron and its derivatives[12,13], the resistance to the effect of technical enzymes[16] and microbial infection, the independence of the bed volume on pH and ionic strength, the easy regeneration and possibility of repeated use indicate the potential of these ion exchangers for separations on a laboratory or technical scale. Preliminary experiments with technical protease have already been carried out (ref. 16, Fig. 2), where it was demonstrated that for reversible sorption of the technical enzyme the bed of the Spheron ion exchanger should have been a broad and low layer on a fritted filter, and desorption results in a peak similar in form to a chromatographic one, although not as perfect as those obtained on chromatographic columns.

The flow-rate for chromatography on Spheron and its ion-exchange derivatives was usually *ca.* 5 ml/cm^2·min, corresponding to *ca.* 30 l/min at a column diameter of 80 cm (using the medium pressures given in this paper). We believe that HPLC of technical enzymes can be carried out even on a semi-pilot plant scale, with short elution times.

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GAS–SOLID CHROMATOGRAPHY ON CATION-EXCHANGE RESINS

DARC TOPOLOGICAL ANALYSIS OF THE BEHAVIOR OF ALKENES ON NICKEL IONS*

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SUMMARY

Retention indices of 44 alkenes in gas–solid chromatography were determined on the Ni^{2+} form of a cation-exchange resin and on the corresponding unsulfonated copolymer matrix. The Ni^{2+} form was prepared from a sulfonated Porapak Q ethylvinylbenzene–divinylbenzene copolymer. DARC topological analysis (DTA) was applied to the Kováts retention indices determined at 175°C. DTA helps to delineate the evolution of specific π bond–Ni^{2+} ion interactions with progressive variations of the structural effects of the alkenes.

INTRODUCTION

Gas–solid chromatography (GSC) on metal-containing surfaces is of continuing interest, particularly for the analysis of unsaturated hydrocarbons[1,2]. Such analyses are selective because of specific interactions due to the possibility of formation of charge-transfer complexes[3]. Attention has also been drawn to the specific interactions between olefins and cations in gas–liquid chromatography when solutions of different salts or metal complexes are used[4]. The high selectivity of stationary phases containing silver nitrate, particularly efficient for the separation of internal olefinic isomers, is well known[5]. Different complexes of rhodium have also been used[6,7]. However, GSC has the advantage of enabling one to work at relatively high temperatures, which significantly reduces the time of analysis. References to the use of

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metal-containing packings in GSC are numerous. Different cationic forms of zeolites[1] and ion-exchange resins[8] have been used for the separation of various types of organic compounds.

The recent development of data processing methods applicable to chromatography, including topological analysis of structural effects[9,10] and factor analysis of trends[11], has led to better quantification of these specific interactions[12–14]. Such information is useful both for improving the rapid analysis of hydrocarbons and for a physico-chemical understanding of catalysis. Indeed, similarities between chromatographic and catalytic parameters, such as the type of support and its effects on adsorbates, are evident. The reactivity of alkenes in catalysis and their chromatographic adsorption on metal-ion-containing surfaces is governed by the strength of the specific interaction between the carbon–carbon double bond and the cation. This interaction is dependent on the nature of the cation, its environment and the structural environment of the carbon–carbon double bond. An example of this dependence is found in the work of Jacobson and Pittman[15], who used polystyrene–divinylbenzene resin anchored nickel complexes for selective oligomerizations and hydrogenations. McMunn *et al.*[16] studied the influence of the environment of the catalyst sites on the selectivity for various nickel and platinum forms as homogeneous and heterogeneous catalysts. Steric control of the alkyl group can also govern the selectivity in metathesis[17,18]. Furthermore, complementary information dealing with the possibilities of approach of alkene molecules at the surface of a catalyst can be deduced from their non-specific adsorption in GSC on graphitized carbon black[19].

Within the framework of our study of GSC on cation-exchange resins[8,20] we have recently reported on trends in the selectivity of various hydrocarbons with the help of correspondence factor analysis (CFA)[11]. The role played by the cross-linked ethylvinylbenzene–divinylbenzene copolymer matrix and the relative chromatographic specificities of different cations (H^+, K^+, Tl^+, Na^+, Ag^+, Ni^{2+}, Zn^{2+}, Cd^{2+}) have been delineated[11]. We have now undertaken a more precise study of molecular structural effects on specific interactions of a homogeneous series of alkenes with selected cations using the principle of DARC topological analysis (DTA) based on the concepts of the DARC topological system (DARC is an abbreviation for description, acquisition, retrieval and computer-aided design[21–24]). In this paper we present the first results, dealing with the specificity of the nickel form of the cation-exchange resin.

EXPERIMENTAL

Apparatus

All chromatographic work was carried out using a Varian Model 200 chromatograph with flame-ionization detectors (Varian Instruments, Palo Alto, CA, U.S.A.). The instrument was modified slightly to accomodate a calibrated thermometer in the oven.

Glass columns (10 cm × 6 mm O.D.) were used. The packing material was held in the column by silanized glass-wool plugs at the ends.

Ultra-high-purity nitrogen (AGL Welding Supply, Clifton, NJ, U.S.A.) was used as the carrier gas. All measurements were performed at 175°C and with a carrier gas flow-rate of 40 ml/min.

Packings

The preparation of lightly sulfonated resins was described previously[11]. The sulfonated resin (0.85 mequiv./g H^+) was converted into the nickel form by passing a measured volume of 0.1 *M* nickel nitrate solution through a glass column filled with dried resin. The converted resin was washed with water and then dried to 80°C in a vacuum oven for 4 h.

The metal content of the resin was determined by atomic-absorption spectrophotometry on acid-digested samples. The particular resin used in this study contained 1.8% of nickel. The unsulfonated form of the resin (UnS) consisted of Porapak Q (80–100 mesh), obtained from Waters Assoc. (Milford, MA, U.S.A.).

Reagents and chemicals

All test compounds were of the highest purity commercially available and were used as received (mainly from Aldrich, Milwaukee, WI, U.S.A., Tridom/Fluka, Hauppauge, NY, U.S.A., and Pfaltz and Bauer, Stamford, CT, U.S.A.). About 50 μl of compound were placed in an air-filled 30-ml butyl-rubber-stoppered bottle (Pierce Hypo-Vial, Pierce Chemical Co., Rockford, IL, U.S.A.). After mixing the contents of the bottle, 10–15-μl samples of the vapor were withdrawn in a disposable hypodermic syringe fitted with a 25-gauge needle and injected into the instrument.

Retention data

Retention times were determined by measurement of distances on the recorder chart tracings, after verifying that the chart speed control was accurate and precise. Kováts retention indices were calculated using the customary method with the normal hydrocarbons as reference compounds. The precision of the Kováts retention indices reported was $\pm$ 3 units based on duplicate injections carried out in random sequence.

Data processing

Factor analysis of Kováts retention indices and other numerical techniques chiefly stress stationary phase behavior. In order to understand fully the interaction between the solutes and the stationary phase with respect to the behavior of the individual solute–carbon atoms, a topological analysis was carried out on a large set of solutes.

Correlation between structure and chromatographic retention were set up by the DARC/PELCO (perturbation of an environment limited concentric and ordered) procedure developed by Dubois and co-workers[21–24]. The principles of this procedure have been described previously with respect to their applications in chromatography[9], which were developed for analytical[9,10,25–27] or physico-chemical purposes[12–14]. This procedure has been applied in other fields such as pharmacochemistry[28–32] and spectroscopy[33–35]. Only salient features will be mentioned here.

Fig. 1 shows the derivation for a set of eight alkenes from ethene, with the carbon–carbon double bond taken as the focus. Each of the alkenes is associated with a graph, with the topological sites of the molecule (the skeletal carbon atoms) corresponding to the nodes of the graph. Superposition of the graphs gives a composite trace characteristic of this set of compounds. Fig. 2a gives the trace for the set of 44 alkenes used in this study (they are listed in Table I). Each site in this

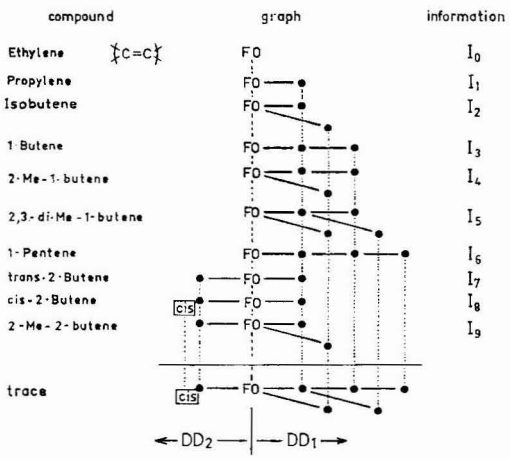


Fig. 1. Principle of superposition of the elementary graphs of some alkenes to give the characteristic trace of the population studied.

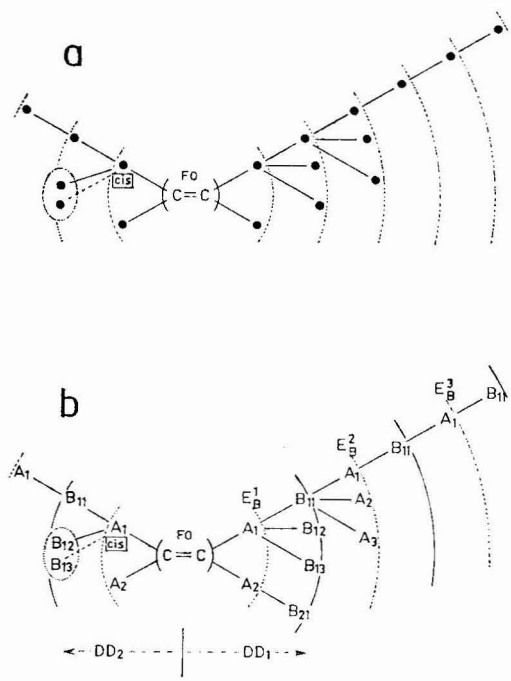


Fig. 2. (a) Characteristic trace of the population of 44 alkenes listed in Table I; (b) topological site ordering of the molecular environment by the ELCO concept for the superposition of the elementary graphs.

trace is specified unequivocally in the environment by a linear labelling order A_i or B_{ij} (Fig. 2b).

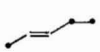
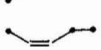
The influence of each site is interpreted as a perturbation term (P). For example, the term A_2 in the first environment E_B^1 is formally equivalent to the differ-

TABLE I

TOPOLOGICAL ANALYSIS OF BEHAVIOR OF ALKENES ON THE UNSULFONATED SUPPORT AND ON THE NICKEL FORM OF THE CATION-EXCHANGE RESIN

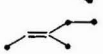
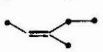
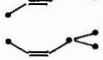
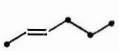
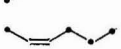
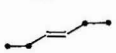
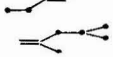
Experimental (1) and (3) and calculated Kováts retention indices at 175°C and Kováts retention index increments (ΔI).

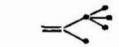
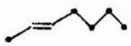
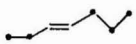
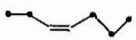
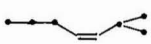
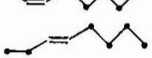
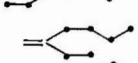
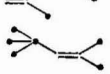
Correlation: No.	1	2	3
Number of compounds used	44	44	44
Number of parameters	19	19	25
Correlation coefficient, R	0.9997	0.995	0.9989
Standard deviation, σ	4.8	18.2	9.5
Average deviation, $\bar{x}$	2.6	8.6	4.1
Exner test, ψ	0.001	0.02	0.005
F-test	1920	120	334

No.	*Compound*	*Graph*	*Unsulphonated support*			*Ni^{2+} form of resin*					*ΔI (3)−(1)*	*B.p.(°C)*
			Exptl. (1)	*Calc. (2)*	*Diff. (1)−(2)*	*Exptl. (3)*	*Calc. (4)*	*Diff. (3)−(4)*	*Calc. (5)*	*Diff. (5)−(3)*		
1	Ethylene		200	200	0	212	212	0	212	0	12	−103.7
2	Propylene		304	306	−2	380	414	−34	394	−14	76	− 47.7
3	1-Butene		404	399	5	493	497	− 4	490	3	89	− 6.3
4	*trans*-2-Butene		410	409	1	486	501	−15	483	3	76	0.9
5	*cis*-2-Butene		418	412	6	499	502	− 3	488	11	81	3.7
6	Isobutene		398	402	−4	537	508	29	541	− 4	139	− 6.9
7	2-Methyl-1-butene		493	495	−2	622	592	30	614	8	129	31.2
8	2-Methyl-2-butene		507	506	1	622	595	27	606	16	115	38.6
9	1-Pentene		493	495	−2	582	585	− 3	583	− 1	89	30.0
10	*trans*-2-Pentene		502	502	0	574	584	−10	579	− 5	72	36.4
11	*cis*-2-Pentene		507	505	2	579	586	− 7	584	− 5	72	36.9
12	2,3-Dimethyl-1-butene		577	570	7	650	674	−24	650	0	73	55.6
13	3,3-Dimethyl-1-butene		550	559	−9	658	632	26	644	14	108	41.2

(Continued on p. 120)

TABLE I *(continued)*

No.	*Compound*	*Graph*	*Unsulphonated support*			Ni^{2+} *form of resin*					*ΔI (3)−(1)*	*B.p. (°C)*
			Exptl. (1)	*Calc. (2)*	*Diff. (1)−(2)*	*Exptl. (3)*	*Calc. (4)*	*Diff. (3)−(4)*	*Calc. (5)*	*Diff. (5)−(3)*		
14	2-Ethyl-1-butene		593	593	0	678	667	11	681	− 3	85	64.7
15	2-Methyl-1-pentene		590	591	−1	675	679	− 4	682	− 7	85	62.1
16	3-Methyl-1-pentene		574	570	4	674	667	7	676	− 2	100	54.2
17	4-Methyl-1-pentene		573	574	−1	673	650	22	666	7	100	53.9
18	2-Methyl-2-pentene		596	598	−2	681	684	− 3	693	−12	85	67.3
19	3-Methyl-*trans*-2-pentene		601	599	2	679	678	1	679	0	78	70.4
20	3-Methyl-*cis*-2-pentene		601	601	0	679	680	− 1	683	− 4	78	67.7
21	4-Methyl-*trans*-2-pentene		573	577	−4	676	667	9	673	3	103	58.6
22	4-Methyl-*cis*-2-pentene		573	580	−7	676	668	8	677	− 1	103	56.4
23	1-Hexene		590	595	−5	673	678	− 5	675	− 2	83	63.5
24	*trans*-2-Hexene		602	598	4	675	672	3	672	3	73	67.9
25	*cis*--2-Hexene		602	601	1	675	673	2	676	1	73	68.9
26	*trans*-3-Hexene		593	595	−2	671	673	− 2	666	4	78	67.1
27	2,4-Dimethyl-1-pentene		664	670	−6	716	745	−29	716	0	52	81.6

28	2,3,3-Trimethyl-1-butene		661	655	6	699	726	−27	699	0	38	77.9
29	2,3-Dimethyl-2-pentene		697	697	0	723	723	0	723	0	26	97.4
30	4-Methyl-1-hexene		680	673	7	750	744	6	757	− 7	70	86.7
31	1-Heptene		692	690	2	767	769	− 2	766	1	75	93.6
32	*trans*-2-Heptene		692	698	−6	760	765	− 5	764	− 4	68	97.9
33	*trans*-3-Heptene		693	691	2	762	761	1	759	3	69	95.7
34	*cis*-3-Heptene		693	694	−1	762	762	0	763	− 1	69	95.8
35	3-Ethyl-2-pentene		695	696	−1	742	754	−12	746	− 4	47	96.0
36	4,4-Dimethyl-*cis*-2-pentene		665	662	3	720	719	1	734	−14	55	80.4
37	2-Methyl-*cis*-3-heptene		767	767	0	850	850	0	850	0	83	112.0
38	1-Octene		796	796	0	861	861	0	861	0	65	121.3
39	*trans*-2-Octene		794	794	0	857	857	0	855	1	63	124.9
40	*cis*-2-Octene		794	796	−2	857	857	0	860	− 3	63	125.6
41	*trans*-3-Octene		794	791	3	857	854	3	851	6	63	123.3
42	2-Ethyl-1-hexene		789	788	1	849	848	1	841	8	60	120
43	2,4,4-Trimethyl-1-pentene		750	750	0	790	790	1	790	0	40	101.5
44	2,4,4-Trimethyl-2-pentene		747	747	0	791	791	1	791	0	44	104.9

ence in behaviors of isobutene and propylene. As this site also appears in other compounds (I_4, I_5 and I_9 in Fig. 1), its perturbation term is estimated by an average value obtained by a multiple regression program that takes into account all the members of a given set of compounds containing this site[13].

These perturbation terms are the components of a vector $\vec{I}(m)$ that characterizes the information for an experimental population made up of m compounds. This vector is defined by the basic topology–information relationship

$$I(\varepsilon) = < \vec{T}(\varepsilon) \mid \vec{I}(m) >$$

where $I(\varepsilon)$ is the contribution from the environment and $\vec{T}(\varepsilon)$ is the topological vector of the environment. $\vec{T}(\varepsilon) = (X_1, X_2, \ldots, X_j, \ldots, X_n)$; thus $X_j = 1$ when the jth site of the environment is occupied and $X_j = 0$ when it is not.

The PELCO method consists of calculating the $\vec{I}(m)$ vector and defining thereby a topology information correlation which will be represented as the diagrams in Figs. 3 and 4. Information concerning a compound X can be calculated from information $I(X_0)$ for a reference compound (here ethylene) and can be expressed as $I(X) = I(X_0) + I(\varepsilon)$. Some examples are shown in Fig. 5.

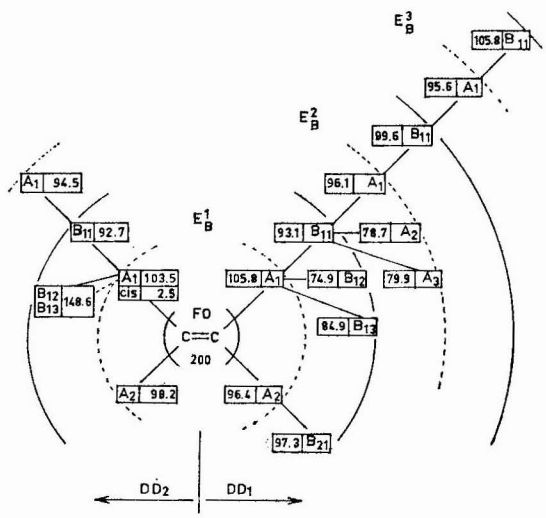


Fig. 3. Topological information diagram for retention on the unsulfonated copolymer matrix.

RESULTS AND DISCUSSION

Retention indices of the alkenes on the unsulfonated support (UnS) and on the nickel form of the cation-exchange resin are given in Table I. Fig. 6 shows a plot of the Kováts retention indices of the alkenes on the unsulfonated ethylvinylbenzene–divinylbenzene copolymer matrix *versus* the boiling point of the compounds. Regular variations are observed for isomeric compound subpopulations defined by carbon atom number.

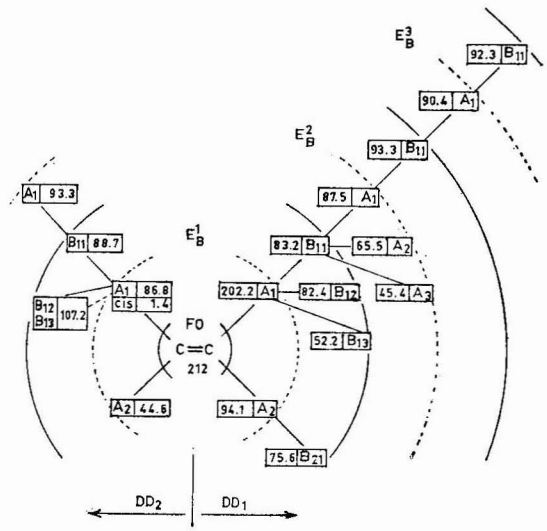


Fig. 4. Topological information diagram for retention on the nickel form of the ion exchanger.

Alkene	*1-Butene*	*2-Methyl-2-pentene*	*cis-2-Pentene*
Graph	FO —●— ●	●—●⟍FO⟋● ●	●— FO —●—● *cis*
Topographic information	200 105.8 93.1	92.7 103.5 105.8 200 96.4	103.5 105.8 2.5 200 93.1
Kovát's retention index	calc. 399	598	505
	exp. 404	596	507

Fig. 5. Calculation of predicted retention index on the unsulfonated copolymer matrix, using the information in Fig. 3.

Fig. 7 gives the Kováts retention indices of the same alkenes on the Ni^{2+} form of the cation-exchange resin *versus* their boiling points. The regular trend for every subpopulation seen in Fig. 6 disappears. Specific structural effects occur within each subpopulation, resulting in a larger dispersion of retentions.

The trends are not the same for butene or pentene as for the higher alkenes. The range of Kováts retention indices for butene or pentene is 20 index units (I.U.) on the UnS column, but it increases to about 50 I.U. on the Ni^{2+} form. This difference is due to the retention of the geminal alkenes (isobutene and 2-methyl-1-butene) and 2-methyl-2-butene. In comparison, the range for the hexenes is 50 I.U. on the UnS column (from the crowded 3,3-dimethyl-1-butene up to the *cis*- and *trans*-2-hexenes

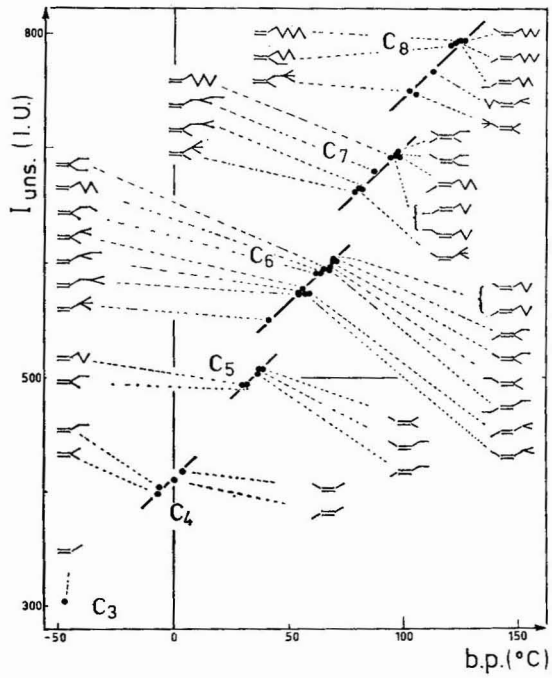


Fig. 6. Retention index of alkenes (I_{uns}) on the unsulfonated copolymer, in index units (I.U.), as a function of boiling point of the alkene. Regular trends are observed for every subpopulation (from C_4 up to C_8).

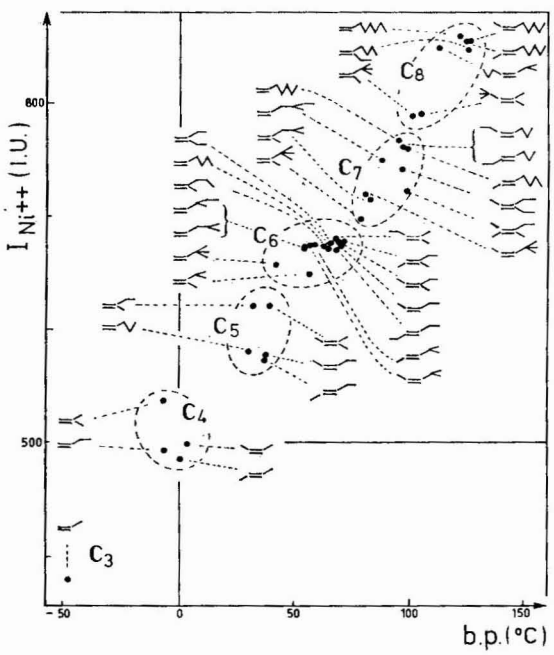


Fig. 7. Retention index of alkenes on the nickel form of the ion exchanger as a function of boiling point of the alkene. The dispersion of the data for each subpopulation suggests, in this instance, a relatively complex law for the structural effects on the adsorption phenomena.

and the *cis*- and *trans*-3-methyl-2-pentenes) decreasing to 25 I.U. on the Ni^{2+} form. For the heptenes the range of Kováts retention indices increases from 40 I.U. on the UnS column to 70 I.U. on the Ni^{2+} form and for the octenes from 50 I.U. on the UnS column to 70 I.U. on the Ni^{2+} form. The increase in Kováts retention index (ΔI) from the UnS to the Ni^{2+} packing is shown in Fig. 8. The ΔI shows the specificity brought by the Ni^{2+} ion at the molecular level. ΔI varies from the unexpectedly low value of 12 I.U. for ethylene up to 139 I.U. for isobutene. Only the previously mentioned geminal 1-alkenes and 2-methyl-2-butene have a ΔI in the range 115–130 I.U. In the range 100–115 I.U. we find some hexene isomers, the crowded 3,3-dimethyl-1-butene, the branched 3-methyl-1-pentene, 4-methyl-1-pentene and *cis*- and *trans*-4-methyl-2-pentene. In addition to ethylene, the lower ΔI values are observed for tetrasubstituted ethylene, 2,3-dimethyl-2-pentene with $\Delta I = 26$ I.U., and for compounds having steric strain in the vicinity of the carbon–carbon double bond. 2,4,4-Trimethyl-1-pentene ($\Delta I = 40$ I.U.) and 2,4,4-trimethyl-2-pentene ($\Delta I = 44$ I.U.) are two compounds that show this property.

The difficulty in discerning other than gross changes by classical means makes the DTA treatment of this set of data particularly attractive as a method for delineating the structural trends and placing them on a quantitative basis.

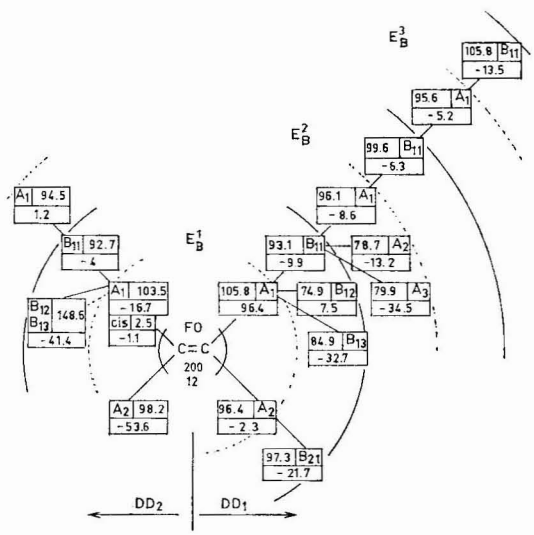


Fig. 8. Extent of specific interactions of alkenes with the nickel form of the ion exchanger. Upper values in each box are retentions on unsulfonated copolymer, lower values are the specific and supplementary contributions due to the nickel form of the ion exchanger.

DTA behavior: unsulfonated support

The DARC topology information diagram (Fig. 3) gives the behavior of alkenes on the unsulfonated matrix and Fig. 5 demonstrates the ease of using the diagram. Every substitution at the ethylenic focus contributes about 100 I.U., and the chain lengthening results in an incremental increase of approximately 100 I.U., as expected. Branching in the first development direction, DD_1, in such compounds as 3,3-dimethyl-1-butene and 2,4,4-trimethyl-1-pentene results in a contribution of

about 80 I.U. In addition, no noticeable specificity is observed for *cis*-alkenes relative to the corresponding *trans*-isomers.

The contribution of topological sites is different here to those values observed in gas–liquid chromatography (GLC) for a similar series of alkenes on the non-polar stationary phases Squalane and Apiezon[13]. The contribution of substitution at the ethylenic focus was slightly higher and the contribution from branching was 10–20 I.U. lower for these liquid phases. The topological values for the UnS column are very close to those obtained on a sulfonated packing in the hydrogen form, the data for which will be published in a subsequent paper. This indicates that the sulfonated and unsulfonated forms of the resin retain the compounds in the same manner. The addition of the metal results in the differences in selectivity.

The statistical significance of the topological analysis is high. Differences between the experimental and the calculated retention indices using $I(\varepsilon)$ are small (the average deviation is 2.6 I.U., the standard deviation 4.8 I.U.) and this reinforces the validity of using the additivity of the contributions of the topological sites.

DTA behavior: nickel form of the resin

The DTA behavior of the alkenes on the Ni^{2+} form of the sulfonated resin is shown in Fig. 4. There are readily observable differences in topological site contributions between this form and the unsulfonated form of the copolymer, particularly with monosubstitution, tetrasubstitution and branching. The differences in values for the various topological sites between Figs. 3 and 4 are due to the specific interactions of the nickel form of the sulfonated support relative to the unsulfonated matrix. The specific interactions due to the nickel form are given in Fig. 8; these will now be examined in detail.

The carbon–carbon double bond, which is the focus in the DTA treatment, shows a slight specificity (+12 I.U.) relative to the unsulfonated matrix. Monosubstitution on the ethylenic focus given by site A_1, in the first environment E_B^1 of the first development direction, labelled DD_1, which refers to the first direction of development of the graph according to a controlled algorithmic growth[22], is very important. This specificity of 96.4 I.U. shows a strong increase in the specific interaction between Ni^{2+} and the carbon–carbon double bond due to the monosubstitution of the sp^2 carbon atom of the ethylenic focus. This is in agreement with the increase of π net charge of -0.0133 *e* calculated by CNDO/2 *ab initio* calculations[34] for propylene relative to ethylene.

Chain lengthening for 1-alkenes results in a decrease of about 10 I.U. from the preceding specific interaction for every topological site. These negative contributions (−9.9, −8.6, −6.3, −5.2 and −13.5) are in agreement with the influence on retention index of the successive addition of a carbon atom to the chain from 1-butene to 1-octene.

Geminal disubstitution, as exemplified by site A_2 in the DD_1 direction in the E_B^1 region, shows no specific interaction (−2.3 I.U.), except for isobutene, which will be discussed later.

Disubstitution with *trans*-isomers is given by A_1 in E_B^1 of the second development direction DD_2. The specific interaction for a carbon in A_1 is −16.7 I.U. The *cis*-isomer shows no improvement in specificity (−1.1 I.U.). Site A_1 also appears in trisubstituted ethylenes such as 2-methyl-2-butene.

Tetrasubstitution, represented by site A_2 in E_B^1 of DD_2, shows a substantial negative interaction with the nickel form of the copolymer (−53.6 I.U.). Actually, all branching seems to diminish the specific interaction between the carbon–carbon double bond and the Ni^{2+} ion.

All of the preceding observations dealing with negative specific interactions, which are most often opposite to electronic effects[34], suggest that increasing the degree of substitution diminishes the possibility of overlapping of the filled π orbital of the alkene with the vacant d orbitals of nickel.

DTA behavior: nickel form of resin with interaction parameters

A decrease in the quality of the statistical tests of the correlations with the Ni^{2+} form of the resin relative to the correlation with the unsulfonated form of the resin is observed. Both of the correlations, calculated in the same way, offer a good example of the differences of the additivity of the structural parameters (*i.e.*, the topological sites) due to the particular physico-chemical effects in the adsorption phenomena.

Differences between experimental and calculated values for correlation 2 in Table I are less than 5%. Most of the major deviating values are for the geminal compounds for which the calculated values can be lower than experimental (compounds 6, 7 and 8) or higher than experimental (compounds 27, 28 and 35).

With the flexibility of choice available for the parameters in DTA[9,10] it is possible to distinguish contributions of linear or branched 1-alkenes from the geminal 1-alkenes when different groups are present simultaneously or independently. Calculation of the interaction parameters between A_2 (in E_B^1 of DD_1) introduced by the geminal alkenes is given in Fig. 9 and offers the possibility of working at a second-order level of precision. Corresponding calculated values with this correlation 3 having 25 parameters are given in Table I. Improved agreement between experimental and calculated retention indices, shown by the statistical tests (average deviation 4.1 I.U., standard deviation 9.5 I.U.) should be emphasized.

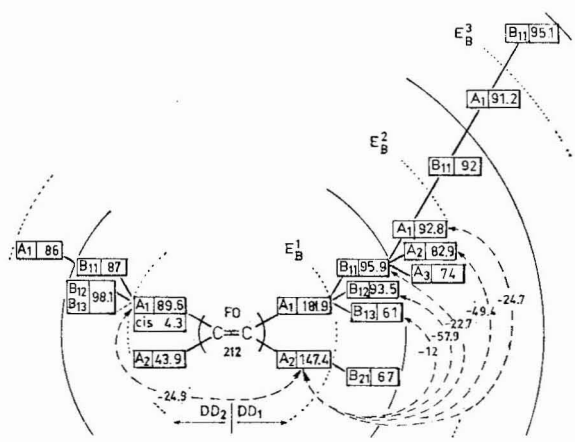


Fig. 9. Topological information diagram for retention of alkenes on the nickel form of the ion exchanger showing some interaction parameters. The negative contribution of these parameters to retention on the Ni^{2+} form underlines the sensitivity of the specific π double bond–Ni interactions to branchings and bulky groups in the homologous series of geminal 1-alkenes.

These new parameters take into account deviations due to adsorption phenomena when different groups or, more specifically, when different topological sites are present simultaneously. It can readily be seen that all of these interactions are negative and contribute to a diminution of the relatively strong influence of site A_2 (in E_B^1 of DD_1) introduced by isobutene (147.4 I.U.). As an example, when site A_2 is present together with a *tert.*-butyl group on the same sp^2 carbon, the weights of the interactions between site A_2 and B_{11}, B_{12} and B_{13} (in E_B^1 of DD_1) are −22.7, −57.9 and −12 I.U., respectively. The sum of these interactions is −92.6 I.U. for the *tert.*-butyl group and helps to explain why a difference of only 41 I.U. is observed between the experimental retention indices of 2,3,3-trimethyl-1-butene and 3,3-dimethyl-1-butene instead of 157 I.U., which is the difference between the experimental values for propylene and isobutene.

The numerical expressions of these interactions are not just a mathematical device; they also have a physico-chemical meaning. They show the decrease of the specific interaction between the carbon–carbon double bond and the Ni^{2+} ion when different sites are present simultaneously. These interactions are in agreement with the Kováts retention index increments, ΔI, which themselves are indicative of the specific interactions, with the Ni^{2+} ion, for the whole molecule. The strong decrease observed in ΔI values for propene, 3,3-dimethyl-1-butene and 2,3,3-trimethyl-1-butene of 139, 108 and 38 I.U., respectively, also shows the influence of the simultaneous presence of a methyl and a *tert.*-butyl group on the same sp^2 carbon. Another example that illustrates this point is the fact that ΔI for 3,3-dimethyl-1-butene is 108 I.U. but only 73 I.U. for the geminal isomer 2,3-dimethyl-1-butene.

Specific interactions; ionization potentials

To evaluate the possibilities of charge transfer between the π bond of alkenes and the empty orbitals of the Ni^{2+} ion, a frontier molecular orbital treatment that would consider the energies and shapes of the highest occupied molecular orbital (HOMO) of the donor and the lowest unoccupied molecular orbital (LUMO) of the acceptor would be attractive[35,36], but difficult owing to the complexity of the studied olefins. Photo-electron spectroscopy has been shown to be a valuable physical method for evaluating substituent effects when reactivity is based on charge-transfer possibilities[36].

The specific interactions of the alkenes with the Ni^{2+} ion, expressed as Kováts retention index increments, ΔI, is plotted in Fig. 10 against the olefin ionization potentials (IP) determined elsewhere by photoelectron spectroscopy[37]. This figure shows that for monosubstituted ethylenes, linear or branched, and for the lower geminal alkenes (isobutene and 2-methyl-1-butene) the degree of specific interaction (ΔI) is closely related to the IP. For 1,2-disubstituted ethylene, trisubstituted ethylene (2-methyl-2-butene), crowded alkenes with a *tert.*-butyl group and tetrasubstituted ethylene (2,3-dimethyl-2-pentene) we can observe successive decreases in ΔI despite the availability of the π electron.

These trends are in good agreement with the DTA results in Fig. 9, in the same way that DTA results dealing with alkenes in GLC have been compared successfully with CNDO/2 *ab initio* calculations[13].

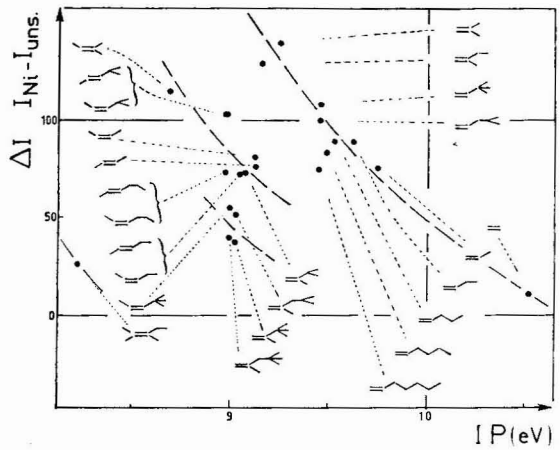


Fig. 10. Specific contribution to retention (ΔI) due to the nickel form of the ion exchanger relative to the unsulfonated copolymer matrix (uns.) as a function of ionization potential of the alkene (IP). Decrease of the specific interaction with degree of substitution, branchings or bulky groups for di-tri- or tetrasubstituted ethylenes suggest a dominant influence of steric effects.

CONCLUSION

DARC topological analysis of the behavior of alkenes in GSC on the Ni^{2+} form of a cation-exchange resin allows a determination of structural effects on the specificity brought by the Ni^{2+} cation relative to the unsulfonated matrix. Furthermore, DTA can be used to aid in explaining the behavior of molecules at the level of their skeleton carbon atom. The contributions of all the possible groups (*n*-alkyl, isopropyl, *tert.*-butyl, neopentyl, etc.) obtained by progressive substitution of ethylene, taken as the reference compound, are easily deduced from the topology information diagrams. DTA shows clearly the influence of structural effects on the ability of the molecules to form charge-transfer complexes between Ni^{2+} and the olefin. Specific adsorption on Ni^{2+} is related to the overlap of the π bonds and is very sensitive to hindrance due to the degree of substitution or to the substitution of bulky groups. This steric effect counteracts the electronic effect that would favor interaction.

The different spectroscopic methods used in the field of catalysis for the study of adsorbed species are generally limited to very simple molecules. DTA of retention indices, however, offers the possibility of studying any molecule that belongs to a coherent series, with the precision of the DTA results being directly related to the precision of the experimental results[14,38]. Indeed, as in every mathematical method, our model is partly conventional. The use of topology–information diagrams needs only a minimum of training, which is easily obtained with some examples showing how to calculate retention indices for compounds whose graphs are included in the trace of the population studied. This paper underlines the complementarity between DTA and factor analysis methods, such as correspondence factor analysis (CFA)[11], for the physico-chemical exploitation of tables of data and analyses of main trends on structural bases.

Work is under way to study and elucidate the specificities brought about by

the use of other cations (Ag^+, Zn^{2+}, etc.) that have shown interesting and complementary effects[11].

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GAS CHROMATOGRAPHIC DETECTION OF SO_2, NO_2, AMINES AND HALOCARBONS USING AN AEROSOL IONIZATION DETECTOR*

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SUMMARY

The highly specific aerosol ionization detector primarily serves to detect halocarbons and basic or acid reacting compounds. The advantages and disadvantages of this detector for the determination of SO_2, NO_2, amines and halocarbons were studied. Applications of the detector in the fields of air pollution measurement and of the monitoring of the air at workplaces are described. Detection limits are given and possibilities of a further improvement of the sensitivity are discussed.

INTRODUCTION

The aerosol ionization detector (AID) developed by Popp *et al.*[1–3] has proved suitable for a selective and sensitive detection of substances or groups of substances. The principle of this detector was described earlier[3]. Aerosols from basic reacting compounds are formed by reaction with trifluoroacetic acid[4], aerosols from acidic reacting compounds by reaction with amines[5–7], aerosols from halocarbons by reaction with hot copper oxide[6–8] and those from metallorganic compounds by pyrolysis[7,8]. A combination of these reactions (*e.g.* oxidation of SO_2 to SO_3 by means of an oven and subsequent reaction with amines) is also possible.

This paper describes the possibilities of the AID for the determination of SO_2, NO_2, amines and halocarbons, pointing out its advantages and disadvantages with respect to the various modes of operation.

THEORETICAL

A theoretical model[3] of the processes occurring in an AID is analogous to the studies of Wentworth *et al.*[9–11] for the electron-capture detector (ECD). The authors[3] showed that the relation

$$\Delta i/i_0 - \Delta i = K^* \cdot a$$

* Presented at the *6th International Symposium "Advances and Application of Chromatography in Industry", Bratislava, September 16–19, 1980.*

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where Δi = decrease of the ionization current in the presence of aerosols, i_0 = basic ionization current, K^* = constant defined in analogy to the electron capture coefficient and a = concentration of sample molecules is also valid for the AID.

Theoretical studies by Grosse[12] for continuously working aerosol ionization analyzers, especially for the ionization chamber using an internal radiation source, provided the relation

$$\frac{\Delta i}{i_0 - \Delta i} \cdot \frac{1}{\Delta(\beta Z)} = -\left[\sqrt{\frac{\alpha q}{1 - i_0/i_s}}\left\{2 - \frac{i_0}{i_s}\left(1 - \frac{i_0}{i_s}\right)\right\} + \beta Z\right]^{-1}$$

where i_s = saturation current, α = ion–ion recombination coefficient, β = attachment coefficient of ions to aerosols, Z = number of aerosol particles per unit volume and q = number of ions formed per unit time and volume.

Grosse defined the sensitivity, S, of an AID to be the quotient of the change, Δi, of the ionization current and the change, $\Delta(\beta Z)$, of the value which is characteristic of the aerosol particles.

Assuming small concentrations of aerosols, S is approximately equal to the expression

$$\frac{i_0}{\sqrt{\alpha q}} \frac{\sqrt{1 - i_0/i_s}}{2 - i_0/i_s}$$

The maximum sensitivity is reached with $i_0/i_s = 0.76$. Using the d.c. mode of AID operation this ratio should be noted.

EXPERIMENTAL

Fig. 1 shows the principle of an AID with a reaction oven. The oven is slid over the unfilled end of the quartz gas chromatographic column or over a small quartz tube connecting the column and the detector of such a size that the dead volume between the column packing and the ionization chamber is less than 1 ml. The quartz tube contains a copper pin, diameter less than 3 mm and length 2 mm. At oven temperatures of 900–980°C, the halocarbons to be analysed are converted

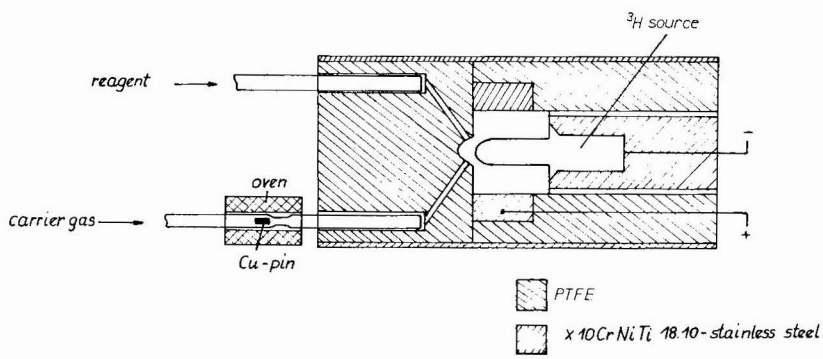

Fig. 1. Scheme of the AID.

into aerosols. The determination of SO_2 takes place at temperatures of *ca.* 900°C (without copper pin) and with the addition of a basic reagent; the acid and basic reacting compounds are determined without heating and with the column filled by the addition of amines and trifluoroacetic acid, respectively. In each case air is used as carrier gas. This has two advantages: (i) the ECD mode of operation cannot affect the selectivity and the sensitivity of the AID; (ii) it is possible to inject large sample volumes without the occurrence of troublesome air peaks.

The detector has a concentric tube geometry, and the radiation source (^3H, ^{63}Ni or ^{85}Kr) serves as an internal electrode. The experiments described in this paper have been performed with an ^3H source. The detector worked in the d.c. mode of operation with a ratio $i_0/i_s = 0.7$ and was inserted in an environmental gas chromatograph constructed at the Central Institute for Isotope and Radiation Research, Leipzig[13,14].

RESULTS AND DISCUSSION

Determination of halocarbons

Halocarbons (especially chloro- and fluoro-containing compounds) are determined by conversion into copper chloride aerosols at 900–980°C, *e.g.*:

$$C_2HCl_3 + CuO \rightarrow CuCl_2 + \text{products}$$

In comparison to the ECD, the AID has the advantage that chloro and fluoro compounds with one or two halogen atoms can also be determined with detection limits in the ppb range; the disadvantage lies in the lower sensitivity compared with the sensitivity of the ECD for CCl_4, pesticides and some other compounds. Bromides are detected with a lower sensitivity than chlorides and fluorides and iodides are undetectable. Fig. 2 shows the connection between the quotient $\Delta i/i_0 - \Delta i$ and the concentration, a, of the sample (in this case Freon 12). The AID has a dynamic range (this applies to all modes of operation) of *ca.* 10^2. After a sharp bend there is an extended range in which there also exists a linear connection between $\Delta i/i_0 - \Delta i$ and a. With adequate calibration we used both regions for measurements.

The detection limits for the following halocarbons determined with the AID lie between 100 ppb* and 500 ppb: ethyl chloride, allyl chloride, *n*-butyl chloride, Freon 12, methylene chloride, *n*-propyl chloride, halan, tetrachloromethane, tetrachloroethane, trichloroethylene. If amines are added behind the reaction oven (in this case the temperature of the detector must not exceed 60°C) it is possible to reach detection limits of *ca.* 10 ppb.

Fig. 3 shows the chromatogram of a sample with 100 ppm of Freon 12, CCl_4 and C_2HCl_3.

Determination of SO_2

The determination of SO_2 was performed at temperatures of *ca.* 900°C and subsequent reaction with a basic reacting compound (*n*-propylamine):

$$SO_2 + 1/2\ O_2 \longrightarrow SO_3 \text{ (oxidation)}$$
$$SO_3 + 2\ RNH_3 + H_2O \rightarrow (RNH_3)_2SO_4$$

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

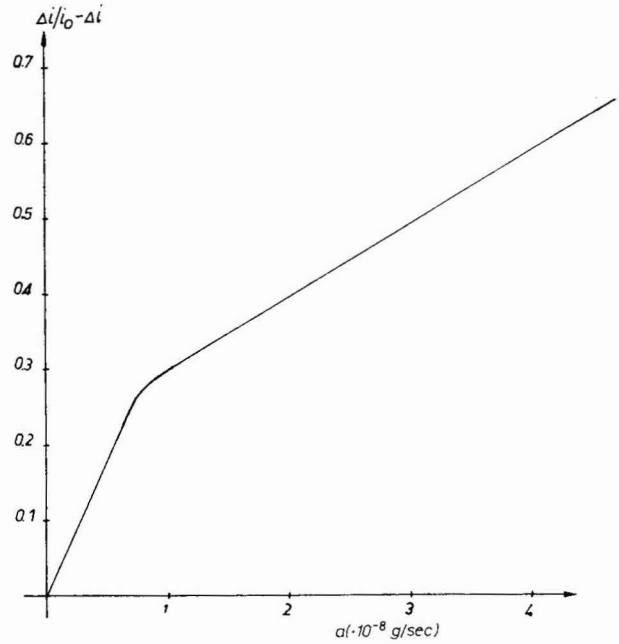

Fig. 2. Linearity of the AID. Sample: Freon 12.

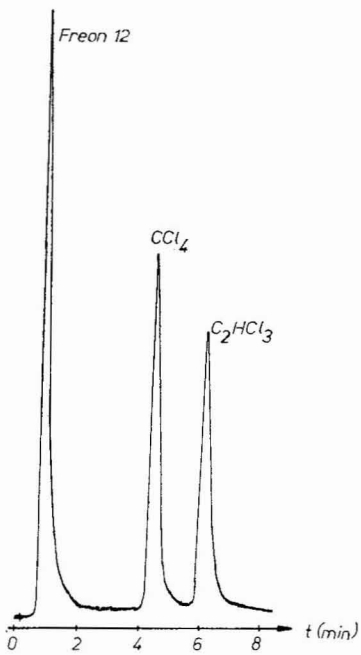

Fig. 3. Analysis of halocarbons in air. Column: 2 m × 4 mm I.D., glass, 10% SE-30 on Chromosorb W AW DMCS. Temperatures: column, 30°C; oven: 960°C. Sample size: 50 μl with 100 ppm Freon 12, CCl_4 and C_2HCl_3.

The concentration of *n*-propylamine in the ionization chamber of the AID was 20 ppm. With this arrangement it was possible to detect 200 ppb of SO_2 in a 10-ml air sample.

Fig. 4 shows a chromatogram of 1 ml of air containing 5 ppm SO_2. A short deactigel column was used.

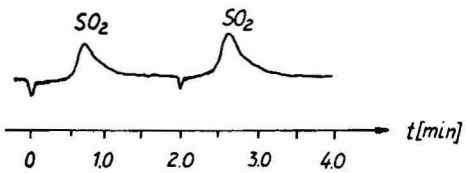

Fig. 4. Determination of SO_2. Column: 25 cm × 4 mm I.D., glass, deactigel. Carrier gas: air. Temperatures: 130°C; oven, 900°C. Concentration of amine: 20 ppm. Sample size: 1 ml air with 5 ppm SO_2.

Air pollution measurements of SO_2 are possible using a cooled pre-column for sample enrichment. With deactigel as column packing no substance passing the column except H_2S causes a detector signal.

The detection limit of the AID in this mode of operation (and also in the others) can be improved by radioactive sources with a higher saturation current (in our case 10^{-9} A), so that an air pollution control without enrichment seems to be possible.

Determination of NO_2 and amines

The determination of NO_2 and amines with the AID is simple in so far as the reaction oven is unheated and only the corresponding reagent diffuses into the ionization chamber of the detector. Yet there are the following difficulties:

(1) For the determination of basic reacting compounds it is necessary to avoid column packings with basic reacting liquid phases. Similarly, for the determination of acid reacting compounds, packings with acidic reacting liquid must be avoided because bleeding causes a strong decrease of the basic ionization current and as a result a decrease of the sensitivity.

(2) The gas–gas reaction which leads to the formation of aerosols can be used only for temperatures below 60°C; higher temperatures cause a change of the steady state in favour of the initial substances. Thus the determination of amines with Chromosorb 103 as column packing makes it necessary to work with a hot column and cold detector, but this may lead to the condensation of the substances in the ionization chamber.

The following two examples show the detection of NO_2 and amines. Fig. 5 shows the determination of NO_2 in a glass blower's workshop with sample enrichment. The column, cooled to −78°C, served for sample enrichment from 130 ml of air. When the column was warmed the NO_2 peak and an asymmetric water peak appeared. Without enrichment a detection limit of 250 ppb (related to a 10-ml sample) was achieved with this arrangement.

Fig. 6 shows the detection of amines in air using Chromosorb 103 as column packing and with the column and the detector at different temperatures. The best results were obtained with a 95:5 mixture of nitrogen and oxygen as carrier gas. The oxygen peak is due to this carrier gas. Further studies with amines are in preparation.

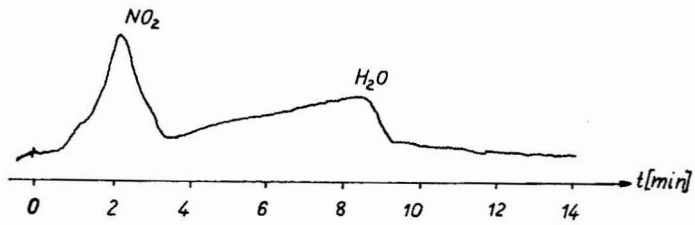

Fig. 5. Analysis of NO_2 in a glass blower's workshop. Column: 1 m × 4 mm I.D., PTFE, 10% Fluorolube-oil on Fluoropak 80. Temperature: 30°C. Concentration of amine: 100 ppm. Sample size: NO_2 enriched from 130 ml. NO_2 room concentration: 1 ppm.

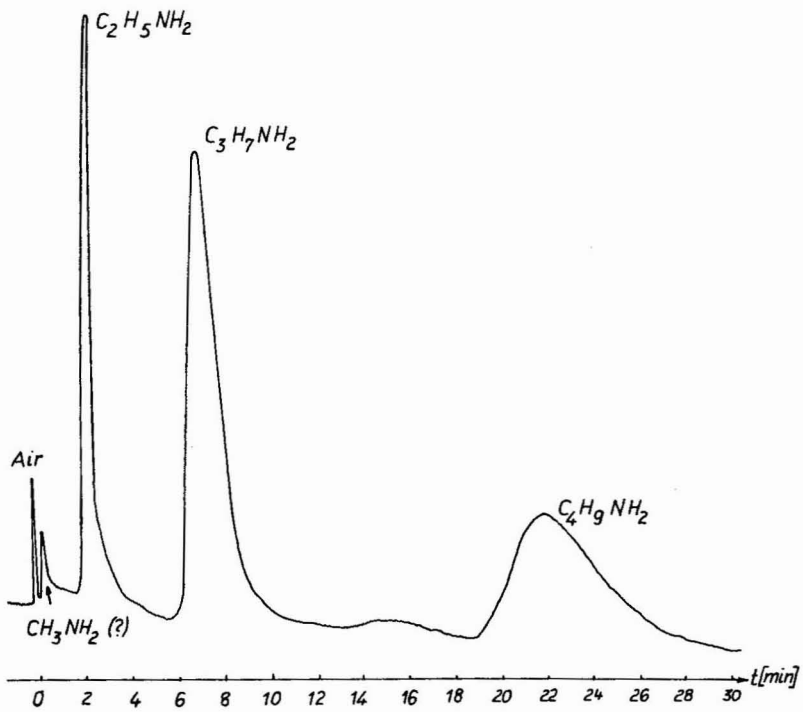

Fig. 6. Determination of amines. Column: 2 m × 4 mm I.D., glass, Chromosorb 103. Carrier gas: nitrogen–oxygen (95:5). Temperatures: column, 120°C; detector, 50°C. Concentration of trifluoroacetic acid: 100 ppm. Sample size: 100 μl air with $7 \cdot 10^{-8}$ g $C_2H_5NH_2$, $5 \cdot 10^{-7}$ g $C_3H_7NH_2$, $5 \cdot 10^{-7}$ g $C_4H_9NH_2$.

CONCLUSIONS

These studies show that a sensitive and selective detection of halocarbons, SO_2, NO_2 and amines with the AID is possible. The detection limits in the most favourable cases (detection of halocarbons with reaction oven and subsequent amine reaction) are 10 ppb, and in other cases (detection of halocarbons with reaction oven, detection of SO_2 and NO_2) lie between 100 ppb and 500 ppb. The dynamic range amounts to 10^2.

Using ^3H sources with a higher saturation current than available in our arrangement (10^{-9} A), and using space charge effects[14–16], an improvement of sensitivity and detection limit can be expected. Detection limits of 1–10 ppb for all the applications seem to be possible.

Difficulties do occur, especially in the detection of amines, owing to the necessity to keep the detector temperature below 60°C.

The greatest advantage of the AID is the very high selectivity in each mode of operation. A great number of applications are possible owing to the various methods for the formation of aerosols.

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ISOLATION AND SEPARATION OF NEW NATURAL LACTAM ALKALOIDS OF ERGOT BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*

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SUMMARY

Separation of lactam derivatives of ergotoxine alkaloids, of which two were newly isolated from natural material, by high-performance liquid chromatography is described. The separation method utilizes LiChrosorb NH_2 as a stationary phase eluted with diethyl ether–ethanol (96:4) as a mobile phase. Alkaloids are detected with a UV detector. The proposed names for the studied lactam derivatives are ergocristam, ergocristinam, ergocornam, ergocorninam, ergocryptam and ergocryptinam.

INTRODUCTION

Natural ergot alkaloids of the peptide type with a cyclol structure were isolated in diastereomeric pairs either from ergot sclerotia of the parasitic fungus *Claviceps purpurea*, or from the mycelium of saprophytic strains of this fungus. One of the groups of cyclol ergot alkaloids (CEA) is composed of ergotoxine-type alkaloids with an isopropyl group in a C-2′ position. The compounds isolated from natural material are ergocristine[1], ergocornine[2] and ergocryptine[3,4], and their dextrorotatory enantiomers. In 1973 Stütz *et al.*[5] described the isolation of a new alkaloid from a lyophilized mycelium of the strain *C. purpurea* which, owing to the altered structure of its peptide moiety, did not belong to any of the above mentioned groups. It was the first representative of the non-cyclol (lactam) group of peptide ergot alkaloids (LEA) with a D-configuration of proline. The structure of this alkaloid corresponded to the cyclol alkaloid ergocristine (Fig. 1).

Isolation of an identical alkaloid from sclerotia of the ergocristine strain *C. purpurea* of a Czechoslovak production and preparation of its semisynthetic

* Presented at the *6th International Symposium "Advances and Application of Chromatography in Industry", Bratislava, September 16–19, 1980.*

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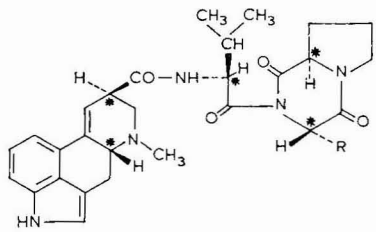

Fig. 1. Structural formula of non-cyclol lactam ergot alkaloids of the ergotoxine group. R = isopropyl (ergocornam), isobutyl (ergocryptam), benzyl (ergocristam).

derivatives was described by Černý *et al.*[6]. The new non-cyclol structure led Floss *et al.*[7] to conclusions concerning the biosynthesis of the peptide moiety of ergot alkaloids. They assumed an effect of a competitive reaction which brings about a reversal of configuration on the optically active carbon of L-proline to the D-configuration. The resulting compound cannot enter the biochemical reactions which lead to the formation of a cyclol structure. The sequence of the presumed biochemical reactions participating in the synthesis of ergocornine, ergocryptine and the corresponding non-cyclol alkaloids was dealt with by Floss *et al.*[7].

The non-cyclol derivatives ergocornine and ergocryptine were obtained during isolation of ergotoxine alkaloids from ergot of Czechoslovak production. Thin-layer chromatography of raw bases of the alkaloids yielded two new spots with properties different from those of the ergotoxine group alkaloids. The alkaloids exhibited properties similar to those of the previously described non-cyclol alkaloid, the structure of which corresponds to ergocristine. The structure of these alkaloids was confirmed by a nuclear magnetic resonance method[8].

High-performance liquid chromatography (HPLC) of ergotoxine ergot alkaloids has been performed by several researchers[9–11]. This study is based on results obtained in an analysis of CEA published earlier[12].

EXPERIMENTAL

Isolation of LEA

Processing of 500 kg of ergocornine–ergocryptine ergot of Czechoslovak production yielded 200 g of bases in which LEA were concentrated. Boiling in ethanol in the presence of phosphoric acid transformed alkaloids into dihydrophosphates of levorotatory forms. After a release of the bases by an aqueous solution of potassium hydrogen carbonate and an extraction into diethyl ether, the alkaloids were concentrated into a foam-like evaporation residue (135 g). Chromatography of this residue on a silica gel column (8.0 kg) using chloroform as the elution agent yielded 35 g of a concentrate containing *ca.* 20% of LEA. Repeated chromatography on a silica gel column (3.5 kg) using an elution mixture of chloroform with 1% methanol yielded, after fraction pooling, oily evaporation residues of N-[N(*d*-lysergyl)-L-valyl]-L-valyl-D-proline-lactam and N-[N(*d*-lysergyl)-L-valyl]-L-leucyl-D-proline-lactam. The proposed names for these compounds are ergocornam and ergocryptam. Precipitation of alkaloids by diethyl ether yielded 0.5 g of raw ergocryptam and 0.9 g of ergocornam. Both alkaloids were obtained in a pure form by repeated chromatography on a silica gel column (200 mg of ergocryptam, 400 mg of ergocornam).

Isolation of N-[N(*d*-lysergyl)-L-valyl]-L-phenylalanyl-D-proline-lactam (proposed name ergocristam) was performed according to Černý *et al.*[6].

Isomerization of LEA

N-[N(*d*-Isolysergyl)-L-valyl]-L-valyl-D-proline-lactam (ergocorninam), N-[N(*d*-isolysergyl)-L-valyl]-L-leucyl-D-proline-lactam (ergocryptinam) and N-[N(*d*-isolysergyl)-L-valyl]-phenylanyl-D-proline-lactam (ergocristinam) were prepared by diluting individual LEA in ethyl acetate acidified by 60% acetic acid at room temperature. After equilibration (*ca.* 5 h) individual solutions were extracted by water, concentrated and chromatographed on a silica gel column using chloroform as an elution agent.

High-performance liquid chromatography

Separation of the mixture of CEA and LEA was performed with diethyl ether (p.a.) and ethanol (UV-spectroscopic purity) from Lachema, Brno, Czechoslovakia, as mobile phases. Standards of all used compounds were prepared in Galena, Opava, Czechoslovakia.

Separation of a mixture of CEA and LEA was performed on a Varian 8500 high-performance liquid chromatograph. Detection was performed by a Variscan LC UV detector, and integration of peaks by a C 111 integrator (Varian Aerograph, Walnut Creek, CA, U.S.A.). The chromatographic column was 25 cm × 2 mm I.D. The stationary phase was silica gel modified by alkylamine, particle size 10 μm. The column was packed in the laboratory[13] with LiChrosorb NH_2 ($N_{agroclavine} = 1070$). The column temperature was 20°C and the pressure 2.5 MPa. Elution was performed with diethyl ether–ethanol (96:4). The flow-rate of the mobile phase was 40 ml/h. The qualitative analysis of substances in the mixture was performed by comparing their elution volumes with those of pure reference substances.

RESULTS AND DISCUSSION

The separation of a mixture of LEA was performed using the results of an analysis of CEA by the HPLC method[12]. Differences in mutual interactions of the screened substances with a stationary and a mobile phase were sufficient for a satisfactory separation of individual components of the mixture of CEA and LEA during isocratic elution. Retention data of diastereomeric pairs of LEA and CEA are given in Table I.

The chromatographic behaviour of the studied substances is markedly influenced by the steric arrangement of the C-8 carbon of the ergolene nucleus. An intramolecular hydrogen bond, N-6···H—N-20, is formed in an axial position of the amide group of derivatives of isolysergic acid (-inines), bringing about a decrease in polarity of these derivatives. Interaction between the imine group of alkaloid and the amine group of the stationary phase decreases, -inines are retained and their elution volumes are smaller than those of -ines, in which the amide group is in an equatorial position.

This fact can be used for separation of biologically effective cyclol derivatives from isolysergic acid derivatives with a negligible biological activity. The same holds for the separation of the LEA diastereomeric pairs. The difference between retention volumes of the LEA isomers is more marked than that between the volumes of the

TABLE I

ELUTION DATA FOR LACTAM ERGOT ALKALOIDS AND CYCLOL ERGOT ALKALOIDS

$r_{l,s}$ = relative retention; k' = capacity factor.

CEA and LEA	*Molecular weight*	*Mobile phase diethyl ether–ethanol (96:4)*	
		$r_{l,s}$	k'
Ergocorninine	561.7	0.88	6.80
Ergocorninam	545.8	0.88	6.80
Ergocornine	561.7	1.36	11.00
Ergocornam	545.8	2.02	16.80
Ergocryptinine	575.7	0.77	5.80
Ergocryptinam	559.8	0.68	5.00
Ergocryptine	575.7	1.45	11.80
Ergocryptam	559.8	1.66	13.60
Ergocristinine	609.7	1.00	7.80
Ergocristinam	593.8	0.86	6.60
Ergocristine	609.7	1.61	13.20
Ergocristam	593.8	1.70	14.00
Retention volume of ergocristinine (ml)		3.12	

CEA isomers (Table I). With respect to the ergolene nucleus the axial position of the peptide is affected by the polarity of the hydroxyl group bound to C-12′. The CEA-inines are therefore more polar than the -inams. The elution rate of the CEA-inines is therefore, under the given conditions, considerably lower than that of the lactam derivatives. The elution rate difference is most clearly manifested with ergocristinine

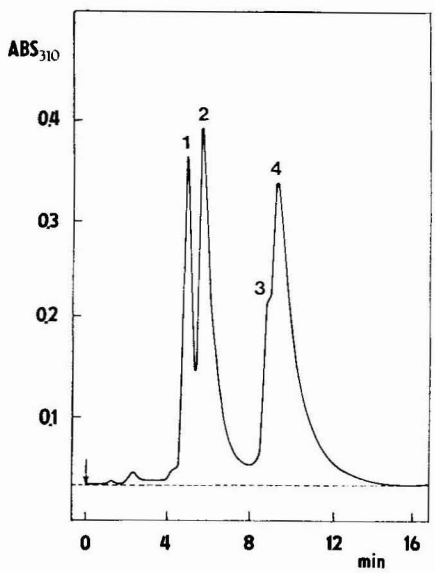

Fig. 2. Chromatogram of a mixture of diastereomeric pairs of ergocristine and ergocristam. Column, LiChrosorb NH_2; mobile phase, diethyl ether–ethanol (96:4); flow-rate of the mobile phase, 40 ml/h; pressure, 2.5 MPa; detector, Variscan UV at 310 nm. Peaks: 1 = ergocristinam; 2 = ergocristinine; 3 = ergocristine; 4 = ergocristam.

and ergocristinam. Separation of the mixture of ergocristine (or ergocristam), ergocristinine and ergocristinam poses no problems. On the other hand, ergocristine and ergocristam are practically inseparable (Fig. 2). In an equatorial arrangement of the CEA molecule (-ines) a hydrogen bond, 0-19···H···0-13′ is formed, not N-6···H—N-20. This explains why the CEA-ines are less polar than the LEA-ams, and why their retention volumes are smaller. The most marked difference between retention volumes was found with the ergocornine–ergocornam pair. Similarly, no problems are met in the separation of a mixture of ergocorninine (or ergocorninam), ergocornine and ergocornam. Retention volumes of ergocorninine and ergocorninam are identical (Fig. 3). The best separation of both pairs of alkaloids is obtained with a mixture of ergocryptine, ergocryptam, ergocryptinine and ergocryptinam, in which all four substances are readily separated (Fig. 4).

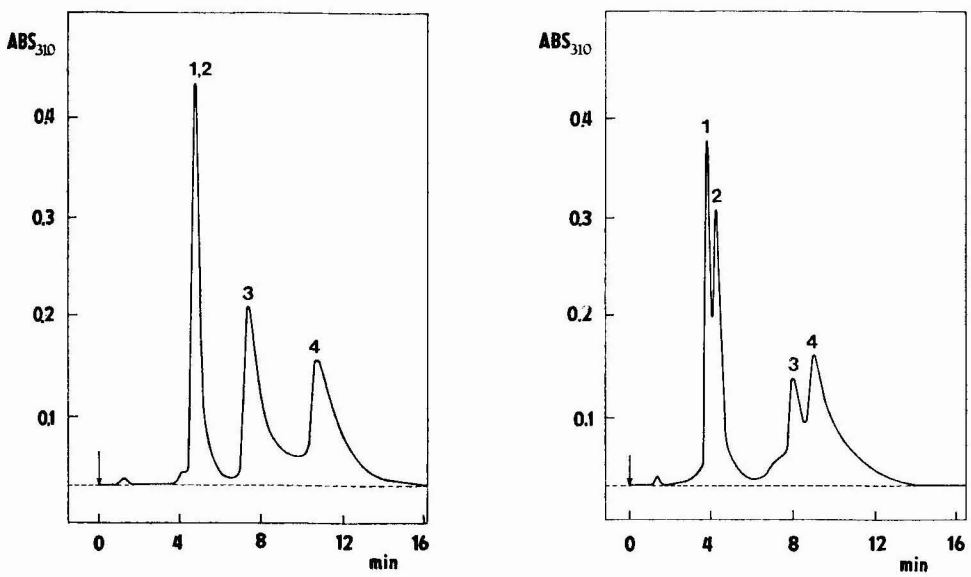

Fig. 3. Chromatogram of mixture of diastereomeric pairs of ergocornine and ergocornam. Column, LiChrosorb NH_2; mobile phase, diethyl ether–ethanol (96:4); flow-rate of the mobile phase, 40 ml/h; pressure, 2.5 MPa; detector, Variscan UV at 310 nm. Peaks: 1 and 2 = ergocorninam and ergocorninine; 3 = ergocornine; 4 = ergocornam.

Fig. 4. Chromatogram of mixture of diastereomeric pairs of ergocryptine and ergocryptam. Column, LiChrosorb NH_2; mobile phase, diethyl ether–ethanol (96:4); flow-rate of the mobile phase, 40 ml/h; pressure, 2.5 MPa; detector, Variscan UV at 310 nm. Peaks: 1 = ergocryptinam; 2 = ergocryptinine; 3 = ergocryptine; 4 = ergocryptam.

The chromatographic study of separation rendered possible the specification of conditions for the application of the HPLC method in the analysis of CEA in a mixture with LEA. The results could be used for a routine determination of the CEA/LEA mixture in natural materials, in the field of biogenesis of alkaloids, and in the control of pharmaceutical preparations.

The authors expect new lactam alkaloids corresponding to the remaining cyclol alkaloids to be found soon. Hence a new terminology is proposed in which the suffixes -ine or -inine for the cyclol alkaloids will be substituted by suffixes -am or -inam for the lactam alkaloids.

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Note

Determination of molecular-weight distribution of polymers by microcolumn exclusion chromatography*

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The use of microcolumn exclusion chromatography (MEC) for the investigation of the molecular-weight distribution (MWD) of polymers seems very promising. Low consumption of the sorbent ($\approx$50 μg) and the solvent ($\approx$50–100 μl) not only greatly reduces the cost of the analysis but also permits the use of a variety of solvents (including very pure solvents and those that are not readily available) and avoids the dangers of toxicity and fire. Taking as an example the separation of a mixture of polystyrene (PS) standards, it has been shown[1] that MEC systems can be developed (column diameter 0.6 mm) the performance of which is as high as that of conventional exclusion chromatographic columns (diameter 4.0 mm).

The aim of the present note is to describe a simple, reliable and precise system of MEC for the determination of the MWD of polymers.

Experiments were carried out with a microcolumn MSFP-3 chromatograph (Novosibirsk Institute of Organic Chemistry, Siberian Department of the Academy of Sciences of the U.S.S.R.). To increase the reproducibility of retention volumes a pump was prepared having a chamber volume of 300 μl and a precision calibrated plunger. A gear with a micrometer screw ensured to within 0.5% a constant volume rate of solvent injection. The columns were prepared from PTFE capillary tubes (0.6–0.65 mm I.D. and 0.7 mm O.D). They were packed with microspherical silica gel sorbents LiChrospher Si 100 (pore diameter = 100 Å, particle diameter = 5 μm) (1) and LiChrospher Si 1000 (pore diameter = 1000 Å, particle diameter = 10 μm) (2), from an aqueous suspension prepared in an ultrasonic disperser as described in ref. 2 at a rate of 67 μl/min (the pressure increasing to 70–80 atm). To increase the efficiency of the columns the sorbents were fractionated by sedimentation and fractions with particle diameter of 7 ± 1 μm were used. The height equivalent to a theoretical plate of the columns was 35 μm.

It has been shown[1] (Fig. 1) that a mixture of sorbents 1 and 2 in the ratio 2:3 permits the separation of PS over the MW range from 10^3 to $2 \cdot 10^6$. To increase the precision and simplify the calculations of MWD, a column was packed with a mixture of these sorbents which gave a linear calibration dependence (LCD) of retention volumes on log M. On the basis of calibration dependences obtained for

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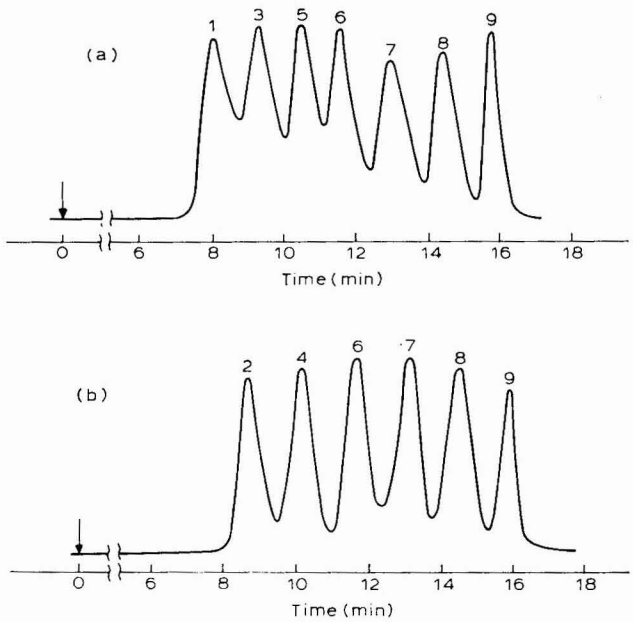

Fig. 1. Chromatograms of two mixtures (a,b) of PS standards. Column (300 × 0.6 mm) packed with a mixture of LiChrospher Si 100 and Si 1000 sorbents (2:3). Eluent: Methylene chloride; flow-rate 4.6 μl/min. Sample volume: 0.5 μl (5 mg/ml). Detection at $\lambda = 260$ nm. M_w: $2.61 \cdot 10^6$ (1); $8.67 \cdot 10^5$ (2); $4.11 \cdot 10^5$ (3); $2.00 \cdot 10^5$ (4); $1.11 \cdot 10^5$ (5); $3.3 \cdot 10^4$ (6); 10^4 (7); $2.1 \cdot 10^3$ (8); 78 (9) (benzene).

each of these sorbents, their ratio in the mixture that gave a LCD to within 0.2% over the range of M from $3 \cdot 10^3$ to 10^6 was calculated[3]. The experimental calibration dependence obtained on a 300 × 0.6 mm column (efficiency 9500 plates) packed with this mixture of sorbents was in good agreement with the theoretical dependence and was strictly linear over this MW range (Fig. 2).

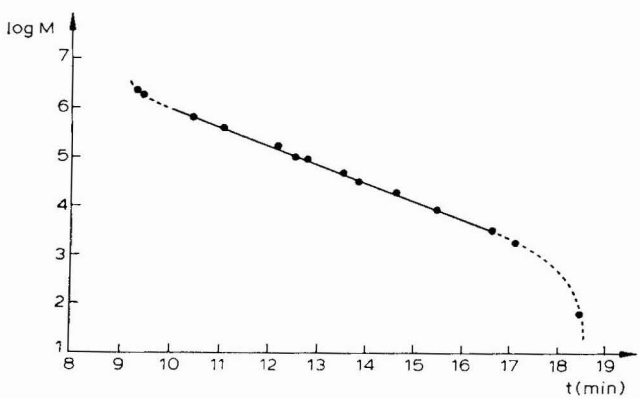

Fig. 2. Retention times *vs.* logarithm of the molecular weight of PS standards. Column (330 × 0.6 mm) packed with a mixture of LiChrospher Si 100 and Si 1000 sorbents. Eluent: methylene chloride; flow-rate 4.6 μl/min. Sample volume: 0.3 μl (1 mg/ml).

The chromatograms of PS standards obtained on this column exhibit asymmetric peaks, resulting in low values of average MW. This asymmetry is probably due to spreading in the detector as is shown by the results of our investigation of spreading in the cell. In separate experiments it was found that the concentration dependence of retention volumes is absent for PS with MW of 867,000 and 10,000 at concentrations of up to 1 mg/ml and 4–5 mg/ml respectively. To obtain true MWD it was necessary to correct for instrumental spreading.

MEC was used to determine the MWD of a PS standard, PS-706 (U.S. National Bureau of Standards)[4]. The MWD of this sample calculated from a chromatogram corrected for instrumental spreading[5] and the values of M_w and M_n found from it are in good agreement with the literature data (Table I). The reproducibility of the determination was $\approx 5\%$.

TABLE I

CHARACTERISTICS OF SAMPLE PS-706

	MEC	*According to ref. 4*
M_n	$(148 \pm 5) \cdot 10^3$	$136.5 \cdot 10^3$
M_w	$(273 \pm 5) \cdot 10^3$	$(278 \pm 5) \cdot 10^3$

It has been shown that MEC can be used to analyse the MWD of polymers with a precision comparable to conventional EC but with an approximately hundredfold decrease in the consumption of the sorbent and the eluent and a corresponding increase in sensitivity.

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Note

Determination of underivatized CNS-stimulants and methadone in urinary extracts by glass capillary gas chromatography

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Recently, reported methods for the determination of stimulants in biological fluids have employed conventional gas chromatography[1–8]. Quartz capillary columns have not yet been applied to the determination of underivatized stimulants or related drugs. Our method, previously used for the detection of stimulants in urine, involves extraction of alkalinized urines with large solvent volumes (diethyl ether), an additional clean-up extraction, back-extraction into 15% formic acid solution and chromatographic analysis of the formic acid extract on an alkalinized Carbowax column (5%KOH, 5% Carbowax 20M TPA on Chromosorb W HP). This technique is time consuming and laborious. Furthermore, the separation power of the column is not sufficient to resolve all the stimulants.

The frequency of use or abuse of amphetamine-related drugs was studied by Jain *et al.*[9]. Amphetamine, phentermine, ephedrine and methamphetamine were the most common stimulants detected in 10,000 screened samples. Aggarwal *et al.*[10] reported a very rapid procedure using a tiny volume of a solvent mixture for the extraction of basic drugs from alkalinized urines. We have now adapted their extraction technique, without any derivatization procedure, and combined it with chromatographic analysis on fused silica capillary columns.

EXPERIMENTAL

Apparatus

A Packard Model 427 gas chromatograph equipped with a flame ionization detector and a split injector was connected to a Sigma 10 Chromatography Data Station (Perkin-Elmer). A fused silica capillary column (25 m × 0.20–0.21 mm I.D., Hewlett-Packard No. 19091-60025) deactivated with Carbowax 20 M and with SP 2100 as the stationary phase was applied.

The glass capillary gas chromatographic (GCGC) conditions were as follows: injection port 230°C; detector 230°C; oven temperature isothermal at 110°C for 4 min, programmed from 110°C to 220°C at 16°/min and then isothermal at 220°C for 4 min. Nitrogen was employed both as carrier gas (flow-rate, 0.8 ml/min) and as the make-up gas (flow-rate, 10 ml/min). Splitting ratio = 1:10.

Reagents

The solid buffer was a mixture of $NaHCO_3$ and K_2CO_3 (3:2, w/w). The extraction solvent was chloroform–isopropanol (4:1, v/v). The internal standard solution

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was prepared by dissolving 10 mg of diphenylamine in 100 ml of ethanol. Drug standards in urine (1–20 μg/ml, as free base) were prepared by adding the drugs amphetamine, phentermine, chlorophentermine, ephedrine, phenmetrazine, diethylpropione, methylphenidate and methadone to a drug-free urine.

Procedure

Approximately 5 ml of urine were saturated with solid buffer (*ca.* 2 g). The mixture was shaken by vortexing and centrifuged for 5 min to remove any insoluble material. A 4-ml volume of the supernatant urine was transferred to a conically tipped culture tube with a PTFE-lined screw cap, followed by 100 μl of the internal standard solution. After mixing, 100 μl of the extraction solvent were added. The mixture was vortexed for 1 min, then centrifuged for 5 min. The supernatant urine was then aspirated and discarded. A 1-μl volume of the organic phase was injected on the gas chromatograph.

RESULTS AND DISCUSSION

Fig. 1 shows typical chromatograms obtained in this procedure. In the upper chromatogram the stimulants, methadone, nicotine and caffein (the last two compounds may be present in many urines) are resolved under the present chromatographic conditions. No interfering peaks were found in chromatograms from blank urines run through the procedure.

Calibration graphs of peak height (or peak area) ratios of the drug and internal standard *versus* the drug concentration were linear in the range 1–10 μg/ml for all the drugs. All the correlation coefficients were greater than 0.97. Table I gives precision data obtained by repeated analysis of a urine to which all the drugs had been added. Detection limits were as follows: stimulants, *ca.* 0.3 μg/ml (except for ephedrine, 1 μg/ml); methadone, *ca.* 0.6 μg/ml.

No interference from barbiturates was found. Other basic or neutral drugs or their metabolites may interfere; however, this problem is easily overcome by adapting the technique to mass spectrometry, which yields additional structural information. No metabolites were studied. Positive samples for each drug should be confirmed by mass spectrometry.

The method is adequate for the screening of samples from suspected stimulant abusers. As the technique is very simple the saving of labour and time is considerable compared to the methods previously used.

TABLE I

PRECISION DATA

In each case $n = 6$.

Compound	*Mean (μg/ml)*	*CV (%)*
Amphetamine	8.9	8.4
Phentermine	2.8	10.0
Chlorophentermine	5.2	3.1
Ephedrine	4.8	10.8
Phenmetrazine	4.9	5.5
Diethylpropione	5.8	4.1
Methylphenidate	3.1	1.9
Methadone	2.6	11.5

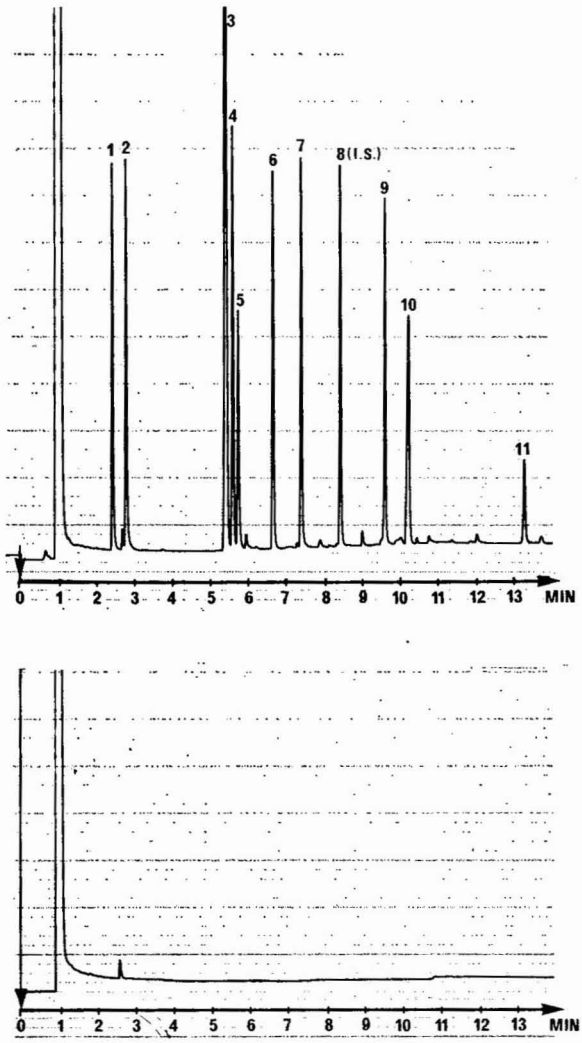

Fig. 1. Upper chromatogram: an analysis of a urine drug standard (5 μg/ml of each drug, except ephedrine 20 μg/ml, as free base). Peaks: 1 = amphetamine; 2 = phentermine; 3 = nicotine; 4 = chlorophentermine; 5 = ephedrine; 6 = phenmetrazine; 7 = diethylpropione; 8 = diphenylamine (internal standard); 9 = methylphenidate; 10 = caffein; 11 = methadone. Retention times are given in Table II. Lower chromatogram: a urine blank without addition of the internal standard.

TABLE II

RETENTION TIMES OF THE STIMULANTS

Peak no.	*Compound*	*Retention time (min)*	*Relative retention*
1	Amphetamine	2.59	0.30
2	Phentermine	2.90	0.34
3	Nicotine	5.57	0.65
4	Chlorophentermine	5.78	0.68
5	Ephedrine	5.93	0.69
6	Phenmetrazine	6.83	0.80
7	Diethylpropione	7.54	0.88
8	Diphenylamine (I.S.)	8.55	1.00
9	Methylphenidate	9.70	1.13
10	Caffein	10.36	1.21
11	Methadone	13.47	1.58

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Note

Thin-layer chromatographic separation of the lower alkanols as the xanthates

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The lower alkanols were first separated as their xanthates by paper chromatography by Lederer and Summerfield[1] using a short ascending development (*ca.* 6 h) on Whatman No. 1 paper with *n*-butanol–water–ammonia solution (50:45:5). Subsequently several workers tried to repeat these separations using overnight descending development and noted considerable decomposition of the xanthates (for a discussion see Gasparič and Borecký[2]); they proposed the use of more alkaline solvents to avoid decomposition of the xanthates.

It occurred to us that with the advent of high-performance thin-layer chromatography (HPTLC) the xanthates should be re-examined, as the decomposition seemed to be mainly a matter of the development time. We report our findings in this paper.

EXPERIMENTAL AND RESULTS

Preparation of xanthates

C_1–C_5 potassium xanthates were prepared by the following procedure. To 1 ml of alkanol were added 0.2 g of powdered potassium hydroxide. The alkaline solution was cooled to 0°C, then 0.3 ml of carbon disulphide were added dropwise with stirring. The precipitated xanthate was filtered off on a Büchner funnel and dissolved in acetone, then filtered again to separate it from solid potassium hydroxide. Finally, the solution was dried under vacuum. A xanthate mixture was obtained by the same procedure, starting with a mixture of alkanols in equal amounts.

A 100-ml volume of methanol–ethanol–water (1:40:59) mixture was examined to test the possible detection of small amounts of methanol in alcoholic beverages. The mixture was first distilled at 82°C to remove water and the first 4-ml fraction was subjected to the above procedure for preparation of the xanthates.

Chromatography of xanthates

About 10 mg of xanthates were dissolved in 2–3 ml of distilled water immediately before the analysis. Polygram CEL 400 (Macherey, Nagel & Co., Düren,

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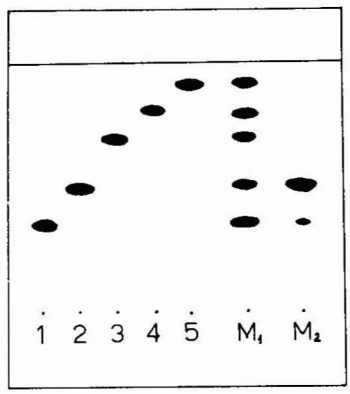

Fig. 1. Thin-layer chromatogram of xanthates of C_1–C_5 aliphatic alcohols on Polygram CEL 400. Solvent: *n*-butanol–water–ammonia solution (5:4:1). Xanthate derivatives from (1) methanol; (2) ethanol; (3) propanol; (4) butanol; (5) amyl alcohol; (M_1) mixture of equal amounts of C_1–C_5-alkanols; (M_2) first fraction from distillation of methanol–ethanol–water (1:40:59).

G.F.R.) thin layers (0.1 mm thick layers of microcrystalline cellulose) with a fluorescence indicator (254 nm) were equilibrated for 10 min before development in a chromatographic tank containing *n*-butanol–water–ammonia (5:4:1) as solvent. Samples of 10 μl were applied as streaks 1 cm apart from each other and 1 cm from the lower edge of the layer. After development the spots were detected by their dark brown fluorescence under ultraviolet light.

As shown in Fig. 1, a short run of 3 cm, developed in 25 min, yielded a complete separation of the C_1–C_5 alkanols; a longer development did not increase the resolution.

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Note

Thin-layer chromatography of chlorinated guaiacols

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In a previous paper[1] we discussed the thin-layer chromatography (TLC) of guaiacol and six chlorinated guaiacols on silica gel with 40 different solvent systems. In that study dichloromethane–benzene–methanol (60:30:10) and acetone were recommended for group separation, whereas light petroleum (b.p. 40–60°C)–ethyl acetate (70:30) and dichloromethane–chloroform (90:10) were shown to be suitable for separation of the individual compounds by one-dimensional TLC.

The work described here was undertaken to synthesize nine new chlorinated guaiacols, study their characteristic colour reactions on the TLC plate, determine the R_F values with different solvent systems and finally calculate the standard deviations[1] and the relative differences[1,2] in the R_F values in order to select the most suitable solvent systems for particular separations.

EXPERIMENTAL

Samples

The compounds used (see Fig. 1) were 4-chloroguaiacol (I), 3-chloroguaiacol (II), 6-chloroguaiacol (III), 3,6-dichloroguaiacol (IV), 3,4-dichloroguaiacol (V), 5,6-

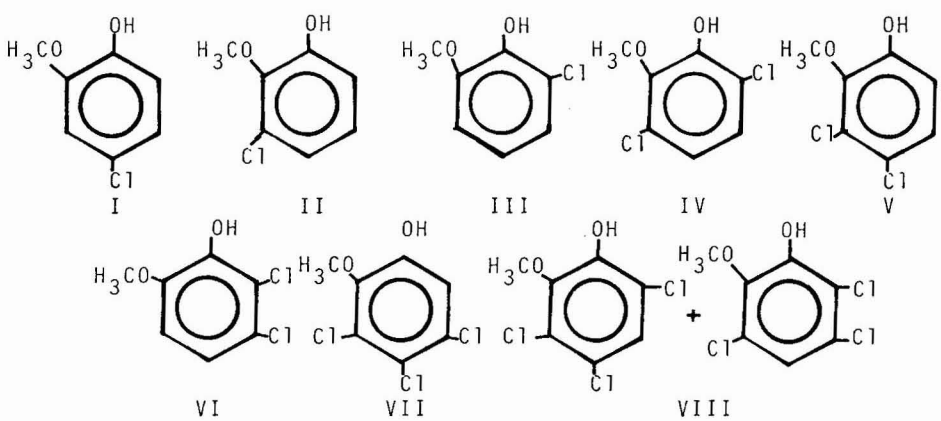

Fig. 1. Structures of 4-chloroguaiacol (I), 3-chloroguaiacol (II), 6-chloroguaiacol (III), 3,6-dichloroguaiacol (IV), 3,4-dichloroguaiacol (V), 5,6-dichloroguaiacol (VII), 3,4,5-trichloroguaiacol (VII) and a mixture (1:1) of 3,4,6-trichloroguaiacol and 3,5,6-trichloroguaiacol (VIII).

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dichloroguaiacol (VI), 3,4,5-trichloroguaiacol (VII) and a mixture (*ca.* 1:1) of 3,4,6-trichloroguaiacol and 3,5,6-trichloroguaiacol (VIII). All compounds were synthesized using limited methylation of the corresponding chlorinated catechols according to the method of Vickery *et al.*[3]. They were purified by silica gel column chromatography (eluent: dichloromethane). The structures were confirmed by infrared (IR), ^{1}H nuclear magnetic resonance (NMR), ^{13}C NMR and mass spectroscopy (MS). The purity of the compounds was checked by glass capillary gas chromatography.

Apparatus and methods

A 0.5% solution of each guaiacol in dichloromethane was prepared. Two microlitres of I–VII and 4 μl of VIII were spotted 1.5 cm from the bottom of the plate to the concentrating zone with spot intervals of 1 cm. Other conditions and apparatus were as presented in ref. 1. A 2% solution of 3,5-dichloro-*p*-benzoquinonechlorimine in toluene[4] was used for spot detection.

Solvent systems

A preliminary screening of eight solvents or solvent mixtures was undertaken. The compositions (v/v) of these were as follows:

(1) dichloromethane
(2) chloroform
(3) dichloromethane–chloroform (90:10)
(4) light petroleum (b.p. 40–60°C)–diethyl ether (70:30)
(5) light petroleum (b.p. 40–60°C)–ethyl acetate (70:30)
(6) acetone
(7) dichloromethane–benzene–methanol (60:30:10)
(8) dichloromethane–ethyl acetate–acetic acid (80:15:5)

TABLE I

CHARACTERISTIC COLOUR REACTIONS OF CHLORINATED GUAIACOLS

Plates were sprayed after different times with a 2% solution of 3,5-dichloro-*p*-benzoquinonechlorimine in toluene and the colours of the spots were compared after different times. Amount of each compound applied: 10 μg.

Compound	*Neutral developing solvent*			*Acidic developing solvent*		
	1 h	*24 h*	*10 days*	*1 h*	*24 h*	*10 days*
I	Brown	Brown	Brown	Orange-brown	Brown	Brown
II	Violet-blue	Violet-blue	Light violet-blue	Light yellow	Light violet	Violet-blue
III	Blue	Brown	Orange-brown	Orange-brown	Brown	Orange-brown
IV	Blue-green	Violet-blue	Violet	Violet-grey	Violet	Violet
V	Light yellow	Greenish grey	Light violet	Light yellow	Light yellow	Light violet
VI	Blue	Red-brown	Red-brown	Red-brown	Red-brown	Red-brown
VII	Light yellow	Light violet	Light violet	Light yellow	Light yellow	Light violet
VIII*	Violet	Violet	Violet	Light yellow	Violet	Violet

* Only one spot was observed.

RESULTS AND DISCUSSION

Colour reactions

The characteristic colour reactions of compounds I–VIII are shown in Table I. With neutral solvent systems (1–7) and 1 h after spraying, different colours for almost all spots were observed. The colour reactions of the 3-chloro-substituted guaiacols were clearly different from those of the others: after 10 days the spots of II, IV, V, VII and VIII were violet-based and those of the other compounds studied were brownish. In addition, II, V and VII were coloured much more slowly and for these compounds only light colours were observed.

Using the acidic solvent system (8) and 1 h after spraying, the spots of 3-chloro-substituted guaiacols (except IV, see Table I) were yellowish and those of the other compounds studied were brownish. Compounds II, IV, V, VII and VIII (especially V and VII) gave much slower and weaker colour reactions and their final colours after 10 days were violet-based, whereas those of I, III and VI were brownish.

R_F values

The R_F values of the spots were measured with an accuracy of better than 0.03. All runs were carried out three times and the average R_F values were calculated. The results are presented in Table II.

TABLE II

R_F VALUES OF CHLORINATED GUAIACOLS ON A SILICA GEL G 60 LAYER WITH DIFFERENT SOLVENT SYSTEMS

Solvent system	*Compound*								*Standard deviation of R_F*	*Development time (min)*
	I	*II*	*III*	*IV*	*V*	*VI*	*VII*	*VIII**		
1	0.38	0.25	0.40	0.33	0.29	0.43	0.29	0.35	0.062	30
2	0.45	0.37	0.48	0.43	0.35	0.46	0.34	0.42	0.053	35
3	0.36	0.25	0.38	0.32	0.28	0.41	0.28	0.34	0.055	30
4	0.18	0.21	0.18	0.26	0.17	0.12	0.16	0.22	0.042	30
5	0.34	0.39	0.34	0.46	0.35	0.26	0.35	0.43	0.062	30
6	0.64	0.63	0.63	0.65	0.64	0.63	0.63	0.63	0.009	25
7	0.57	0.54	0.58	0.58	0.53	0.57	0.54	0.57	0.020	30
8	0.68	0.68	0.70	0.72	0.66	0.69	0.68	0.72	0.021	45

* Only one spot was observed.

All these solvent systems (1–8) have previously[1] been used for selecting the most suitable solvent systems for particular separations of guaiacol and six chlorinated guaiacols. Under the present experimental conditions with solvent system 3 the R_F value of 5-chloroguaiacol in the three runs was less than 0.03 units smaller than the corresponding value presented in ref. 1. Hence we conclude that the present results are comparable with those obtained previously.

Standard deviations, s, of the R_F values (see ref. 1) and relative differences, x, between R_F values (see refs. 1 and 2) of I–VIII in each run were calculated to estimate the separation power of each solvent system. Additional information on the separation of the components was obtained from x_{ij} matrixes and $\bar{x}$ and Σx values (see Table III).

TABLE III

RELATIVE DIFFERENCES, x, BETWEEN R_F VALUES OF GUAIACOLS ON SILICA GEL G 60 WITH SELECTED SOLVENT SYSTEMS

Each value of x is calculated by dividing the difference of two R_F values by their average. The averages, $\bar{x}$, and sums, Σx, of x for each run are also given.

Solvent system	*x values*								*Average,* $\bar{x}$	*Sum,* Σx
	—	*II*	*III*	*IV*	*V*	*VI*	*VII*	*VIII*		
1	I	0.413	0.051	0.141	0.269	0.123	0.269	0.082	0.222	6.218
	II		0.462	0.276	0.148	0.529	0.148	0.333		
	III			0.192	0.319	0.072	0.319	0.133		
	IV				0.129	0.263	0.129	0.059		
	V					0.389	0.000	0.188		
	VI						0.389	0.205		
	VII							0.188		
2	I	0.195	0.065	0.045	0.250	0.022	0.278	0.069	0.156	4.371
	II		0.259	0.150	0.056	0.217	0.085	0.127		
	III			0.110	0.313	0.043	0.341	0.133		
	IV				0.205	0.067	0.234	0.023		
	V					0.272	0.029	0.182		
	VI						0.300	0.090		
	VII							0.211		
3	I	0.361	0.054	0.118	0.250	0.130	0.250	0.057	0.206	5.762
	II		0.413	0.246	0.113	0.485	0.113	0.305		
	III			0.171	0.303	0.076	0.303	0.111		
	IV				0.133	0.247	0.133	0.061		
	V					0.377	0.000	0.194		
	VI						0.377	0.187		
	VII							0.194		
4	I	0.154	0.000	0.364	0.057	0.400	0.118	0.200	0.269	7.523
	II		0.154	0.213	0.211	0.545	0.270	0.047		
	III			0.364	0.057	0.400	0.118	0.200		
	IV				0.419	0.737	0.476	0.167		
	V					0.345	0.061	0.256		
	VI						0.286	0.588		
	VII							0.316		
5	I	0.137	0.000	0.300	0.029	0.267	0.029	0.234	0.198	5.531
	II		0.137	0.165	0.108	0.400	0.108	0.098		
	III			0.300	0.029	0.267	0.029	0.234		
	IV				0.272	0.556	0.272	0.067		
	V					0.295	0.000	0.205		
	VI						0.295	0.493		
	VII							0.205		
6	I	0.016	0.016	0.016	0.000	0.016	0.016	0.016	0.012	0.347
	II		0.000	0.031	0.016	0.000	0.000	0.000		
	III			0.031	0.016	0.000	0.000	0.000		
	IV				0.016	0.031	0.031	0.031		
	V					0.016	0.016	0.016		
	VI						0.000	0.000		
	VII							0.000		

The following conclusions can be made from Tables II and III. With all solvent systems studied, 3,4,6-trichloroguaiacol and 3,5,6-trichloroguaiacol had the same R_F values (see Table II). The use of chloroform (2) always resulted in x_{ij} values which

were different from zero and also in relatively high s, $\bar{x}$ and Σx values. Thus this solvent is recommended for the separation of I–VIII by one-dimensional TLC. Solvent systems 1, 3, 4 and 5 give one (or two) x_{ij} values which differ from zero (see Table III) and large s, $\bar{x}$ and Σx values. Hence, these solvent systems could be used only as the first stage in a two-dimensional TLC procedure in which the final separation of I, III, V and VII is carried out with an another solvent system. The highest $\bar{x}$ and Σx values were observed for solvent system 4. However, the value of s is relatively small (0.042) and one x_{ij} value is equal to zero. Thus, dichloromethane (system 1) or dichloromethane–chloroform (90:10) (system 3) could be applied as with system 4 to the separation of I–VIII by two-dimensional TLC. Finally, solvent system 6 (acetone) gives almost identical R_F values (0.63–0.65) and the smallest s, $\bar{x}$ and Σx values. Hence it is the best solvent system for group separation of I–VIII.

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CHROM. 13,517

Book Review

Wilson & Wilson's Comprehensive analytical chemistry, Vol. X, Organic spot test analysis; The history of analytical chemistry, edited by G. Svehla, Elsevier, Amsterdam, Oxford, New York, 1980, XIV + 282 pp., price US$ 83.00, Dfl. 170.00 (subscription price US$ 73.25, Dfl. 150.000), ISBN 0-444-41859-8.

Like *Rodd's Chemistry of carbon compounds*, *Comprehensive analytical chemistry* is intended to stand halfway between the one-volume treatise, or text-book, and the thirty-volume treatises. This is a formidable undertaking and so it has taken over 10 years to reach Volume 10. Usually the second edition is started soon after the completion of the first, because by that time the first volumes are already out of date. The reviewer would thus like to address these remarks both to the potential buyer and to the Editors of the (hopefully) second edition.

The first part of this volume is exactly what one would expect to find in such a treatise. It is, in fact, an up-to-date condensed version of Feigl's *Organic spot tests*, 54 pages long, with 211 references. That most of the papers quoted are earlier than 1970 is due to the fact that little work has been done in this field since then.

The second part of the book deals with the history of analytical chemistry and is about 200 pages long. It seems doubtful whether this is of such importance as to justify almost all of one volume, out of a ten-volume treatise. Several histories of analytical chemistry have appeared in recent years, one of which is by one of the authors of this volume (F. Szabadvary). However, this history makes rather good reading. It is full of anecdotes (although most I have read already in "Bunge") and of unintentional humour, such as:

"He (*i.e.*, Liebig) had noticed that cattle there (*i.e.*, in the Argentine) were slaughtered for their hides, the meat being left to rot. The Liebig meat cube resulted from this enterprise".

There are also sentences which give food for thought, such as "Polarography is simply electrolysis with a dropping mercury electrode." I wonder which reader will profit from this profundity?

Then there are unforgivable (to some chromatography fanatics) howlers, such as on page 257 about M. Tswett:

"Born in 1972 in Russia, Tsvett moved with his family to Switzerland..."

To sum up: it might be a good idea to omit the history in future editions.

Lausanne (Switzerland) MICHAEL LEDERER

PUBLICATION SCHEDULE FOR 1981

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

MONTH	N 1980	D 1980	J	F	M	A	M	J	J	A	S	O	N	D
Journal of Chromatography			203 204 205/1 205/2	206/1 206/2 206/3	207/1 207/2 207/3	208/1 208/2 209/1		The publication schedule for further issues will be published later.						
Chromatographic Reviews							220/1							
Biomedical Applications	221/1	221/2	222/1	222/2	222/3	223/1	223/2							

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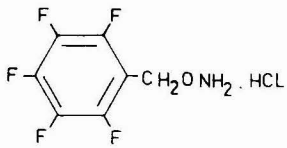

A sensitive derivatizing agent for EC-GC analysis of keto steroids:

1) K.T. Koshy, D.G. Kaiser, A.L. VanDerSlik, J. Chromatogr. Sci. **(1975)** *13,* 97
2) T. Nambara, K. Kigasawa, T. Iwata, M. Ibuki, J. Chromatogr. **(1975)** *114,* 81
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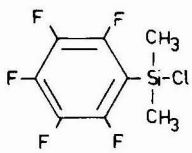
Pentafluorophenyldimethylchlorosilane (Flophemesyl chloride)



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1) P.M. Burkinshaw, E.D. Morgan, C.F. Poole, J. Chromatogr. **(1977)** *132,* 548 (and references cited therein)
2) A.J. Francis, E.D. Morgan, C.F. Poole, J. Chromatogr. **(1978)** *161,* 111
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14660	**1,3-Bis(chloromethyl)-1,1,3,3-tetramethyldisilazane purum** ~99%(GC)	1 lt ≈ 1.05 kg	5 ml 21
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17615	**(Bromomethyl)dimethylchlorosilane, BMDMCS purum** ~98%(GC); B.P. 133-136°	1 lt ≈ 1.38 kg	10 ml 18
25117	**(Chloromethyl)dimethylchlorosilane, CMDMCS purum** >99%(Cl); B.P. 110-113°	1 lt ≈ 1.08 kg	10 ml 20
36510	**1,3-Dichloro-1,1,3,3-tetrafluoroacetone, DCTFA purum** 98%(GC); B.P. 43-46°	1 lt ≈ 1.52 kg	5 ml 20
65945	**N-Methyl-bis-trifluoroacetamide, MBTFA purum** 97%(GC); B.P. 121-122°	1 lt ≈ 1.55 kg	5 ml 32
76690	**2,3,4,5,6-Pentafluorobenzaldehyde, PFBA purum** ~99%(GC); M.P. 22-25°; B.P. 170-173°		5 g 29
76732	**2,3,4,5,6-Pentafluorobenzoyl chloride puriss.** B.P. 158-159°	1 lt ≈ 1.67 kg	5 ml 50
17910	**2,3,4,5,6-Pentafluorobenzyl bromide, PFB-Br purum** >99%(GC); B.P. 110-115°		5 g 42
76735	**O-(2,3,4,5,6-Pentafluorobenzyl)hydroxylamine, HCl puriss.** p.a. >99%(Cl)		250 mg 17
76750	**Pentafluorophenyldimethylchlorosilane purum** >98%(Cl); $B.P._{12}$ 72-74°	1 lt ≈ 1.40 kg	5 ml 67
77253	**Perfluorobutyric anhydride, HFBA puriss.** p.a. >99%(GC); B.P. 109-111°	1 lt ≈ 1.67 kg	10 ml 45
77292	**Perfluoropropionic anhydride, PFPA purum** >99%(GC); B.P. 70-72°	1 lt ≈ 1.59 kg	5 ml 29
91719	**Trifluoroacetic anhydride, TFAA puriss.** >99%(GC); B.P. 40-42°	1 lt ≈ 1.51 kg	10 ml 20

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