JOURNAL
of
CHROMATOGRAPHY

INTERNATIONAL JOURNAL ON CHROMATOGRAPHY, ELECTROPHORESIS AND RELATED METHODS

VOL. 38 1968

ELSEVIER PUBLISHING COMPANY

AMSTERDAM

PRINTED IN THE NETHERLANDS

CHROM. 3713

SILICONE GREASE/SILICONE OIL STATIONARY PHASE FOR THE SEPARATION OF 3-METHYLBUT-1-ENE FROM 1,1-DIMETHYLCYCLOPROPANE

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(Received July 15th, 1968)

SUMMARY

A novel type of stationary phase acting in the capacity of both a solvent and an adsorbant is reported here. The advantages of this phase over other stationary phases discussed in the literature as suitable for the separation of two closely boiling C_5H_{10} isomers of interest are considered.

During the kinetic study of the gas-phase pyrolysis of neopentyl chloride[7,8] it became necessary to analyse by gas chromatography the reaction products which included methane, methyl chloride, isobutene, 1-chloro-2-methylpropene, 1,1-dimethylcyclopropane and the three isomeric methylbutenes. A slight excess of gaseous ammonia was added to the reaction system prior to collection of samples to remove the hydrogen chloride eliminated from neopentyl chloride in order to prevent it from combining with the secondary and tertiary olefins produced in the pyrolysis[8]. Because of the presence of ammonia, the columns chosen for the analysis had to be resistant to prolonged exposure to ammonia.

Separation of the low boiling products and the isomeric methylbutenes was readily achieved on a squalane column, but separation of 3-methylbut-1-ene from 1,1-dimethylcyclopropane was very poor. The following stationary phases have been reported as suitable for the chromatographic separation of these two substances: 2,5-hexadione in series with a short column of silver nitrate in ethylene glycol[3–5], dimethylsulpholane[6], tetramethylsilane[6], and dioctyl phthalate[6]. Of these, only the first stationary phase gave satisfactory performance while tetramethylsilane was only fair. When an ethyl acetoacetate*/silver nitrate in ethylene glycol column, similar to that reported by FREY[3] was used (in series with a short diglycerol precolumn to remove excess ammonia from the sample) under equivalent operating conditions, 3-methyl-

* Since 2,5-hexadione was not readily available to us, ethyl acetoacetate was used instead. A later check confirmed that the two materials give identical separation of the compounds in question.

but-1-ene could be separated from 1,1-dimethylcyclopropane but its peak exhibited extensive "tailing" to an extent dependent upon the concentration of the olefin. Similar "tailing" was noted in the case of isobutene and 2-methylbut-1-ene, but not with 2-methylbut-2-ene. Activated alumina, poisoned with 2% w/w paraffin oil, when tested for the separation of the C_5 isomers was found to give adequate results with almost symmetrical peaks, but retention times were not reproducible and increased markedly with the aging of the column.

A 1:1 mixture of silicone grease/silicone oil as a stationary phase on Celite gave excellent separation of 3-methylbut-1-ene from 1,1-dimethylcyclopropane yielding symmetrical peaks for 3-methylbut-1-ene, isobutene and 2-methylbut-1-ene. This mixed phase was also found capable of efficient separation of some other hydrocarbons from the corresponding olefins, better than the separation on silicone grease alone, reported by AVERILL AND ETTRE[2], but not as good as that reported by FREY[3] on 2,5-hexadione.

EXPERIMENTAL

Stationary phases

Squalane, ethyl acetoacetate* and diglycerol were of commercial origin. The alumina was of chromatographic grade (B.D.H.). The other materials used were: Silicone Oil SF96 (1000 centistokes) from General Electric, high vacuum silicone grease and silicone fluid 200 (1000 centistokes) from Dow Corning, Atpet 80 and Tween 20 (Atlas Powder Co.) and Alkaterge T (Commercial Solvents Corp.). The silver nitrate solution in ethylene glycol was prepared by leaving an excess of silver nitrate crystals in contact with ethylene glycol for one week, the mixture being shaken occasionally.

Columns

The columns were constructed of aluminium tubing $^3/_{16}$ in. external diameter ($^1/_8$ in. internal diameter). The stationary phase was deposited by the usual "slurry" technique onto Celite 545 (100–120 mesh BSS). The Celite used for the squalane and silicone grease/silicone oil column was dry-sil treated.

The squalane column (4 ft.) was prepared with 20% w/w squalane plus 1% w/w Tween 20 as a "tailing reducer"**. The ethyl acetoacetate column (10 ft.) was prepared with 20% w/w of ethyl acetoacetate*** and was followed by a silver nitrate/ethylene glycol column (1.8 ft.) with 20% w/w stationary phase. The silicone grease/silicone oil column (12 ft.) was prepared with 5% w/w silicone grease and 5% w/w Silicone

* See footnote, p. 1.

** Control experiments have shown that peak "tailing" is greatly reduced in the presence of Tween 20 in the case of the squalane column and with a mixture of Atpet 80 and Alkaterge T in the case of the silicone grease/silicone oil column.

*** A recent check in our laboratory, after this study was completed, on the performance of a 2,5-hexadione/silver nitrate series column revealed that peak "tailing" can be practically eliminated if the support material (*i.e.*, Celite 545) used in the preparation of both sections of the column is carefully deactivated by silanising with dichlorodimethylsilane. FREY's results[3] are readily reproduced on a column of 2,5-hexadione (12 ft.) followed by a silver nitrate/ethylene glycol column (1.5 ft.), each made up with 20% w/w of stationary phase on treated Celite. A chromatogram of the separation on this column is not shown here because the analysis was carried out with a different instrument and with slightly different operating conditions. Only the separation of 3-methylbut-1-ene from 1,1-dimethylcyclopropane and from the other two methylbutenes was examined.

Oil SF96, 0.3% w/w Atpet 80, and 0.1% w/w Alkaterge T. In the case of the aceto-acetate column the ratio of the lengths of the acetoacetate/silver nitrate columns was found to be important, best results being observed with a ratio of 5.6/1, respectively, which is similar to that used by FREY[3] (*i.e.*, 8:1). The ratio of the silicone grease to silicone oil was also found to be important with optimum results being observed with a 1:1 ratio of the mixed phases. Dow Corning Silicone Fluid 200, alone, gave similar separation as squalane, with negligible separation of 3-methylbut-1-ene from 1,1-dimethylcyclopropane.

PROCEDURE

The gas chromatograph with an all-glass flame-ionisation detector used for this study was built in the chemistry school workshop. The operating conditions are tabulated in Table I. Flow rates were measured with a soap bubble flow meter. Hydro-

TABLE I

OPERATING CONDITIONS FOR FLAME-IONISATION CHROMATOGRAPH

	Squalane	*Ethyl acetoacetate/ silver nitrate*	*Silicone oil/ silicone grease*
Nitrogen flow rate (ml/min)[a]	22.9 ± 0.2	20.0 ± 0.2	16.1 ± 0.2
Inlet pressure (cm Hg)[b]	37	60	57
Column temperature (°C)	40.0 ± 0.2	0.0 ± 0.5	0.0 ± 0.5

[a] Air flow rate at 700 ± 50 ml/min and hydrogen flow rate at 28.5 ± 0.5 ml/min were constant for all columns.

[b] Outlet pressure was atmospheric for all columns.

carbon mixtures were prepared on a high vacuum line. Vapour samples of 0.1 to 2.0 ml were removed for analysis by means of a gas-tight syringe from a vapour reservoir (*ca.* 900 ml).

RESULTS AND DISCUSSION

Separation of neopentyl chloride pyrolysis products on the three columns is illustrated in Figs. 1–3. It is evident from Fig. 1 that the squalane column is only suitable for separation of substances which differ considerably in their boiling points, the substances being eluted from the column according to their boiling points. The performance of the ethyl acetoacetate/silver nitrate column is shown in Fig. 2. Here the substances are separated according to their polarity, as well as their volatility. It is clearly unsatisfactory for quantitative analysis because of the extensive peak "tailing" mentioned previously (refer to footnote***, p. 2). Separation of the same substances on the silicone grease/silicone oil column, shown in Fig. 3, indicates that while this stationary phase behaves as a non-polar one, separation is superior to that on the squalane column.

A likely explanation of the improved separation on this column is that the silica flour contained in the silicone grease acts as an adsorbent material, so that the mixed

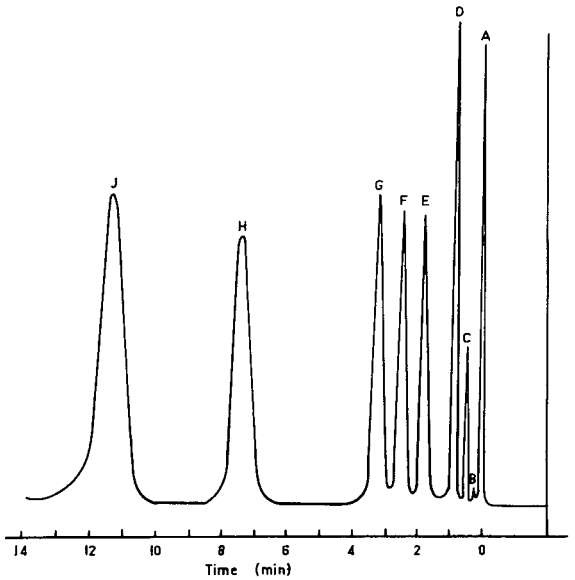

Fig. 1. Neopentyl chloride pyrolysis products on a squalane column. A = Methane; B = ethane/ethylene; C = methyl chloride; D = isobutene; E = 1,1-dimethylcyclopropane/3-methylbut-1-ene; F = 2-methylbut-2-ene; G = 2-methylbut-2-ene; H = 1-chloro-2-methylpropene; J = neopentyl chloride.

stationary phase has the dual character of both a partition column and an adsorption column. AVERILL AND ETTRE[2] have first commented that the separation of light hydrocarbons on silicone grease can be attributed to the adsorptive properties of the

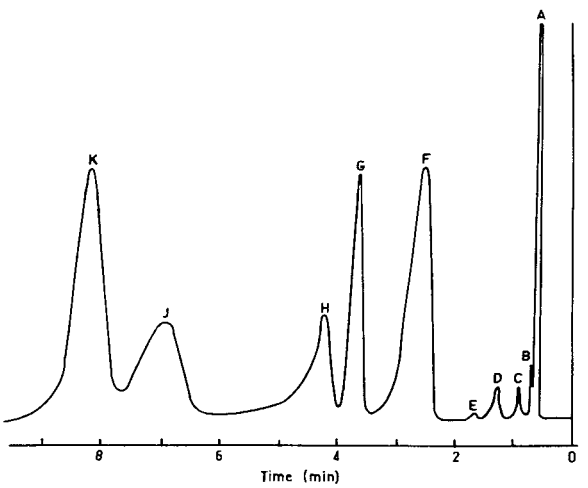

Fig. 2. Neopentyl chloride pyrolysis products on an ethyl acetoacetate/silver nitrate column. A = Methane; B = ethane/ethylene; C = propane/propylene; D = methyl chloride; E = unknown; F = isobutene; G = 1,1-dimethylcyclopropane; H = 3-methylbut-1-ene; J = 2-methylbut-1-ene; K = 2-methylbut-2-ene.

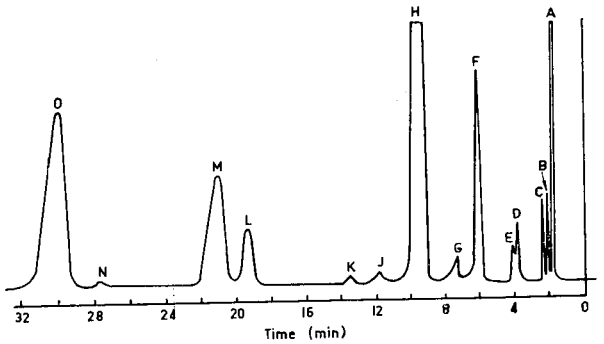

Fig. 3. Neopentyl chloride pyrolysis products on silicone oil/silicone grease column. A = Methane; B = ethylene; C = ethane; D = propylene; E = propane; F = methyl chloride; G = unknown; H = isobutene; J = neopentane; K = unknown; L = 3-methylbut-1-ene; M = 1,1-dimethylcyclopropane; N = unknown; O = 2-methylbut-1-ene.

silica flour present in the grease. SMITH[9] has shown that the silica flour in silicone grease behaves as a strong adsorbent. Addition of small amounts of silicone fluid (7.2%) is reported by him to reduce the adsorptive activity of the inorganic silica flour.

We added a much larger amount of silicone oil to the grease than SMITH to reduce the viscosity of the grease, and to diminish the magnitude of the liquid phase mass transfer term at the low temperatures used. Alkaterge T and Span 80 were added to act as "tailing" reducers as suggested by AVERILL[1].

The advantage of the silicone grease/silicone oil stationary phase over 2,5-hexadione/silver nitrate is that a single, short column performs equally well as the series column of the latter, thus resulting in a reduction of retention times. An added versatility of the mixed column is that it can be used with a temperature programming analysis of high boiling materials after separation of C_2–C_5 hydrocarbons and olefins is achieved at 0°. Since there is negligible loss of stationary phase which could contribute to a temperature-dependent background the use of this column is recommended if the eluted substances are to be trapped for the purpose of further identification by physical methods such as mass spectrometry or infra-red spectroscopy. This stationary phase is also more resistant to gradual poisoning by traces of moisture and ammonia than both ethyl acetoacetate (or 2,5-hexadione)/silver nitrate, and alumina, and does not require a precolumn to remove moisture or ammonia from the sample. Indeed, no change in retention times has been observed after continued use of the column for over one year. In the case of the alumina column, the drift in retention time on aging, despite precaution to eliminate traces of ammonia, suggests that atmospheric moisture may be the responsible factor.

ACKNOWLEDGEMENTS

We gratefully acknowledge gifts of methylbutenes from Professor S. V. STIMSON of the University of New England, and of Alkaterge T and Atpet 80 from Chemical Materials P/L (Sydney), and of Silicone Oil SF96 from Brown and Dureau P/L,

Chemical Division (Sydney). We thank Professor A. MACCOLL of University College, London for helpful criticism during the preparation for publication. One of us (J.S.S.) is indebted to C.S.I.R.O. for the award of a Post Graduate Senior Fellowship.

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CHROM. 3693

MOLECULAR WEIGHT FRACTIONATION OF POLYETHYLENE GLYCOL BY GAS CHROMATOGRAPHY

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(Received June 3rd, 1968)

SUMMARY

The authors carried out temperature-programmed, gas chromatographic, molecular-weight fractionation of various derivatives of polyethylene glycol, with the general formulae $RO(CH_2CH_2O)_nR$ (R = Me, Ph, TMS) and $R(CH_2CH_2O)_n$-CH_2CH_2R (R = Cl, PhS), in order to determine which derivative has the highest volatility and thermal stability and the lowest energy of adsorption on the support, thus making it the most suitable for the routine analysis of polyethylene glycol products with a molecular weight of less than 1000. Although the dimethyl derivatives are the most volatile, the bis-trimethylsilyl derivatives are the most suitable for the analysis of polyethylene glycol by GLC under the conditions used.

INTRODUCTION

Polyethylene glycols are important surfactants, and yet little work has been done to determine the distribution of the degree of polymerization. These compounds have the general formula $RO(CH_2CH_2O)_nR'$, where R and R′ denote *e.g.* hydrogen atoms, alkyl groups, or alkylaryl groups. Amongst the chromatographic techniques proposed for the analysis of surfactants (see ref. 1 for a bibliography), GLC is the most promising for the rapid determination of the distribution of the degree of polymerization since it entails the molecular weight fractionation of the products and the quantitative estimation of the resulting molecular-weight fractions by a suitable detector. With polyethylene glycol[2,3] and its monononylphenyl[4] and monoalkyl[5] ethers, however, low volatility restricts this analysis to the lower members. New possibilities were opened up by the discovery that the relative retention volume decreases as the two primary OH groups are successively substituted by methoxy groups[6]. The acetylation of the OH group has recently been suggested for the analysis of monoalkyl ethers[7], and has also been utilized in the analysis of polyglycerols[8], since the acetylated derivatives have a higher volatility and thermal stability.

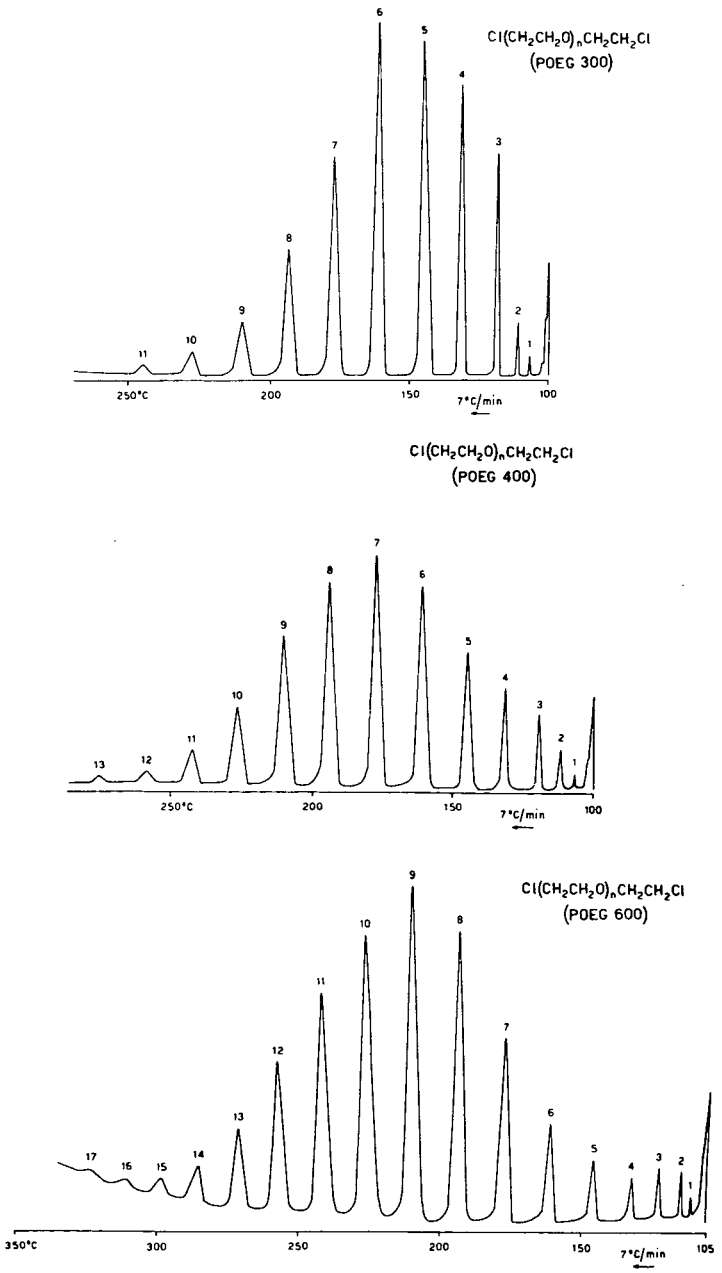
Cl(CH2CH2O)nCH2CH2Cl
(POEG 300)
250°C
200
150
7°C/min
100
Cl(CH2CH2O)nCH2CH2Cl
(POEG 400)
250°C
200
150
7°C/min
100
Cl(CH2CH2O)nCH2CH2Cl
(POEG 600)
350°C
300
250
200
150
7°C/min
105

Fig. 1.

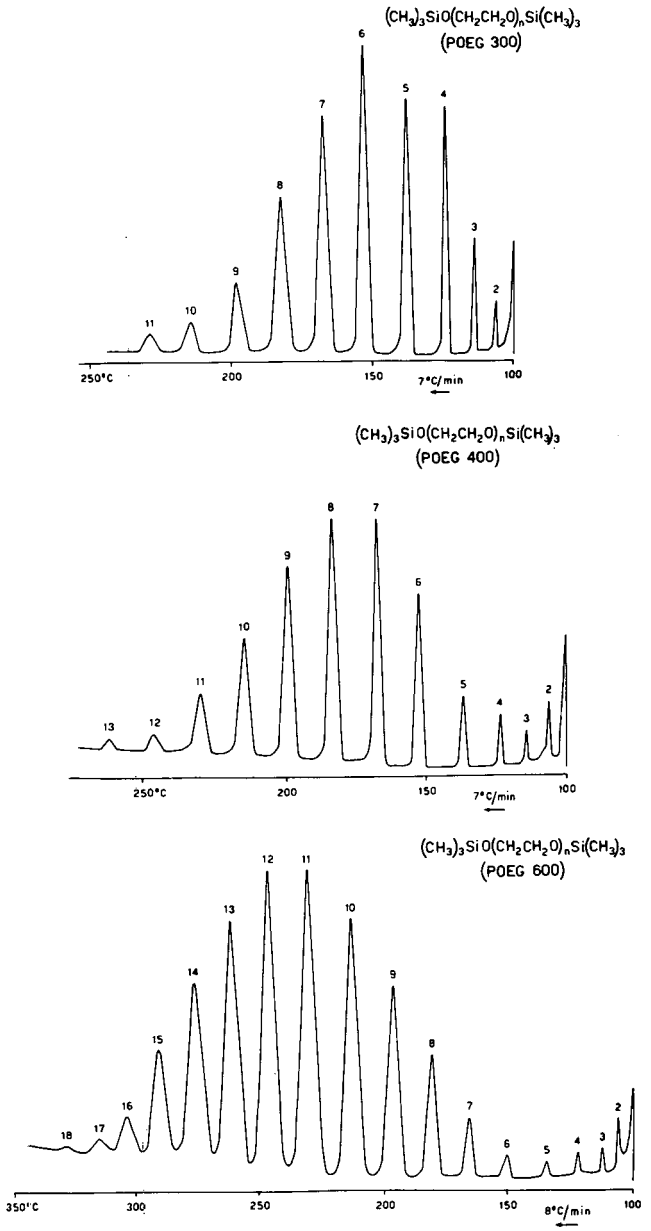

Fig. 1 *(continued)*.

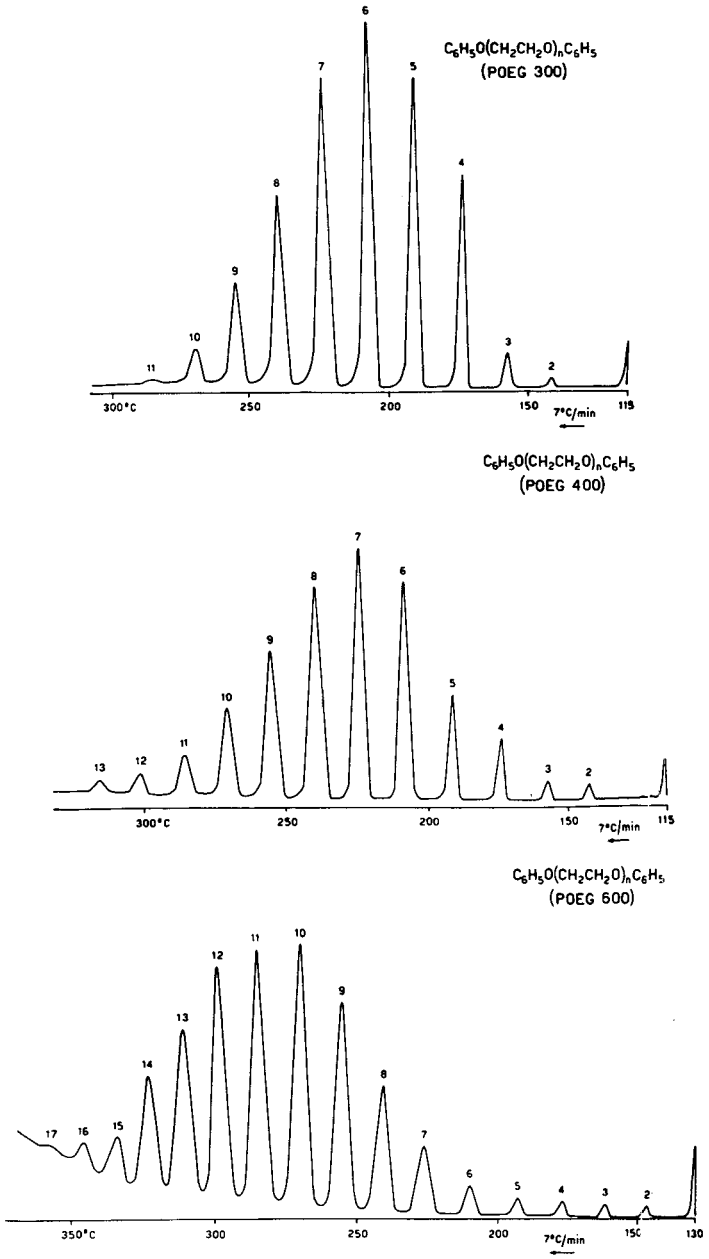

Fig. 1 *(continued)*.

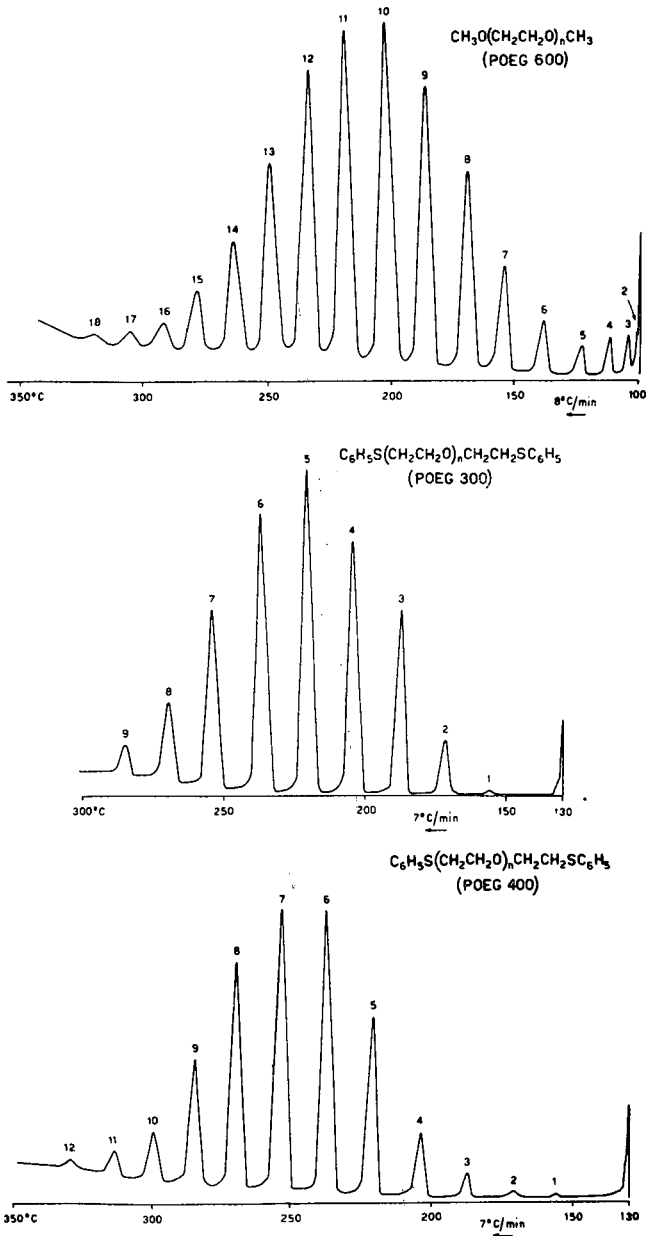

Fig. 1. Gas chromatograms of the various derivatives of polyethylene glycol products with a mean molecular weight of 300, 400, and 600 (PEG 300, PEG 400, and PEG 600). The number of peaks gives the value of n.

This paper deals with the gas chromatographic determination of the volatility and the thermal stability of polyethylene glycol and its dimethyl, diphenyl, and bis-trimethylsilyl ethers as well as $Cl(CH_2CH_2O)_nCH_2CH_2Cl$ (the replacement of the OH group by chlorine raises the volatility) and $PhS(CH_2CH_2O)_nCH_2CH_2S\,Ph$ (to find out what effect the introduction of sulphur has on the chromatographic behaviour).

EXPERIMENTAL

The work was done with a Wilkens Aerograph 1521-1 fitted with a flame-ionization detector and a linear temperature programmer. We used a stainless steel column (22 cm in length and $\frac{1}{8}$ in. internal diameter), packed with 60–80 mesh silanized Chromosorb W impregnated with 5% of silicone-gum rubber SE-30. The injection point temperature varied with the compounds between 375 and 400°, the detector temperature being 375°. The temperature was raised from 100 to 350° at a rate of 7 or 8°/min. The flow rate of the carrier nitrogen was 54 ml/min.

To minimize the retention time of the higher molecular-weight products, we chose the shortest column that still separated the fractions. Under the analytical conditions used, the column selected could resolve alkanes differing by one methylene group. Furthermore, we chose a low-activity support and a low-volatility stationary phase. The amount of the latter should not be less than 5%, since otherwise the support adsorbs, particularly in the case of higher molecular-weight samples. It was found essential to stabilize the column at 300° for at least 24 h before use, possibly by using the temperature programmer several times. The chosen flow rate of nitrogen gave the best results. With this column, the effective peak number[12] for the n-C_{22}/n-C_{23} paraffins was 0.3.

The three polyethylene glycol samples were industrial products (ex Chemische Werke Hüls) with stated mean molecular weights of 300, 400, and 600. Hexaethylene glycol, prepared by the method of FORDYCE, *et al.*[9], was converted into the appropriate derivatives which were used as internal standards. Thionyl chloride was used to prepare the dichloro derivatives[9], and these were then converted into the dimethyl, the diphenyl, and the bis-thiophenoxy derivatives by condensation with sodium methoxide, phenoxide, and thiophenoxide by the general method of CRETCHER AND PITTENGER[10]. The TMS derivatives were prepared with the aid of hexamethyldisilazane and trimethylchlorosilane[11]. The reagent was always in excess to maximize the conversion of the polyethylene glycol. Moisture was removed from the reagents and the equipment, the polyethylene glycol being dried for 6 h at 80° at a reduced pressure of 1 mm Hg. The preparations are briefly described below.

To prepare the dichloro derivatives, a solution of 0.01 mole of polyethylene glycol in 5 ml of pyridine was treated in a 50 ml flask with 0.03 mole (50% excess) of thionyl chloride, added dropwise and with vigorous agitation. The reaction mixture was kept for $\frac{1}{2}$ h at 80°, and then cooled. The residue was extracted with three 10-ml portions of diethyl ether, and the ether extracts were combined, treated with 0.5 g of sodium carbonate, and filtered. The filter was washed with 10 ml of diethyl ether, and the filtrate was freed from the solvent and impurities by heating at 80° and 10 mm Hg.

To prepare the bis-trimethylsilyl derivatives, 0.1 ml of hexamethyldisilazane and 0.05 ml of trimethylchlorosilane were introduced into a small test-tube containing

5 mg of polyethylene glycol in 0.2 ml of pyridine. The reactants were mixed and allowed to stand for 5 min.

The dimethyl derivatives were prepared by the dropwise addition of 0.005 mole of the dichloro derivative to 0.015 mole (50% excess) of sodium methoxide (prepared by mixing 0.35 g of metallic sodium with 15 ml of methanol). The solution was agitated for 10 min, the solvent distilled off, and the residue heated for 2 h at 70°. Cooling was followed by extraction with two 10-ml portions of diethyl ether, filtration, and the removal of the solvent by heating for 2 h at 40° and 0.5 mm Hg.

The diphenyl derivatives were prepared by mixing 0.02 mole of phenol with 0.015 mole of a methanolic solution of sodium methoxide (prepared as described above). The methanol was taken off under vacuum, and 0.005 moles of the dichloro derivative were added dropwise. The mixture was heated for 2 h at 120°, cooled, and extracted with five 5-ml portions of diethylether. The extracts were combined and freed from the solvent and phenol at 60° and 0.1 mm Hg.

To prepare the bis-thiophenoxy derivatives, 0.02 moles of thiophenol were introduced into 0.015 mole of a methanolic solution of sodium methoxide (prepared as described above). The methanol was distilled off, 0.005 moles of the dichloro derivative were added, and the reaction mixture was heated at 80° for 4 h. The excess thiophenol was then distilled off at 100–120° and 0.5 mm Hg.

RESULTS

The chromatograms in Fig. 1 show the molecular-weight fractionation of the various derivatives of polyethylene glycol (PEG) samples with a mean molecular weight of 300, 400, and 600. Table 1 contains the values for the elution temperature

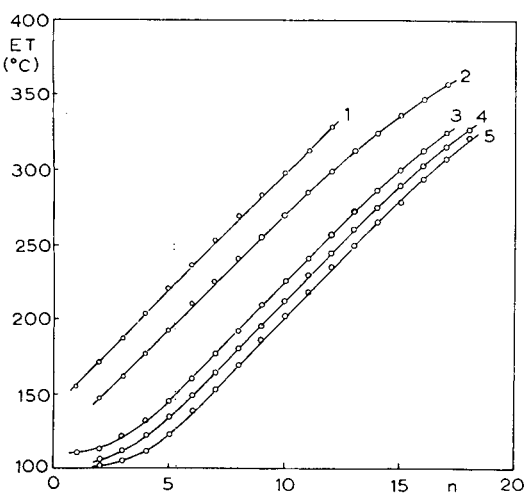

Fig. 2. Variation of the elution temperature ET with the degree of polymerization n for the bis-thiophenoxy (1), diphenyl (2), dichloro (3), bis-trimethylsilyl (4), and the dimethyl (5) derivatives of polyethylene glycol.

TABLE I

GAS CHROMATOGRAPHIC RESULTS OF THE MOLECULAR-WEIGHT FRACTIONATION OF POLYETHYLENE GLYCOL DERIVATIVES

n = degree of polymerization; ET = elution temperature (°C); A = peak area (%); x_n = mole fraction (%); tr = traces.

n	$Cl(CH_2CH_2O)_nCH_2CH_2Cl$									$(CH_3)_3SiO(CH_2CH_2O)_nSi(CH_3)_3$								
	PEG 300			PEG 400			PEG 600			PEG 300			PEG 400			PEG 600		
	ET	A, %	x_n, %	ET	A, %	x_n, %	ET	A, %	x_n, %	ET	A, %	x_n, %	ET	A, %	x_n, %	ET	A, %	x_n, %
1	106	tr	—	106	tr	—	111	tr	—	—	—	—	—	—	—	—	—	—
2	111	0.94	1.74	111	0.80	1.72	114	0.44	1.16	106	0.80	1.37	106	0.70	1.40	106	0.33	0.86
3	119	6.22	9.34	119	2.13	3.71	122	0.82	1.76	114	3.01	4.40	114	0.89	1.52	113	0.31	0.68
4	131	12.09	15.26	132	4.34	6.35	132	0.99	1.78	124	10.36	13.17	123	1.86	2.76	123	0.33	0.63
5	144	21.28	23.15	145	9.79	12.34	146	1.90	2.94	137	16.38	18.42	136	4.01	5.28	135	0.29	0.50
6	161	22.81	21.81	161	16.29	18.05	161	3.72	5.07	153	22.75	22.93	152	10.60	12.50	150	0.60	0.92
7	177	17.03	14.52	177	19.86	19.62	177	8.16	9.91	167	21.14	19.33	167	18.90	20.21	165	1.92	2.65
8	193	10.67	8.21	193	19.18	17.10	193	14.62	16.02	182	15.02	12.56	182	22.15	21.65	181	4.72	5.96
9	209	5.10	3.57	210	14.45	11.74	210	19.25	19.22	197	6.08	4.68	198	19.00	17.11	196	9.04	10.52
10	226	2.21	1.42	226	7.68	5.73	226	19.19	17.60	213	2.57	1.88	213	12.49	10.43	213	14.18	15.29
11	243	1.65	0.98	242	3.38	2.33	241	13.81	11.71	228	1.89	1.26	229	6.62	5.15	230	17.58	17.65
12	—	—	—	259	1.16	0.75	257	9.17	7.23	—	—	—	245	1.78	1.30	245	17.02	16.01
13	—	—	-	273	0.94	0.56	272	4.39	3.24	—	—	—	262	1.00	0.69	261	14.18	12.54
14	—	—	—	—	—	—	286	2.12	1.46	—	—	—	—	—	—	275	10.53	8.84
15	—	—	—	—	—	—	299	0.92	0.60	—	—	-	-	—	—	290	6.16	4.87
16	—	—	—	—	—	—	312	0.50	0.30	—	—	—	—			303	2.06	1.55
17	—	—	—	—	—	—	324	tr	—	—	—	—	—	—	—	315	0.75	0.53
18	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	327	tr	—

TABLE I *(continued)*

n	$CH_3O(CH_2CH_2O)_nCH_3$			$C_6H_5O(CH_2CH_2O)_nC_6H_5$									$C_6H_5S(CH_2CH_2O)_nCH_2CH_2SC_6H_5$					
	PEG 600			*PEG 300*			*PEG 400*			*PEG 600*			*PEG 300*			*PEG 400*		
	ET	*A*, %	x_n, %	*ET*	*A*, %	x_n, %	*ET*	*A*, %	x_n, %	*ET*	*A*, %	x_n, %	*ET*	*A*, %	x_n, %	*ET*	*A*, %	x_n, %
1	—	—	—	—	—	—	—	—	—	—	—	—	156	tr	—	156	tr	—
2	102	tr	—	141	0.29	0.49	143	0.69	1.30	147	0.39	0.93	173	3.06	4.26	172	0.43	0.69
3	105	0.51	1.40	158	2.94	4.26	158	1.24	2.00	162	0.51	1.05	188	11.78	14.50	187	1.37	1.96
4	112	0.81	1.78	174	10.36	13.09	174	3.16	4.44	177	0.66	1.18	205	18.45	20.34	204	4.21	5.38
5	124	0.78	1.43	191	17.63	19.77	191	6.86	8.56	193	0.82	1.30	221	24.61	24.57	221	12.80	14.81
6	139	1.79	2.83	208	22.63	22.80	208	16.46	18.46	211	1.63	2.33	237	21.45	19.57	237	23.32	24.66
7	154	4.08	5.64	224	22.20	20.31	224	21.53	21.93	226	4.31	5.59	253	13.12	11.02	253	23.63	23.10
8	170	8.90	10.94	240	14.26	11.95	239	21.54	20.08	241	7.84	9.30	269	5.51	4.28	269	18.16	16.38
9	187	13.77	15.23	255	7.22	5.56	255	15.17	13.04	255	13.03	14.26	284	2.02	1.46	283	9.81	8.24
10	203	17.59	17.70	270	2.47	1.77	270	7.77	6.20	270	16.91	17.16	—	—	—	298	3.78	2.97
11	219	16.73	15.44	284	tr	—	285	3.20	2.38	285	16.55	15.68	—	—	—	312	1.88	1.39
12	235	14.42	12.29	—	—	—	300	1.43	0.99	299	15.20	13.49	—	—	—	328	0.60	0.42
13	250	9.56	7.57	—	—	—	316	0.95	0.62	313	10.88	9.08	—	—	—	—	—	—
14	265	5.49	4.05	—	—	—	—	—	—	324	7.14	5.63	—	—	—	—	—	—
15	279	3.07	2.13	—	—	—	—	—	—	336	2.65	1.98	—	—	—	—	—	—
16	294	1.28	0.83	—	—	—	—	—	—	347	1.22	0.86	—	—	—	—	—	—
17	307	0.89	0.55	—	—	—	—	—	—	357	0.26	0.18	—	—	—	—	—	—
18	321	0.33	0.19	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

(ET), the relative peak area (A), and the molar fraction (x_n) of the constituents. Hexaethylene glycol derivatives were used as internal standards.

Maximum volatility and thermal stability and minimum energy of adsorption on the support are the characteristics of the most suitable type of derivative for the determination of n by GLC. The volatility of derivatives with the same value of n decreases as follows: dimethyl > bis-trimethylsilyl > dichloro > diphenyl > bis-thiophenoxy derivatives. The elution temperature within a series varies linearly with n over a wide range (see Fig. 2). The deviations from linearity at high n values are probably due to difficulties in keeping the temperature-programming linear and the flow rate of the carrier gas constant at high temperatures. In the linear domain between about 150 and 300°, the graphs for all the derivatives have the same slope of about 15°/n. In addition, using the more volatile derivatives (dimethyl and bis-trimethylsilyl derivatives), one can separate compounds with an n of up to 18, *i.e.* up to a molecular weight of about 900.

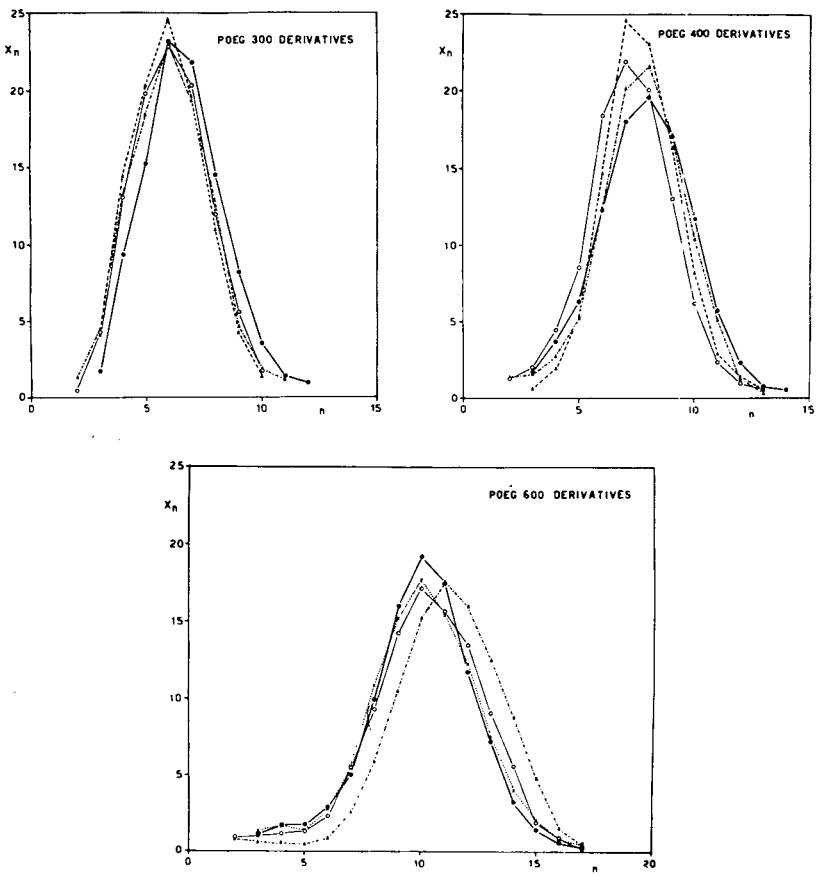

Fig. 3. Distribution of the degree of polymerization n for the dichloro (●), bis-trimethylsilyl (▲), dimethyl (×), diphenyl (○), and the bis-thiophenoxy (△) derivatives of polyethylene glycol 300, 400, and 600 (in the case of the dichloro and the bis-thiophenoxy derivatives, n refers to the starting polyethylene glycol product).

The replacement of oxygen by sulphur in the molecule lowers the volatility, even if the fact that it also reduces the value of n by one is taken into account. However, even conversion into the bis-thiophenoxy derivatives facilitates the resolution of compounds with a molecular weight exceeding 750.

The derivatives generally have a fairly good thermal stability, permitting an evaporator temperature of 375° in all cases. With the bis-trimethylsilyl, the dimethyl, and the diphenyl derivatives, this temperature can be raised to 400° without any pyrolysis occurring.

Adsorption on the support, which is particularly a hazard with compounds having a higher n value and less volatile substituents, was found the smallest in the case of the bis-trimethylsilyl and the dimethyl derivatives.

The most suitable type of derivative can be chosen on the basis of the distribution curves in Fig. 3, though it must be ascertained in each particular case that polyethylene glycol is quantitatively converted into the derivative. The curves for the derivatives of PEG 300 are superimposable, and so are those for the derivatives of PEG 400. In the case of the derivatives of PEG 600, on the other hand, the distribution curve for the bis-trimethylsilyl derivatives is displaced towards higher n values, probably because of the higher volatility and because of the fact that these derivatives were prepared in a single step. Although the dimethyl derivatives are more volatile, they were prepared *via* the dichloro derivatives, and the possibility of a systematic shift in the distribution during this reaction cannot be excluded. The good agreement between the curves for the dimethyl derivatives and those for the dichloro derivatives indicates that the conversion into the dimethyl compounds is almost quantitative, which agrees with the high yields; the chlorination of polyethylene glycol must therefore be less complete.

These results show that, under the present experimental conditions, the bis-trimethylsilyl derivatives are the most suitable for the gas chromatographic analysis of polyethylene glycol products, since they have a high volatility, high thermal stability, low energy of adsorption on the support, and a simpler synthesis which probably ensures a more complete conversion.

ACKNOWLEDGEMENT

The authors wish to thank Chemische Werke Hüls (Marl/Kreis Recklinghausen, Germany) for supplying the polyethylene glycol samples.

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CHROM. 3738

RECOVERY OF ^{14}C-LABELED SUGAR AND ALCOHOL DERIVATIVES IN GAS CHROMATOGRAPHY

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(Received August 5th, 1968)

SUMMARY

The recovery of trimethylsilyl (TMS) derivatives in gas chromatography was determined by using ^{14}C-labeled compounds. Under the conditions used approximately one-half of the cholesterol derivative was recovered. Four-fifths of the glycerol derivative and one-fourth of the stearyl alcohol derivative were recovered. Approximately one-fourth of the TMS derivatives of glucose, fructose and sucrose were recovered. Rechromatography of the TMS derivative of glucose resulted in the same loss as found initially. With the TMS derivative of glucose-^{14}C, radioactivity was found throughout the length of the column. Thus decomposition and/or deposition of the derivative occurred throughout the column.

The recovery of the trifluoroacetyl derivative of glucose was less than that of the TMS derivative.

INTRODUCTION

The quantitative analyses of sugars as their trimethylsilyl (TMS) ether derivatives have been based upon the use of internal standards[1,2]. The same is true for the gas chromatography of acetylated alditols derived from sugars[3]. Little effort has been devoted to the determination of the absolute recovery of such derivatives.

SIMMONDS AND LOVELOCK[4] used the ionization cross-section detector as an absolute detector to determine the absolute recovery in the gas chromatography of 32 steroids. They obtained essentially complete recovery only with the hydrocarbon steroid androstane. With cholesterol only 49 to 66% recovery was obtained, depending upon the liquid phase employed. Considerable variations in the percentage recovered of the other steroids examined were observed.

The use of ^{14}C-labeled sugars and alcohols permitted the determination of the absolute recovery in gas chromatography. The recoveries of the TMS derivatives of ^{14}C-labeled sugars and alcohols and the trifluoroacetyl (TFA) derivative of glucose were determined and are reported in this paper. It was found that the absolute re-

coveries of these derivatives were considerably less than quantitative and that this was due to decomposition of the derivatives and retention of decomposition products throughout the length of the columns.

MATERIALS AND METHODS*

Sucrose-^{14}C (u.l., 5 mC per mmole), fructose-^{14}C (u.l., 2 mC per mmole), glucose-^{14}C (u.l., 3 mC per mmole), stearyl-1-^{14}C alcohol (5.3 mC per mmole), cholesterol-4-^{14}C in benzene solution (50 mC per mmole) and glycerol-2-^{14}C in water solution (14 mC per mmole) were purchased from New England Nuclear Corp., Boston, Mass. Silicone Oil SF 96-50, Carbowax 20 M, nonyl-phenoxypolyoxyethylene-ethanol (Igepal) and Chromosorb G were purchased from Varian Aerograph, Walnut Creek, Calif. Sil-Prep ampules containing 1 ml of reagent (pyridine–hexamethyldisilazane–trimethylchlorosilane, 9:3:1) were obtained from Applied Science Laboratories, Inc., State College, Pa.

Radioactivities of ^{14}C samples were determined by the liquid scintillation system in naphthalene–dioxane scintillation solution of BUTLER[5] in a Tri-Carb spectrometer manufactured by the Packard Instrument Co., Inc., La Grange, Ill.

A Model 800A gas chromatographic system manufactured by the Packard Instrument Co., Inc. was used. Detection was by the electron capture detector with argon as the carrier gas. All columns were glass coils, 6 ft. by 3 mm I.D. Acid-base washed Chromosorb G 100/120 mesh was the support for the stationary phase. Stationary phases were 2% SF 96-50, 2% SF 96-50 + 0.005% Igepal and 2% Carbowax 20 M with respect to the chromosorb. New columns were conditioned overnight at a temperature slightly above the maximum intended to be used. The argon flow through the columns was approximately 40 cc per min. Injections were made with a Hamilton one-microliter syringe. Collection of the gas effluent was accomplished by means of a short piece of teflon tubing leading directly from the detector outlet into the scintillation solution contained in a vial. Collection was continued for several minutes beyond the end of the detection of the derivative. The teflon tubing was added to the scintillation solution. The radioactivity contained in hexane or tetrahydrofuran solutions of the derivatives was determined by adding aliquots directly to the scintillation solution.

The TMS derivative of cholesterol was prepared with 25 mg of unlabeled cholesterol to which had been added approximately 1 μC of cholesterol-4-^{14}C. The mixture was added to 1.0 ml tetrahydrofuran containing 0.2 ml hexamethyldisilazine and 0.1 ml trimethylchlorosilane. After 20 h at 25° the reaction mixture was evaporated to dryness in vacuum. The reactants were treated with hexane. The solution was filtered, the hexane evaporated and the TMS dissolved in 0.2 ml of tetrahydrofuran and so used in gas chromatography. All other TMS derivatives were prepared with Sil-Prep. One-milliliter ampoules were used with 16.5 mg of unlabeled glycerol to which was added approximately 1 μC of glycerol-2-^{14}C, 15 mg stearyl alcohol containing 1 μC stearyl-1-^{14}C alcohol, 5 mg sucrose containing approximately 3 μC of labeled sucrose, 5 mg fructose containing approximately 1 μC of fructose-^{14}C, and 5 mg of

* Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

glucose containing from 2 to 20 μC of glucose-^{14}C. Several glucose preparations were used within this range of radioactivity. In every case the TMS derivative after evaporation in vacuum was treated with hexane, filtered, evaporated, and dissolved in approximately 0.2 ml of hexane for gas chromatographic analyses.

The TFA derivative of glucose was prepared by the procedure of TAMURA AND IMANARI[6]. Two preparations were used, one containing approximately 2 and the other 6 μC of glucose-^{14}C per 5 mg of glucose. Hexane solutions of the derivative were used in gas chromatography analyses. In every instance, 1 λ of the hexane solutions of TMS and TFA derivatives was injected into the gas chromatography columns.

RESULTS

The recoveries of the TMS derivatives of ^{14}C-labeled cholesterol, glycerol and stearyl alcohol are given in Table I. Approximately one-half of the TMS derivative of cholesterol was recovered. This recovery was essentially the same as that obtained with cholesterol by SIMMONDS AND LOVELOCK[4]. Better recoveries (80%) were obtained with the TMS derivative of glycerol. The same recovery was obtained at 105°, 135° and 170°, thus the retention of the glycerol derivative on the column was not temperature dependent. The effect of temperature on the other derivatives was not determined. With the TMS derivative of stearyl alcohol-^{14}C only one-fourth of the derivative reached the detector.

TABLE I

RECOVERY OF TRIMETHYLSILYL DERIVATIVES OF CHOLESTEROL-^{14}C, GLYCEROL-^{14}C AND STEARYL ALCOHOL-^{14}C IN GAS CHROMATOGRAPHY

TMS derivative	*Stationary phase*	*Column temperature*	*C.p.m. injected*	*C.p.m. recovered*	*Per cent recovery*
Cholesterol	SF 96-50	250°	12,300	5,300	43
			12,300	5,500	45
			15,200	7,200	47
			15,200	8,200	54
			15,200	8,500	56
Glycerol	Carbowax	105°	7,200	5,800	80
			7,200	5,900	82
			7,200	5,700	79
Glycerol	Carbowax	135°	6,800	4,900	72
			6,800	5,900	87
			6,800	5,700	84
Glycerol	Carbowax	170°	6,800	5,400	79
Stearyl alcohol	SF 96-50	200°	9,400	2,400	26
			9,400	2,500	27
Stearyl alcohol	Carbowax	200°	8,900	2,500	28
			8,900	2,700	30
			8,900	2,100	24
			8,900	2,000	23
			8,900	2,700	30

TABLE II

RECOVERY OF TRIMETHYLSILYL DERIVATIVES OF ^{14}C-LABELED SUGARS IN GAS CHROMATOGRAPHY

TMS derivative	*Stationary phase*	*Column temperature*	*C.p.m. injected*	*C.p.m. recovered*	*Per cent recovery*
Glucose	SF 96-50	225°	187,000	44,500	24
			232,000	55,200	24
			278,000	74,500	27
Glucose	Carbowax	200°	26,300	6,600	25
Fructose	SF 96-50	225°	7,800	2,000	26
			7,800	2,800	36
			7,800	2,100	27
Fructose	Carbowax	200°	7,800	1,800	23
Sucrose	SF 96-50	225°	30,400	9,400	31
			30,400	9,400	31
			34,400	7,400	22
			36,700	10,800	29
			36,700	10,800	29
Sucrose	Carbowax	200°	39,400	10,400	26

The recoveries obtained with the TMS derivatives of sugars (Table II) were less than one-third of that injected. However, the results were reproducible, thus validating the use of internal standards for the analyses of mixtures of sugars as their TMS derivatives.

The recovery of the TMS derivative of glucose-^{14}C on rechromatography was investigated. For this purpose the condensable effluent from the chromatograph of TMS-glucose-^{14}C was collected in a capillary tube. The condensate was combined from several injections. The condensate was dissolved in hexane and rechromatographed. The retention time of the recovered derivative was identical to that observed with the primary hexane solution of the derivative. The recoveries on rechromatography are given in Table III. They were the same as those observed on the first chromatography (Table II). Hence, the material issuing forth from the column was the same as that entering and decomposition apparently occurred in the second chromatography to the same extent as the first.

The site of retention of ^{14}C on the column was determined. For this purpose a

TABLE III

RECOVERY OF THE TRIMETHYLSILYL DERIVATIVE OF GLUCOSE-^{14}C ON RECHROMATOGRAPHY

Column: SF 96-50 + Igepal; temperature: 225°.

C.p.m. injected	*C.p.m. recovered*	*Per cent recovery*
105,000	28,200	27
	27,000	26
	33,500	32

TABLE IV

RETENTION OF RADIOACTIVITY OF THE TRIMETHYLSILYL DERIVATIVE OF GLUCOSE-^{14}C ON THE COLUMN

The stationary phase was Carbowax and the column temperature 200°.

Segment No.	*Amount (cm³)*	*C.p.m.*[a]
1[b]	1	1900
2	2	1600
3	2	1100
4	2	690
5	2	520
6	2	480
7	2	280
8	2	240
9	2	200
10	2	170
11[c]	2	130

[a] Corrected for radioactivity contributed by the corresponding amount of stationary phase.
[b] Injection end.
[c] Exit end.

newly prepared and conditioned column was used. The TMS derivative of glucose-^{14}C in hexane solution was injected several successive times. The total amount of ^{14}C injected corresponded to 720,000 c.p.m. The column was cooled and the packing removed in segments beginning at the injection end. The radioactivity in the various segments was determined (Table IV) in thixotropic gel containing scintillation solution*. The ^{14}C content was greatest at the injection end but nevertheless was found throughout the entire length of the column. Thus decomposition and deposition of the TMS derivative of glucose occurred throughout the column. The total amount of radioactivity found on the column corresponded to only slightly more than 1% of that injected. This figure was minimal because of two considerations. Firstly, the stationary phase *per se* caused considerable quenching when measured by either an

TABLE V

RECOVERY OF THE TRIFLUOROACETYL DERIVATIVE OF GLUCOSE-^{14}C IN GAS CHROMATOGRAPHY

Column	*Temperature (°C)*	*C.p.m. injected*	*C.p.m. recovered*	*Per cent recovered*
Carbowax	150	70,000	9,800	14
Carbowax	170	70,000	3,600	5
		23,700	2,300	10
		23,700	2,500	11
SF 96-50	150	23,700	810	3
		23,700	670	3
SF 96-50	190	23,700	1,300	5

* The radioactivity of 2 cm³ of either the support or the stationary phase in thixotropic gel containing scintillation solution was found to be 100 c.p.m.

internal or external standard. Secondly, the state of deposition of the decomposition products on the stationary phase and the effect thereof on counting efficiency are not known. Answers to these considerations can only be obtained by further and rather extensive research. Of significance was the fact that the ^{14}C was distributed all along the column and that decomposition was occurring throughout the column.

A comparison was made between the recovery of TFA and TMS derivatives of glucose-^{14}C. The results showed the TFA derivative (Table V) to be inferior to the TMS derivative (Table II). More of the TFA derivative was decomposed and/or retained by the column.

DISCUSSION

The recoveries of the TMS derivatives of sugars and alcohols were found to be reproducible and sufficiently large to warrant their use in the quantitative determination of such compounds by gas chromatography wherein internal standards are employed. However, because of the low recoveries and the fact that recovery varies from one sugar or alcohol derivative to another, this technique may not be particularly suitable for the separation and identification of an unknown mixture of sugars or alcohols. For example, the method was not satisfactory for the separation of radioactive components contained in the sugar fraction resulting from the metabolism of ethylene-^{14}C by avocado[7] because radioactivity material balances could not be made.

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CHROM. 3674

A SENSITIVE, LOW BACKGROUND DETECTOR FOR RADIO GAS–LIQUID CHROMATOGRAPHY*

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(First received May 2nd, 1968; revised manuscript received June 28th, 1968)

SUMMARY

The design of a novel detector assembly for radio gas–liquid chromatography is described. The assembly comprises a gas flow proportional counter, plastic phosphor anti-coincidence guard counter and graded gamma shield. The net background count rate is 1.01 c.p.m.

The operational advantages of low background detector systems are discussed and the merits of digital display of counting information described.

INTRODUCTION

Radio gas–liquid chromatography (radio-GLC), a technique which seems likely to prove one of the most powerful available to the biochemist concerned with problems of metabolism, has been the subject of recent reviews by JAMES[1], KARMEN[2], and SCOTT[3].

The methods used to monitor the effluent stream from a GLC column for radioactivity fall into two main groups, those depending on the intermittent trapping of the effluent and those using continuous flow monitors. The former method, in which, characteristically, an automatic fraction collector is used to trap the effluent in cartridges filled with silicone-coated anthracene[4] or *p*-terphenyl crystals[5] which are then counted in a liquid scintillation spectrometer, has the advantage that samples may be counted over long periods of time. It suffers from the fact that the resolution offered by the fraction collector is intrinsically much lower than that of the GLC column; further disadvantages of this method lie in the risk of loss of material during fraction changing and in the high cost of the necessary ancillary liquid scintillation spectrometer. Continuous flow counters, in contrast, have the commanding merit that radio assay is made simultaneously with mass determination; comparison of the profiles of the mass and radio peaks, which is thus facilitated, provides a valuable indication of radiochemical purity and identity. A further advantage of most con-

* An apparatus of this design is now being manufactured by Panax Equipment Ltd.

tinuous monitors lies in the slightness of the losses encountered on dealing with carrier-free radio-labelled compounds. The major limitation inherent in the method is that the time during which effluent materials are available to the counter is restricted by the need to select such flow-rate counter–volume parameters as will prevent mixing of adjacent peaks.

Flow counting procedures in which the effluent peaks from the GLC column are absorbed continuously in liquid scintillation phosphor[6,7] or silicone oil-coated anthracene crystals[8] and the accumulated materials monitored by single or twin photomultiplier tubes operating in coincidence have been described; they share the serious disadvantage that statistical considerations make it impossible to detect minor peaks following major radioactive components. True flow-counting methods have been based on the use of ionisation chambers[9–11], scintillation spectrometers[5,12] and gas flow proportional counters[13] to detect the passage of radioactive peaks. The latter procedure has been extensively developed by JAMES *et al.*[1,14–16], who have advocated combustion and reduction of the effluent radio-peaks to $^{14}CO_2$ and $^{3}H_2$; the argon carrier is mixed with metered quantities of 'cold' CO_2, and the counting gas thus formed is fed to a gas flow proportional counter. By this means, the disadvantages of risk of contamination and of inherent instability of proportional counters operating at high temperatures are avoided. A variant of this procedure, in which the effluent is reduced to $^{14}CH_4$ and argon–methane used as the counting gas, has been described[17].

The resolution of a flow counter is determined by its volume and by the carrier gas flow rate; for any given set of resolution parameters, the sensitivity is determined by the counting efficiency and by the background current or count rate. The ionisation detectors used for radio-GLC offer counting efficiencies for ^{14}C and tritium of 33% and 75% respectively and show background currents of approximately 3×10^{-16} A, equivalent to the signal produced from a sample of activity 780 d.p.m.[11]. Large volume ionisation chambers offer higher counting efficiencies, but because of their lower resolution have found application only in interrupted elution radio-GLC[18]. The flow scintillation counter gives efficiencies of 70% and 20% for $^{14}CO_2$ and $^{3}H_2$ with background count rates in the respective channels of 15 and 50 c.p.m.[12]. Proportional counters used for radio-GLC are known to have counting efficiencies approaching 100% for $^{14}CO_2$ and in excess of 60% for $^{3}H_2$; background count rates are, of course, dependent on the type of construction and the extent of shielding but are commonly in the region of 25–50 c.p.m.[13,16,19,20]. The continuous flow, cylindrical window proportional counter which has been described[21] has a low counting efficiency for soft β emitters but has the advantage of ease of decontamination.

The present communication describes the design and performance of an anti-coincidence, gas flow proportional counter, having a background count rate of approximately 1 c.p.m. and able, in consequence, to detect low levels of radioactive materials in the effluent of GLC systems. It is currently in use in these laboratories for the identification and analysis of trace quantities of ^{14}C- and ^{3}H-labelled steroid metabolites in fish tissues.

APPARATUS

The detection assembly consists of a gas flow proportional counter, a cosmic guard counter, a graded shield, an anti-coincidence gate circuit and the data display

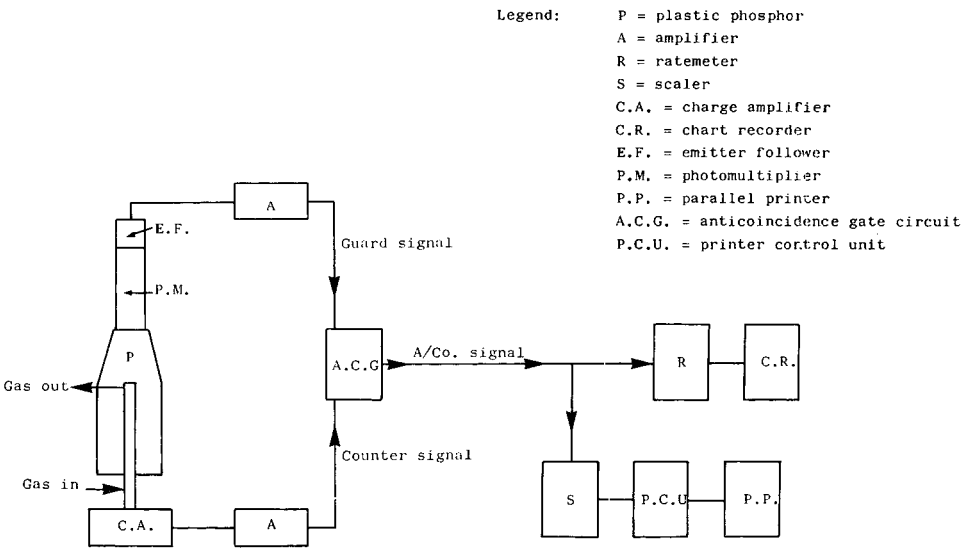

Fig. 1. Block diagram of counter and data presentation modules.

modules. These are described in detail below, and illustrated in block diagram form in Fig. 1. The signal from the proportional counter is fed via a charge amplifier and pulse amplifier to the input of the anti-coincidence gate. The cosmic guard counter comprises a cylinder of plastic phosphor, optically coupled to a photomultiplier whose output signal is fed, via an emitter follower and pulse amplifier, to the gate input of the anti-coincidence circuit. The latter rejects those signals from the proportional counter which are time-coincident with those from the guard channel. The anti-coincidence signal is then fed to a ratemeter and chart recorder. When accurate quantitation of the radio peaks is desired, this form of data display is supplemented by printed digital presentation of counts from a scaler/timer which may be interrogated manually, or automatically at pre-determined time intervals. The signal and guard counter are surrounded by a gamma ray shield of graded construction.

Details of the construction of the gas-flow counter are given in the dimensioned diagram, Fig. 2. The cathode of the counter is formed by a length of oxygen-free, high-conductivity copper (B.S.S. 1861). A tapered copper ring, fixed with epoxy resin at the top of the counter, provides a seat for the end shield; the lower flange forms the upper half of the gas seal. The counter is electroplated with high-purity nickel to prevent "tailing" of tritium peaks and the inside is polished by lapping. The anode wire (tungsten; 0.002 in. diam.) is located by two nickel-plated copper supports which serve, additionally, to reduce the electric field in the region of the teflon insulators. It is secured, at the upper end, by a teflon plug pressing in to a tapered hole in the anode support; a teflon cap is interposed between this plug and the end shield and covers the end of the wire. The wire is secured at its lower end by the central pin of a standard Gremar connector (Nuclear Chicago). The plated counter tube is a press-fit into a nickel-plated copper barrel which is threaded at its lower end to accept the Gremar connector coupling the counter to the charge amplifier. This arrangement

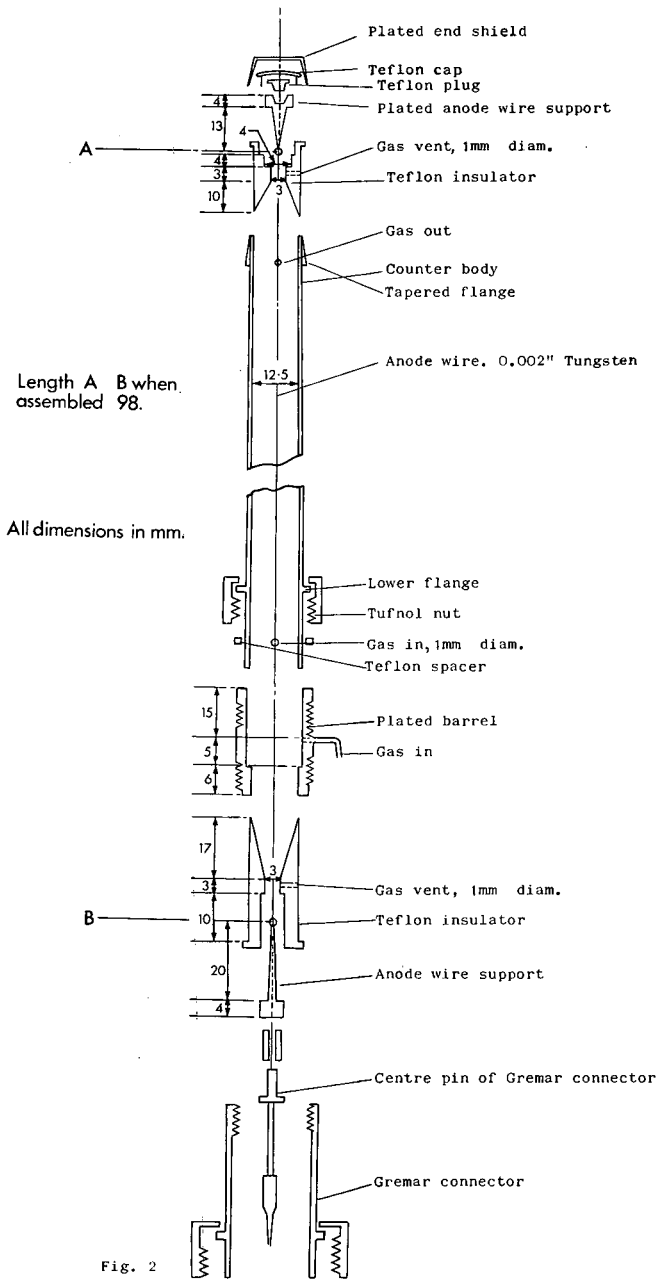

Fig. 2. Gas flow proportional counter.

ensures minimum degradation of pulse shape due to stray capacitances. The barrel conducts counting gas to the detector via a tapered teflon insulator; a Tufnol nut compresses a teflon spacer between the upper face of the barrel and the lower face of the counter flange to provide a gas-tight seal. The volume of the cell, 12 ml, was chosen as offering a satisfactory compromise between the competing requirements of achieving high count rates and of maintaining reasonable resolution.

The guard counter consists of a rod (8 in. × 3 in. diam.) of plastic phosphor (Naton 102A; Nuclear Enterprises) tapered over the upper 3 in. of its length to form an upper face, $1\frac{3}{4}$ in. diam., to which a photomultiplier (E.M.I.; type 6097S) is optically coupled by silicone oil. An axial hole ($\frac{5}{8}$ in. diam. × 5 in.) forms an inverted well for the gas flow counter from which exit gases are ducted via a radial hole, $^1/_{16}$ in. diam. The circumference and base of the phosphor are coated with titanium oxide paint to improve light collection by the photomultiplier.

The phosphor and photomultiplier are contained in separate, mating housings of tubular steel (pre-1945; wall thickness, $\frac{7}{8}$ in.), the lower of which is lined with high conductivity copper, $\frac{1}{4}$ in. thick. The proportional counter is inserted through this housing and into the well of the phosphor via a light-tight seal. Lead annuli (wall thickness, 2 in.) surround the two component housings and are supported by a lead-lined steel cave. An axial hole ($\frac{1}{4}$ in. diam.) drilled in one of the annuli allows an external ^{137}Cs source to be inserted, permitting the ready determination of counter plateaux. The shielding arrangements are illustrated in Fig. 3.

The emitter follower, charge amplifier, pulse amplifiers and ratemeter are standard, commercially available modules (Panax Equipment). The anti-coincidence

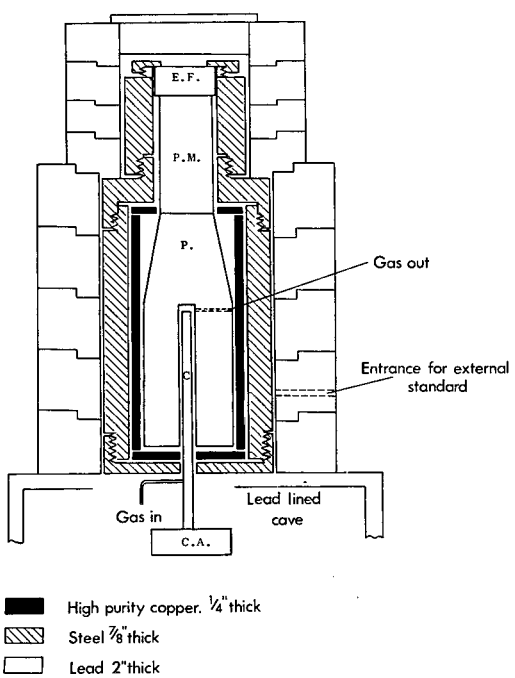

Fig. 3. Flow counter, cosmic and gamma guards.

circuit (Panax Equipment) is adjusted to provide optimum performance with the present counters; the signal pulse delay is 1 μsec and the width 1.5 μsec. The guard pulse width is stretched to 6 μsec.

Where digital presentation of counts is desired, to supplement the rate-meter–chart recorder display, a high-speed printer (Kienzle type KN66) is used[22] in conjunction with scaler, timer and printer control modules (Panax Equipment). This arrangement of digital display resembles that described by STÖCKLIN *et al.*[23].

PERFORMANCE

Counter characteristics

An investigation of the dependence of count rate on applied H.T. voltage was made using argon–CO_2 (95:5) as the counting gas and the external ^{137}Cs source. A count rate plateau was observed extending effectively (15% variation) from 1450 V to 1825 V; in the central region of this (1600–1750 V) the variation of count rate with H.T. did not exceed 1%/100 V. An exactly similar plateau was obtained when an internal source of poly(methyl-^{14}C) methacrylate was used in place of the external gamma standard.

The counter efficiency was determined by releasing into the argon–CO_2 gas stream known quantities of $^{14}CO_2$, prepared by injecting standardised aqueous $Na_2{}^{14}CO_3$ into dilute sulphuric acid contained in "bubbler" set in the gas stream. The counting efficiency (E) was calculated from the expression:

$$E\,[\%] = \frac{\text{observed counts} \times 100\,F}{\text{applied activity [d.p.m.]} \times V}$$

where

F = flow rate (ml/min)
V = counter volume

The results of replicate determinations indicated that the efficiency for ^{14}C was 94.5% (S.D. $\pm$ 1%). The apparent efficiency, which was derived when the active volume (volume of the cylinder whose axis is the anode wire) was used in place of the total cell colume in the above expression, was 101.5%; this value reflects the fact that electrons from gas particles in the cone sections are detected, though at less than 2π geometry.

Efficiencies of ^{3}H were determined by injecting standardised solutions of *n*-hexadecane-1,2T(*n*) on to the gas chromatograph; the leg from the effluent splitter to the flame detector was blanked off during these determinations. The mean, observed efficiency was 64% (S.D. $\pm$ 1.5%).

Background count rate

The background count rate of the unshielded proportional counter was found to be 38.7 c.p.m. (S.D. $\pm$ 0.3 c.p.m.); this was reduced to 15.4 c.p.m. (S.D. $\pm$ 0.3 c.p.m.) by the lead–steel–copper shield. Determinations of count rate during a period of 48 h indicated that this background was reduced to 1.01 c.p.m. (S.D. = $\pm$ 0.02 c.p.m.) by the anti-coincidence guard; the coincidence component, presumably originating largely in cosmic events, was approximately 8.6 c.p.m. This proportion of cosmic to

total counts is in substantial agreement with values quoted by other workers[24–26]. Table I summarises the effects of shielding and cosmic guarding on the background count rate. The results of Pearson's Chi square[27] test, shown in column 4 (P), indicate that the background counts fit a Poissonian distribution. Changes in the H.T. supply voltage to the photomultiplier which caused changes in the count rate of the guard detector from 200 c.p.m. at 800 V to 1680 c.p.m. at 1150 V did not significantly alter the anti-coincidence background count rate.

TABLE I

EFFECTS OF SHIELDING AND COSMIC GUARDING ON THE BACKGROUND COUNT RATE

Shield	*Background (c.p.m.)*	*S.D. (c.p.m.)*	*P*
—	38.7	± 0.3	
Steel–copper	15.4	± 0.3	
Ditto + anti-coincidence guard	7.05	± 0.3	
Lead–steel–copper	9.61	± 0.2	
Ditto + anti-coincidence guard	1.01	± 0.02	0.2

In an early form of the counter which had no plated copper end cap, the anti-coincidence count rate was low (≃ 0.3 c.p.m.), but at the expense of a sharply lowered counting efficiency. The fact that the background rate was dependent on both counter and photomultiplier H.T. (decreasing with increasing voltage) made clear the necessity of an end cap to prevent photons in the proportional counter from being "seen" by the photomultiplier and causing a loss in desired counts.

Results of experiments with a variety of counters of different arrangements of anode wire location, showed the importance of the plated copper supports in reducing spurious events, presumably by reducing the electrical field in the region of the insulators. In the absence of these supports, the anti-coincidence background count rate was raised to approximately 4 c.p.m.

Operation

The counter is currently in use with an F & M 400 Biomedical gas chromatograph, fitted with an effluent splitter. The output from the rear port is combusted and reduced in a furnace tube containing copper oxide and iron, as described by JAMES *et al.*[1,14–16]. Carbon dioxide (5%) is added at the auxiliary port to form a counting gas from the argon carrier and hydrogen is injected into the furnace train to maintain the iron in a reduced form[5].

DISCUSSION

The confidence with which the events counted by a radio-detector may be expected to lie within stated deviations from the expected value is given by the familiar expression:

$$d^2 = \frac{10^4 K^2}{T}\left(\frac{1}{NE} + \frac{2B}{N^2E^2}\right)$$

where

d = % deviation
K = confidence constant
T = counting time
B = background count rate
N = sample disintegration rate
E = counting efficiency

When the radioactive effluent of a GLC is monitored discontinuously, by trapping, the background count rate and efficiency of the counter do not, in principle, set limits to the sensitivity of the radio-detection system since appropriately long counting times may be chosen. Under flow detection conditions, however, T is the mean residence time of gas particles in the counter, expressed by V/F where V is the counter volume and F the flow rate. Given that these parameters are fixed by the need to obtain some desired resolution and that the efficiency of a counter is normally an inherent property, the sensitivity of detection of radioactive materials from a GLC column by flow counting may be improved only by reducing the background count rate. Flow counting techniques have such commanding advantages, save in sensitivity, over discontinuous methods that reduction of the background is of considerable importance.

Of the three types of radio-detectors—ionisation chamber, scintillation spectrometer and proportional detector—which have been used as continuous-flow counters, the last seemed most likely to respond to efforts to reduce the background count rate. This background is compounded of contributions from spurious events associated with the counter insulation, from the natural radioactivity of the counter construction materials, from cosmic events and from ambient gamma radiation. The counter described in the present communication was constructed of materials of the lowest intrinsic radioactivity and spurious events were minimised by reducing the electrical field in the region of the insulators. Its gamma shield was of compound form, constructed of materials of decreasing Z number, in order to reduce secondary gamma radiation originating in the shielding as a result of cosmic events; the reduction in the background of the proportional counter caused by this shield was comparable with that effected by single shields of much heavier construction[28]. Plastic scintillator materials have found recent application as anti-coincidence shields[28–30]; the choice of this type of cosmic guard counter in the present detector assembly was dictated by its considerable advantage in ease of maintenance and convenience of geometry over the conventional Geiger-Müller umbrella and by its useful, though small, guarding efficiency for residual gamma radiation. The short width of the guard pulse generated in the anti-coincidence circuit, ensured that, over the range of guard count rates encountered, the loss of desired counts from the proportional detector is negligible ($< 0.05\%$).

The sensitivity of detection of radio peaks from a gas chromatograph depends, of course, on the retention volume of the material being examined. In the following comparison of the sensitivities of different detectors, it is assumed that the active material enters the counter as a bolus which retains a flat-topped, rectangular activity profile as it passes through the counter—conditions which are only approximately realised under actual conditions; it is further supposed that the gas flow rate is adjusted to provide a mean residence time in the counter of 1 min. The counter described in the

present communication has a mean background of approximately 1 c.p.m. and the probabilities that during any single measuring interval of 1 min, 0, 1, 2, 3 .....r counts are recorded are given by e^{-1}, $e^{-1}/1!$, $e^{-1}/2!$, $e^{-1}/3!$ .....$e^{-1}/r!$. There is thus a probability, given by $\sum_{r=4}^{\infty} e^{-1}/r!$ and approximately equal to 0.02, that a count of 4 or more, during any single minute, is due merely to background. Assuming that the efficiency of the counter is 100%, the probability that a bolus of radioactive material, of mean disintegration rate m d.p.m., will cause a count of 4 or more in any single minute is given by $\sum_{r=4}^{\infty} (m+1)^r e^{-(m+1)}/r!$. From this, it may be calculated that the smallest radioactive sample for which there is a 95% probability that it will be detected (by causing 4 or more counts per minute) is 6.7 d.p.m. Using the same probabilities as before, a counter whose mean background count-rate is 50 c.p.m. would detect a radioactive sample of 30 d.p.m. Lowering the background of the counter from 50 to 1 c.p.m. has effected therefore an approximately five-fold improvement in its sensitivity

Data from a radio-GLC system are commonly displayed by a ratemeter and chart recorder; this form of presentation, though in many ways attractively direct, has some inherent disadvantages. The factors which determine the ability of a ratemeter to present faithfully the profiles of radioactivity in the effluent of a chromatograph are the normal statistical variation of radioactive emission, the volume of the counter, the rate of gas flow and the electrical and mechanical inertia of the ratemeter and recorder. These are strictly inter-related. If too short a ratemeter time constant is chosen, counting information will be obscured by the rapid changes in the displayed count rate caused by the random nature of radioactive emission; if too long a time constant is chosen, counting information will be irretrievably lost by the failure of the ratemeter to respond with sufficient speed to changes in the frequency of events within the counter. These factors may be considered in detail as follows. A ratemeter is essentially an averaging instrument which takes a finite time, t, to respond to changes in the rate of input pulses. This time is determined by the expression

$$t = RC\,[\tfrac{1}{2}\log_e 2\,n'RC + 0.394]$$

where

RC = ratemeter time constant
n' = change in input count rate

In practice, it has been found that this may be approximated to

$$t \geqslant 4\,RC$$

Clearly this equilibrium time t must be less than the mean residence time, t_1, of gas in the counter if the ratemeter is to reproduce faithfully the profile of counts registered by the detector. RC in turn determines the probability that the ratemeter reading lies within certain defined deviations from the mean count rate, according to the expression

$$RC = \frac{K^2\,10^4}{2N\,d^2}$$

from which it follows that

$$d^2 \geqslant \frac{2K^2\,10^4}{Nt_1}$$

In contrast, consideration of the statistics of data presentation by a scaler leads to the expression

$$d^2 = \frac{K^2\,10^4}{Nt_1}$$

Thus the size of sample necessary to obtain a determination of radioactivity to within the same limits of deviation and with the same counter-volume and gas flow rates is, with scaler presentation, just half that necessary when the data are displayed by a ratemeter.

Although integrating ratemeters do not suffer the statistical disadvantages of the normal ratemeter and have been used by some workers to display radio-GLC data, their operation is considerably less convenient than the digital system described in the present communication.

ACKNOWLEDGEMENTS

The work described in this communication forms a part of the programme of the Marine Laboratory of the Department of Agriculture and Fisheries for Scotland. The author thanks the Director of the Torry Research Station of the Ministry of Technology for the provision of accommodation in his laboratories and acknowledges his indebtedness to Mr. C. PURVES for his care in constructing the counters and to Mr. J. A. POPE for his advice on the statistical treatment of low count numbers.

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CHROM. 3721

QUANTITATIVE ESTIMATION OF GLYCEROL IN LIPIDS AS ITS TRIMETHYLSILYL ETHER BY GAS–LIQUID CHROMATOGRAPHY

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(Received July 29th, 1968)

SUMMARY

Glycerol has been quantitatively estimated by gas–liquid chromatography of its trimethylsilyl ether, using hexadecane as an inert internal standard. The glycerol contents of some vegetable oils are reported. Kamala seed oil and kusum oil have a low glycerol content. Triglyceride groups separated by argentation chromatography could be estimated from glycerol determination by gas–liquid chromatography.

INTRODUCTION

Since glycerol plays an essential role in the metabolism of triglycerides, phospholipids, and other materials in both animal and plant tissues, a quick and accurate method for its quantitative estimation is of great importance. Earlier methods used to estimate glycerol quantitatively include, among others, periodic acid oxidation[1–7], copper complex formation[8], an enzymatic procedure (ref. 9 and references cited therein), microbiological determination[10] and paper chromatography[11]. Methods involving oxidation with periodic acid are not very accurate because of interference by biological compounds such as glucose, glycerophosphate and ethanolamine[12]. Microbiological, enzymatic and paper chromatographic methods are rather tedious. More recent methods are based on gas–liquid chromatography (GLC)[13–17]. A procedure for the simultaneous quantitative determination of glycerol and fatty acid contents of fats and oils involved interesterification of fat with methanol and analysis of the methyl esters and of glycerol as isopropylidene-glycerol by GLC[13]. In other methods[14–17], the acetyl derivative of glycerol was analysed by GLC against an internal standard such as butane-1,4-diol or hexadecanyl acetate. The different rate of acetylation of a hydroxyl-containing standard and glycerol could perhaps influence the quantitation procedure, and, therefore, use of an inert internal standard seemed preferable. In this paper we report a rapid and quantitative method for the estimation of glycerol as its trimethylsilyl ether by GLC, using hexadecane as an inert internal standard. The method has been applied to determine the glycerol content of vegetable oils and of fractions separated from them on silver nitrate-impregnated silica gel thin-layer plates.

MATERIALS AND METHODS

Apparatus

This consists of an F & M Model 1609 gas chromatographic unit with a hydrogen flame ionization detector system. A 2-ft. × $^3/_{16}$ in. I.D. coiled stainless steel column packed with 2% SE 30 (silicone rubber gum) on Chromosorb W 60–80 mesh was used.

Materials

Petroleum ether AR (b.p. 40–60°) was not purified further.

Pyridine AR (BDH) was refluxed over barium oxide, distilled and kept over solid potassium hydroxide.

Glycerol AR (BDH) was rendered anhydrous by heating it to 120° before use with continuous stirring, and kept under anhydrous condition. Glycerol standards were prepared in dry pyridine to give concentrations from 1.0–3.0 mg/ml.

Methanol GR (Merck) grade was used.

n-Hexadecane was purified by percolation through a column of activated silica gel (BDH quality). A stock solution of 0.5 g hexadecane in 50 ml dry pyridine was prepared (1 ml = 10 mg).

Anhydrous methanolic HCl (5%) was prepared by passing dry hydrogen chloride into methanol till the requisite concentration was obtained.

Hexamethyldisilazane (Peninsular Chemresearch, Gainesville, Fla., U.S.A.) and *trimethylchlorosilane* (K.K. Labs, Plainview, N.Y., U.S.A.) were used without further treatment.

Potassium hydroxide (Merck) pellets.

Tripalmitin was synthesised by refluxing a solution of palmitoyl chloride and glycerol (4:1) in a pyridine and chloroform medium for 4 h[18], followed by column chromatographic purification using the procedure of QUINLIN AND WEISER[19]. The product melted at 65.5°.

Vegetable oils. Triglycerides were obtained from the oils by chromatography on a silica gel column according to QUINLIN AND WEISER[19]. Kamala seed oil and kusum oil (*Schleichera trijuga*) were used as such after removing moisture.

Methods

Silylation procedure. Silylation was carried out by the method of SWEELEY *et al.*[20] incorporating the improvements of WOOD and co-workers[21] as follows: Aliquots of a standard solution of glycerol equivalent to 1.0, 2.0 and 3.0 mg were taken in duplicate in 15-ml glass-stoppered test tubes. To each tube was added successively 30 μl of hexadecane solution, 0.2 ml of hexamethyldisilazane and 0.1 ml of trimethylchlorosilane. The reaction mixture was shaken vigorously for 1 min and set aside for 10 min. At the end of this period 5 ml distilled water and 5 ml petroleum ether were added to the contents of the test tube. After shaking thoroughly, the layers were allowed to separate whereby the silyl ether of glycerol passed into the petroleum layer. Extraction of the aqueous phase was repeated twice with 5 ml aliquots of petroleum ether. The petroleum extracts were combined and, after drying over anhydrous sodium sulphate, the solvent was removed on a water bath until free from the odour of pyridine. The final volume was *ca.* 0.2 ml. Until GLC analysis, these silylated products were stored in a deep-freezer.

Liberation of glycerol from triglycerides. Tripalmitin (12.5 and 25 mg samples, in duplicate) was refluxed for 2 h with 2 ml of 6% methanolic potassium hydroxide. The solution was cooled, neutralised with a solution of 5% methanolic HCl (to Congo red) and the solvent removed on a water bath. The liberated glycerol was taken up in 2 ml pyridine, and the contents mixed thoroughly and silylated by the procedure described above, again incorporating hexadecane (30 μl) as the internal standard.

The vegetable oils (10 mg samples) or glyceride fractions were similarly hydrolysed and silylated.

Analysis by gas–liquid chromatography. Injection port temperature, 170°; detector block temperature, 160°; flow rates of hydrogen, nitrogen and compressed air were respectively, 40, 100 and 400 ml/min; attenuation, 1600; chart speed, 30 in./h. The column was run isothermally at 100° till the glycerol derivative emerged, and thereafter the temperature was increased to 110° by programming at the rate of 5°/min, after which it was again run isothermally till hexadecane emerged. Peak areas were measured by triangulation.

RESULTS AND DISCUSSION

Standardisation experiments with glycerol and with tripalmitin

Fig. 1 shows that silylated glycerol and hexadecane were well separated. Peak areas of silylated glycerol were measured with respect to hexadecane (Table I) and the ratios were found to be close to simple multiples. Standardisation with tripalmitin likewise gave ratios proportional to the amounts taken. These values (ratio of the

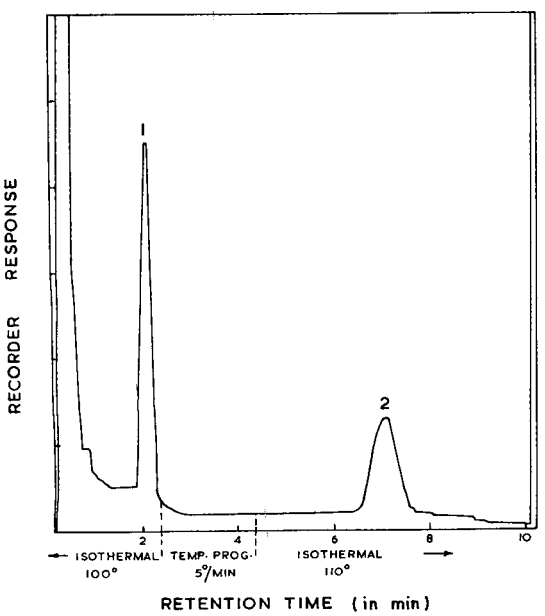

Fig. 1. GLC separation of silylated glycerol (peak 1) and hexadecane (peak 2); conditions are given in the text.

TABLE I

ANALYSIS BY GAS–LIQUID CHROMATOGRAPHY OF GLYCEROL AS ITS TRIMETHYLSILYL ETHER IN STANDARD GLYCEROL SOLUTION AND TRIPALMITIN USING HEXADECANE AS INTERNAL STANDARD

Material added (mg)	*Pyridine solution of hexadecane (μl)*	*Area of glycerol-derived peak (α)*	*Area of hexadecane peak (β)*	*α / β*
Glycerol				
1.0	30	5.60	4.77	1.17
2.0	30	3.06	1.50	2.04
3.0	30	9.35	3.15	2.97
Tripalmitin				
12.5	30	2.14	1.28	1.66
25.0	30	3.90	1.21	3.22

peak area of silylated glycerol to that of hexadecane) were later used when determining glycerol in glyceride groups isolated from cocoa butter by argentation chromatography.

Glycerol estimation in vegetable oils

Most of the vegetable oils were purified by QUINLIN AND WEISER's procedure[19]. Kamala seed oil is unusual in that it contains triglycerides of exceptionally high molecular weight[22], and such chromatographic purification was not feasible. Kusum oil contains nonglyceridic compounds and, therefore, it also was not purified by column chromatography. All the vegetable oils (10 mg samples) were treated for glycerol recovery, and subsequently silylated with addition of the internal standard as outlined in the experimental section. From the relative peak areas of known quantities of silylated pure glycerol and hexadecane, and the relative areas of the silylated glycerol derived from the vegetable oil and hexadecane, the percentage glycerol in the oil can be calculated. For example, using 1 mg glycerol and 0.35 mg hexadecane, the ratio of the peak areas for silylated glycerol and hexadecane was 5.60/4.77 = 1.17. When 10 mg groundnut oil was taken, and 0.35 mg hexadecane added during silylation of the glycerol released, the ratio of the peak areas obtained was 5.21/4.20 = 1.24. Hence the glycerol present in 10 mg groundnut oil is 1.24/1.17 = 1.06 mg, *viz.* 10.6%.

The amounts of glycerol obtained from vegetable oils are given in Table II. Present values for the normal vegetable oils are in good agreement with those reported in the literature[22–24]. This seems to indicate that the method is of general applicability. The higher value for refined cottonseed oil, which was consistently obtained cannot be explained. Kamala seed oil consists of glycerides in which kamlolenic acid (18-hydroxy-9-*cis*, 11-*trans*, 13-*trans*-octadecatrienoic acid) is believed to occur in linked chains terminated by a normal fatty acid. As a result, the mean molecular weight of the oil is around 1800 (ref. 22), and a low glycerol content, as now obtained, was also earlier observed by chemical analysis[25]. Kusum seed oil (from *Schleichera trijuga*) is unique among vegetable oils in that it contains only 37% triglycerides, the rest being non-glyceridic components[23]. The reported glycerol content of this oil is 3.7%, which is in fair agreement with the value of 3.8% now obtained.

TABLE II

GLYCEROL CONTENT OF VEGETABLE OILS

Oil	*Glycerol (%) by other methods*[a]	*Glycerol (%) by the present method*
Safflower oil	10.7	10.9
Groundnut oil	10.8	10.6
Cottonseed oil	10.8	11.9
Cocoa butter	10.8	10.3
Mustard oil	9.9	9.7
Kamala seed oil	3.6	3.4
Kusum oil	3.7	3.8

[a] Refs. 22–24.

Quantitation of glyceride groups in oils by determination of glycerol

The amounts of glycerides present in fractions, into which the parent fats have been resolved by argentation chromatography, have been indirectly estimated by calculation based on the glycerol content estimated in each of these fractions using the periodate oxidation procedure[26,27]. This has now been done using the present method of glycerol estimation for cocoa butter, which was separated into glyceride groups by preparative TLC on silver nitrate-coated silica gel plates using an ether–petroleum ether solvent system (25:75). The separated zones, consisting of glycerides containing 0, 1, 2 and 3 double bonds, were visualised with a dichlorofluorescein spray and viewed by ultraviolet light. Bands were marked lightly. Each fraction was separately scraped off and the fatty material extracted in a continuous solvent extractor using ether[28]. The extracts were dried and suitable aliquots refluxed with 2 ml of 6% methanolic potassium hydroxide solution. The glycerol was liberated, hexadecane added and GLC carried out after silylation. In a separate run, a standard glycerol solution representing 1 ml glycerol was also silylated and analysed by GLC.

The amounts of the various glyceride groups in cocoa butter calculated from

TABLE III

EXAMINATION OF GLYCERIDE GROUPS IN COCOA BUTTER BASED ON GLYCEROL ESTIMATION

Glyceride group, no. of double bonds	*Glyceride present based on glycerol estimation (%)*	*Glyceride present based on weight eluted by column chromatography*[a] *(%)*
0	traces	1.4
1	76.2	73.8
2	16.1	18.1
3	7.7	6.7

[a] Ref. 29.

their glycerol contents determined in this way are shown in Table III. Also given are the proportions of glyceride groups reported by SUBBARAM AND YOUNGS[29] for this fat using the argentation column chromatographic method of DE VRIES[30] for the separation and estimation by weight of glyceride groups. Fully-saturated glycerides, which occur in trace amounts, could not be definitely estimated. The results for other groups agree to within $\pm$ 2.5% with those obtained by column chromatography on a different sample.

Although only analysis of vegetable oils is now reported, the method should also be suitable for the quantitative estimation of glycerol in animal fats, mono- and diglycerides, and other derivatives of glycerol.

ACKNOWLEDGEMENTS

This work was carried out under the joint sponsorship of the Indian Council of Agricultural Research and the Council of Scientific and Industrial Research.

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CHROM. 3740

GEL CHROMATOGRAPHY OF TETRACYCLINE AND DERIVATIVES OF TETRACYCLINE

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(Received August 14th, 1968)

SUMMARY

The elution characteristics of tetracycline (TC), anhydrotetracycline (ATC) and 4-*epi*-anhydrotetracycline (EATC) were studied on Sephadex gel under various conditions of pH and salt concentration. The adsorption of the compounds was strong in acid solvents but diminished as the pH was elevated. In the alkaline region of pH 8.5 to 9.5 the ATC and EATC epimer eluted at different rates from the column. An increase in the salt concentration resulted in stronger adsorption of the compounds to the gel, but did not influence their separation efficiency. The differential elution of the compounds appears to be due to a chromatographic effect distinct from the molecular sieve effect commonly associated with Sephadex.

INTRODUCTION

In a previous study[1], the anhydrotetracycline (ATC) and 4-*epi*-anhydrotetracycline (EATC) derivatives of tetracycline (TC) were found to exhibit adsorption to a column of Sephadex G-25 which enabled the derivatives to be separated from the TC. Under the conditions of these experiments, no separation between the ATC and EATC was found, although data did indicate that when the products were passaged separately over a column they displayed slightly different elution rates.

In the present work, the adsorption characteristics of the TC, ATC and EATC on Sephadex were studied under various conditions of pH and salt concentration. The chemical structures of these compounds are outlined in Fig. 1 (A, B and C, respectively).

REAGENTS AND MATERIALS

Compounds of tetracycline and derivatives

Tetracycline hydrochloride of a high degree of purity was used. Purified compounds of anhydrotetracycline hydrate and 4-*epi*-anhydrotetracycline sulfate were supplied by the Bristol Laboratories.

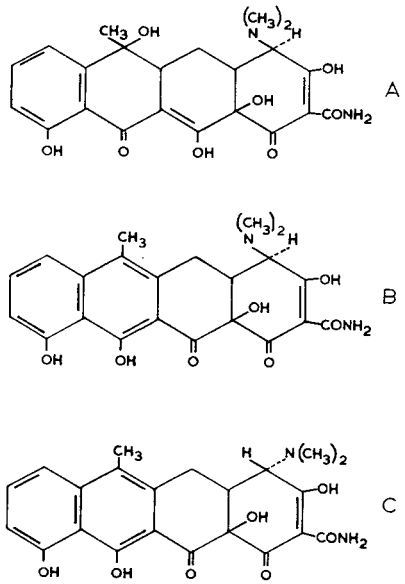

Fig. 1. Diagram of the chemical structures of tetracycline (A), anhydrotetracycline (B) and 4-*epi*-anhydrotetracycline (C).

Gel chromatography

Sephadex G-25 (fine), particle size 20–80 μ, was used to prepare a gel bed 1.8 × 25 cm. Three columns of similar bed dimensions were equilibrated with the following solvents: (1) Dilute HCl, pH 2.5; (2) 0.04 *M* phosphate buffer, pH 7.7; and (3) 0.05 *M* tris buffer, pH 8.5. The column with the tris buffer was re-equilibrated with several solvents of tris buffer of varied pH and/or molarity. Collection of samples was carried out with the LBK Radi Rac fraction collector and 4.4 ml aliquots were collected.

Preparation of sample

Small quantities of the ATC and EATC were weighed and dissolved to give final concentrations of 210 and 200 μg/ml, respectively, (calculated as the hydrochlorides). The tetracycline hydrochloride was prepared in a relatively higher concentration of 6.25 mg/ml to correspond to the approximate sample size analyzed in pharmaceutical preparations. The products (either singly or combined) were dissolved in distilled water and diluted with an equal volume of buffer solution. When the buffer was alkaline, column separation was immediately carried out since the TC on prolonged standing at room temperature developed a precipitate. A volume of 0.5 ml of the solution was applied to the column for analysis.

Determination of the partition coefficient K_d

The calculation briefly follows the formula by GELOTTE[2]:

$$K_d = \frac{V_e - V_0}{V_i}$$

Where $V_i = a \cdot W_r$ and a = dry weight of Sephadex necessary to pack a column bed 1.8 × 25 cm and

W_r = water regain of Sephadex,

V_e = elution volume of test substance at the point of maximum adsorption 273 mμ,

V_0 = eluton volume of haemoglobin measured at 540 mμ.

No correction was made for water of hydration of Sephadex.

RESULTS

In Fig. 2 are shown the elution diagrams of mixtures of tetracycline (TC), anhydrotetracycline (ATC) and 4-*epi*-anhydrotetracycline (EATC) under varied conditions of pH. At pH 2.5 the ATC and EATC displayed equal and maximum retardation on the column but did not show evidence of separation one from the other. Similar elution rates were obtained at pH 1.5. As the pH was raised the elution rates of the ATC and EATC increased, but differentially, so as to effect a separation between the compounds. At pH 9.0 and 9.5, the TC and the derivatives produced constant elution patterns and optimal separation between the ATC and EATC. The effect was eliminated when the compounds were passaged on a column equilibrated with 0.01 *N* NaOH. Under these conditions, the ATC and EATC formed a slight shoulder adjacent to the TC peak (data not shown).

Fig. 3 depicts the elution diagram for the TC and derivatives passaged separately on Sephadex columns equilibrated with phosphate buffer solvent pH 7.7 and tris buffer solvent pH 9.0. It is noted that the positions of elution of the separate compounds agree closely with the admixture elutions in Fig. 2. The sensitivity of the

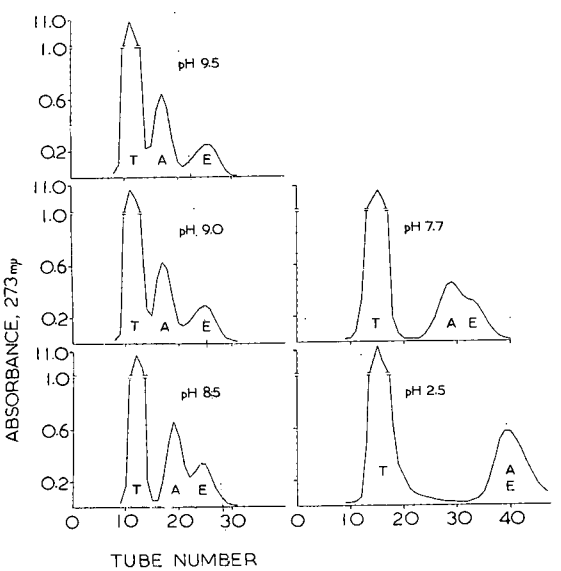

Fig. 2. The gel chromatography of mixtures of tetracycline (T), anhydrotetracycline (A) and 4-*epi*-anhydrotetracycline (E) in solvents of different pH.

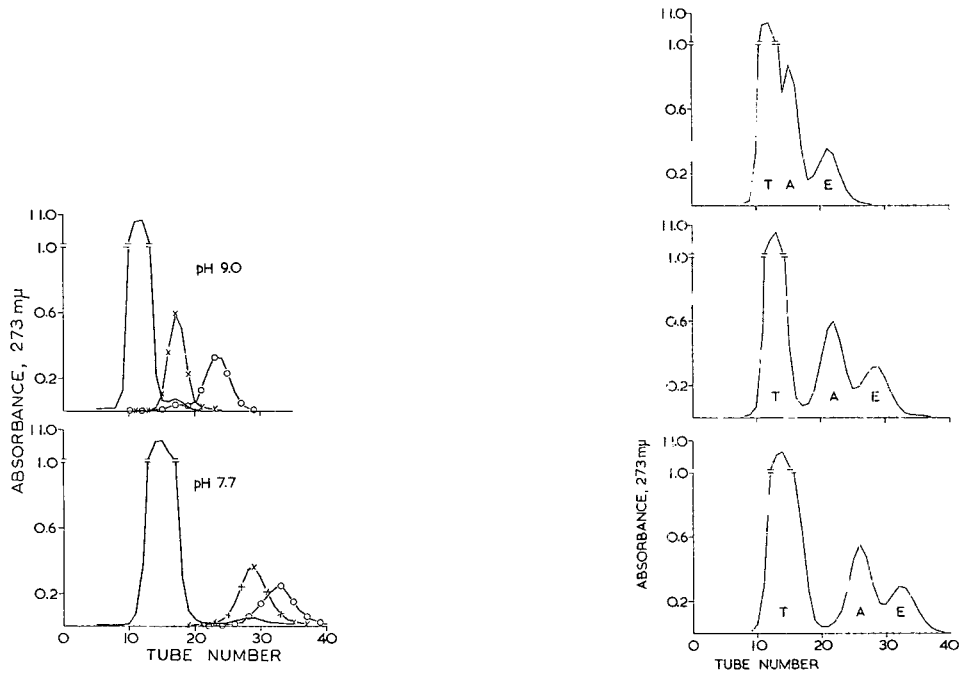

Fig. 3. The gel chromatography of tetracycline (———), anhydrotetracycline (×---×) and 4-*epi*-anhydrotetracycline (○—○) passaged separately on columns of Sephadex at pH 7.7 and 9.0.

Fig. 4. The gel chromatography of tetracycline (T), anhydrotetracycline (A) and 4-*epi*-anhydrotetracycline (E) in solvents of different salt concentration. (A) = 0.025 *M* tris buffer, pH 9.0; (B) = 0.050 *M* tris in 0.025 *M* NaCl, pH 9.0; (C) = 0.050 *M* tris in 0.2 *M* NaCl, pH 9.0.

separation of the components is evident since traces of ATC impurities could be detected in the TC and EATC products.

With low molarity of salt and constant pH of 9.0, the ATC and EATC showed weak adsorption to the column (Fig. 4). As the salt concentration was increased, the ATC and EATC exhibited stronger adsorption as evidenced by their delayed elution (Fig. 4, Table I). Evidence of broadening of the TC peak in the tris–NaCl solvent (C) was observed (Fig. 4).

The TC is subject to oxidation in the alkaline region of pH 9.0. The column eluates of the TC after standing at room temperature for several hours developed a brown discoloration which may be evidence of oxidation. The narrow elution bands obtained for the TC in the region of pH 9.0 as opposed to the broad bands at pH 7.7 and 2.5 may be further evidence of oxidation of the TC.

The ATC and EATC which are important from the analytical point of view were found to be stable by U.V. absorption criteria for periods up to 4 h at pH 9.0. The absorptivities of the compounds (calculated as the hydrochlorides) at pH 9.0 agreed with previous values determined at pH 7.7 (ref. 1). Also in agreement with the reported values, the absorptivity of the EATC was significantly lower than that of the ATC. Higher concentrations of 1 mg/ml of the ATC and EATC were recovered quantitatively with K_d values similar to those of the lower concentrations. Con-

TABLE I

THE INFLUENCE OF pH AND MOLARITY OF SOLVENTS ON THE ADSORPTION OF TC, ATC AND EATC ON SEPHADEX

An increase in numerical magnitude of the K_d values indicates stronger adsorption to the gel matrix. Figures in brackets in top portion of the Table indicate the salt concentration (in molarity) and those in the bottom part indicate the pH of the solutions.

pH	K_d		
	TC	*ATC*	*EATC*
2.5* (<0.01)	2.0	6.9	7.1
7.7* (0.04)	2.0	4.7	5.7
8.5 (0.05)	1.4	2.8	3.8
9.0 (0.05)	1.0	2.4	4.0
9.5 (0.05)	1.0	2.4	4.2
Molarity	*TC*	*ATC*	*EATC*
0.025 (9.0)	1.4	2.0	3.2
0.075 (9.0)	1.6	3.4	4.7
0.250 (9.0)	1.8	4.2	5.3

* The K_d values at these pH readings were calculated from the elution volumes of the individual compounds chromatographed separately.

versely, a dilute solution of TC (200 μg/ml) showed no difference in elution rate from the relatively high quantities routinely passaged on the column.

DISCUSSION

The adsorption effect as it applies to tetracycline (TC) and its derivatives is distinct from the molecular sieve effect which is commonly associated with gel filtration on Sephadex. The separation of the compounds appears to be related to a pure chromatographic effect since they differed only in minor chemical or stereochemical properties.

The TC although retarded in its elution from the column was relatively refractory to the diverse conditions of pH and salt concentration. The anhydro- and 4-*epi*-anhydrotetracycline derivatives (ATC and EATC) exhibited strong and similar adsorption at low pH with K_d values of 6.9 and 7.1, respectively (Table I). With an increase of the pH to the alkaline range, the adsorption of the ATC and EATC diminished and their respective elution rates were almost constant at pH 9.0 and 9.5. In this narrow range, the ATC and EATC eluted at different rates and could thus be separated. As the salt concentration of the solvent was increased at the constant pH of 9.0, the K_d values of the ATC and EATC increased (Table I), but the separation efficiency between the compounds was unchanged. This indicates that the pH is the important variable in separating the compounds.

GELOTTE[2] has studied the secondary adsorption properties of Sephadex. A distinction was drawn between substances which were weakly adsorbed to Sephadex in the absence of electrolyte and those adsorbed in the presence of electrolytes. The tetracycline derivatives fall into the latter category which applies to aromatic and heterocyclic compounds.

The findings on the separation of the ATC and EATC epimer products on Sephadex are of theoretical and practical importance since they reveal a refined chromatographic mechanism operative under optimal solvent conditions. The results are of immediate practical interest since the toxic nature of the EATC (ref. 3) requires its analytical determination in pharmaceutical preparations for human use.

ACKNOWLEDGEMENT

Sincere thanks are extended to Mr. R. BRUNET for his excellent technical assistance.

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CHROM. 3679

THIN-LAYER CHROMATOGRAPHIC/SPECTROPHOTOMETRIC ANALYSIS OF CERTAIN COMPONENTS IN AGED DOUBLE-BASE PROPELLANT

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(Received July 1st, 1968)

SUMMARY

A combination of thin-layer chromatography and spectrophotometry provides a reliable means for measuring typical plasticizers and stabilizers in aged double-base propellants. The use of a thin-layer chromatographic separation eliminates analysis errors encountered in other methods. Both nitrated stabilizers and nitroglycerin breakdown products are removed before measurement of the unused stabilizers and nitroglycerin. Satisfactory thin-layer separations and recoveries were demonstrated even with an application sample size of up to 40 mg. The analysis time was not significantly longer than with other methods and acceptable accuracy and precision were shown in a reliability study.

INTRODUCTION

The determination of nitroglycerin and stabilizers in the presence of degradation products such as lower nitrate esters of nitroglycerin and nitrated stabilizers in aged double-base propellant and in micro propellant samples has been a problem for some time. GRINDLEY AND JEACOCKE[1] and SCHROEDER *et al.*[2], have shown the presence of considerable amounts of nitration products of the stabilizers, resorcinol and 2-nitrodiphenylamine, in aged composite modified double-base propellant. In unpublished work, samples of aged propellant (stored at ambient for five years) were examined for stabilizer breakdown. The main breakdown products observed were resorufin and resazurin. These seemed to be the primary stabilizer nitration products formed. In some samples, very small quantities of 2,4- and 2,4'-dinitrodiphenylamine were observed. Denitration of nitroglycerin in propellant samples appeared to result in the formation of 1,2- and 1,3-dinitroglycerin and the 1- and 2-mononitroglycerin.

Most of these degradation products, if not removed, interfere in the determination of the original components from which they were derived. All procedures currently used for the determination of 2-nitrodiphenylamine[3,4], resorcinol[3,5,6] and nitroglycerin[6-8] in composite modified double-base propellant fail to distinguish between the compound of interest and many of the nitrated/denitrated derivatives. Furthermore, these procedures require samples varying in weight from 0.5 to 5 g. An analytical scheme based on spectrophotometric measurement of double-base propellant

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components following a Soxhlet extraction was reported recently[6], and was shown to reduce analysis time by 70% as compared to wet methods. Although the spectrophotometric scheme is very rapid and reliable when used in connection with relatively fresh propellant, it does not provide for the removal of analytically interfering species in aged propellant. A rapid, yet reliable method for the quantitative determination of nitroglycerin and stabilizers in the presence of degradation products was needed.

Several methods for the removal and separation of degradation products from the original components before analysis have been reported[9,10]. Most of these include separations employing column or thin-layer chromatography (TLC) and were at most semi-quantitative. Quantitative TLC analysis involves either on-plate measurement or removal of the support followed by elution and measurement. However, according to BOBBITT[11], TLC methods involving off-plate U.V. and visible spectrophotometric measurements gave 2–3% accuracy and seemed appropriate for our requirements.

HARTOG AND SHAFER[12] recently succeeded in separating a mixture of nitroglycerin, dinitrotoluene, and diethylene glycol dinitrate by TLC using toluene–chloroform (90 : 10). Following the separation, the three components were eluted from the support and assayed spectrophotometrically, which resulted in 90% recovery. This recovery, although considered excellent in TLC analysis, did not seem sufficient for our purposes since precision of instrumental methods was far superior. Furthermore, the above separation did not provide for the removal of breakdown products.

This investigation reports a technique for rapidly separating a complex mixture while using plate overloading to obtain sufficient sample from a TLC plate for spectrophotometric measurements of nitroglycerin, triacetin, 2-nitrodiphenylamine, and resorcinol utilizing the U.V., visible, and I.R. regions of the spectrum. Only the stabilizers (2-nitrodiphenylamine and resorcinol) were U.V.-visible active while the other two components of interest (nitroglycerin and triacetin) were U.V.-visible inactive and to be determined quantitatively would require either some relatively time consuming color development method[13,14] or collection of sufficient material from the plate for I.R. analysis. To develop a suitably rapid method it was necessary to separate sufficient material on one plate for direct I.R. measurements of all components of interest.

For over a year this technique has been successfully used by this laboratory for the determination of the above components in composite modified double-base propellant containing decomposition products such as mono- and dinitroglycerins and nitrated and nitrosated diphenylamines and resorcinols. However, it is not within the scope of this report to actually measure these breakdown products.

EXPERIMENTAL

Apparatus and reagents

Precoated silica gel TLC plates (E. Merck, A. G., Catalog No. 5715) containing the sample were eluted in a developing tank (Kensington Scientific Corp., Catalog No. K-4097). For U.V. and visible measurements, solutions were read in a 1 cm silica cell against a matched reference on a Beckman DK-2A spectrophotometer. I.R. measurements were performed on a Beckman IR-7 spectrophotometer in a 0.2 mm calcium fluoride liquid cell against a matched reference. All reagents used were reagent grade.

Procedure

Standard solutions were prepared in concentration ranges shown in Table I and scanned against a solvent reference using conditions as indicated in Table II. The absorptivity for each component was determined by plotting net absorbance against concentration and determining the slope of the calibration curve.

Using diethyl ether, enough propellant was Soxhlet extracted such that 40 mg of extract was obtained. After a 24-hour extraction, the ether was allowed to evaporate from the extract and the residue redissolved in about 5 ml of ethyl acetate.

TABLE I

STANDARD SOLUTIONS

Component	*Concentration range (mg/ml)*	*Solvent*
Nitroglycerin	1–5	1,2-Dichloroethane
Triacetin	2–10	1,2-Dichloroethane
2-Nitrodiphenylamine	0.004–0.020	Ethyl acetate
Resorcinol	0.004–0.020	Ethyl acetate

The ethyl acetate solution containing the propellantextract was applied with a syringe evenly across a TLC plate and a chromatogram developed by the ascending method using benzene–ethyl acetate (85 : 15). Following the development the strip of support containing the separated components of interest was removed from the plate and the components eluted from the support with ethyl acetate. The ethyl acetate was evaporated and the individual components quantitatively dissolved in the appropriate solvent. Nitroglycerin and triacetin were measured in the I.R., resorcinol

TABLE II

OPERATING CONDITIONS

Component	*Scanning range*	*Base line*	*Peak*	*Cells*	*Reference*
Nitroglycerin	1600–1950 cm^{-1}	1900 cm^{-1}	1659 cm^{-1}	0.2 mm calcium fluoride	8
Triacetin	1700–1950 cm^{-1}	1900 cm^{-1}	1745 cm^{-1}	0.2 mm calcium fluoride	8
2-Nitrodiphenylamine	400–750 mμ	700 mμ	420 mμ	1 cm silica	4
Resorcinol	250–350 mμ	300 mμ	272 mμ	1 cm silica	6

in the U.V., and 2-nitrodiphenylamine colorimetrically. The absorbance of the solutions was determined as outlined in Table II and corresponding percentages of each were obtained.

DISCUSSION

In developing a satisfactory chromatographic procedure for the separation of nitroglycerin, triacetin, 2-nitrodiphenylamine and resorcinol, it was necessary to (1)

completely separate from each other and from interferences all components to be spectrophotometrically measured on one plate, (2) confine each separated component to a narrow range of R_F values, *i.e.*, to avoid "streaking" in the chromatogram, and (3) separate enough material for quantitative measurement. Incomplete separation of components on the plate may cause interference when measured spectrophotometrically depending on the components involved and their analytical wavelengths. Streaking of a component complicates the recovery operation due to the large amount of support material that must be eluted.

To optimize the developer to be used for the desired separation a test solution containing purified nitroglycerin, triacetin, resorcinol, and 2-nitrodiphenylamine was applied to a TLC plate and developed using a variety of solvents. Benzene alone was found to provide an excellent separation of nitroglycerin and 2-nitrodiphenylamine but failed to move the triacetin and resorcinol out of the origin. Incremental additions of ethyl acetate to the benzene increased the R_F values of all components. A benzene–ethyl acetate (85 : 15) mixture allowed for satisfactory separation of the above components. Further increase of ethyl acetate caused resorcinol and nitroglycerin to approach the R_F values of triacetin and 2-nitrodiphenylamine, respectively.

To determine the optimum range of sample sizes that could be handled by the TLC plate, six synthetic mixtures containing nitroglycerin, triacetin, resorcinol, and 2-nitrodiphenylamine were prepared by combining aliquots from standard solutions containing the above components. Aliquots of the combined standard solution were then chromatographed, and each component determined spectrophotometrically. The results are shown in Table III. Although the recoveries of triacetin and 2-nitrodiphenylamine were relatively independent of the amounts in the sample, nitroglycerin

TABLE III

PLATE LOADING AND RECOVERY DATA

Mix No.	*Nitroglycerin, TLC/I.R.*			*Triacetin, TLC/I.R.*		
	Nominal (mg/ml)	*Determined (mg/ml)*	*Recovery (%)*	*Nominal (mg/ml)*	*Determined (mg/ml)*	*Recovery (%)*
1	36.02	34.65	96.2	3.21	3.17	98.8
2	32.30	31.53	97.6	6.44	6.48	100.6
3	29.02	28.15	97.0	9.70	9.77	100.7
4	26.92	26.40	98.1	12.85	12.86	100.1
5	22.81	22.62	99.2	16.04	16.12	100.5
6	20.15	20.12	99.9	19.31	19.44	100.7
	Resorcinol, TLC/U.V.			*2-Nitrodiphenylamine, TLC/Visible*		
	Nominal (mg/ml)	*Determined (mg/ml)*	*Recovery (%)*	*Nominal (mg/ml)*	*Determined (mg/ml)*	*Recovery (%)*
1	0.404	0.385	95.3	0.400	0.398	99.5
2	0.808	0.734	90.8	0.400	0.997	99.5
3	0.404	0.381	94.4	0.800	0.792	99.0
4	1.212	0.991	81.8	0.400	0.400	100.0
5	0.404	0.396	98.1	1.200	1.174	97.8
6	0.404	0.402	99.5	0.400	0.398	99.5

and resorcinol recoveries were severely affected by the amount contained in the chromatographed aliquot. Aliquots containing up to 40 mg of components were satisfactorily separated; however, further increase in sample size resulted in streaking and low recoveries of nitroglycerin and resorcinol. Samples containing less than 20 mg did not yield sufficient material for reliable I.R. analysis of nitroglycerin and triacetin.

Precision data for the methods were obtained from an ethyl acetate solution containing nitroglycerin, triacetin, 2-nitrodiphenylamine, and resorcinol. Six 1 ml aliquots, each containing about 40 mg of sample were developed and the separated components spectrophotometrically measured using the described procedure. Relative standard deviations found were 0.59%, 1.91%, 0.76%, and 1.13% for nitroglycerin, triacetin, 2-nitrodiphenylamine, and resorcinol, respectively (Table IV).

TABLE IV

PRECISION DATA FOR THE METHODS

Run	*Nitroglycerin (mg)*	*Triacetin (mg)*	*2-Nitrodiphenyl-amine (mg)*	*Resorcinol (mg)*
1	36.1	2.77	0.391	0.406
2	36.1	2.91	0.395	0.400
3	36.4	2.80	0.396	0.414
4	36.5	2.84	0.391	0.404
5	36.4	2.77	0.390	0.407
6	36.6	2.84	0.395	0.406
Average	36.4	2.82	0.393	0.406
S. D.	0.21	0.05	0.003	0.005
Rel. S.D. (%)	0.59	1.91	0.76	1.13

Emphasis had to be placed on the careful application of the sample to the plate. The success or failure in obtaining a satisfactory chromatogram from a sample of this magnitude lies primarily in the application. The sample solution was applied in a thin uniform band about 5 mm wide across the plate, about 2 cm from the bottom.

Of the components to be analyzed, only 2-nitrodiphenylamine and some nitration products were visually detected. To locate the remaining components, several spray reagents were investigated. Although specific spray reagents were available for the detection of the components of interest, visualization of the non-colored components could be affected by spraying with phosphomolybdic acid[11]. Since the components separated on the plate were to be spectrophotometrically measured, spraying the plate containing the sample with phosphomolybdic acid was not feasible. Instead a test strip was spotted and developed along with the sample plate. After development, the test strip was sprayed and the separated components located. The precoated plates used were of sufficient uniformity that changes in R_F values of the components from plate to plate were negligible.

Removal of the support containing the separated components and elution of the components from the support was a simple task. The efficiency of the described

separation was insured by qualitatively and quantitatively determining each recovered component. I.R. spectra were obtained of each separated component and found to be identical to the I.R. spectra of nitroglycerin, triacetin, the stabilizers. In addition, the U.V. spectra obtained from the separated stabilizers were identical to those of the pure components.

A recent study performed on aged propellant samples showed that resorcinol was the major stabilizer in ammonium perchlorate containing propellant. The first nitration product was 4-nitroresorcinol which then appeared to react rapidly with more resorcinol to form resorufin and resazurin. Finally, the 2,4-dinitroresorcinol began to appear and was the dominating species just prior to auto-ignition. No 2-nitroresorcinol was found in any of the aged propellants.

The 2-nitrodiphenylamine nitrated more slowly than resorcinol in propellant. Nitration of 2-nitrodiphenylamine did not occur until most of the resorcinol was reacted. In resorcinol-depleted samples, 2-nitrodiphenylamine yielded on nitration 2,4-, 2,4′- and 2,2′-dinitrodiphenylamine.

From the results of this study, the conclusion can be made that as long as a sufficient level of resorcinol exists in the propellant, nitrated derivatives of 2-nitrodiphenylamine need not be expected. In this case only resorufin and resazurin appear to be the major stabilizer nitration products. After exhaustion of resorcinol, however, 2-nitrodiphenylamine undergoes nitration to form the previously mentioned products.

TABLE V

R_F VALUES OF COMPOUNDS TO BE ANALYZED AND LIKELY DECOMPOSITION PRODUCTS

Adsorbent: Silica gel (precoated TLC plates, E. Merck, A.G., Cat. No. 5715). Developer: benzene–ethyl acetate (85:15).

Compound	*R_F value*
Nitroglycerin	0.74
Triacetin	0.52
2-Nitrodiphenylamine	0.82
Resorcinol	0.26
1,2-Dinitroglycerin	0.30
1,3-Dinitroglycerin	0.47
1-Mononitroglycerin	0.02
2-Mononitroglycerin	0.05
2,4-Dinitrodiphenylamine	0.79
2,4′-Dinitrodiphenylamine	0.69
2-Nitroresorcinol	0.84
2,4-Dinitroresorcinol	0.00
2,4-Dinitrosoresorcinol	0.00
Styphnic acid	0.00
Resorufin	0.00
Resazurin	0.00

Table V lists the R_F values of several common products obtained from the breakdown of composite modified double-base propellant which, if not removed, interfere in the determination of the primary stabilizers and nitroglycerin. The separation using benzene–ethyl acetate (85 : 15) was excellent for samples containing no nitration products of 2-nitrodiphenylamine. If the latter are present, a change of

solvents from benzene–ethyl acetate (85 : 15) to benzene has been shown to separate the nitrated diphenylamines satisfactorily. However, using benzene as the developer, resorcinol and triacetin remain in the origin and an additional separation employing benzene–ethyl acetate (85 : 15) is necessary to move these components.

We have shown that although some overlapping occurs, particularly with 1,3-dinitroglycerin and triacetin, and 1,2-dinitroglycerin and resorcinol, the R_F interferences caused by these lower nitrates do not affect the absorbances of the compounds to be determined.

The methods were considered accurate for the determination of nitroglycerin, triacetin, 2-nitrodiphenylamine, and resorcinol in double-base propellant without sacrificing precision and accuracy. The TLC separation described above provided the analyst with a method of removing undesirable decomposition products which usually interfere with the determination of nitroglycerin and stabilizers.

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CHROM. 3759

THIN-LAYER SOLUBILIZATION CHROMATOGRAPHY

II. KETONES

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SUMMARY

A series of eight high-molecular weight ketones is separated by development on thin layers of starch-bound anion-exchange resin with wash liquids composed of aqueous methanol or acetone. The results are compared to those on ion-exchange papers, and the effects of a number of variables on the results are studied.

INTRODUCTION

The first paper of this series[1] introduced a new method for the chromatographic separation of organic nonelectrolytes by development with aqueous solutions of organic compounds on thin layers composed of a polystyrene ion-exchange resin plus binder. The technique was termed thin-layer solubilization chromatography and was applied to the separation of a group of phenols on starch-bound layers of Dowex 50W-X8(H^+) with aqueous methanol as the wash liquid.

Although other workers have used thin layers containing ion-exchange resins, we are aware of no other work employing layers composed of only strongly-acidic or strongly-basic polystyrene resin plus binder. BERGER *et al.* originally employed thin layers containing equal parts of ion-exchange resin and cellulose powder plus plaster binder for separations of halides and organic dyes[2]. They later omitted the binder and reported that layers formed from an aqueous slurry of cellulose MN 300 and resin (1:6, w/w) adhered well after air drying[3]. They also used layers of chelating resins to separate alkali-metal ions[4] and juxtaposed layers of anion- and cation-exchange resins (including cellulose) to separate various mixtures of ions[5,6]. HUETTENRAUCH[7] separated the components of vitamin B complex on a layer of Wofatit CP 300 weakly-acidic resin, PARIHAR *et al.*[8] separated various nitro organic compounds on layers of Amberlite 400 or 45 containing no binder and DRAGULESCU *et al.*[9] separated niobium and tantalum oxalates on layers of MN 300 DEAE-cellulose mixed with Dowex 1.

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This paper reports the application of our method to the separation of a series of uncombined high molecular-weight ketones, studies of several variables involved in the method and a comparison of the results with those obtained when the same ketones were subjected to solubilization chromatography on ion-exchange papers containing the same resin[10].

EXPERIMENTAL

Procedures for the preparation of the layers, spotting of the initial zones and ascending development of the plates in saturated N-chambers were exactly as described before[1].

In most cases, the strongly-basic quaternary-ammonium anion-exchange resin, Amberlite CG-400, 200–400 mesh (Rohm and Haas Co.), was used as supplied in the chloride form. Layers were 200 μ thick on 8 × 8 inch glass plates. For conversion of the resin to other forms, an appropriate amount of resin was stirred in a large beaker with distilled water, the bulk of the resin was allowed to settle and the fines were decanted off. This procedure was repeated 3 times. The resin was then packed into a column[11] through which an appropriate ionic solution was passed until selective qualitative tests indicated that conversion was complete. The column was washed with distilled water, the resin removed and used to prepare the layers. For comparison, some runs were also made with layers prepared from chloride-form resin from which the fines had been removed.

Developing solutions were prepared by proper dilution of reagent-grade solvents. Individual test solutions of the ketones were 1% in methanol (v/v), except for Nos. 3 and 5 (see Table I) which were 0.5%. Mixtures were prepared on the plate by successive application of individual samples to the same area of the layer, with drying in between.

TABLE I

VALUES OF R_F ON CG-400 ANION-EXCHANGE LAYERS

(a) R_F of front; (b) R_F of rear.

Ketone		*Methanol concn.:*					*Acetone concn.:*	
		12.0 M	*14.0 M*	*17.0 M*	*19.0 M*	*22.0 M*	*6.0 M*	*8.0 M*
(1) Phenyl-2-propanone	(a)	0.42	0.53	0.68	0.72	0.76	0.66	0.75
	(b)	0.32	0.45	0.59	0.62	0.68	0.48	0.61
(2) 4′-Methylacetophenone	(a)	0.37	0.48	0.70	0.74	0.81	0.58	0.73
	(b)	0.27	0.40	0.60	0.64	0.75	0.42	0.63
(3) *trans*-4-Phenyl-3-buten-2-one	(a)	0.25	0.35	0.59	0.63	0.69	0.57	0.57
	(b)	0.15	0.26	0.47	0.52	0.61	0.37	0.04
(4) 1-Phenyl-1,3-butanedione	(a)	0.13	0.21	0.38	0.42	0.60	0.37	0.55
	(b)	0.06	0.16	0.31	0.33	0.52	0.33	0.39
(5) Hexanophenone	(a)	0.15	0.27	0.58	0.64	0.83	0.18	0.57
	(b)	0.09	0.20	0.50	0.54	0.76	0.00	0.30
(6) Phenyl-2-thienyl ketone	(a)	0.11	0.17	0.35	0.38	0.58	0.26	0.56
	(b)	0.04	0.11	0.30	0.31	0.50	0.14	0.36
(7) 4′-Phenylacetophenone	(a)	0.05	0.15	0.34	0.38	0.59	0.21	0.46
	(b)	0.00	0.06	0.25	0.29	0.51	0.13	0.33
(8) 2-Tridecanone	(a)	0.07	0.20	0.70	0.85	1.0	0.16	0.77
	(b)	0.01	0.12	0.57	0.73	0.90	0.00	0.08

Five μl of each ketone solution was developed over a distance of 15 cm. Development times were approx. 60 min for layers prepared from the commercial resin and 30–40 min for layers prepared from resin with the fines removed.

All of the ketones except 4 and 6 were detected by spraying the air-dried plates with a fresh, basic solution of 3,5-dinitrobenzoic acid in methanol[1,12]. The zones initially appear blue on a red-purple background. The background fades with time, and the zones become more prominent. Although the blue color is stable with time, it is best to mark the zone positions immediately because the zones spread somewhat after spraying. Ketones 4 and 6 were detected before spraying by viewing under ultraviolet light in the dark. They appear as light brown spots on a bright yellow background.

RESULTS AND DISCUSSION

R_F values are reported for the leading and trailing edges of each zone so that separation possibilities are apparent from the data. The ketones will often be referred to by the numbers listed in Table I.

Preliminary studies and separations

Methanol was initially chosen as the wash liquid because it had proved successful for the separation of a series of ketones by column solubilization chromatography[13] and for the separation of this same series of ketones by paper solubilization chromatography[10]. Other wash liquids were evaluated, but none proved so good as methanol for achieving the desired separations. Acetone is a stronger developer than methanol on a molar basis, and two pairs of ketones not separable with any molarity of methanol were separated with 6 *M* acetone (see below). Higher molarities of acetone gave irregularly shaped zones, however. No useful degree of differential migration was found with any other wash-liquid system, and many caused cracking of the layers. Those tried include various molarities of methyl ethyl ketone and acetic acid and other more complex three- and four-component systems.

Table I shows R_F values for the individual ketones on chloride-form layers prepared from the resin as received with different molarities of methanol and acetone as the developer. In both cases there is a general increase in the R_F value for each ketone as the molarity of wash liquid increases. This trend was expected and is consistent with earlier results of column[13], paper[10,14,15] and thin-layer[1] solubilization chromatography. The sequence of the ketones was not the same with each molarity of methanol, however. The developed zones had lengths of approx. 1.5 cm in the methanol wash liquids and were regularly shaped. With acetone, the zones were less compact.

Comparison between R_F values for the same ketones on the Amberlite CG-400 resin layers and paper loaded with Amberlite IRA-400(Cl^-) resin[1] with methanol wash liquids shows that at a given molarity of methanol, the resin paper has less affinity for each ketone and yields generally more diffuse zones. This is to be expected because the ion-exchange paper is in effect an ion-exchange layer diluted with cellulose. The selectivity for and sequence of the ketones in the two media are also quite different, indicating that interactions with the cellulose in the resin paper may be significant.

Based on the results in Table I, separations of binary mixtures of most of the

ketones were planned and performed. Table II shows the molarity of methanol or acetone which was used in each case to achieve these separations. It is seen that only 4 out of 28 pairs of ketones could not be separated reliably with either 14, 19 or 22 *M* methanol, and that 2 of these pairs are separable by development with 6 *M* acetone. Some ternary mixtures were also resolved, for example ketones 2, 3 and 7 with 14 *M* methanol and ketones 5, 6 and 8 with 19 *M*. In every case, the R_F values of the ketones were the same whether a pure sample or in a mixture with one or more other ketones.

TABLE II

WASH LIQUIDS GIVING RELIABLE SEPARATIONS OF KETONE PAIRS

Ketone mixture[a]	*Methanol concn. (M)*	*Acetone concn. (M)*
1–2	—	—
1–3	14	
1–4	14 or 19	
1–5	14	
1–6	14 or 19	
1–7	14 or 19	
1–8	14	
2–3	22	
2–4	14 or 19	
2–5	14	
2–6	14 or 19	
2–7	14 or 19	
2–8	14	
3–4	14 or 19	
3–5	22	
3–6	14 or 19	
3–7	14 or 19	
3–8	19 or 22	
4–5	19	
4–6	—	6
4–7	—	6
4–8	19	
5–6	14 or 19	
5–7	14 or 19	
5–8	22	
6–7	—	—
6–8	19	
7–8	19	

[a] See Table I for identification of ketones.

In the earlier study with this same group of ketones on ion-exchange paper, only ketones 7 and 8 could not be separated from each other[1]. This pair is easily separated with 19 *M* methanol on ion-exchange layers. Mixtures of ketones 1–2 and 6–7, neither of which are separated on layers with either methanol or acetone, can be separated with 8 *M* and 14 *M* methanol, respectively, on ion-exchange paper[1]. Because of the similarity between the techniques used to develop the layers and papers, these differences in selectivity are most probably due to the differences in the nature of the stationary phases.

Variables affecting R_F values

The R_F values in Table I are the average of 5 "identical" runs for each ketone with 14 and 19 *M* methanol and at least 2 replications for the other wash liquids. The reproducibility of the runs was excellent, as indicated by the fact that the extremes of the R_F values (*i.e.*, the highest R_F value for the front of the zone and the lowest R_F for the rear in any of the replications for a given ketone) were very similar to the means. For example, the respective extremes for ketones 1–8 in 14 *M* methanol for 5 runs were 0.55, 0.44; 0.49, 0.40; 0.39, 0.23; 0.21, 0.15; 0.27, 0.20; 0.19, 0.08; 0.16, 0.06; and 0.27, 0.09 (compare to means in Table I).

To determine the effect of the size of the initial zone, 5, 10 and 15 μl spots of each test solution were developed with 14 and 19 *M* methanol. The spots were applied in 5-μl increments with drying in between. A slight, general increase in both the front and rear R_F values with increasing concentration was noted.

By use of the variable-thickness spreader, layers of 500 μ and 100 μ were cast for comparison with the usual 200-μ layers. In the thicker layers, zones developed with 14 and 19 *M* methanol were both longer and thinner and had generally higher R_F values. The ketones with the highest R_F values showed a relatively greater increase in movement. Development times were the same. In the thinner layers, the initial and developed ketone zones were more diffuse, and R_F values were generally decreased. Development times for 14 and 19 *M* methanol increased from 60 to 115 min.

After preparation, the layers were normally allowed to stand in air overnight before use. However, no change in R_F values was noted if the layers were stored for 1 or 2 weeks before use or if they were used as soon as 50 min after casting. Any shorter waiting period resulted in a non-adherent layer.

TABLE III

VALUES OF R_F ON LAYERS IN DIFFERENT IONIC FORMS (FINES REMOVED) WITH 14 AND 19 *M* METHANOL AS WASH LIQUIDS

(a) R_F of front; (b) R_F of rear.

Ketone[a]		R_F							
		Cl^- form		*SO_4^{2-} form*		*Br^- form*		*I^- form*	
		14.0 M	*19.0 M*	*14.0 M*	*19.0 M*	*14.0 M*	*19.0 M*	*14.0 M*	*19.0 M*
(1)	(a)	0.63	0.85	0.62	0.81	0.54	0.73	0.38	0.57
	(b)	0.51	0.73	0.50	0.70	0.41	0.65	0.27	0.46
(2)	(a)	0.57	0.87	0.55	0.86	0.50	0.78	0.40	0.63
	(b)	0.48	0.76	0.45	0.74	0.40	0.69	0.29	0.52
(3)	(a)	0.50	0.82	0.51	0.80	0.42	0.72	0.27	0.55
	(b)	0.40	0.67	0.39	0.70	0.31	0.60	0.16	0.47
(4)	(a)	0.26	0.55	0.29	0.44	0.30	0.55	0.22	0.50
	(b)	0.16	0.48	0.15	0.32	0.18	0.45	0.00	0.00
(5)	(a)	0.38	0.82	0.60	0.78	0.50	0.73	0.39	0.61
	(b)	0.29	0.71	0.50	0.68	0.41	0.67	0.29	0.53
(6)	(a)	0.25	0.57	0.32	0.65	0.21	0.48	0.13	0.40
	(b)	0.15	0.45	0.21	0.57	0.11	0.38	0.06	0.26
(7)	(a)	0.20	0.57	0.26	0.67	0.16	0.46	0.13	0.37
	(b)	0.09	0.46	0.10	0.55	0.09	0.36	0.05	0.21
(8)	(a)	0.30	1.0	0.30	0.92	0.37	0.95	0.32	0.85
	(b)	0.11	0.82	0.15	0.82	0.15	0.82	0.03	0.66

[a] See Table I for identification of ketones.

To determine the effect of changing the ionic form of the resin, a column procedure, described above, was used to effect conversion from chloride to the desired form. In order to get an adequate flow of solution, even with pressure applied to the top, it was necessary to decant the fines from the resin before packing it into the column. With both 14 and 19 *M* methanol as the wash liquid, the ketones moved generally farther but in about the same sequence on layers prepared from chloride-form resin with the fines removed. This can be seen by comparing the data in Table III, which are the average values for five separate runs, to those in Table I. The order of decreasing affinity for the layers is, with some exceptions, $I^- > Br^- > Cl^-$, the same as on anion-exchange papers in these forms. There is no clear trend evident on the sulfate-form layers. The sequence of the R_F values for the ketones is about the same in every case, and separation possibilities are not improved by changing the form of the resin layer. In every case, however, development times were reduced to about 30 min on these converted layers.

Preliminary evaluation of cation-exchange layers

A few preliminary results have been obtained with the same ketones on layers of starch-bound Amberlite CG-120 sulfonic acid cation-exchange resin. The preparation of consistently smooth layers composed of this resin has been more difficult because the consistency of slurry seems to be more critical. Moreover, neither the 3,5-dinitrobenzoic acid spray nor numerous other reagents we have tried will detect the ketones on these layers. Some success has been achieved with 10% ethanolic phosphomolybdic acid plus heating, but even this is not entirely reliable. Runs we have made with methanol wash liquids indicate some differential migration of the ketones in relatively compact zones.

Mechanism of thin-layer solubilization chromatography

Thin-layer solubilization chromatography is a partition process closely related to paper solubilization chromatography, the mechanism of which has been previously discussed[10]. The major difference is the absence of the cellulose matrix in the layers and the presence instead of a relatively small percentage of starch binder. It is likely that differences between the two techniques are due to interactions between the solutes and/or wash liquids with the cellulose in the ion-exchange papers, interactions which are absent in the ion-exchange layers.

ACKNOWLEDGEMENT

D. A. G. and R. L. were participants in Lafayette's N.S.F.U.R.P. program. DONALD UPSON contributed some results incorporated in this paper.

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CHROM. 3724

THIN-LAYER GEL FILTRATION OF PROTEINS

I. METHOD

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(Received July 31st, 1968)

SUMMARY

Several aspects of thin-layer gel filtration have been re-examined. By the use of standard densitometric equipment, a quantitative evaluation of the thin-layer gel filtration pattern has been made possible. The recovery of proteins by the print technique has been studied using a number of proteins of different molecular weights.

For three Sephadex gels, G-75, G-100 and G-200, a linear correlation was found between the R_M value (defined as the ratio of the migration distance of the tested protein to that of myoglobin, which was used as a standard) and the molecular weight or the molecular radius of a number of globular proteins.

Fibrinogen fitted well into the straight line of the R_M–log molecular radius relationship, but not into that of the R_M–log molecular weight relationship. Apoferritin did not fit into the R_M–log molecular weight relationship and its molecular radius of 80 Å is in conflict with values found by other methods.

On Bio-Gel P-300 ferritin was separated into three fractions which on disc electrophoresis were found to differ in the content of the electrophoretically distinct α, β and γ-components.

The use of thin-layer filtration for the separation of proteins has been described in many papers[1–21]. For a number of proteins a correlation has been obtained between migration, referred to a standard protein, and molecular weight[3–5,7–10] However, despite its simplicity and potential usefulness for the determination of molecular weights of proteins on the microgram scale, the method has not yet found wide application, especially when compared with the column technique of gel filtration and with other thin-layer chromatographic methods. This appears to be due to technical limitations concerning reproducibility of results, a lower accuracy, as compared with the column technique for determination of molecular weight, and the lack of a quantitative approach in the evaluation of the gel filtration pattern.

Currently, two different types of apparatus are generally used for thin-layer gel filtration. Usually the plates coated with the gel are placed in a moist chamber at angles differing for each particular gel and chosen so as to obtain the optimal flow

rate[2,4]. Alternatively, the sandwich chamber may be used[5]. The separation usually requires several hours depending on the gel used, the length of the plate and the angle at which the plate is inclined. Subsequently, the proteins are detected either directly on the plate[1,3,10] or by taking a print with filter paper which can be dried and stained by any of the dyes found convenient in paper electrophoresis[2,4,5]. In most of the thin-layer gel filtration techniques reported, the proteins are applied as spots. While localization of the spots can easily be achieved, densitometry has not been attempted and cannot be readily accomplished because of the irregular geometry of the spots. When using the spot application technique in studies with heterogeneous systems, the components present in smaller amounts are not easily detected.

An attempt has been made, therefore, to improve thin-layer gel filtration with the aim of getting more reproducible results. Standard densitometric equipment has been used for the quantitative evaluation of the results. Several aspects of the method have been re-examined, including preparation of the plates, application of the sample and detection of the proteins. Three Sephadex gels, G-75, G-100 and G-200, were employed, and in addition, two Bio-Gel preparations, P-60 and P-300, were tested for their suitability for thin-layer gel filtration. Quantitative relationships were established between the migration rates referred to a standard protein and the logarithms of the molecular weight or the Stokes radius of a number of well defined proteins. The extension of the fractionation range by the use of Bio-Gel P-300 is demonstrated by the separation of ferritin.

EXPERIMENTAL

Materials

Sephadex gels G-75, G-100 and G-200, all "superfine", were obtained from Pharmacia, Uppsala, Sweden. Several batches of each gel were used. Polyacrylamide gels, Bio-Gel P-60 and P-300, both with particle size 400 mesh, were obtained from Bio-Rad Laboratories, Richmond, Calif., U.S.A. Bovine serum albumin, human serum albumin and bovine γ-globulin were obtained from Behringwerke, Marburg, Germany, while catalase, creatinephosphokinase, peroxidase and cytochrome c (horse heart) came from Boehringer und Söhne, Mannheim, Germany. Human ceruloplasmin and transferrin were supplied by KABI, Stockholm, Sweden, sperm whale myoglobin, ferritin (2 × crystallized) and protamine sulfate (salmon and herring) came from Koch and Light, Colnbrook, England, while α-chymotrypsin, haemoglobin, β-lactoglobulin, ovalbumin (5 × cryst.) and ribonuclease (5 × cryst.) were from Serva. Heidelberg, Germany.

Methods

Preparation of the gel plates: A gel suspension was prepared by mixing 8 g of Sephadex G-75, 6 g of the G-100 and 4 g of G-200 or 5.4 g of Bio-Gel P-60 and 3.5 g of P-300 with 100 ml of 0.5 *M* NaCl containing 0.02 *M* phosphate buffer (KH_2PO_4–Na_2HPO_4), pH 7.2–7.4. The gel suspension was kept at 4° for two to three days and was evacuated briefly with a water pump before coating the plates. Two thin-layer spreaders were tried out, one from Camag A.G., Muttenz, Switzerland and the other from Desaga, Heidelberg, Germany. The latter was found more suitable for obtaining evenly coated plates. Glass plates 20 × 20 cm in size were used, except for a few

experiments in which 40 × 20 cm plates were employed. A 0.5 mm layer was used throughout, since a 0.3 mm layer was found unsuitable due to rapid evaporation at the edges. A slight excess of solvent was used in order to obtain a smooth surface on spreading. The plates were either allowed to dry at room temperature for 5–15 min and then transferred to a moist chamber where they could be stored for about two weeks, or they were used immediately after appropriate pre-equilibration as described below.

The apparatus consisted of a perspex chamber, 50 × 25 × 10 cm, with a glass plate cover. The arrangement of the plates was essentially as described by MORRIS[4]. Two 20 × 20 cm plates or one 40 × 20 cm plate could be used per chamber; the angle of the gel plates was fixed by a number of 20 × 5 cm glass plates at the lower end of the gel plates. A 20 × 6 cm Whatman No. 3 paper strip was used to establish contact between the buffered solution and the gel layer. At the lower end of the gel plate another 20 × 2 cm Whatman No. 3 strip was placed to prevent the collection of drops at the end of the plate and thereby to obtain a more even flow. Before each run, the gel plates were mounted in the apparatus in the evening and a flow of solvent through the gel was established during the night for pre-equilibration of the gel. This was found to be a necessary step for obtaining reproducible results.

For application of the substance, the pre-equilibrated plates were removed from the chamber and mounted horizontally. A line was marked on the glass plate beneath with a felt-marker at a distance of 3 cm from the upper end. The substance was applied as a streak: 10–20 μl of a 0.2–2% protein solution was placed on the edge of a microscopic cover slide (18 × 18 mm) and held against the gel surface. In a few seconds the solution had soaked into the gel, most rapidly with Sephadex G-75, slower with Sephadex G-200 and slowest with Bio-Gel P-300. Care had to be taken to touch the gel surface with the whole length of the edge of the cover slide, otherwise a drop forms giving rise to an irregular starting zone. To avoid displacement of the samples during application, all samples were first prepared on the cover slides and then quickly applied. On a 20 cm broad plate five to six samples can be run simultaneously. When applied at a distance of 2–2.5 cm from the edge, no distortion of the zones due to an edge effect was noted. Myoglobin was included as a reference protein in each run and was placed always on the middle of the plate. The plates, after application of the samples, were replaced in the apparatus and the paper wick was pushed slightly downwards to ensure good contact with the gel layer. With a particular gel the flow rate depends on the angle at which the plate is inclined and the level of the solvent in the reservoir vessel. The liquid level was kept constant at 1 cm from the upper end of the vessel. The angles were $\sim 5°$ and 10° for the G-75 and G-200 Sephadex gels, respectively. The optimal angle had been found empirically, so as to obtain a flow at which the myoglobin migrates at a rate of 1 cm per hour. The runs were usually complete in 4–7 h.

After completion of the run, the plates were taken from the chamber, and the contact paper wicks and the paper strip at the lower end of the plate were removed. Filter paper Whatman No. 3 was then used to take a print from the gel; Whatman paper No. 1 could be used for Sephadex G-75 and Bio-Gel P-60. A piece of filter paper, 20 × 17 cm, was rolled onto the gel layer from the edge near the starting line, which was marked on the paper with a felt-marker by viewing against light. The liquid phase was soaked off from the gel layer by the paper, which after 30–60 sec

was stripped away and dried in an oven at 110° for 15 min. Drying the paper on the glass plate has been found to distort the zones strongly due to uneven evaporation of water from the plate.

Staining

Proteins were stained either with Amido Black 10B or with Coomassie Brilliant Blue R250, which was the dye of choice when sensitivity[22] was important. A saturated solution of Amido Black 10B (E. Merck AG, Darmstadt, Germany) in methanol–glacial acetic acid (9 : 1, v/v) was used; the staining time was 5–10 min. For destaining, two volumes of the 9 : 1 methanol–acetic acid solution were mixed with one volume of water. The first two washing solutions were rejected; the subsequent ones were decolorized with charcoal and then re-used. Coomassie Blue (Serva, Entwicklungslabor, Heidelberg, Germany) was used as a 0.25% solution in methanol–acetic acid (9 : 1, v/v). After staining for 10 min, the paper was destained first for a few minutes with tap water until no dye could be washed off, then with a mixture of methanol–acetic acid and water (50 : 10 : 50, v/v). The first washing solution of the latter was rejected, and the subsequent ones were decolorized with charcoal.

Densitometry was accomplished with the Chromoscan recording and integrating densitometer (Joyce, Loebl & Comp. Ltd., Gateshead, England) with the thin-layer scanning attachment. The instrument was operated in reflectance with 10 × 1 mm apertures. The 595 or 620 filters were used with Amido Black staining and Coomassie Blue staining.

For molecular weight determinations, the distance from the starting line to the middle of each zone was measured with an accuracy of 0.05 mm either directly on the print or on the densitogram. The results were expressed by the R_M value defined as the ratio of the migration distance of the tested protein d_P to that of myoglobin d_M, which was used as the reference protein:

$$R_M = \frac{d_p}{d_M}$$

Calculations of the constants of the equations describing the relationship between the R_M value and the molecular weight or Stokes radius were performed with an IBM computer.

Gel electrophoresis

Electrophoresis of ferritin in polyacrylamide gel was performed as described by CLARKE[23]. A Tris–glycine buffer pH 8.2–8.3 was employed. Two gel layers, 5% (6 cm) and 2.5% (TEMED free, 0.5 cm), were used. A 0.1% solution of $K_4Fe(CN)_6$ in 0.1 *M* HCl was employed for staining ferritin. The stained gel was kept in 10% acetic acid.

RESULTS

The separation of two protein mixtures on Sephadex G-200, as shown in Fig. 1, illustrates the efficiency of the method. Two mixtures containing sperm whale myoglobin, bovine serum albumin, bovine γ-globulin and horse spleen ferritin (mixture I) and cytochrome c, ovalbumin and bovine serum albumin (mixture II) were resolved on a 20 × 20 cm plate in a run of 6.5 h. The mixtures contained 1% of each of the

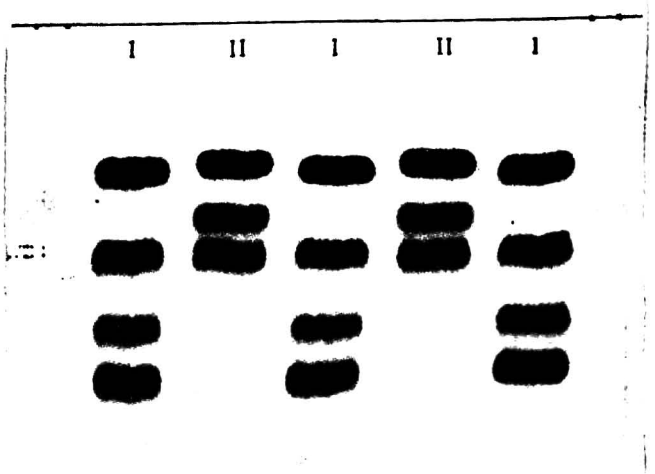


Fig. 1. Thin-layer gel filtration on Sephadex G-200. Proteins (from top to bottom): I myoglobin, bovine serum albumin, bovine γ-globulin and ferritin; II cytochrome c, ovalbumin and bovine serum albumin. Sample: 10 μl of the protein mixtures containing 1% of each protein. Plate: 20 × 20 cm. Separation time: 6.5 h. Staining with Amino Black 10B.

proteins, 10 μl being applied on the plate. The corresponding densitometric curves are shown on Fig. 2. Identical migration distances, with reference to the starting line, of the protein common to both mixtures, namely bovine serum albumin, can be clearly seen both on the plates and on the densitograms. Distinct differences in the migration of cytochrome c and myoglobin can also be observed.

A number of factors were found to influence zone size, *viz.* flow rate, protein concentration and sample volume. A flow rate of 1 cm per hour for myoglobin was optimal and yielded well defined zones without the distortion which was frequently observed when the flow rate was doubled. At the same angle of the plates, there was a decreasing flow rate for the three Sephadex gels studied, G-75, G-100 and G-200. With Bio-Gel P-60, the flow rate was comparable to that of Sephadex G-75. With the other polyacrylamide gel tested, P-300, the flow rates were extremely slow and could not be improved even by increasing the angle of the plates up to 40–50°. This is in contrast to the Sephadex gels examined, which all showed a marked dependence of the flow rate on the inclination angle. Improved flow rates could be obtained with

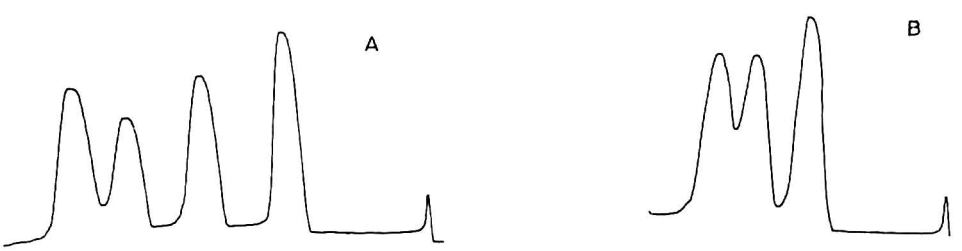


Fig. 2. Densitograms of the two protein mixtures of Fig. 1. A Mixture I; B mixture II. The small peak on the right corresponds to the starting line.

Bio-Gel P-300, however, when the plates were coated with suspensions swollen for about ten to fourteen days at 4°, instead of for only two to three days, which was the standard procedure for other gels. Even when gels of P-300 were treated in this manner, the running time had to be increased two to three times to obtain a migration distance of myoglobin comparable to that on Sephadex G-200.

The effect of protein concentration and sample volume on zone size was investigated using sperm whale myoglobin and bovine serum albumin solutions. With 1% protein solutions applied in 10 μl quantities with a cover slide (18 mm edge), as described under *Methods*, the zones obtained after migrating 5–12 cm were usually 8–10 mm wide and 22–23 mm long (Fig. 1). The effect of concentration on the width of the zones was studied on Sephadex G-75 and G-200 with myoglobin solutions of 0.5, 1.0, 2.0 and 3.0%. All solutions were applied in a volume of 10 μl. After gel filtration for 6–7 h with a flow rate of about 1 cm per hour, prints were taken and stained with Amido Black. A 1% solution was run on each plate; its width was 1.0. With the 0.5% solution, a narrowing of the zones was observed by a factor of 0.5–0.6 of the control. With solutions of higher concentration a broadening of the zone width by a factor of 1.15–1.2 for the 2% and 1.3–1.5 for the 3% solution, respectively, was obtained.

Similarly zone size was found to depend on the volume in which the sample was applied. A 1.5 increase of zone width was observed when the sample volume was increased from 10 μl to 40 μl. There was only a small variation in the length of the zone by a factor of no more than 1.1–1.2 of the control, when either protein concentration or sample volume were increased up to 3% or 40 μl.

Besides these differences in the size of the zones, the highest protein concentration (3%) and sample volume (40 μl) caused a slight decrease in the R_M value in experiments with sperm whale myoglobin and bovine serum albumin on two of the Sephadex gels studied, G-75 and G-200. The effect of protein concentration on the R_M value was also studied with haemoglobin and β-lactoglobulin, because both of these proteins may be eluted from gel columns with elution volumes which are concentration-dependent[3]. With thin-layer gel filtration on Sephadex G-200, the R_M value obtained for haemoglobin was constant (1.31) for a 1–2% solution. For β-lactoglobulin there was no difference in the R_M value (1.26) in the 0.5–2% range, while a slight decrease (1.23) was observed with a 3% solution.

The print technique was used throughout this work. Preliminary experiments had shown that much better results were obtained when the paper print was removed after a brief contact with the gel layer (10–30 sec being sufficient) than when the paper was dried on the glass plate in contact with gel, a procedure employed by a number of workers. In order to estimate the amount of protein removed by the print technique, different mixtures of proteins containing 1% of each of the protein were run on Sephadex G-75 and G-200 gel plates. After completion of the run, the first print was taken either after a contact of 30 sec or after 10 min contact. Immediately after removal of the first print, a second print was taken, the paper being kept in contact with the gel for an additional 5 min. All the prints were dried as usual, stained with Amido Black and evaluated densitometrically. Since no additional material could be removed by a third print, the area of the curves of these two prints was taken as 100%.

The amount of protein removed did not appreciably depend on the time of

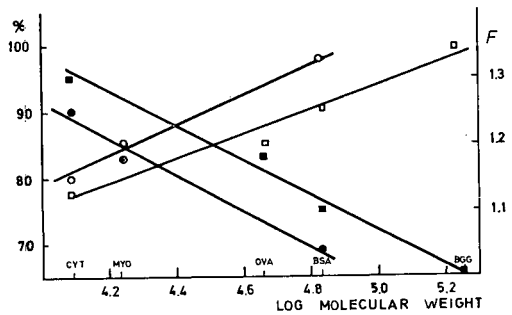
TABLE I

RECOVERY OF PROTEINS (%) BY THE PRINT TECHNIQUE ON SEPHADEX G-75 AND G-200[a]

Protein	*Sephadex G-75*		*Sephadex G-200*	
	First print	*Second print*	*First print*	*Second print*
Protamine	53	47	54	46
Cytochrome c	80	20	77	23
Myoglobin	85	15	—	—
Ovalbumin	—	—	85	15
Serum albumin (bovine)	96	4	90	10
γ globulin (bovine)	—	—	100	0
Ferritin	—	—	100	0

[a] Print taken with filter paper Whatman No. 3. Contacts with the gel layer: first print, 30 sec.; second print, 5 min. Staining with Amido Black 10B, followed by planimetric evaluation of the densitogram.

contact of the paper with the gel; a short contact of 30 sec was almost as effective as a contact of 10 min. The amount of protein removed did, however, depend on its molecular weight. The results of these experiments are presented in Table I. On Sephadex G-200 ferritin and γ-globulin were removed completely with the first print by a short contact, while with all the other proteins an additional amount could be removed by a second print. Both on Sephadex G-75 and G-200, the quantity removed by this print was highest with the three low molecular weight proteins myoglobin, cytochrome c and protamine. With cytochrome c and myoglobin there was again little difference in the recovery of the protein resulting from short or long contact of the first print with the gel. Only with protamine, a strongly basic protein, could a 10–20% increase of the recovery be obtained with 10 min contact.



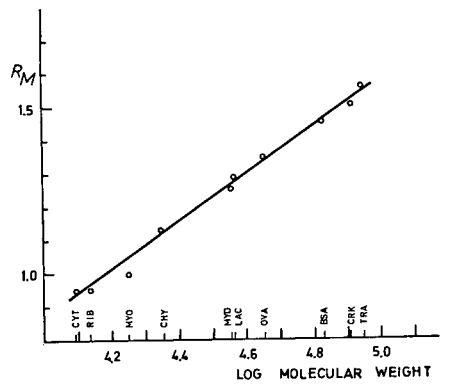


Fig. 3. Recovery of proteins by the print technique. (○——○) Sephadex G-75; (□—— □) Sephadex G-200; F = factor correcting recovery to 100%. (●——●) Sephadex G-75; (■——■) Sephadex G-200.

Fig. 4. R_M as a function of the logarithm of molecular weight for thin-layer gel filtration on Sephadex G-75.

In Fig. 3 the recovery of proteins differing in molecular weight removed by the first print on Sephadex G-75 and G-200 is shown together with the curves for the correction factors calculated to obtain a 100% recovery.

The R_M value was not found to depend on the swelling time of the suspension. This was proved for ferritin and fibrinogen, both proteins of high molecular weight, for which differences would be expected on the basis of experiments with column gel filtration[24]. Plates coated with Sephadex G-200 suspensions kept before spreading for three days at 4°, which was the standard procedure, or for three weeks under the same conditions resulted in identical R_M values for both proteins.

On the basis of these experiments, the following operational parameters were found to yield optimal results: (a) with single proteins, 0.2–2% protein solutions should be used, while in mixtures of proteins the concentration of the individual components should also be of the same order; (b) a sample volume of 10–20 μl per 18–20 mm (cover slide edge); (c) a flow rate of 1 cm per hour and a final migration distance of 5–6 cm for sperm whale myoglobin; (d) prints taken after a short contact of 30–60 sec with the gel.

Relationship between molecular weight or molecular radius and R_M value

For a number of proteins of known molecular weight, as listed in Table II, the R_M values were determined. 1% solutions of a single protein, or mixtures of several proteins containing 1% of each, were prepared in 0.5 M NaCl containing 0.02 M phosphate buffer. Of these solutions, 10 μl were applied to the plates. Five samples were run on each plate with sperm whale myoglobin in the middle. At least twelve samples were run for each individual protein. The arithmetic mean, the standard deviation and the standard error are given in Table II. A good linear relationship between R_M value and logarithm of the molecular weight was obtained for all the Sephadex gels studied. The slopes and the intercepts of the regression lines were calculated by the method of least squares. For the proteins used in these calculations,

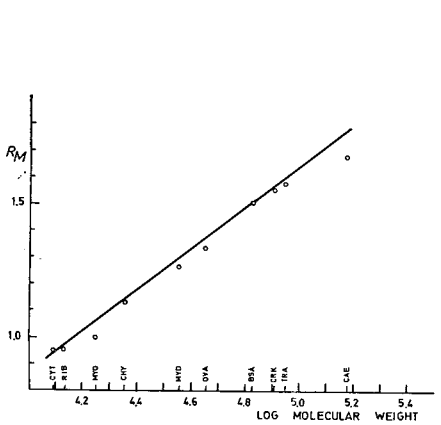


Fig. 5. R_M as a function of the logarithm of molecular weight for thin-layer gel filtration on Sephadex G-100.

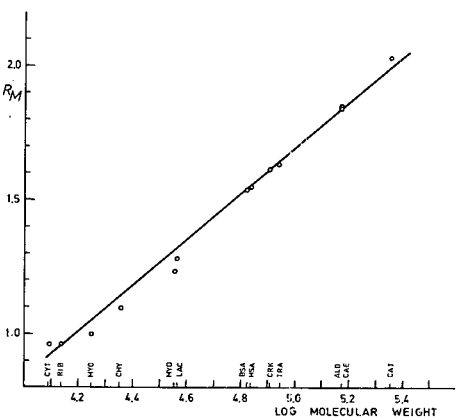


Fig. 6. R_M as a function of the logarithm of molecular weight for thin-layer gel filtration on Sephadex G-200.

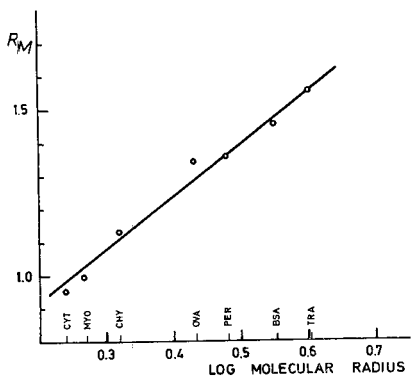


Fig. 7. R_M as a function of the logarithm of molecular radius for thin-layer gel filtration on Sephadex G-75.

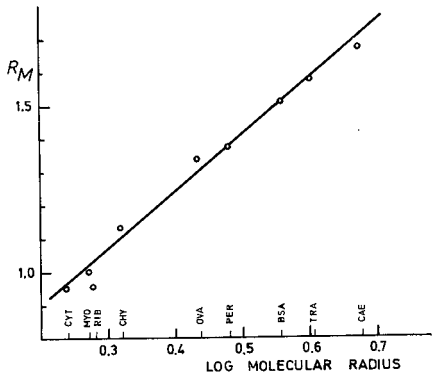


Fig. 8. R_M as a function of the logarithm of molecular radius for thin-layer gel filtration on Sephadex G-100.

the results are graphically presented for Sephadex G-75, G-100 and G-200 in Figs. 4, 5, and 6, respectively. With Sephadex G-75, the upper limit for linearity was 90 000. Creatinekinase and transferrin still fitted into the straight line. With Sephadex G-100, the same upper limit for linearity was found. On Sephadex G-200 linearity could be obtained up to a molecular weight of 240 000.

The equations[8] describing the relationship between the logarithm of the molecular weight and the R_M value, calculated according to the method of least squares, were found to be:

$$\text{G-75:} \quad \log M = 1.402\, R_M + 2.776 \tag{1}$$

$$\text{G-100:} \quad \log M = 1.281\, R_M + 2.896 \tag{2}$$

$$\text{G-200:} \quad \log M = 1.172\, R_M + 3.015 \tag{3}$$

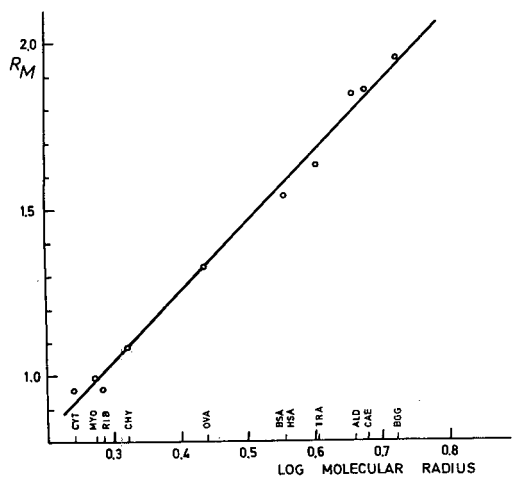


Fig. 9. R_M as a function of the logarithm of molecular radius for thin-layer gel filtration on Sephadex G-200.

TABLE II

R_M VALUES, STANDARD DEVIATIONS AND STANDARD ERRORS OF PROTEINS IN THIN-LAYER GEL FILTRATION ON SEPHADEX G-75, G-100 AND G-200

Protein	*Code*	*Molecular weight*[a] $\times 10^{-3}$	*Molecular radius*[a] ($m\mu$)	*G-75*			*G-100*			*G-200*		
				R_M	σ	$\sigma/\sqrt{n}$	R_M	σ	$\sigma/\sqrt{n}$	R_M	σ	$\sigma/\sqrt{n}$
Cytochrome c	CYT	12.4	1.74	0.9522	0.0470	0.0100	0.9515	0.0184	0.0042	0.9597	0.0299	0.0049
Ribonuclease	RIB	13.7	1.92	0.9529	0.0248	0.0045	0.9590	0.0214	0.0064	0.9642	0.0205	0.0044
Myoglobin	MYO	17.8	1.88	1.0			1.0			1.0		
α-Chymotrypsin	CHY	22.5	2.09	1.1323	0.0298	0.0082	1.1366	0.0210	0.0040	1.0952	0.0188	0.0043
Myoglobin dimer	MYD	35.6		1.2549	0.0014	0.0004	1.2628	0.0007	0.0002	1.2337	0.0013	0.0004
β-Lactoglobulin	LAC	36.5		1.2908	0.0118	0.0034				1.2821	0.0369	0.0084
Peroxidase	PER	40.0	3.02	1.3599	0.0286	0.0065	1.3766	0.0244	0.0081	1.3241	0.0326	0.0094
Ovalbumin	OVA	45.0	2.73	1.3467	0.0615	0.0123	1.3390	0.0294	0.0064	1.3318	0.0092	0.0023
Serum albumin (bovine)	BSA	67.0	3.60	1.4547	0.0923	0.0201	1.5090	0.0293	0.0088	1.5434	0.0400	0.0078
Serum albumin (human)	HSA	69.0	3.61							1.5457	0.0573	0.0153
Creatinekinase	CRK	81.0		1.5027	0.0420	0.0126	1.5508	0.0117	0.0033	1.6185	0.0696	0.0186
Transferrin (human)	TRA	89.0	4.02	1.5572	0.0828	0.0249	1.5781	0.0047	0.0014	1.6358	0.0305	0.0088
Alcohol dehydrogenase (yeast)	ALD	150.0	4.55							1.8459	0.0524	0.0165
Caeruloplasmin	CAE	150.0	4.73				1.6719	0.0205	0.0065	1.8574	0.0525	0.0131
γ-Globulin (bovine)	BGG	169.0	5.25							1.9552	0.1564	0.0326
Catalase	CAT	240.0	5.22							2.0362	0.0566	0.0141
Fibrinogen	FIB	330.0	10.07							2.4850	0.0593	0.0182
Apoferritin	APF	465.0	6.00							2.3373	0.0510	0.0117

[a] Molecular weights and radii were taken from refs. 8, 24, 31, 34, 39, 40 and 41.

Sephadex G-75 and G-100 gave a better linearity for the proteins of lower molecular weight than the G-200 gel.

A linear correlation was also obtained between the Stokes radii and the R_M values. In Figs. 7, 8 and 9 the results are shown for Sephadex G-75, G-100 and G-200. The corresponding equations relating the R_M value and the logarithm of the molecular radius calculated for the proteins shown in Figs. 7–9 were:

$$\text{G- 75:} \quad \log r = 0.627\, R_M - 0.376 \tag{4}$$
$$\text{G-100:} \quad \log r = 0.588\, R_M - 0.329 \tag{5}$$
$$\text{G-200:} \quad \log r = 0.479\, R_M - 0.202 \tag{6}$$

Comparison of the separation efficiency of Sephadex G-200 and Bio-Gel P-300

Experiments with ferritin have demonstrated that with Bio-Gel P-300 the fractionation range can be considerably extended. When crystalline ferritin was separated on Sephadex G-200, a single zone was obtained which, on densitometry, showed a slight asymmetry in the leading part (Fig. 10). On Bio-Gel P-300 three zones were detected, which could be densitometrically evaluated without staining the ferritin due to its intense brown colour. They were found to amount to 80%, 16% and 4% of the total material in the order of increasing migration distance. These figures, compared with those obtained by other workers on gel electrophoresis[25–27],

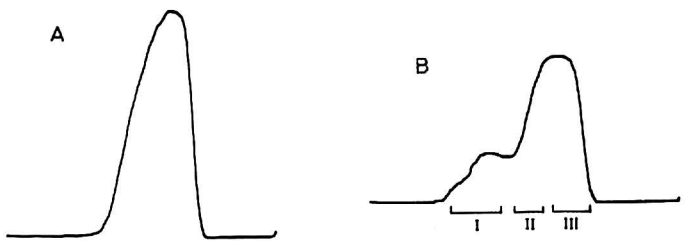


Fig. 10. Thin-layer gel filtration of ferritin on (A) Sephadex G-200 and (B) Bio-Gel P-300. Fractions I, II and III were isolated by preparative thin-layer gel filtration and used for electrophoresis in polyacrylamide gel.

strongly suggested that the fractions obtained on thin-layer gel filtration correspond to the ferritin components found on gel electrophoresis in polyacrylamide and starch.

A preparative thin-layer gel filtration method followed by polyacrylamide gel electrophoresis was employed to test this hypothesis. 120 μl of a 10% ferritin solution were applied by means of a 12 cm long glass plate to a standard 20 × 20 cm glass plate with a 0.5 mm layer of Bio-Gel P-300. An angle of 20° was used. The separation could be followed easily because of the intense colour of ferritin. After a run of 24 h, a print was taken with filter paper Whatman No. 3. Three strips were cut perpendicularly to the direction of separation (as indicated in Fig. 10) and were eluted with distilled water in a moist chamber by placing one end of the strip in a beaker filled with distilled water. The eluates were dialyzed and concentrated by vacuum evaporation to 1–2% protein concentration. The result of gel electrophoresis of these fractions is shown in Fig. 11. Distinct differences in the separation of the α, β and γ-

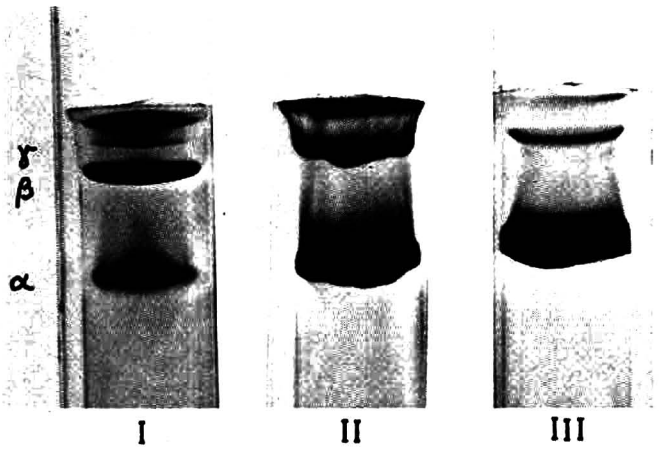


Fig. 11. Disc electrophoresis of ferritin fractions isolated by preparative thin-layer gel filtration on Bio-Gel P-300. For description of fractions I, II and III, see Fig. 10.

ferritin in the thin-layer fractions can be seen. The most rapid fraction (FI) on thin-layer gel filtration consists predominantly of β and γ-ferritin with some α-ferritin. Fractions FII and FIII are composed of α-ferritin with only small amounts of the electrophoretically slower β and γ-ferritins.

DISCUSSION

Many of the claims for the advantages of thin-layer gel filtration over the column technique are fully justified. Simplicity and speed, need of a minimal amount of material in the microgram range, high resolution and good reproducibility are among the features of the method. Application of the substance as a streak has made possible subsequent densitometric evaluation of the pattern thus providing a means for a quantitative approach. The possibility of running different samples simultaneously on the same plate is a useful property of the thin-layer technique, which should prove of value in the analysis of heterogeneous systems and fractions isolated during preparative work. Thin-layer gel filtration is basically an analytical method; however, preparative separations with amounts up to 10 mg of proteins can be easily accomplished on standard 20 × 20 cm plates with a 0.5 mm layer and the protein load could probably be increased on plates with thicker layers. Besides this, in preparative work thin-layer gel filtration presents a useful supplementation of the column gel filtration technique for establishing optimum separation parameters (gel type and solvent).

The possibility of taking a print after completion of the run is a valuable characteristic of thin-layer gel filtration. Thus a permanent record is obtained which can be handled as easily as paper chromatograms and electropherograms and which can be evaluated by standard densitometric equipment. Protein detection by the print technique has many advantages over direct detection on the glass plate. While incomplete removal of the proteins of low molecular weight appears to be a drawback of the print technique (Table I), a complete removal is not a critical factor in many

experiments. When quantitative evaluation of systems containing proteins of different molecular weight is attempted, incomplete removal will introduce an error for the components of low molecular weight which can however be compensated for either by applying a correction factor (Fig. 3) or by taking two consecutive prints.

Thin-layer gel filtration has proved to be equally well suitable for a qualitative analysis of homogeneous proteins and of heterogeneous systems, as well as for the determination of the molecular weight of proteins. While thin-layer and column gel filtration differ in many respects, a comparison of the results obtained by both techniques demonstrates that they are basically identical.

The suitability of gel filtration for the determination of molecular weight of globular proteins has been fairly well established[3,8,24,28,29]. Good correlations were found between molecular weight and gel filtration behaviour for a number of globular, carbohydrate-free proteins on a variety of different gels. With more information on the gel filtration behaviour of different proteins, some of the limitations of the method are being better realized[24,30]. Simultaneously, new potentialities of gel filtration for determination of other molecular parameters are being recognized[24,31,32].

While most of the results on the determination of molecular weight by gel filtration have been obtained with the column technique, there are already a few reports that a good correlation exists also between gel filtration behaviour and molecular weight on thin layers[3-5,7-10]. For all the Sephadex gels studied, G-75, G-100 and G-200, a good linear relationship between R_M value and molecular weight was obtained (Figs. 4–6). The upper limit for the linear correlation for Sephadex G-75 was about 90 000, a higher value than that calculated for the exclusion limit of this gel in column gel filtration[8]. For the G-100 gel also 90 000 was found as an upper limit for the linear correlation. Caeruloplasmin and yeast alcohol dehydrogenase, both with a molecular weight of 150 000, did not fit well into the straight line of the R_M–log molecular weight relationship. However, no proteins with an intermediate molecular weight between 90 000 and 150 000 were tested. For the G-100 gel an extension of the upper limit above the value of 90 000 appears to be possible. For the G-200 gel the upper limit for the linear correlation was 240 000. This value is also higher than that found in column gel filtration, though linearity up to a range of 500 000 and even higher has been claimed for the G-200 gel in columns depending on the gel batch and the swelling time[24].

No evidence for linearity was obtained in thin-layer gel filtration on Sephadex G-200 in the range above 240 000. None of the three proteins tested, with molecular weights higher than that of catalase (240 000), namely fibrinogen, apoferritin and serum macroglobulins, fitted into the straight line of the R_M–log molecular weight relationship. The anomalous gel filtration behaviour of fibrinogen can be explained in terms of its strong molecular asymmetry. The molecular weight found for apoferritin was 575 000, a value much higher than those found by other methods. Some of the possible reasons for the deviation of apoferritin will be discussed later. For the serum macroglobulins an R_M value of 2.35 was obtained[33] with a corresponding molecular weight of 600 000, which is much lower than the accepted value of 820 000–1 000 000 (see ref. 34).

There is only very limited information on the gel filtration behaviour of proteins with molecular weights higher than 240 000 on Sephadex G-200 thin layers. In the range above 240 000, linearity of a relationship between the logarithm of the molecu-

lar weight and the migration rate was obtained with thyroglobulin (mol.wt. 650 000) in one laboratory[4], whereas a deviation from linearity was observed for the same protein in another laboratory[10]. One possible explanation for these conflicting results may be differences in the technique employed for detecting the proteins. Drying the paper print in contact with the gel layer on the glass plate, a technique used by MORRIS[4] has been found in this laboratory to cause a displacement of the zones strongly affecting the zones located at the ends of the plates. In addition, thyroglobulin has been shown to be heterogeneous[24,35], another possible reason for irregular gel filtration behaviour.

Obviously further work is needed with well defined proteins of molecular weights higher than 240 000, to establish the quantitative relationship in this range. Non-linearity of the relationship between the logarithm of the molecular weight and the migration rate in this range would not present a limiting factor for molecular weight or Stokes radii determinations since it has been already demonstrated in column gel filtration that the non-linear part of the calibration curves (reduced elution volume and log molecular weight) may also provide a useful basis for these determinations[24].

Although it has been repeatedly stated that the accuracy of the molecular weight determinations by the thin-layer method is lower than that of the column technique, the results reported in this paper indicate that this is not necessarily so. Although it has not been possible to eliminate fluctuations in migration of the same protein on the same plate, statistical evaluation of several runs (10–15) of a single protein or a protein mixture which can easily be obtained on two to three plates of the standard size shows good reproducibility of the R_M values. An accuracy of $\pm$ 10% for the determination of molecular weight was calculated for the Sephadex G-200 gel. A value of 10% for the uncertainty in molecular weight determinations of carbohydrate-free, globular proteins by the column gel filtration technique has been considered to be appropriate[24]. The effect of fluctuations in migration on thin-layer gel filtration can be reduced by using an internal standard whereby the reference protein is run in a mixture with the unknown protein, provided that there is a sufficient difference in molecular weight for the distinct separation of both and that there is no interaction between them. The early claims for differences in standard curves for thin-layer molecular weight estimations with various batches of Sephadex[9] could not be confirmed. Identical R_M values were obtained with different batches of Sephadex G-75, G-100 and G-200.

All the proteins proposed for calibration of gel filtration columns proved to be suitable for establishing the quantitative relationship between migration rate and molecular weight on thin layers. Globular proteins, which on column gel filtration yield values consistent with those obtained by other methods, gave similar values also on thin-layer gel filtration, while carbohydrate-rich proteins, known to deviate on column gel filtration[3,24,28,36], migrate anomalously also on thin layers.

For some of the proteins a different behaviour was observed with different gel types. Thus peroxidase, a carbohydrate-containing enzyme[37,38], with a molecular weight of 40 000 was found to have a molecular weight of 48 000 and 46 000 by thin-layer gel filtration on Sephadex G-75 and G-100, respectively. By thin-layer gel filtration on Sephadex G-200, however, a value of 41 000 was obtained for the molecular weight. Ovalbumin, another glycoprotein, fitted well into the straight-line correlation between the molecular weight and the R_M value for the G-75 and G-100 gel;

it was however repeatedly found to deviate from the straight line on Sephadex G-200. The reason for this anomalous behaviour on certain gel types is not known.

The molecular weight obtained for bovine γ-globulin was 200 000, which is very close to the value obtained on column gel filtration[24], but is considerably higher than the accepted molecular weight of 169 000 (see ref. 39). Haemoglobin has been shown to dissociate on column gel filtration[3,28] and this dissociation has also been observed in thin-layer gel filtration[4]. The R_M value obtained for a 1–2% protein concentration corresponds to a molecular weight of 35 600, a value very close to one-half of that obtained by sedimentation and X-ray crystallographic data. The concentration-dependent dissociation of β-lactoglobulin, found on column gel filtration[3], however, was not observed when 0.5–3% solutions of this protein were separated by thin-layer gel filtration of Sephadex G-75 and G-200.

While there is general agreement that the gel filtration behaviour of proteins is correlated to molecular size, there is some uncertainty as to which size parameter is decisive. In a theoretical treatment of the gel filtration process a relationship between the gel filtration behaviour of proteins and their Stokes radii has been deduced[31,40]. For most proteins studied, useful empirical correlations have been obtained between gel filtration behaviour and molecular weight or gel filtration behaviour and Stokes radii or diffusion coefficients. The basis for this is both the similar frictional ratios and the partial specific volumes of the proteins used[3,24,28,29,31,37]. For ferritin, urease and fibrinogen, it has been claimed that the gel filtration behaviour is a function not of the molecular weight but of the Stokes radius[32]. This claim may be criticized because there is no general agreement on the molecular weight of ferritin or urease and because fibrinogen belongs to the group of carbohydrate-containing proteins for which a deviation from normal behaviour on gel filtration has been observed[8].

When ferritin and fibrinogen with myoglobin added as an internal standard were run on the same plates of Sephadex G-200, a higher R_M value was found for the latter. With an R_M value of 2.485 fibrinogen does not fit into the linear relationship between the R_M values and the molecular weight, but it correlates fairly well with the R_M–Stokes radius relationship (Fig. 9, eqn. (6)). Ferritin and apoferritin, prepared from it by reduction with sodium dithionite, gave identical R_M values when run on the same plates with myoglobin added as an internal standard. Since the molecular weight of ferritin depends on the iron content, its iron-free protein component, apoferritin, with a defined molecular weight of 465 000 and the same molecular diameter as ferritin, is much better suited for determining if it is the molecular weight or the Stokes radius which determines gel filtration behaviour. With an R_M value of 2.34, apoferritin did not fit into the straight line of the R_M value–molecular weight relationship (Fig. 6, eqn. (3)). The Stokes radius obtained for this value corresponded to 80 Å, a value very close to that calculated in one of the papers postulating gel filtration behaviour to be a function of the Stokes radius[32]. This value, however, is in conflict with the available data on the diameter of apoferritin, derived from X-ray diffraction measurements on wet crystals, by which a diameter of 122 ± 6 Å was obtained[41]. A diameter of about 100 Å was determined by electron microscopy[42–44]. The difference may be due to molecular shrinkage caused by loss of water. Thus the results obtained with ferritin, apoferritin and fibrinogen are not consistent. A possible reason for the deviation observed for apoferritin appears to be the heterogeneity of ferritin and the apoprotein derived from it, which has been well established in gel

electrophoretic experiments and has also been confirmed in this paper by thin-layer gel filtration on Bio-Gel P-300.

On starch gel and polyacrylamide gel electrophoresis, crystalline ferritin and apoferritin have been shown to occur as several distinct components, termed α, β and γ, according to decreasing electrophoretic mobility[25–27,45]. By a number of methods, including column gel filtration on Sephadex G-200, an enrichment of some of the components has been achieved[45,46]. Ultracentrifugal analysis provided evidence that the electrophoretically distinct apoferritin components differ in molecular size and probably correspond to the dimer, trimer and even higher polymers of a monomeric unit with the molecular weight of 465 000 (see refs. 45 and 47). Whereas crystalline ferritin gave on thin-layer gel filtration with Sephadex G-200 only a single zone, with Bio-Gel P-300 three zones were detected (Fig. 10), which on densitometry were found to amount in the order of increasing migration rate, to 80%, 16% and 4% of the total material, respectively. These figures compare well with those obtained on gel electrophoresis[27], where 75–85% of α-ferritin, 10–15% of β-ferritin and about 10% of γ-ferritin have been found. Thin-layer gel filtration on Sephadex G-200 of the three ferritin fractions isolated by the preparative method on Bio-Gel P-300 yielded different R_M values; the highest R_M value was obtained for Fraction I, the lowest for Fraction III.

The results obtained with the three fractions, isolated after preparative thin-layer gel filtration, indicate that a separation of the electrophoretically distinct components of ferritin can also be achieved by means of gel filtration, provided a suitable gel is chosen. It is an unsolved question whether the small amounts of α-ferritin present in Fraction I and the β- and γ-ferritin present in Fractions II and III are due to overlapping or whether they reflect association–dissociation phenomena[48].

ACKNOWLEDGEMENTS

This study was carried out at the Institute of Biology and Agriculture, Reactor Center, Seibersdorf, Austria, within the framework of the International Programme on Food Irradiation (Seibersdorf Project). The skilful technical assistance of Miss K. LENTSCH is gratefully acknowledged. The author is much indebted to Mr. H. DELINCÉE for the calculations on an IBM computer. This work was generously supported by the Deutsches Bundesministerium für Wissenschaftliche Forschung.

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CHROM. 3725

THIN-LAYER GEL FILTRATION OF PROTEINS

II. APPLICATIONS

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(Received July 31st, 1968)

SUMMARY

Human and animal serum proteins have been separated into three fractions on Sephadex G-200. The percentage contents of these fractions in human serum were estimated after densitometric evaluation.

Glycoproteins of horse and human serum have been separated on Bio-Gel P-300 thin layers and detected by periodic acid–Schiff staining.

Soluble proteins from mammalian cells grown *in vitro* (normal human fibroblasts and HeLa cancer cells) have been separated on Sephadex G-200 into six fractions. The quantitative contents and the molecular weights of these fractions have been determined.

Five fractions have been detected in soluble leaf proteins from spinach, the molecular weights ranging from 18 600 to more than 700 000.

The molecular weight of *o*-diphenol oxidase from mushrooms was 50 000 for a mixture of polyphenols and tyrosine used as a substrate. In addition to this enzyme, in mushrooms with an open cap another enzyme appeared with a molecular weight of 115 000. The molecular weight of a commercial tyrosinase preparation was 135 000.

In the previous paper several aspects of thin-layer gel filtration of proteins were examined and an improved method was described[1]. Linear relationships between migration, referred to a standard protein (myoglobin), and molecular weight or Stokes radius of a number of well defined globular proteins were established, thus providing a basis for estimation of these size parameters. Application of the sample as a streak and subsequent densitometric evaluation of the pattern has made possible a quantitative approach which was considered to be of value in analyzing heterogenous protein systems. In the present paper some applications of this modified method are described, including separation of protein systems of both animal and plant origin. In addition to separation of serum proteins on Sephadex G-200, separation of serum glycoproteins in thin layers of the polyacrylamide Bio-Gel P-300 is also described. The usefulness of thin-layer gel filtration for the rapid determination of the molecular weight of enzymes is illustrated by the results obtained with mushroom *o*-diphenol oxidase (EC 1.10.3.1).

EXPERIMENTAL

Materials

Sephadex gel G-200 "superfine" was obtained from Pharmacia, Uppsala, Sweden. The polyacrylamide gel Bio-Gel P-300 came from Bio-Rad Laboratories, Richmond, Calif., U.S.A. Myoglobin was supplied by Koch and Light, Colnbrook, England and tyrosinase was obtained from Sigma, St. Louis, U.S.A. Human and animal sera were used without added preservatives within a few days after preparation.

METHODS

Preparation of the plates, application of the sample, separation, staining and densitometric evaluation were carried out as described in the previous paper[1].

Protein-bound carbohydrates

Protein-bound carbohydrates were detected by the periodic acid–Schiff reaction (PAS reaction). The Schiff reagent[2] was diluted before use to 1:1, v/v with 95% ethanol[3]. The stained strips were washed in a $NaHSO_3$ solution acidified with HCl and subsequently with acetone to which 1% trichloroacetic acid had been added[4]. For densitometry a 550 filter was used.

Soluble proteins from mammalian cells grown in vitro

HeLa cells and human fibroblasts after 10–20 passages (Institute of Histology, University of Vienna) were cultivated as monolayers either in a lactalbumin hydrolysate medium (HeLa) or in Parker's medium 199 (fibroblasts) supplement by 5% chicken embryonic extract and 10–20% human umbilical cord serum with the addition of antibiotics[5]. The cells were detached from the glass by EDTA treatment, washed four times with 0.9% NaCl solution containing 0.02% KCl and $CaCl_2$. Before use the washed cells were kept as a sediment at −30°. The cells (50–200 × 10^6) were homogenized in a Potter–Elvehjem homogenizer, in 1–3 ml 0.02 *M* phosphate buffer (pH 7.2–7.4), supplemented by three freeze-thawing cycles at −30°. The homogenate was centrifuged at 34 000 *g* for 15 min and the supernatant concentrated by evaporation *in vacuo* to about 2–3% protein concentration.

Leaf proteins

Leaf proteins were isolated from spinach. *Step 1*: Ten grams of fresh spinach leaves were homogenized with 50 ml of a 4 *M* $(NH_4)_2SO_4$ solution in 0.05 *M* phosphate buffer (pH 7.2–7.4) in a MSE homogenizer operated at full speed for 3 min, the vessel being cooled in an ice bath. The resulting suspension of protein precipitate and cell debris was filtered through a Büchner funnel and the precipitate washed on the filter with 4 *M* $(NH_4)_2SO_4$ solution. *Step 2*: After washing, the precipitate was extracted with 0.1 *M* phosphate buffer (pH 7.2–7.4) and centrifuged at 34 000 *g* for 15 min. The sediment was once again extracted with 0.1 *M* phosphate buffer and centrifuged as above. *Step 3*: The supernatants from both centrifugations were combined and the soluble proteins salted out with ammonium sulphate (70 g/100 ml). The protein precipitate was separated by high-speed centrifugation and the sediment obtained was dissolved in 0.1 *M* phosphate buffer (pH 7.2–7.4). The protein solutions were used

in 2–4% concentration. Sometimes the solutions were found to have an exceptionally high viscosity, but this could be reduced by repeating Step 3.

o-Diphenol oxidase

Common cultivated mushrooms, either fresh or frozen at −20°, were employed as a starting material for the preparation of *o*-diphenol oxidase. In most experiments a single mushroom was used. It was homogenized at 2–4° in a porcelain mortar for 3–5 min with an equal volume of pre-chilled, deaerated 0.1 *M* phosphate buffer, pH 7.2–7.4. In some preparations ascorbic acid was added to the phosphate buffer to obtain a final concentration of 50 m*M* in the homogenate. The homogenates were centrifuged immediately in the cold at 34 000 *g* for 15 min. After centrifugation, 10–20 μl of the clear, yellow supernatant were placed without delay on a Sephadex G-200 plate with myoglobin as a standard in the middle. After a run of 5–7 h, a print was taken with Whatman No. 3 paper impregnated with a mixture of polyphenols and tyrosine (mixed substrate) or with tyrosine alone. The mixed substrate, prepared in deaerated 0.02 *M* buffer phosphate pH 7.2–7.4, contained caffeic acid (0.0015 *M*), DOPA (3,4-dihydroxyphenylalanine, 0.0015 *M*), pyrocatechol (0.01 *M*), pyrogallol (0.01 *M*) and tyrosine (0.0015 *M*); the latter was also used alone at the same concentration. The paper was dipped into the substrate and was subsequently dried at room temperature. To detect enzyme activity, the paper was left in contact with the gel for about 5–10 min, then removed from the plate and dried at room temperature. Maximum colour usually developed after 20 min. A decrease in colour was observed during the next few hours; the remaining colour was stable.

RESULTS

Serum proteins

A separation on Sephadex G-200 of normal serum proteins from pooled human and animal sera is shown in Fig. 1. Three fractions can be clearly distinguished in both the human and the animal sera, corresponding to the three principal classes of proteins with sedimentation constants of 4*s*, 7*s* and 19*s*. Densitometric tracings of two of the sera are shown in Fig. 2.

Fig. 1. Thin-layer gel filtration of serum proteins on Sephadex G-200. Sample: 10 μl of undiluted serum proteins. Plate 20 × 20 cm. Separation time: 6 h. Staining with Amido Black 10B. From left to right: human, horse, pig and bovine serum. From top to bottom: 4*s*, 7*s* and 19*s* components of serum.

Fig. 2. Densitograms of (A)-human and (B)-horse serum proteins separated on Sephadex G-200 From left to right: 19*s*, 7*s* and 4*s* serum components.

While all the sera were qualitatively similar, distinct quantitative differences in the pattern of human and horse serum can easily be seen in the content of the 7*s* fraction.

The R_M values for the three fractions were determined in experiments using the method of internal standard. The sera were diluted with 0.5 *M* NaCl containing 0.02 *M* phosphate buffer (pH 7.2–7.4), myoglobin being added to a final concentration of 1%. Ten microliters of the mixture were applied to the plate. In Table I the R_M values measured on the densitometric tracings for the maxima of the peaks are shown. Included also are the percentage contents obtained on planimetry of the area in the densitogram for several dilutions, after staining with Coomassie Blue and Amido Black. The latter values were corrected for incomplete removal of albumin by the print[1].

The periodic acid–Schiff staining method (PAS reaction) cannot be applied to prints of Sephadex thin layers because of heavy background staining. As an alternative, thin-layer gel filtration on the polyacrylamide Bio-Gel was tried and found to be suitable. Two preparations of Bio-Gel P-60 and P-300 having different optimal fractionation ranges were employed. With the latter, serum glycoproteins were separated into three to four zones (Figs. 3 and 4). In order to compare the glycoprotein

TABLE I

R_M VALUES AND PERCENTAGE CONTENTS OF HUMAN SERUM PROTEINS SEPARATED BY THIN-LAYER GEL FILTRATION ON SEPHADEX G-200

Component	R_M *value*	*Serum dilution*[a]					
		Coomassie Blue			*Amido Black 10 B*		
		1 + 4	1 + 6	1 + 8	1 + 2	1 + 3	1 + 4
4*s*	1.49	77.9	76.8	84.6	70.5	75.9	78.1
7*s*	1.99	20.2	21.3	13.8	25.9	21.0	19.7
19*s*	2.35	1.9	1.9	1.5	3.6	3.1	2.2

[a] Dilution with 0.5 *M* NaCl + 0.02 *M* phosphate buffer, pH 7.2–7.6.

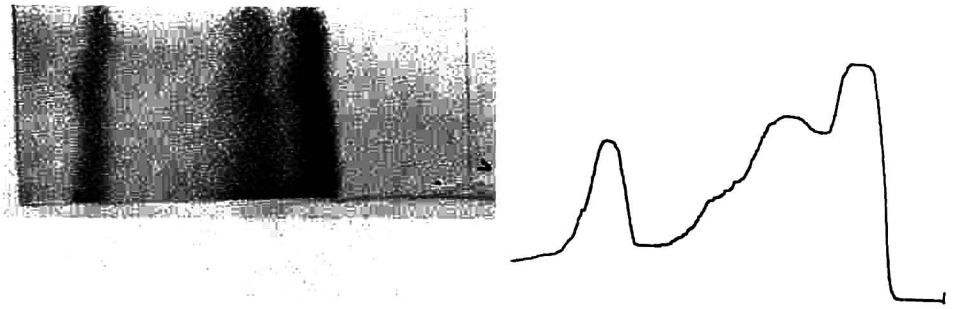

Fig. 3. Thin-layer gel filtration of glycoproteins on Bio-Gel P-300. Sample: 10 μl per cm of undiluted horse serum. Periodic acid–Schiff staining. From left to right: 19*s*, 7*s* and 4*s* components.

Fig. 4. Densitogram of horse serum glycoproteins separated on Bio-Gel P-300.

pattern with that of the proteins, 70 μl of undiluted serum were applied by means of a microscopic slide (length 7.5 cm) to a 20 × 20 cm plate, two samples being run onto one plate. After a run of 25–30 h, a print was taken with filter paper Whatman No. 3 and dried as usual at 110°. Afterwards the print was cut lengthwise along each separated sample and one half was stained with Amido Black for protein detection; the other half was stained by the PAS reaction. The protein pattern was very similar to that obtained on Sephadex G-200. Three zones corresponding to the 4*s*, 7*s* and 19*s* components were detected. The zone of the 7*s* fraction, however, was broader. It was composed of a weakly stained leading fraction and a strongly stained fraction migrating more slowly. PAS staining also revealed three to four zones in human and animal sera. Quite a considerable fraction of the PAS-stained material resides in the 19*s* fraction. The PAS-stained material parallels that stained with Amido Black for protein detection; the relative distribution, obtained with both methods, however, is quite different.

Soluble proteins of mammalian cells grown in vitro

Soluble proteins were isolated from normal human fibroblasts and HeLa cancer cells, the 34 000 *g* supernatant being used for the gel filtration experiments. Since rather limited amounts of cells grown *in vitro* are available, Coomassie Blue was tried as a stain and found to be much superior to Amido Black due to its increased sensitivity. When fibroblast and HeLa soluble proteins were separated on Sephadex G-200 on the same plates, a complex pattern of six fractions was observed (Figs. 5 and 6). There

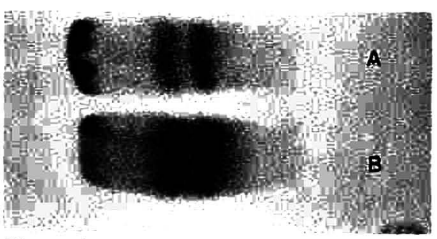

Fig. 5. Thin-layer gel filtration on Sephadex G-200 of soluble proteins from (A) normal human fibroblasts and (B) HeLa cancer cells. Staining with Coomassie Blue.

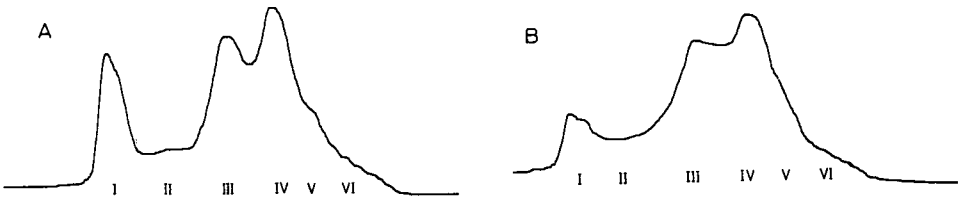

Fig. 6. Densitograms of soluble proteins from (A) normal human fibroblasts and (B) HeLa cancer cells (see Fig. 5).

was a basic similarity in both patterns, but quantitative differences in the contents of different fractions can easily be noted in the densitometric tracings. The R_M values for the particular fractions were determined from the densitograms by extrapolating the peaks to Gaussian curves and drawing a perpendicular from the top of the peak to the base line. The distance of this perpendicular from the starting line was measured and divided by the distance for myoglobin similarly obtained. In this way, R_M values for the weak fractions can be estimated with greater accuracy than by direct measurement on the plate. For the weakest fractions, measurements directly on the plate become impossible. The R_M values, the corresponding molecular weights[1] and the quantitative percentage contents of these fractions obtained in eight runs of each material are shown in Table II. The percentage contents of fractions III to VI were corrected for incomplete removal by the print technique as described previously[1].

TABLE II

PERCENTAGE CONTENTS, R_M VALUES AND MOLECULAR WEIGHTS OF SOLUBLE PROTEINS FROM MAMMALIAN CELLS GROWN *in vitro* AFTER THIN-LAYER GEL FILTRATION ON SEPHADEX G-200

Fraction	*Fibroblasts*			*HeLa cells*		
	% content	*R_M value*	*Molecular weight*	*% content*	*R_M value*	*Molecular weight*
I	17.6	2.36	⩾600,000	11.5	2.35	⩾600,000
II	8.5	2.02	240,000	9.6	2.10	300,000
III	28.8	1.72	107,000	32.1	1.76	120,000
IV	31.4	1.48	56,000	30.4	1.50	59,000
V	9.4	1.26	31,000	11.6	1.26	31,000
VI	4.3	1.09	19,600	4.8	1.09	19,600

Soluble plant proteins

Soluble leaf proteins from spinach leaves could be separated into five fractions (Fig. 7). The R_M values of these fractions after staining with Coomassie Blue were determined from the densitometric tracings after extrapolation to Gaussian curves as described above for the soluble proteins derived from mammalian cells. The molecular weights of these fractions are shown in Table III together with their percentage contents corrected for incomplete removal by the first print as described previously[1]. A few experiments were performed with soluble proteins isolated from potato tubers. Again a complex pattern of three to four fractions was observed.

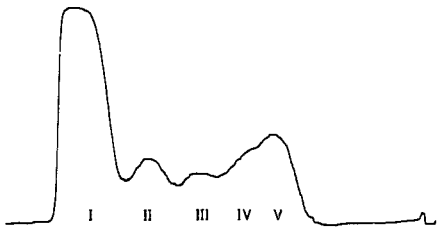

Fig. 7. Soluble leaf proteins from spinach separated by thin-layer gel filtration on Sephadex G-200. Densitogram after staining with Coomassie Blue.

TABLE III

PERCENTAGE CONTENTS, R_M VALUES AND MOLECULAR WEIGHTS OF SOLUBLE LEAF PROTEINS SEPARATED BY THIN-LAYER GEL FILTRATION ON SEPHADEX G-200

Fraction	*% content*	R_M *value*	*Molecular weight*
I	38.1	2.42	$\geqslant$700,000
II	14.0	1.95	200,000
III	11.3	1.62	82,000
IV	10.8	1.36	40,000
V	25.8	1.07	18,600

Enzymes

Thin-layer gel filtration has been used to determine the molecular weight of *o*-diphenol oxidase (EC 1.10.3.1) from mushrooms. A homogenate prepared from mushrooms frozen at −20°, as described under Methods, contained enough enzyme activity to be easily detected on the gel. The homogenate was applied to Sephadex G-200 plates immediately after its preparation to avoid any storage effect. After a run of 4–7 h, a print was taken with a dry filter paper Whatman No. 3 impregnated previously with a mixture of polyphenols containing caffeic acid, DOPA, pyrocatechol, pyrogallol and tyrosine. Alternatively a paper treated with tyrosine only was used.

The substrate-impregnated paper was left in contact with the gel for about 5–10 min. An intensive reaction could be observed within well defined symmetric zones comparable in size to those of standard proteins after staining with Amido Black (Fig. 8). The distances of the enzyme zones from the starting line were measured directly on the plates, as well as the distance of myoglobin, which was sufficiently distinct to be evaluated without staining. The R_M values obtained for a number of different preparations were 1.42, corresponding to a molecular weight of ~50 000. There was no difference in the R_M values obtained with mixed substrate or with tyrosine.

Homogenates prepared in the presence of ascorbic acid sometimes gave qualitatively different pictures from those prepared in its absence. The ascorbic acid homogenates contained, in addition to the R_M 1.42 component, a second component visible as a more intensive narrow zone (3–4 mm broad) in the trailing part of the main zone. This zone could be detected both with the mixed substrate and with tyrosine. The R_M value obtained was 1.30, corresponding to a molecular weight of 35 000.

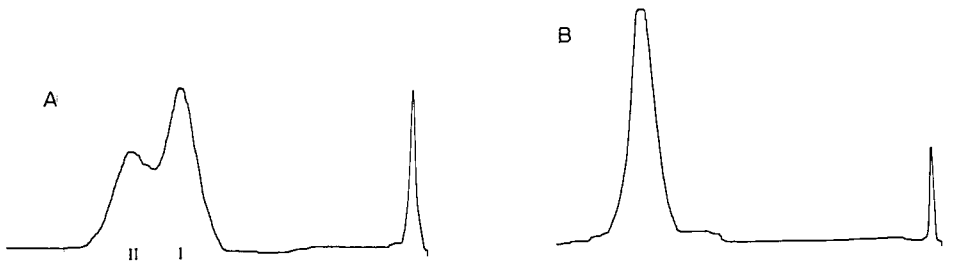

Fig. 8. *o*-Diphenol oxidase separated by Sephadex G-200 thin-layer gel filtration. Densitogram of a print taken with paper impregnated with a mixed substrate containing caffeic acid, DOPA, pyrocatechol, pyrogallol and tyrosine. A — Homogenate of a mushroom with an open cap; B — commercial "tyrosinase". Molecular weight (I) ~50 000; (II) ~115 000. Peaks on right: starting line.

Homogenates prepared without or with the addition of ascorbic acid and stored overnight at 4° in a closed vessel without access of air, showed an appreciable loss of activity, but the R_M values remained unchanged. Salting out of the proteins from the freshly prepared homogenate with ammonium sulphate at 4° at a saturation of 0.66, or precipitation with acetone at −30° and subsequent redissolving of the precipitate to the original volume of the homogenate, had no influence on the R_M value. However, heating the homogenate at 60–65° for a few minutes yielded preparations which could be resolved by thin-layer gel filtration into two zones of activity. In addition to the zone present in the untreated homogenate a component with a higher R_M value was also observed.

With a commercial preparation of "tyrosinase" (Sigma Chemical Company, St. Louis, U.S.A.) only one zone of activity was observed with an R_M value of 1.80, both for the mixed substrate and tyrosine, corresponding to a molecular weight of 135 000, which is much higher than that obtained with the freshly prepared homogenates.

Using mushrooms of differing maturity, however, a striking difference was observed. Mushrooms with a closed cap showed only one zone of enzyme activity with an R_M value corresponding to the 50 000 molecule, whereas homogenates of mushrooms with a cap already open yielded two zones of activity, one corresponding to the 50 000 molecule, the other with an R_M value of 1.75, corresponding to a molecular weight of 115 000 (Fig. 8A).

DISCUSSION

Some of the applications of thin-layer gel filtration described in this paper serve to emphasize the versatility of the method. Separation of serum proteins using gel columns has become an established procedure, and it was also the object of early applications of thin-layer gel filtration[6–10]. A simple and reproducible method of separating serum proteins should be of considerable clinical importance. Three fractions could be distinguished in both human and animal sera corresponding to the three principal classes of proteins having the sedimentation constants 4s, 7s and 19s. The assignment of the components to ultracentrifugal classes has been demonstrated in column gel filtration experiments[11].

The R_M value determined for the 4s fraction on thin-layer gel filtration differs from that of isolated albumin[1], the discrepancy probably being due to the heterogeneity of this peak. Albumin is the main component of the 4s peak, but it is accompanied by other components differing in molecular weight[12]. For the 19s fraction an R_M value of 2.35 would correspond to a molecular weight of about 600 000, assuming that the R_M value–molecular weight relationship in this range is still linear. It has been pointed out, however, that there is insufficient evidence for linearity of the R_M value–molecular weight relationship in the range of molecular weights above 240 000 (see ref. 1).

The percentage contents of the three classes of serum proteins, after planimetric evaluation, clearly depend on dilution of the serum (Table I). With increasing protein concentration, the values for the 4s fraction decrease. For some of the dilutions, the percentage contents are fairly constant with both of the dyes tested. Depending on the aim of the analysis two dilutions will probably yield more accurate values for the 4s/7s and 7s/19s ratios than can be obtained with only one dilution. The values for the percentage contents of the three classes of serum proteins obtained on thin-layer gel filtration with subsequent densitometric evaluation are very similar to those obtained by elution of proteins stained with Amido Black from a paper print[9], the main difference being in the values for the 19s fraction.

The data on the quantitative distribution of the three classes of serum proteins in thin-layer gel filtration (Table I) may be compared with those obtained on ultracentrifugation. For the 4s, 7s and 19s, peak percentages of 83–86, 11–13 and 3–4, respectively, are accepted[12,13]; however, values which are lower for the 4s and higher for the 7s components were obtained in recent ultracentrifugal estimations[14,15].

The densitogram of the stained serum proteins separated by thin-layer gel filtration is distinct from the pattern obtained by column gel filtration. The observed difference is due to the different detection methods. In column gel filtration, proteins are detected either by ultraviolet absorption at 280 mμ or shorter wavelengths with some of the flow analyzers, or by the Folin reaction. With all these methods, the results depend strongly on the amino acid content of the proteins. Great differences in the specific extinction coefficients for the three classes of serum proteins have been demonstrated[14].

Gel filtration has already been successfully applied to the analysis of paraproteins[14,16–18]. Recently characteristic column gel filtration patterns for a number of pathological conditions have been described and their value for differential diagnosis stressed[19]. The usefulness of thin-layer gel filtration for the separation of pathologic sera has already been demonstrated[6,7,9,20]. The method described here should provide a basis for a simplified quantitative approach to the analysis of abnormal serum proteins differing in molecular size. Due to the small amount of material needed for analysis, some of the protein-poor body fluids, in addition to serum proteins, could be easily analyzed by this method.

While lipoprotein detection has been successfully performed after thin-layer gel filtration of serum proteins[6], there are no reports on the separation of glycoproteins. This is probably due to technical difficulties with Sephadex gels. Thin-layer gel filtration on Bio-Gel P-300 proved to be a useful approach. The results obtained by this method are in agreement with the physicochemical characteristics of some of the major serum glycoproteins[12]. In view of the importance of glycoprotein analysis for

clinical diagnosis, it would be interesting to study the glycoprotein distribution of pathological sera.

Thin-layer gel filtration of soluble plant proteins on Bio-Gel P-60 with subsequent PAS and Amido Black staining, has made it possible to separate proteins from polysaccharides[21]. The presence of polysaccharides in protein preparations of plant origin presents a problem in the separation of both macromolecules by different fractionation procedures. Thin-layer gel filtration could in such cases offer a rapid answer about the efficiency of these procedures.

Due to the microgram quantities of material necessary for analysis, thin-layer gel filtration may be anticipated to be particularly useful in cases where only small amounts of material are available. With mammalian cells grown *in vitro*, the amount of material appears to be a limiting factor and an attempt was made, therefore, to separate by thin-layer gel filtration the soluble proteins derived from normal human fibroblasts and HeLa cancer cells. It has been previously shown that HeLa proteins can be separated on Sephadex G-200 columns[5]. A much improved separation can be achieved by thin-layer gel filtration on Sephadex G-200, by which also a comparison with soluble proteins from human fibroblast run simultaneously on the same plate has been made possible (Fig. 5).

The molecular weights determined for the fibroblast and HeLa soluble proteins (Table II) bear a striking similarity to the values obtained on the analysis of soluble rat liver proteins[22]. The molecular weight of the soluble liver proteins has been interpreted in terms of a hypothetical size regularity. The unit of size was assumed to be about 10,000 (u) and 30,000 ($3u$). More than 80% of the soluble liver proteins was demonstrated to belong to size classes whose approximate molecular weights are multiples of these units. The same regularity can be seen in the distribution of the molecular weights of mammalian cells grown *in vitro* (Table II). About 75% of HeLa and fibroblast soluble proteins can be accounted for in terms of the hypothetical size regularity postulated for the soluble liver proteins.

There is an increasing number of papers dealing with the electrophoretic properties of soluble leaf proteins[23–28], but there is only very limited information on their size parameters. This is due partially to technical difficulties in the isolation of soluble leaf proteins which become easily precipitated by some of the components present in plant cells, thus necessitating the use of protective agents[29,30]. Among the better characterized soluble leaf proteins is the so-called "Fraction I protein" which is a major component found in leaf extracts of various plants[29,31–36], and with which a number of enzyme activities are associated[32,33,36]. Column gel filtration has already been successfully applied for the isolation and purification of "Fraction I protein". The molecular weight of "Fraction I protein" is about 600,000 (see ref. 36). Both the high R_M value and the high percentage content of our Fraction I (Fig. 7), amounting to 40% (Table III), found on thin-layer gel filtration of soluble leaf proteins suggest that this fraction is probably identical with the "Fraction I protein" studied by other workers.

The molecular weight for Fraction I on thin-layer gel filtration is about 700,000, a value close to those accepted for "Fraction I protein". In view of the uncertainty in the molecular weight estimations in the range above 240,000 (see ref. 1), this value is to be considered only an approximation. Calculation of the Stokes radius for Fraction I according to eqn. 6 (see ref. 1) gives a value of ~180 Å for the diameter, which is much

higher than that of 120 Å found in electron microscopy[35]. Molecular shrinkage, due to water loss during preparation for electron microscopy, or aggregation of the "Fraction I protein", which has been well established during purification and storage[32], or reversible association–dissociation phenomena could be the explanation for this discrepancy.

In addition to the high molecular weight Fraction I, four other fractions of lower molecular weight in the range from 20 000 to 200 000 could be detected on thin-layer gel filtration of soluble leaf proteins (Fig. 7, Table III). Similar to soluble proteins from mammalian cells grown *in vitro*, three of the fractions show size regularity with a unit size of about 20 000. It is uncertain whether the size classes of 40 000 and 80 000 represent molecules already present in the cell or whether they arise during their isolation as a result of interaction with other cell components. Comparative studies with different isolation procedures, especially those employing various protective agents[37], could help to clarify this question. Thin-layer gel filtration of soluble leaf proteins could prove of value in studies on protein changes associated with leaf aging, as well as with phytopathological processes, since only small amounts of material are needed and single leaves will yield sufficient material for analysis.

One of the potential advantages of thin-layer gel filtration is the possibility of detecting enzymes by using a paper impregnated with the appropriate substrate to give a coloured reaction, or by spraying the paper with the substrate. Enzymes can be specifically located by this means. If no colour reaction is available for detection, an alternative approach is to elute the substance from the paper print with an appropriate buffer, enzyme determinations being then performed with the eluates[38]. As with the column gel technique, the thin-layer method may also be applied to studies of labile enzymes which could be damaged on purification. Besides the small amounts of material required, an additional advantage of the thin-layer method is its greater speed, when compared with the column. Within a few hours after the preparation of the crude enzyme extract, the run can easily be completed. In addition to speed, separation of interfering substances may prove to be another useful property of the method. Preparative thin-layer gel filtration[1] of enzymes can easily provide sufficient material for detecting enzyme activity and also for application of other microanalytical methods, *e.g.* disc electrophoresis or immunodiffusion tests.

The experiments with *o*-diphenol oxidase from mushrooms described in this paper were intended to illustrate some of the potentialities of thin-layer gel filtration of enzymes. There are conflicting data on the molecular weight of this enzyme in a number of papers; most of them agree on a molecular weight of 100 000–130 000 for the purified enzyme[38–41]. The results of thin-layer gel filtration give an estimated molecular weight of 50 000 for the enzyme in the homogenate and of 135 000 for a commercial preparation of "tyrosinase", thus strongly suggesting the presence of enzymatically active subunits that combine under certain conditions to yield a product of higher molecular weight. It is not clear whether this association of smaller units to a component of higher molecular weight is a physiological phenomenon or whether it represents a preparative artifact.

There was a striking difference when homogenates of mushrooms of differing maturity were analyzed. An additional component of higher molecular weight was consistently observed in older mushrooms in which the cap was already open. This need not necessarily reflect the state of the enzyme in the cell, but could also be an

isolation artifact due to interaction of the enzyme with other components appearing in mushrooms of greater maturity.

There was no change in the molecular weight of the 50 000 enzymes, either by precipitation from the homogenate with acetone or by salting out with ammonium sulphate, both of which are treatments routinely employed in the purification of this enzyme. The even greater complexity of the situation is stressed by the presence of two components (35 000 and 50 000) which sometimes could be detected when the homogenate was prepared with the addition of ascorbic acid. Recombination of these subunits would give enzymes with a molecular weight in the range between 100 000–130 000, which would compare well with the molecular weight of purified enzyme preparations. Recombination of subunits could also provide an explanation for the electrophoretic and chromatographic heterogeneity of polyphenol oxidase preparations[42–44].

ACKNOWLEDGEMENTS

This study was carried out at the Institute of Biology and Agriculture, Reactor Center, Seibersdorf, Austria, within the framework of the International Program on Food Irradiation (Seibersdorf Project). The excellent technical assistance of Miss K. LENTSCH is gratefully acknowledged.

The author wishes to thank Dr. G. KELLNER for providing the mammalian cells grown *in vitro*. This work was generously supported by the Deutsches Bundesministerium für Wissenschaftliche Forschung.

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CHROM. 3734

PHOTODENSITOMETRY IN THE THIN-LAYER CHROMATOGRAPHIC ANALYSIS OF NEUTRAL LIPIDS

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(First received May 3rd, 1968; revised manuscript received July 11th, 1968)

SUMMARY

A procedure for photodensitometric quantitation of thin-layer chromatograms is described which can give a complete analysis of a mixture of neutral lipids in a single chromatogram without the necessity for reference mixtures.

A variety of methods is available for the quantitation of lipids separated by thin-layer chromatography[1,2]. Potentially, the most rapid and precise analyses would be obtained by photodensitometry of the chromatograms after the organic materials have been charred, particularly as described by investigators at the Hormel Institute[3–8]. However, several factors complicate the procedure so that it becomes a tedious and painstaking operation. Usually, several chromatograms are necessary to produce an analysis, and there is a constant need for comparison with reference mixtures. Ideally, it would be possible to obtain a complete analysis of a lipid mixture with one uninterrupted scan over a single thin-layer chromatogram. This ideal has now been attained by careful consideration of the principles which affect the photodensitometry of thin-layer chromatograms.

GENERAL PRINCIPLES

Photodensitometry implies measurement of the proportion of the incident light which is transmitted by a semi-opaque material. The method is relatively insensitive when the proportion of light transmitted falls below 5%, so that it is usual to work with optical densities below 1.3. It is obvious that for a spot on a chromatogram this limitation should apply not to the reading obtained on the photodensitometer, but to the actual density over all parts of the spot.

Provided one is working within the practical limits of optical density, spot size should be inversely proportional to spot density for any given quantity of substance. This will be true if the photodensitometer is in each case measuring only that light which is passing through the spot, and not light which is passing through blank areas

of the plate, although it is probably acceptable if the same proportion of the incident light passes through blank areas in each case. BLANK *et al.* recommended a slit 25% longer than the diameter of the spot[7]. This presumably meant that the slit had to be adjusted for each spot on a chromatogram, since the spots vary in size according to both quantity of substance and distance of migration. It would certainly mean that the maximum optical density recorded would be about 0.7, since even with a totally-absorbing spot there would still be at least 20% transmission between the edges of the spot and the ends of the slit. Under these circumstances it will not be noticed when density of a spot is beyond the measurable limit, in which case the readings would bear little relationship to density. However, with a fixed slit length the readings would be proportional to spot area, and since spot area is in turn proportional to the quantity of organic compound in the spot, at any particular R_F value, the readings may still be an effective means of quantitation. When the densitometer is thus used as a planimeter, the variation of spot size with R_F value, and other factors, necessitates the constant use of reference materials. Moreover, a reference compound is required for each class of compound in the chromatogram[9]. As many as seven chromatograms may have to be scanned to produce one analysis[10].

When the density over all or a major proportion of a spot is greater than the practical limit, further increase in density will be largely undetected. This will mean, firstly, that differences in charring yield will be eliminated. This may explain why many investigators have not detected differences in carbon yield between compounds of widely differing carbon content. Secondly, the inverse relationship between spot size and spot density will be lost, giving rise to the commonly-observed effect of increased "degree of charring" with increased distance of travel on the chromatogram. Both of these effects are apparent in the results reported by LOUIS-FERDINAND *et al.*[9].

In addition to the effect of variation in spot size, a number of factors will affect the density of the spot produced by a given amount of a compound after charring. The principal of these will be the charring reaction itself, which is achieved by a succession of oxidations and dehydrations to produce something approaching elemental carbon. There is the possibility of great variation in the efficiency of this conversion with any one compound, but the present stage of development indicates that conditions can be found under which yields are reproducible.

Basically, the proportion of carbon which a compound contains (the so-called "carbon-density") will govern the maximum yield of elemental carbon it can produce on charring. In any technique which involves spot density this factor must be taken into account, but it is one which involves a constant mathematical relationship.

Of more concern are factors which can vary for any particular compound. For instance, compounds which are not readily attacked by the charring agent are capable of evaporating from the thin-layer plate before they can be converted to carbon, although the excessive temperature of 360° has been cited[6] for instances where this has occurred. To avoid such evaporation it is common practice to include a powerful oxidizing agent, such as potassium dichromate, in the primary charring agent, which is usually sulphuric acid. It was recognized by PRIVETT *et al.*[8] that the dichromate reagent is capable of oxidizing organic compounds to carbon dioxide, with a consequent decrease in the yield of carbon, but there is no report that this possibility was investigated.

The foregoing principles have been taken into account in devising the present

procedure for quantitative thin-layer chromatography. In particular, the quantities of lipid in each chromatogram were limited to that which would produce spots of measurable optical density. This factor also minimized spot size, and the maximum size of spots was further defined by the use of narrow lanes on the chromatographic plates. The slit length was made slightly less than the width of the lanes, and with this arrangement there was an almost constant relationship between slit length and spot width. The charring procedure was carried out in a manner calculated to avoid both over-oxidation and evaporation. Development was performed in such a way as to resolve the mixtures of lipids on a single chromatogram.

Application of the technique to some representative mixtures of lipids indicates that it can achieve the major objectives of convenience and accuracy. It must be emphasized that the present application is confined to determination of the relative amounts of constituents in a mixture rather than the absolute amounts of lipid applied to the plates.

EXPERIMENTAL

Preparation of the thin-layer plates

The 20 × 20 cm glass plates were spread with a 0.25 mm layer of Silica Gel G (E. Merck and Co.) using a Quickfit-Reeve Angel plate-leveller and spreader. The layers were dried at 120° for 3 h and then stored in glass tanks. Before use the plates were developed overnight in ether to remove contaminants. The adsorbant layer was then divided into vertical lanes 7 mm wide. Immediately before application of the samples the plates were re-activated by heating at 130° for 30 min.

Development of the chromatograms

For a simple mixture of lipids, development in a single solvent mixture was usually adequate. For a more complex mixture of lipids however, a system of multiple development was employed. This consisted of:

(1) development with hexane to a line 1 cm below the top of the plate;

(2) after drying at room temperature for 10 min the plate was developed to the line with benzene;

(3) after drying again the plate was developed to the 10 cm level in a mixture of hexane–ether–acetic acid (70:30:1).

Multiple development techniques in which the more polar solvent systems precede the less polar should be avoided because the more mobile components form seriously-distorted spots under such conditions.

Charring of the chromatograms

The developed chromatogram was normally sprayed with 50% sulphuric acid, using a pressure pack can fitted with a polypropylene spray head and a glass reagent jar (Crown Industrial Products, Hebron, Ill.). The acid was sprayed as evenly as possible until the first appearance of fine spots of dampness on the silica gel. Actual wetness of the layer was rigorously avoided. The plate was then set upon a cold aluminium plate (8 × 8 × 0.25 in.) lying on a 6 × 6 in. hot plate (Autemp Heater, Fisher Scientific). Heating was commenced by turning the hot plate control to 5·5. The temperature rose from 20° to a maximum of 220° during a period of approximately

30 min. Under these conditions even saturated lipids began to char at about 180°, and by the time full temperature was reached, maximum darkening was achieved and most of the sulphuric acid had evaporated. A further 10 min heating was allowed for complete vaporization of the acid.

Scanning the chromatograms

The photodensitometer (Photovolt Corp., New York, Model 52-C) was fitted with a thin-layer chromatography search unit, a Varicord Recorder (Model 42 B) and an Integraph integrator (Model 49). A substage slit was installed which limited the incident light to a beam of 1 × 4 mm. A mask over the photocell was provided with a 0.02 × 4 mm slit.

The dimensions of the lanes on the thin-layer plate allowed it to be aligned on the stage of the search unit so that the beam of incident light passed symmetrically through each spot of the series of spots produced by each lipid sample. In this way it was possible to scan each lane in a single uninterrupted run.

Analysis of synthetic mixtures

A standard solution of each individual pure lipid (Applied Science Labs., Inc.) was prepared, and appropriate mixtures of these lipids were then obtained by combination of accurately-measured volumes of the standard solutions. The resulting mixtures contained between 0.3 and 3 μg per ml of each lipid, and usually between 1 and 2 μl of a solution was applied to a lane of the plate. The chromatograms were then developed, charred, and scanned on the densitometer as described. Normally, it was possible to calculate percentage composition directly from the integrator output on the recorder chart. Where variations in the baseline interfered with accurate

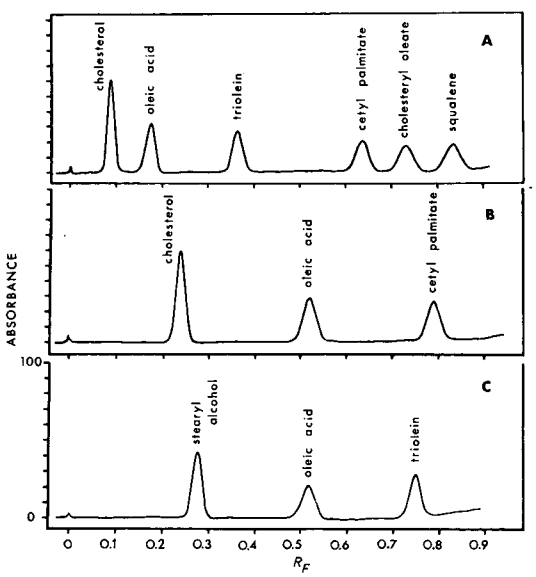

Fig. 1. Photodensitometer records of the thin-layer chromatograms of some synthetic mixtures of lipids. (A) Resolution obtained in the treble-development system. (B, C) Resolution in hexane–ether–acetic acid (70:30:1).

integration of a peak, its area was determined by triangulation and converted to integrator units by means of a factor determined experimentally. The number of integrator units for each peak was then adjusted by a factor derived by dividing the molecular weight of the respective molecule by the sum of the atomic weights of the carbon atoms in the molecule. The proportion of each constituent was then calculated from the adjusted integrator units.

RESULTS

Fig. 1A is a representative example of the densitometer output in scanning a chromatogram of a mixture containing equal weights of the various lipids. The resolution in this instance was obtained with the multiple development technique. It is apparent that the carbon yield for cholesterol was far greater, in relation to the other lipids, than would be expected on the basis of its carbon density. It was therefore necessary to determine whether this was due to chemical structure or to the position of cholesterol in the chromatograms, where it was the constituent closest to the origin. Mixtures containing cholesterol were chromatographed in the hexane–ether–acetic acid solvent system, which in this instance was allowed to migrate to the top of the plates, so that the cholesterol travelled a greater distance than usual, as shown in Fig. 1B. This did not affect the relative carbon yield. To determine whether it was the steroidal structure or the hydroxyl function which was responsible for the high carbon yield, a reference mixture containing a non-steroidal fatty alcohol, stearyl alcohol, was chromatographed, and a high carbon yield was obtained from this compound also (Fig. 1C). Finally, a mixture containing equal weights of cholesterol and stearyl alcohol was chromatographed, and the carbon yields for the two alcohols were virtually identical.

It can, therefore, be accepted that the hydroxyl function causes a greatly

TABLE I

ANALYSIS BY THIN-LAYER CHROMATOGRAPHY OF REFERENCE MIXTURES USING CORRECTION FACTORS FOR THE CARBON CONTENT OF THE RESPECTIVE MOLECULES AND FOR THE HYDROXYL GROUP EFFECT

7 mm lane width and 0.02 × 4 mm slit.

Constituents	R_F *value*[a]	*Composition (weight %)*			
		Sample 1		*Sample 2*	
		Found[b]	*Known*	*Found*[c]	*Known*
Cholesterol	0.09	16.6 ± 0.68	16.6	2.3 ± 0.34	2.5
Oleic acid	0.19	17.2 ± 0.53	16.6	26.4 ± 1.34	25.0
Triolein	0.36	17.3 ± 0.38	16.6	30.4 ± 1.51	30.0
Cetyl palmitate	0.63	17.5 ± 0.90	16.6	24.0 ± 1.59	25.0
Cholesteryl oleate	0.72	15.2 ± 0.90	16.6	2.4 ± 0.28	2.5
Squalene	0.82	16.1 ± 0.89	16.6	14.6 ± 1.23	15.0

[a] Distance travelled/height of plate in the treble development system.

[b] Mean and standard deviation for eight consecutive chromatograms on the same thin-layer plate.

[c] Mean and standard deviation for fifteen consecutive chromatograms on the same thin-layer plate.

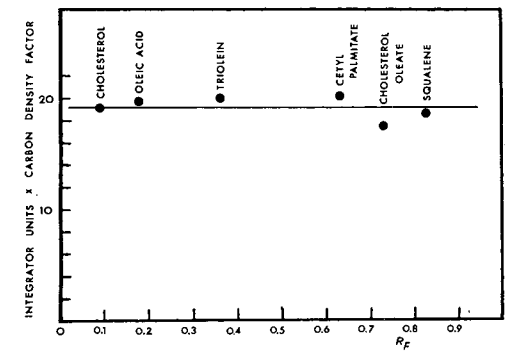

Fig. 2. Demonstration of the independence of the carbon yield from the distance of migration.

enhanced carbon yield compared with non-hydroxylated compounds, and it is necessary to allow for this effect in calculating the composition of mixtures containing free alcohols. The appropriate factor was derived experimentally from the relationship between the average of the peak areas for the non-hydroxylic compounds, adjusted for carbon content of the respective molecules, and the area of the cholesterol peak, also adjusted for carbon content. This showed that the area of the peak for an alcohol must be reduced by the factor 0.66, and this has been applied in the calculation of the results reported here.

Typical results are shown in Table I, which were obtained with a 7 mm lane width and 4 mm slit length. Fig. 2 illustrates the remarkable constancy of the carbon yield over the full extent of the chromatograms and for all of the lipids chromatographed, provided only that the correction factor for the high yield from free alcohols is applied and that the calculations are based on the proportion of carbon in the original molecules. Because of the constancy of the carbon yield it is possible to construct a graph of peak area against the weight of carbon for each compound chromatographed. This produces a straight line passing through the origin, as in Fig. 3.

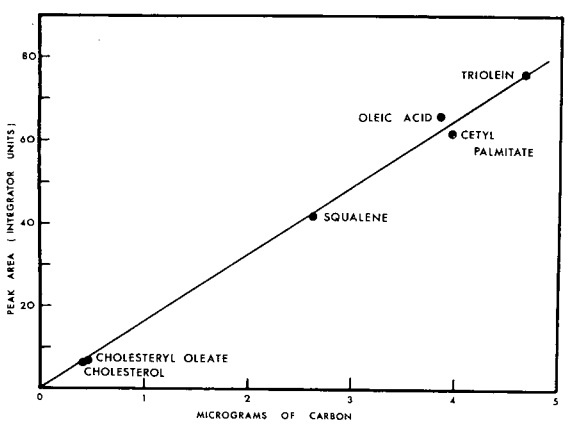

Fig. 3. Relationship between the weight of lipid carbon applied to a chromatoplate and the area of the peak produced in the photodensitometer scan.

TABLE II

EFFECT OF A WIDER LANE AND A LONGER SLIT ON THE ANALYSIS OF THE REFERENCE MIXTURES

14 mm lane width and 0.02 × 7 mm slit.

Constituents	R_F *value*	*Composition (weight %)*			
		Sample 1		*Sample 2*	
		Found[a]	*Known*	*Found*[b]	*Known*
Cholesterol	0.09	5.4 ± 0.30	16.6	2.3 ± 0.41	2.5
Oleic acid	0.20	13.0 ± 0.62	16.6	17.0 ± 1.51	25.0
Triolein	0.35	14.8 ± 0.56	16.6	20.8 ± 1.43	30.0
Cetyl palmitate	0.65	27.2 ± 1.10	16.6	34.5 ± 1.56	25.0
Cholesteryl oleate	0.76	20.3 ± 0.69	16.6	3.1 ± 0.36	2.5
Squalene	0.85	19.2 ± 0.69	16.6	22.4 ± 2.39	15.0

[a] Mean and standard deviation for eleven consecutive chromatograms on one thin-layer plate ruled with 14 mm lanes and scanned with a 7 mm slit length.

[b] Mean and standard deviation for twelve consecutive chromatograms on one thin-layer plate.

To demonstrate that the narrow lanes were a significant factor in maintaining a constant densitometer response over the length of the chromatograms, series of analyses were conducted in which wider (14 mm) lanes and a 7 mm slit length were employed. The results shown in Table II, which were obtained in this way, bear little relation to the actual composition of the reference mixtures. The mixtures were the same as those which produced the satisfactory results with narrower lanes shown in Table I. In addition to the tendency for a greater densitometer response with increasing R_F values the results obtained with wide lanes varied erratically from one compound to the next. This appeared to be due to differences in the shapes of the spots. An

TABLE III

EFFECT OF POTASSIUM DICHROMATE SPRAY REAGENT ON THE ANALYSIS OF A REFERENCE MIXTURE OF LIPIDS

Constituents	*Composition (weight %)*		
	Found[a]		*Known*
	50% H_2SO_4	*$K_2Cr_2O_7$–70% H_2SO_4 (ref. 8)*	
Cholesterol	2.9 ± 0.37	3.3 ± 0.18	2.5
Oleic acid	24.3 ± 1.20	28.4 ± 1.00	25.0
Triolein	28.8 ± 1.58	29.5 ± 1.14	30.0
Cetyl palmitate	26.0 ± 0.79	26.4 ± 1.80	25.0
Cholesteryl oleate	2.6 ± 0.28	1.9 ± 0.32	2.5
Squalene	15.4 ± 0.62	10.6 ± 0.79	15.0
Total peak area (integrator units)	263 ± 15	170 ± 9	

[a] Mean and standard deviation for six consecutive chromatograms in each series, all on the same thin-layer plate.

elongated spot tended to give a smaller densitometer response than one which was more round. Narrower lanes minimized variation in spot shape as well as in spot size.

Table III compares two series of results derived from analyses carried out on the same thin-layer plate, on which each of the lanes was spotted with the same amount of the reference mixture. One half of the plate had been sprayed with the standard 50% sulphuric acid solution, and the other half with potassium dichromate–70% sulphuric acid reagent. The lanes which had been sprayed with the dichromate reagent produced a very much lower overall response from the densitometer, as shown by the sums of the peak areas. Those compounds which normally showed the more diffuse spots, because of their higher R_F values, showed the greatest decrease in carbon yield.

DISCUSSION

The constant yield obtained over almost the full extent of the chromatograms is in marked contrast to the results of other investigators. The usual results are exemplified by those of LOUIS-FERDINAND *et al.*[9], who reported a continual increase in "degree of charring" with distance of migration. PRIVETT *et al.* avoided this effect to some extent by fixing a constant ratio of slit length to spot diameter[7]. This allowed them a constant carbon yield between R_F 0.4 and 0.8, but since this was too narrow a range in which to resolve most lipid mixtures they required several chromatograms to obtain an analysis. The present additional range of usable R_F values makes it possible to analyze many lipid mixtures in a single chromatogram. This advantage has been gained by working within the practical limits of optical density, by ensuring a constant ratio of slit length to spot diameter, and by observing charring conditions under which compounds of widely differing reactivity give a constant yield of carbon. A further major advantage of the present technique is the capability of conducting up to 25 analyses on one standard size (20 × 20 cm) thin-layer plate.

It has been shown in this investigation that the addition of potassium dichromate to the sulphuric acid spray reagent can result in a decreased yield of carbon in the charred spots. This presumably is the result of part of the organic compounds having been oxidized to carbon dioxide. It was noticed that the greater the R_F value the greater was the decrease in carbon yield when sprayed with the dichromate reagent. This effect is to be expected, since the greater the area covered by a spot the greater is the amount of oxidizing agent to which it is exposed.

Most investigators have demonstrated the reliability of their particular analytical technique by constructing a graph relating the weight of compound to the thin-layer spot area or the photodensitometer peak area. This usually produces a straight line passing through the origin, but with a different slope for each compound and for each chromatographic system in which the compound is developed. In the present technique such deviations have been eliminated, so that a single line is produced for a wide range of simple lipids. The cause of the deviation of free alcohols from this condition is unknown and is under investigation. It would seem probable that similar effects will be found for other classes of compound, but it appears that once a correction factor has been derived it can be applied without further recourse to reference mixtures.

ACKNOWLEDGEMENT

This investigation was supported in part by Grant No. AM 07388 from the National Institute of Arthritis and Metabolic Diseases, U.S. Public Health Service.

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CHROM. 3729

ISOLATION OF PORPHYRINS FROM PORPHYRIA URINE BY PREPARATIVE THIN-LAYER CHROMATOGRAPHY*

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(First received July 1st, 1968; revised manuscript received August 5th, 1968)

SUMMARY

A preparative method of thin-layer chromatography for the isolation of porphyrins from bovine or human porphyric urine, is presented. The isolated uroporphyrin, 7-, 6-, and 5-carboxylic porphyrins and coproporphyrin from bovine urine were crystallized and identified as their methyl esters. Adaptation of this method for analytical work is described and examples are given of data from human and bovine porphyria samples.

Many methods[1–11] have been described for the identification and isolation of urinary porphyrins but in the main these have been used for small quantities and have not been preparative in type. Normal natural sources rarely provide sufficient amounts of porphyrins for preparation in any considerable quantity; in most cases previous methods have been concerned with isolation, purification, identification and quantitative determination in the micro- or, at most, milligram range. A few semi-preparative methods have been described for separation and crystallization of uroporphyrins, coproporphyrins and protoporphyrins from natural sources. The earlier techniques for the ether-soluble porphyrins involved extraction and fractionation with varying concentrations of hydrochloric acid[1]. These are tedious and highly difficult for large quantities and they do not permit efficient separation.

It is well known that the urine in human or bovine erythropoietic porphyria[9,12,13] has a remarkably high content of various porphyrins, especially uro-, hence is a rich source for an appropriate method of preparative isolation. Over many years a variety of methods have been used in this laboratory but none have proved adequate for this purpose. Recently extensive study has been made of high-voltage electrophoresis[13] and of solvent partition[1], with moderate success. These have now been compared with a preparative or analytical method of thin-layer chromatography (TLC) which

* Aided by a grant from the American Cancer Society (P-402), and the John and Mary Briggs Porphyria Research Fund.

has been found to be simpler and more efficient, hence preferable to any method previously used by us or of which we are aware.

MATERIALS AND METHODS

Preparation of crude porphyrin mixture

Urine was collected from a number of bovine porphyrics[12] over a period of three months. After adjusting the pH to 5 with glacial acetic acid, each collection of approximately 25 gallons was mixed with 10 lb. of talc in a 30-gallon plastic drum. The foaming which occurred was controlled by addition of acetone. Several times over a two-day period the talc was stirred vigorously and finally allowed to settle. Most of the supernatant urine was decanted and the talc recharged with a fresh batch of acidified urine. The supernatant urine was again decanted and the remainder was filtered on a large Buchner funnel. After washing with distilled water, the porphyrin was eluted from the talc with dilute ammonium hydroxide and acidified to pH 3.2 with concentrated HCl. After standing overnight at 4° the precipitated porphyrins were collected on a sintered glass funnel and esterified with methanol–sulfuric acid (20:1, v/v). The esters were extracted into chloroform and worked up in the usual way[1]. Direct elution from the talc with methanol–sulfuric acid (20:1, v/v) was carried out with smaller volumes of urine from human cases of porphyria, including three of congenital erythropoietic (EP) type, one of hepatic porphyria cutanea tarda (PCT), and one of hepatic acute intermittent type (AIP).

After concentration to a small volume, the crude bovine prophyrin extract was

TABLE I

ANALYTICAL THIN-LAYER CHROMATOGRAPHY; COMPOSITION OF TOTAL URINE PORPHYRIN IN BOVINE AND HUMAN PORPHYRIA

a = Fluorometer; b = densitometer. For other abbreviations, see text.

	Bovine				*Human*				
	Erythropoietic				*Erythropoietic*			*PCT*	*AIP*
					M.M.	*M.H.*	*D.H.*	*R.V.*	*C.S.*
Amount of crude porphyrin applied	100 mg	1 mg	43 μg		10 μg	10 μg	10 μg	10 μg	10 μg
			a	b					
Amount recovered from TLC	63 mg	0.83 mg	26 μg	—	6.7 μg	8.5 μg	7.0 μg	7.8 μg	7.5 μg
Proportion of recovered porphyrins (%):									
Copro-(4-COOH)	31.6	28.9	20.4	27	40.8	22.8	25.3	1.3	38.9
5-COOH	8.1	7.8	6.9	3	4.3	5.3	4.4	2.3	5.3
6-COOH	0.6	0.5	0.3	—	0.9	0.7	0.6	3.1	1.8
7-COOH	1.4	1.2	1.2	1	1.9	3.0	1.8	22.7	1.8
Uro- (8-COOH)	50.0	53.2	63.1	50	40.3	59.3	55.0	53.0	41.6
Origin (unesterified) (see text)	8.3	8.4	8.1	19	11.8	8.9	12.9	17.6	10.6

subjected to preliminary purification by three precipitations, each time in 50 volumes of petroleum ether (30–60°). The petroleum ether filtrate was yellow (see below under RESULTS), and had an intense green fluorescence with a strong absorption band at 400 mμ. The latter undoubtedly contributes to erroneously high porphyrin values in quantitative spectrophotometric determinations on the initial chloroform solution, which depend on the Soret band absorption. The amount of total porphyrin in the chloroform extract was determined spectrophotometrically using an uroporphyrin standard. This amount includes any absorption due to impurity as mentioned above and in the following. The various amounts applied for TLC (see Table I) were determined in the same way except for the 100 mg sample, which was weighed.

Silica Gel G plates (20 × 20 cm) were prepared according to directions in the Desaga–Brinkmann manual and using their applicator for making varying thicknesses (0.25–1.0 mm). Activation of plates is unnecessary and air drying overnight or for 24 h is sufficient. The amount of absorbed water on the plate has some influence on the quality of separation.

For preparative TLC the isolated porphyrin esters in chloroform solution were applied with the Rodder Streaker* on the plates (5–15 mg per 20 × 20 cm plate). Larger amounts, up to 30 mg per plate, have been separated satisfactorily on 40 × 20 cm plates. As noted below, the use of multiple plates permits processing of 1 g of crude porphyrin in one day. For analytical work the porphyrin esters were applied with a capillary tube or micropipet (see Table I).

The plates were developed in tanks (25 × 25 × 12 cm), using petroleum ether–chloroform (1:5, v/v) with 10% ammonia atmosphere (in a 25 ml cylinder). Previous saturation of tanks for 15–30 min was helpful. Other solvent systems found satisfactory were benzene–chloroform (1:5, v/v) with 10% ammonia atmosphere, and decane–chloroform (1:18, v/v).

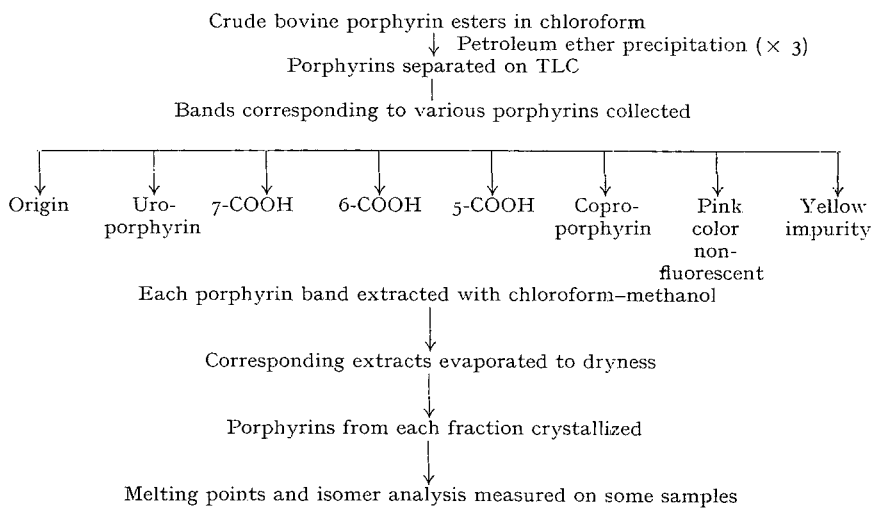

Fig. 1. Schematic pathway for the separation and isolation of bovine porphyrins by TLC on Silica Gel G in petroleum ether–chloroform (1:5) in ammonia atmosphere (10%).

* Rodder Instruments, Los Altos, Calif.

Development times varied with plate thickness. For analytical separation on a 0.25 mm plate 1 h was sufficient to have the solvent front run 11 cm. On 0.25–0.5 mm plates with 5–15 mg amounts of porphyrin 2–4 h were required. Thicker plates (1 mm) needed even more time and the zones were not as distinct because the plates were not as efficiently saturated with the ammonia atmosphere, which facilitates the separation.

After development the preparative plates were dried and the separate porphyrin bands were collected from many plates with the Brinkmann Vacuum Zone Collector. Each zone was extracted on a medium sintered glass filter, using methanol–chloroform (1:4). The eluates were taken to dryness on a Buchler flash evaporator, reconstituted in chloroform and the porphyrin esters crystallized from methanol (Fig. 1). Six plates were developed at a time, thus 1 g of crude porphyrin ester can be separated into its components in four days. By using 40 × 20 cm plates this can be reduced to one day.

Solvent partition was employed when the main objective was to obtain gram quantities of semi-purified uro- or coproporphyrin quickly. This procedure involves pouring the concentrated chloroform solution of porphyrin ester into 25 volumes of ethyl ether, producing an insoluble (mainly uro-) and a soluble (mainly copro-) fraction. These may then be purified further by TLC, if desired.

Previously described methods of paper chromatography[14,15] for isomer analysis were used. The copro- isomer ratios were determined in the Turner door fluorometer*[16]. Melting point determinations were made with a Fisher-Johns micro-melting point apparatus. Uncorrected temperature values are given.

RESULTS

The preparative separation of the total porphyrin esters from bovine porphyria

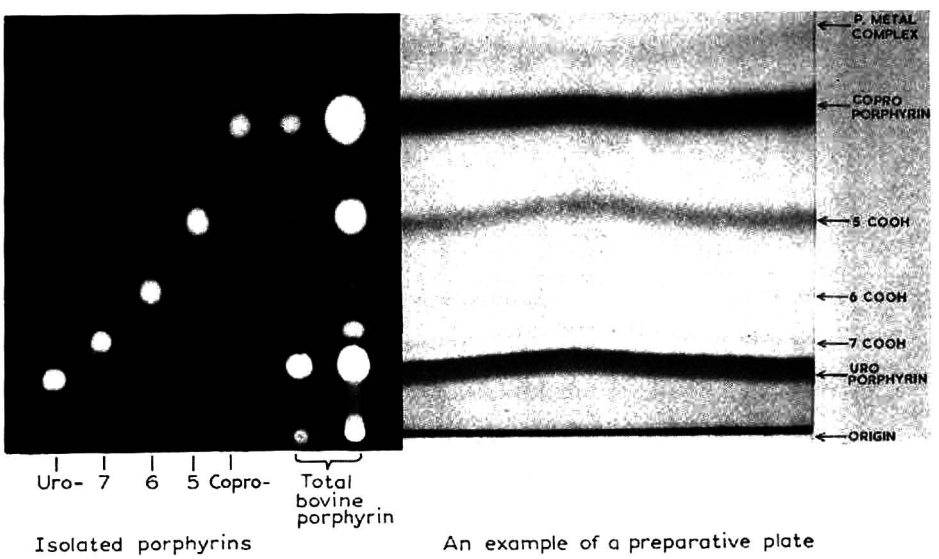

Fig. 2. Plates of Silica Gel G, developed with petroleum ether–chloroform (1:5) in ammonia atmosphere (10%).

* G. K. Turner Associates, Palo Alto, Calif.

urine is shown in Fig. 2. The isolated porphyrins were rerun analytically and proved to be homogenous (Fig. 2). The total bovine porphyrins were run on the same plate to demonstrate the separation achieved. On the primary plate a small amount of the yellow impurity ran to the front. This was identical to the pigment in the above described filtrate from petroleum ether precipitation which on crystallization gave a m.p. of 140–146°. This substance is as yet unidentified. The next band was a pink non-fluorescent one, a coproporphyrin metal complex, followed by the porphyrin bands (Figs. 1 and 2). The brown zone which remained at the origin was dissolved in methanol–sulfuric acid (20:1), extracted into chloroform and rechromatographed, all as described in the foregoing. A small amount of uro- and trace amounts of copro- to 7-COOH porphyrin* were found but the majority remained at the origin as unidentified brown pigment.

TABLE II

PREPARATIVE THIN-LAYER CHROMATOGRAPHY OF BOVINE PORPHYRINS

Porphyrin type	*Absorption maxima of porphyrin esters in* $CHCl_3$[a]					*Melting points (uncorr.) (°C)*
Copro-	622	568	533	499	400	239–245
5-COOH	623	569	536	500	401	218–223
6-COOH	624	568	538	503	403	230–240
7-COOH	625	570	537	501	404	238–243
Uro-	626	572	536	502	406	285–288

[a] Hitachi, Perkin-Elmer Model 139 spectrophotometer.

The absorption maxima and the melting points from the separated porphyrins are presented in Table II. The melting points shown are affected by minor proportions of type III isomer by the CORNFORD–BENSON[14] and ERIKSEN[15] methods. This was determined to be 15% and 14%, respectively, for uro- and copro-, and 18% for 7-COOH (after decarboxylation to copro-[17]).

Quantitative determination of porphyrin in each zone separated on TLC was made in two ways: (1) removal of each zone from the plate with a spatula, extraction from the silica gel with methanol–chloroform (1:4), followed by ordinary fluorometry[18]; (2) directly on the plate using the Photovolt densitometer. (See Table I for percentages of each porphyrin from the original sample.) Preliminary comparisons of direct densitometric determination on the plate indicates that the values compare favorably with fluorometric measurement of the eluted porphyrins. As yet the uro- and 7-COOH porphyrins have been determined in terms of an uroporphyrin standard, the copro-, 5- and 6-COOH porphyrins with a coproporphyrin standard (Table I). The present TLC method could be applied analytically and preparatively to any urinary porphyrins (adaptable to fecal), *e.g.*, the five cases of human porphyrias were investigated analytically and the representative values given in Table I are in agreement with those reported[9].

* uro- = uroporphyrin: 7-COOH, 6-COOH, 5-COOH = hepta-, hexa- and pentacarboxylic; copro- = coproporphyrin.

DISCUSSION

The present TLC method provides a simple rapid separation of porphyrin esters. It has been used for preparative isolation of porphyrins from porphyrin rich urine, either human or bovine (EP). The latter often contains upwards of 1 g of porphyrin in 24 h, mainly uro-, while the urine of the comparable human disease generally contains about 50–100 mg. Both are, in porportion, excellent sources of smaller amounts of copro-, 5-, 7- and 6-COOH porphyrins, in approximately that order of amount. Used simply as an analytical method, the total porphyrin mixture in the range of 1–50 μg is readily separated on the TLC plate and each spot can be determined quantitatively with an appropriate densitometer. As yet our experience with the densitometric method is limited but it appears to be quite satisfactory. In the absence of a densitometer the individual spots are readily eluted as in the preparative method for quantitative determination in a sensitive photofluorometer.

Preliminary solvent partition is useful to obtain quickly a 90–95% pure uroporphyrin (with a small amount of 7-COOH) and 80% pure coproporphyrin (with small amounts of 5- and 6-COOH porphyrins). These may be further purified by TLC.

High-voltage electrophoresis was also tried for preparative separation of porphyrins but has been found far inferior because only small quantities can be isolated over long periods, as compared with TLC; there is too much overlapping of bands and considerable loss of porphyrin.

ACKNOWLEDGEMENT

We are very grateful to Dr. W. COHEN of the Department of Obstetrics and Gynecology, University of Minnesota, for making the Photovolt densitometer available for our studies and for his helpful instruction.

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ZUR TRENNUNG UND IDENTIFIKATION DER FREIEN PORPHYRINE UND DEREN ISOMERE

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(Eingegangen am 1. Juli 1968)

SUMMARY

Separation and identification of free porphyrins and their isomers

Complex porphyrin mixtures can be analyzed by means of extraction chromatography combined with thin-layer chromatographic and absorption spectrophotometric measurements. The fractions obtained in the partition system (tri-*n*-butyl phosphate/hydrochloric acid) may be identified as regards the number of carboxylic groups n by determining the peak position of the Soret band (λ_p using the correlation between n and λ_p. The isomeric structure of the porphyrins can be established by thin-layer chromatography with 2,6-lutidine/water of the corresponding coproporphyrin derivatives obtained by decarboxylation.

Using the methods described, nearly all porphyrin compounds in the reaction chain from uroporphyrin to coproporphyrin of the isomeric series I and III could be identified in a porphyrin sample isolated from the urine of a patient with a hyporegenerative anaemia. Furthermore, several fractions were observed after the extraction and thin-layer chromatographic separations which could not be related to the porphyrins of the isomeric types I and III normally found in porphyrin samples isolated from urine. This was proved by the fact that the λ_p values of the single fractions did not agree with the expected values. Therefore, it was assumed that these compounds, belonging to the isomeric series I and III, differ from the normal porphyrins by their substituents. However, further investigations are still necessary to elucidate the structure and determine the assumed substituents of the isolated porphyrins. There is no evidence that porphyrins of the isomeric types II and IV occur in the porphyrin mixture studied.

EINLEITUNG

Die Biosynthese des dem Häm vorausgehenden Protoporphyrins verläuft, von Bernsteinsäure und Glycin ausgehend, über zahlreiche Reaktionsstufen, die, durch ein komplexes enzymatisches System gesteuert, miteinander in einem stationären Gleichgewicht stehen. Abweichungen von dem in Normalfällen zu beobachtenden Hämpräkursorenmuster erlauben oft wertvolle Rückschlüsse auf die in dem jeweiligen

Falle vorliegende Hämsynthesestörung. Besondere Beachtung bei klinischen Betrachtungen erlangte die Bestimmung der auf natürlichem Wege ausgeschiedenen dominierenden Porphyrine, Uroporphyrin und Coproporphyrin, während die partiell decarboxylierten Zwischenglieder (Hepta-, Hexa- und Pentacarboxylporphyrine) hierbei, von Ausnahmen abgesehen, bisher keine Rolle spielten.

Die analytische Erfassung dieser Glieder der Porphyrinkette kann mit den derzeit bekannten papierchromatographischen[1,2], säulenchromatographischen[3], hochspannungselektrophoretischen[4] und dünnschichtchromatographischen[5–7] Verfahren durchgeführt werden, die das durch die variierende Anzahl der Carboxylgruppen der Porphyrinmolekel bestimmte abgestufte Verhalten zur Auftrennung ausnutzen. Eine entscheidende Einschränkung in der Anwendung erfahren diese Methoden dadurch, dass lediglich Gemische von Porphyrinen des gleichen Isomerentyps analysiert werden können. Treten dagegen, wie in pathologischen Fällen fast stets zu beobachten, Porphyrine der verschiedenen Isomerenreihen, vorwiegend vom Typ I und III, auf, so ist eine eindeutige Zuordnung nicht mehr möglich. Zu ihrer Identifikation müssen daher weitergehende Untersuchungen durchgeführt werden, die eine Bestimmung des Isomerentyps der einzelnen Porphyrine gestatten.

In der vorliegenden Untersuchung wird über eine Zerlegung eines aus dem Urin einer hyporegenerativen Anämie (Patient U.) isolierten ausserordentlich komplexen Porphyringemisches berichtet, wobei zur Identifikation der einzelnen Fraktionen die folgenden Untersuchungen herangezogen wurden:

(1) Extraktionschromatographische Auftrennung der freien Porphyrine und Identifikation der einzelnen Fraktionen über eine Bestimmung der Peaklage λ_p der Soretbande.

(2) Dünnschichtchromatographische Trennung der extraktionschromatographisch erhaltenen Fraktionen, vor und nach der Decarboxylierung.

(3) Versuche zur Identifikation einzelner Porphyrine über deren I.R.-Spektren.

Über weitere analytische Aspekte bei der Auftrennung des gleichen Porphyringemisches wurde bereits ausführlich an anderer Stelle berichtet[8–10].

EXPERIMENTELLER TEIL

Material

Uroporphyrin-I (UP-I). Uroporphyrin-I wurde als Oktamethylester von den Koch-Light Laboratories, Colnbrook, England, bezogen. Nach dem Verseifen in 5 *N* HCl wurde der Hämpräkursor dünnschichtchromatographisch im 2,6-Lutidin/Wasser-System[6] gereinigt, mit 1 *N* HCl eluiert und anschliessend aus schwach salzsaurer Lösung (c_{HCl} = 0.01–0.1 *N*) mit Tri-*n*-butylphosphat (TBP) extrahiert. Hieraus wurde UP-I mit 5 *N* HCl rückextrahiert. Die in der wässrigen Phase verbleibenden geringfügigen TBP-Reste wurden durch mehrmaliges, sukzessives Auswaschen mit Benzol p.A. (5 × 5 ml) vollständig entfernt. Verluste an UP-I sind unter diesen Bedingungen nicht zu befürchten. Die zu den einzelnen Untersuchungen eingesetzte salzsaure Stammlösung enthielt den Hämpräkursor in einer Konzentration von 100 μg/ml 5 *N* HCl. Alle Pipettierungen wurden mit einer Aglaspritze vorgenommen, die bei quantitativem Arbeiten ein genaues Dosieren gestattet. Die eingesetzte Stammlösung zeigte, im Kühlschrank bei 4° aufbewahrt, selbst im Verlauf von mehreren Wochen keinerlei Zersetzung.

Uroporphyrin-III (UP-III). Dieses Uroporphyrinisomer wurde ebenfalls in Esterform von den Koch-Light Laboratories, Colnbrook, England, bezogen ("Ex turaco feathers"). Die Reinigung und Aufarbeitung erfolgte wie vorangehend für UP-I beschrieben. Die salzsaure Stammlösung enthielt das Porphyrin in einer Konzentration von 100 μg/ml 5 N HCl.

Coproporphyrin-I (CP-I). Zur Herstellung von CP-I wurde UP-I nach der Methode von EDMONDSON UND SCHWARTZ[11] decarboxyliert. Die Aufarbeitung wurde, wie nachstehend unter Methoden beschrieben, vorgenommen. Die CP-I-Lösungen wurden mit einer Konzentration von 20–50 μg/ml 5 N HCl (je nach Präparation) zu den Untersuchungen eingesetzt.

Coproporphyrin-III (CP-III). CP-III wurde, analog wie CP-I, aus UP-III durch Decarboxylierung[11] erhalten. Die Aufarbeitung und Herstellung der Stammlösung wurde wie bei CP-I vorgenommen.

Benzol p.A. Handelsübliches Produkt (Hersteller: Fa. E. Merck, Darmstadt; Kat.-Nr. 1783).

Tri-n-butylphosphat (TBP). TBP wurde durch den Handel bezogen (Hersteller: Fa. E. Merck, Darmstadt; Kat.-Nr. 8354) und ohne zusätzliche Reinigung zu den Untersuchungen eingesetzt.

Polytrifluormonochloräthylen (Hostaflon C 2). Zur Aufnahme des Extraktionsmittels TBP wurde Hostaflon C 2 (Hersteller: Farbwerke Höchst AG, Frankfurt/Main) verwendet. Hierzu wurde die bei Aufarbeitung des in Pulverform vorliegenden Produktes erhaltene Siebfraktion mit einer Korngrösse von 50–100 μ eingesetzt. Bei der Imprägnierung wurde TBP tropfenweise unter intensivem Rühren in einem Gewichtsverhältnis von Hostaflon C 2 : TBP = 2 : 1 zugegeben.

Salzsäure p.A. Zur Elution wurden aus Titrisolkonzentraten (Hersteller: Fa. E. Merck, Darmstadt; Kat.-Nr. 9970/0001) angesetzte Lösungen von 1 N HCl verwendet, die in heissem Zustand mit TBP bis zur Sättigung geschüttelt wurden. Die Elutionslösung wurde vor jedem Versuch zur Entgasung für *ca.* 30 min ausgekocht.

Methoden

Decarboxylierung der Porphyrine. Zur Identifikation der extraktionschromatographisch erhaltenen Fraktionen bezüglich des Isomerentyps werden diese durch Decarboxylierung in die entsprechenden Coproporphyrinderivate überführt. Diese werden, zusammen mit den reinen Bezugssubstanzen CP-I und CP-III, dünnschichtchromatographisch im 2,6-Lutidin/Wasser-System[6] entwickelt und anhand der R_F-Werte identifiziert.

Hierzu werden, nach der Methode von EDMONDSON UND SCHWARTZ[11], 3 ml der salzsauren Porphyrinlösung ($c_{HCl} = 0.3\ N$) in Duranglasröhrchen (Länge: 150 mm; Innerer Durchmesser: 10 mm; Wandstärke: 1 mm) bei einem Druck von $p \leqq 1$ Torr (Gasballastpumpe) eingeschmolzen und bei 200° für 4 h im Trockenschrank decarboxyliert. Die entstandenen Reaktionsprodukte werden anschliessend, nach Einstellen der Lösung auf eine Säurekonzentration von $c_{HCl} = 0.01$–$0.1\ N$ mit 1 N NaOH, mit 1 ml TBP extrahiert. Hieraus erfolgt Rückextraktion mit 0.1–0.2 ml 5 N HCl. Die organische Phase wird daraufhin in 5 ml Benzol aufgenommen, und, nach dem Zentrifugieren, abpipettiert. Die wässrige Phase wird anschliessend nochmals sukzessive mit 4 × 5 ml Benzol ausgewaschen, um anhaftende TBP-Reste vollständig zu entfernen. Die Lösung wird in dieser Form zu den DC-Trennungen eingesetzt.

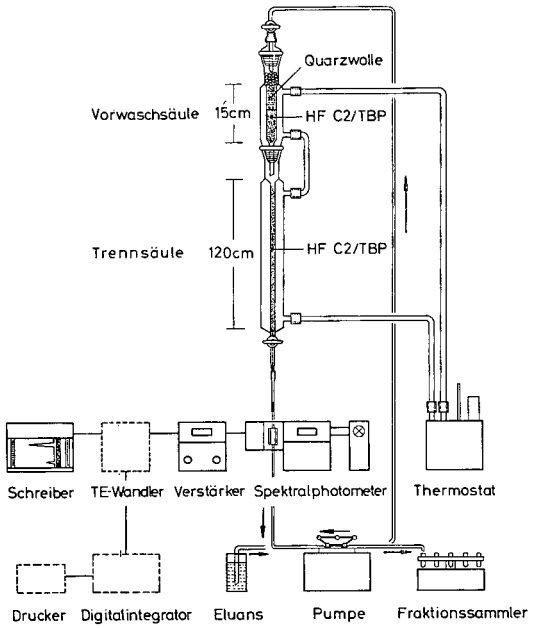

Fig. 1. Apparatur zur extraktionschromatographischen Auftrennung komplexer Porphyringemische mit simultaner quantitativer Bestimmung der einzelnen Fraktionen.

Extraktionschromatographische Auftrennung der freien Porphyrine im TBP/HCl-System. Die zur Durchführung dieser Untersuchungen verwendete, weitgehend automatisierte Apparatur ist in Fig. 1 dargestellt[9,10]. Hauptteil dieser Anordnung ist die eigentliche Trennkolonne (Länge: $L_s = 120$ cm; Innerer Durchmesser: $D_s = 5$ mm), die zusammen mit der Vorwaschkolonne (Länge: $L_{vs} = 15$ cm; Innerer Durchmesser: $D_{vs} = 15$ mm) durch einen Ultrathermostaten (Hersteller: Fa. Gebr. Haake KG, Berlin) auf die gewünschte Versuchstemperatur T_s gehalten wird. Beide Säulen wurden mit dem gleichen Füllmaterial (Hostaflon C 2/TBP) beschickt. Die Vorwaschsäule hat hierbei die Aufgabe, das in die Trennsäule eintretende Elutionsmittel mit TBP abzusättigen. Hierdurch können Auswaschverluste sowie die meist sehr störende Blasenbildung in der eigentlichen Trennkolonne selbst bei extremen Versuchsbedingungen ($T_s = 60°$; $c_{HCl} = 3\ N$) weitgehend ausgeschaltet werden. Die Elutionslösung wurde über eine Proportionierpumpe (Lieferfirma: Technicon GmbH, Frankfurt/Main) sowohl in die Vorwaschsäule unter leichtem Überdruck eingedrückt, als auch von der Trennsäule bei gleichem Schlauchdurchmesser abgesaugt. Hierdurch wird das Auftreten eines Druck- bzw. Soggradienten innerhalb der Trennsäule weitgehend ausgeschaltet, so dass eine bessere Konstanz des Durchflusses im Vergleich zu der an anderer Stelle beschriebenen Anordnung[9] erreicht wird. Die aus der Trennsäule austretende Elutionslösung wird durch eine Durchflussküvette (1 cm MT 4 D, Hersteller: Fa. C. Zeiss, Oberkochen) geleitet, wo kontinuierlich die Transmission der Lösung bei $\lambda_{gem.} = 403$ nm gemessen (Zeiss Spektralphotometer PMQ II mit Monochromator M 4 Q III) und mit einem Potentiometerschreiber (Servo/Riter II, Texas

Instruments, Houston, U.S.A.) registriert wird. Die einzelnen Fraktionen werden in einem zeitgesteuerten Fraktionssammler aufgefangen und können für weitere Untersuchungen (Bestimmung der Peaklage der Soretbande λ_p; Aufnahme von I.R.-Spektren, u.a.) wieder eingesetzt werden. Eine quantitative Bestimmung der in der Probe vorliegenden Porphyrinmenge kann über eine Auswertung des durch den Potentiometerschreiber registrierten Transmissions- bzw. Extinktionsverlaufes durchgeführt werden[10]. Von der geplanten Erweiterung der Versuchsanordnung (Fig. 1) wird eine beträchtliche Reduzierung des derzeit noch erforderlichen Zeitaufwandes bei der quantitativen Auswertung erwartet[10].

Zur Isolierung und Auftrennung von im Urin auftretenden Porphyringemischen wird wie folgt vorgegangen: Der schwach salzsaure Urin (pH 1–3) wird bei einem Volumen von 20–200 ml (je nach Porphyrinkonzentration) mit 1–10 g imprägniertes Filterbodenmaterial (Kieselgel: TBP = 2 : 1) versetzt, kurz geschüttelt und anschliessend über eine Glasnutsche (Porenweite: G 2; Durchmesser: 50–100 mm) gesaugt. Das Filtrat wird stets noch ein- bis zweimal über den gleichen Filterboden gegeben. Anschliessend wird mit 0.01–0.1 *N* HCl gründlich nachgewaschen. Die durch den Filterboden extrahierten Porphyrine werden nun mit 3 × 3 ml 5 *N* HCl sukzessive eluiert, die Lösungen in ein 20 ml Messkölbchen überführt und mit Aqua dest. bis zur Marke aufgefüllt. Ein Aliquot hiervon wird, nach Einstellen der Azidität auf eine Säurekonzentration c_{HCl} = 0.01–0.1 *N* mit 1 *N* NaOH, zur Auftrennung der Porphyrine auf der Säule eingesetzt. Hierzu wird die Lösung in den Vorraum der Trennsäule eingefüllt. Bei kleinem Durchfluss (D = 10 ml/h) erfolgt hierbei vollständige Absorption der Porphyrine in der obersten Schicht des Füllmaterials ($K_{DB} \geqq 1000$ (refs. 9, 10)), deren Höhe, je nach der vorliegenden Porphyrinmenge, 1–10 mm beträgt. Anschliessend wird mit 5–10 ml 0.1 *N* HCl nachgewaschen, die Vorwaschsäule, die mit dem Elutionsmittel (1 *N* HCl) bis zum Ausgang gefüllt ist, aufgesetzt und die Elution durch Einschalten der Pumpe eingeleitet.

Dünnschichtchromatographische Trennung der freien Porphyrine im 2,6-Lutidin/Wasser-System. Die zur Identifikation der einzelnen Porphyrine durchgeführten dünnschichtchromatographischen Trennungen wurden nach einer an anderer Stelle beschriebenen Methode[6] vorgenommen. Hierzu wurden bei 120° für 2 h aktivierte Kieselgelschichten eingesetzt. Als Fliessmittel wird ein Gemisch von 2,6-Lutidin–Wasser (5 : 3) verwendet, das mit Ammoniak (Einstellen von 100 ml konz. Ammoniaklösung in die Trennkammer) gut vorzusättigen ist. Die Menge der einzelnen, von der Säule eluierten Fraktionen lag zwischen 1–50 μg. Die Aufarbeitung und Überführung in kleinste Lösungsvolumina (20–200 μl) setzt die Anwendung der in der Mikroanalyse üblichen Techniken voraus (Pipettierungen mit ausgezogenen Teflonspitzen; Zentrifugieren in speziellen Mikroröhrchen; etc.). Porphyrinmengen von *ca.* 1 μg waren ausreichend, um die zur Identifikation erforderlichen Untersuchungen:

(1) DC-Trennung und Bestimmung der Peaklage λ_p vor der Decarboxylierung,

(2) Decarboxylierung,

(3) DC-Trennung und Bestimmung der Peaklage λ_p nach der Decarboxylierung,

durchführen zu können.

Bestimmung der Peaklage λ_p der Soretbande der Porphyrinfraktionen, vor und nach der Decarboxylierung. Zur weiteren Charakterisierung der bei der extraktionschromatographischen Trennung erhaltenen Porphyrinfraktionen wird eine Bestimmung der Peaklage der Soretbande λ_p vorgenommen (Fig. 2). Hierzu wird der Ab-

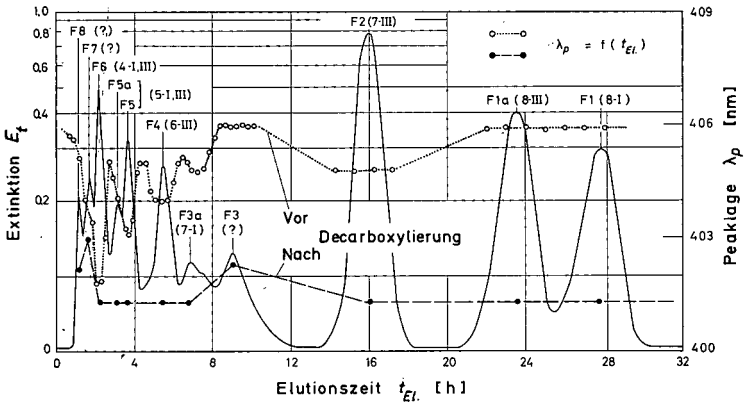

Fig. 2. Elutionschromatogramm des aus dem Urin von Patient U. isolierten Porphyringemisches, aufgenommen mit der beschriebenen Apparatur (Fig. 1). Versuchsbedingungen: Säulentemperatur: $T_s = 20°$; Säulenlänge: $L_s = 120$ cm; Durchfluss: $D = 20$ ml/h; Säurekonzentration: $c_{HCl} = 1\ N$; Messwellenlänge: $\lambda_{gem.} = 403$ nm. Die Peaklage λ_p der in den verschiedenen Fraktionen aufgefangenen Porphyrine wurde vor ($\lambda_p = f(t_{El.})$: vor Decarboxylierung) und nach ($\lambda_p = f(t_{El.})$: nach Decarboxylierung) der Überführung in die entsprechenden Coproporphyrinderivate durch Decarboxylierung bestimmt.

sorptionsverlauf der salzsauren Porphyrinlösung ($c_{HCl} = 1\ N$) im Bereich von 380–450 nm bei kleiner Spaltbreite gemessen. Durch graphische Differentiation der Soretbande im Bereich von 390–410 nm (refs. 6, 12, 13) wird λ_p mit hoher Genauigkeit erhalten. Zur Durchführung der Bestimmung werden, dank der hohen Empfindlichkeit des Porphyrinnachweises, Mengen von min. 0.2 μg (!) benötigt, um λ_p auf $\pm$ 0.1 nm genau bestimmen zu können (5 cm-Mikroküvette; MT 4; Hersteller: Fa. C. Zeiss, Oberkochen). Auf die Einhaltung der Salzsäurekonzentration ($c_{HCl} = 1\ N$) ist hierbei streng zu achten[9].

Aufnahme der I.R.-Spektren einiger Porphyrinfraktionen in Esterform. Die I.R.-Spektren der in Esterform vorliegenden Porphyrine, UP-I, UP-III, CP-I, CP-III, F1, 1a, 2 und f 8,9 (Fig. 2,5), wurden mit einem I.R.-Spektrographen der Fa. E. Leitz, Wetzlar (Modell: III G, mit Gitter III) aufgenommen. Die Substanzen wurden in KBr-Mikropresslingen, die nach einer standardisierten Vorschrift hergestellt wurden, untersucht.

Zur Präparation der Ester wurde die salzsaure Lösung der Porphyrine im Feinvakuum bis fast zur Trockene eingeengt, mehrfach mit Benzol ausgewaschen und anschliessend in 5 ml Methanol/Konz. Schwefelsäure (19:1) aufgenommen. Nach 24-stündigem Stehenlassen bei Raumtemperatur wurde die Lösung in ein auf 0° vorgekühltes Gemisch von 5 ml Aqua dest./1 ml Chloroform eingegossen, in einem Scheidetrichter kräftig geschüttelt und die wässrige Phase abgetrennt. Durch Waschen mit 1 N NH_4OH werden restliche unveresterte Porphyrinanteile entfernt. Anschliessend wird mit Aqua dest. mehrfach nachgewaschen, bis die Waschlösung neutral reagiert. Die organische Lösung der Ester wird nun in ausgezogene Mikroröhrchen überführt und das Lösungsmittel vorsichtig im Wasserstrahlvakuum abgesaugt. Die auskristallisierten Ester werden über KOH für mehrere Tage getrocknet.

Zur Herstellung des Mikropresslings werden 10 mg gut vorgetrocknetes KBr in

das Mikroröhrchen eingefüllt, mit einem Mikrospatel mit dem Ester gut vermischt und in einem Achatmörser anschliessend fein zerrieben. Der Mikropressling wird mit dem kommerziell erhältlichen Presswerkzeug der Fa. Leitz unter standardisierten Bedingungen erhalten (Durchmesser des KBr-Presslings: 2 mm; Durchmesser der Al-Trägerscheibe: 20 mm). Die zur Aufnahme von I.R.-Spektren erforderliche Porphyrinkonzentration lag bei 3–5 μg Ester/mg KBr, so dass unter den genannten Bedingungen 30–50 μg des betr. Esters zur Herstellung eines Presslings benötigt werden. Bei der Präparation von KBr-Presslingen mit Porphyrinen in Säureform trat merkliche Zersetzung ein, so dass auf eine Registrierung dieser I.R.-Spektren verzichtet wurde.

Bei der Herstellung der Porphyrinester ist sorgfältig darauf zu achten, dass eine Kontamination durch Oel- bzw. Fettspuren, die beim Arbeiten mit einer Gasballastpumpe trotz einer grossflächigen, auf — 40° abgekühlten Kühlfalle stets zu befürchten war, absolut ausgeschaltet wird. Daher wurden nach dem Einengen der salzsauren Porphyrinlösungen die auftretenden Rückstände mehrfach mit Benzol ausgewaschen. Freie Porphyrine sind in Benzol völlig unlöslich, so dass Verluste hierbei nicht zu befürchten sind.

ERGEBNISSE UND DISKUSSION

Die extraktionschromatographische Analyse komplexer Porphyringemische ist gegenüber anderen Verfahren von Vorteil dadurch, dass eine Trennung und Bestimmung der Hämpräkursoren in einem Arbeitsgang erfolgen kann[9,10]. Die aus dem Urin über eine Filterbodenextraktion isolierten Porphyrine können in freier Form unmittelbar zur Trennung eingesetzt und anschliessend für weitere Untersuchungen (z.B.: Bestimmung der Peaklage der Soretbande λ_p; Aufnahme von I.R.-Spektren; u.a.) wieder verwendet werden. Die hierbei auftretenden Verluste können, wie frühere Untersuchungen für Coproporphyrin und Uroporphyrin selbst unter extremen Versuchsbedingungen zeigten[9], praktisch vernachlässigt werden. Voraussetzung für eine quantitative Auswertung ist jedoch, dass die Versuchsanordnung unter Versuchsbedingungen mit reinen Bezugssubstanzen geeicht, die spezifische Extinktion bzw. die korrespondierende Extinktionsfläche der einzelnen Porphyrine somit bekannt ist. Weiterhin ist hervorzuheben, dass mit dem beschriebenen Verfahren freie Uroporphyrinisomere, die mit anderen Methoden bisher nicht zerlegt werden konnten, aufgetrennt und ebenfalls quantitativ bestimmt werden können[10].

Ein Beispiel einer solchen Auftrennung eines an anderer Stelle bereits eingehend untersuchten komplexen Porphyringemisches (Patient U.)[8–10] ist in Fig. 2 dargestellt. Während über die Identifikation von F 1, 1a und 2 (UP-I, UP-III und 7-III) bereits ausführlich berichtet wurde[10], soll in der vorliegenden Arbeit der jeweilige Isomerentyp und Decarboxylierungsgrad der Fraktionen F 3–8 bestimmt werden.

Einordnung der Porphyrine anhand der Peaklage λ_p der Soretbande

Als zuverlässigstes Kriterium für die Einordnung von Hämpräkursoren nach Anzahl der Carboxylgruppen n hat sich die Peaklage der Soretbande λ_p erwiesen, die innerhalb einer engen Fehlergrenze ($\pm$ 0.1 nm) selbst mit sehr geringen Porphyrinmengen (min. 0.2 μg) noch bestimmt werden kann. Zur Identifikation der Porphyrine in dem vorliegenden Porphyringemisch wurden die von der Trennsäule kommenden

Fraktionen in einem zeitgesteuerten Probenwechsler aufgefangen, und die Peaklage der eluierten Porphyrine in der beschriebenen Weise bestimmt. Das Ergebnis einer solchen Auswertung ist in Fig. 2 dargestellt ($\lambda_p = f(t_{El.})$: vor Decarboxylierung). Anschliessend wurden die in den Fraktionen um die Elutionsmaxima herum aufgefangenen Porphyrine decarboxyliert und die Peaklage der resultierenden Reaktionsprodukte erneut bestimmt ($\lambda_p = f(t_{El.})$: nach Decarboxylierung).

Diese Auswertung zeigt deutlich die zwischen den Elutionsmaxima x_p und der Peaklage λ_p bestehende Abhängigkeit an. Da die Peakfolge bei der extraktionschromatographischen Zerlegung mit der bei der dünnschichtchromatographischen Auftrennung erhaltenen übereinstimmt (Fig. 2 und 5), letztere jedoch bei der sehr ähnlichen papierchromatographischen Trennung mit dem n-Wert in Korrelation gesetzt wird[1,2], muss auch für die vorliegende Aufzeichnung des Elutionsverlaufes eine

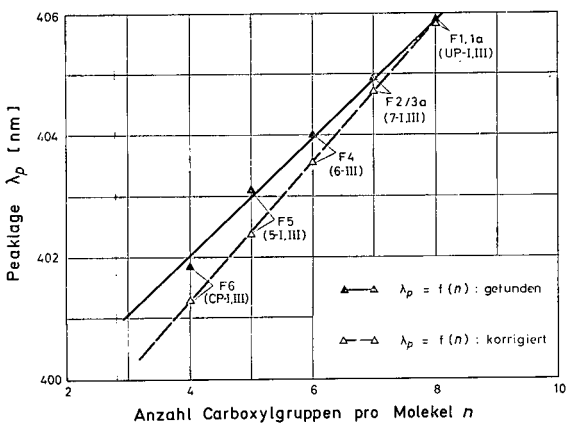

Fig. 3. Abhängigkeit der Peaklage der Soretbande λ_p von der Anzahl der Carboxylgruppen n für eine Säurekonzentration von $c_{HCl} = 1\ N$. Die empirisch erhaltenen λ_p-Werte ($\lambda_p = f(n)$: gefunden) mussten bezüglich der in den Fraktionen enthaltenen Anteile höher carboxylierter Porphyrine korrigiert werden ($\lambda_p = f(n)$: korrigiert).

solche Abhängigkeit zwischen den beiden Parametern λ_p und n angenommen werden.

Die für die im Elutionsmaximum jeweils erhaltenen Peaklagen λ_p sind in Abhängigkeit von den angenommenen n-Werten in Fig. 3 aufgetragen ($\lambda_p = f(n)$: gefunden). Da der für F 6, die dünnschichtchromatographisch eindeutig als CP-I/CP-III-Gemisch identifiziert wurde (Fig. 5), gemessene λ_p-Wert ($\lambda_p = 401.80$ nm) deutlich über dem bei reinen CP-Proben erhaltenen ($\lambda_p = 401.30$ nm) liegt, musste angenommen werden, dass diese Fraktion durch Anteile mit höheren λ_p-Werten (F 1, 1a und 2) kontaminiert ist. Diese Annahme wird gestützt durch den in Fig. 2 eingetragenen Verlauf von λ_p: Die bei ungünstigem Peak/Talverhältnis zwischen den einzelnen Elutionspeaks stets zu beobachtende Erhöhung der λ_p-Werte im Vergleich zu den in den Elutionsmaxima erhaltenen kann nur so gedeutet werden, dass die in hohem Überschuss vorliegenden Fraktionen 1, 1a und 2 in merklichem Umfange in den Fraktionen F 3–8 auftreten. Eine Abschätzung der Höhe der Kontamination von F 1, 1a und 2 in F 6 über eine Bestimmung der Verschiebung der Peaklage nach einem an anderer Stelle beschriebenen Verfahren[14] ergab einen Anteil von *ca.* 10%.

Diese Überlagerung ist offenbar darauf zurückzuführen, dass die Kapazität der Säule überschritten und Anteile von F 1, 1a und 2 sukzessive in die Elutionsphase übergehen. Bei einem günstigeren Verhältnis von F 3–8/F 1–2 bzw. bei einer vorangehenden Abreicherung von F 1–2 können Störungen dieser Art sicherlich völlig vermieden werden. Bei der Auftrennung von reinen CP–UP-Gemischen konnte bei Mengen von 1–20 μg eine solche Überlagerung mit der gleichen Versuchsanordnung nicht beobachtet werden[9]. Die in F 3–8 vorliegenden Anteile von F 1–2 liessen sich auch dünnschichtchromatographisch nachweisen (Fig. 5).

Die λ_p-Werte der extraktionschromatographisch erhaltenen Fraktionen mussten daher unter Zugrundelegung der bei reinen Proben von CP und UP gemessenen entsprechend korrigiert werden (Fig. 3, $\lambda_p = f(n)$: korrigiert). Die für die einzelnen

TABELLE I

EXTRAKTIONSCHROMATOGRAPHIE BZW. DÜNNSCHICHTCHROMATOGRAPHIE VON PORPHYRINEN VOR UND NACH DECARBOXYLIERUNG

Zusammenstellung der bei der extraktionschromatographischen bzw. dünnschichtchromatographischen Auftrennung für die verschiedenen Fraktionen erhaltenen Elutionszeiten $t_{El.}$, R_F-Werte sowie die vor (λ_p, vD) und nach (λ_p,nD) der Decarboxylierung absorptionsspektrophotometrisch gemessenen Peaklagen der Soretbande. Die empirisch erhaltenen Werte (λ_p) sind bezüglich der Anteile höher carboxylierter Porphyrine korrigiert (λ^c).

F	$t_{El.}$ *(h)*	R_F-*Werte*	λ_p, *vD (nm)*	λ_p^c, *vD (nm)*	λ_p, *nD (nm)*	λ^*, *nD (nm)*	*n*	*Zuordnung*	
F 1	28.3	0.02	405.90	405.80	401.50	401.30	8	8-I	UP-I
F 1a	23.5	0.02	405.90	405.80	401.45	401.30	8	8-III	UP-III
F 2	16.0	0.05	404.90	404.70	401.30	401.30	7	7-III	
F 3	9.0	0.03	406.00		402.30			?	
F 3a	6.8	0.10	405.00	404.70	401.50	401.30	7	7-I	
F 4	5.4	0.14	404.00	403.50	401.30	401.30	6	6-III	
F 5	3.6	0.29	403.10	402.40	401.30	401.30	5	5-I,III	
F 5a	3.0	0.34	403.10	402.40	401.30	401.30	5		
F 6	2.6	0.40	401.80	401.30	401.35	401.30	4	4-I	CP-I
		0.43						4-III	CP-III
F 7	1.6	0.46	403.90		403.00			?	
F 8	1.0	0.46	405.70		402.20			?	

decarboxylierten Hämpräkursoren bei einer Säurekonzentration von $c_{HCl} = 1.00\ N$ angenommenen bzw. erhaltenen Peaklagen λ_p sind in Tabelle I zusammengestellt.

Die zwischen der Elutionszeit $t_{El.}$ und der Anzahl der Carboxylgruppen n bestehende Abhängigkeit ist in Fig. 4 dargestellt. Hieraus geht hervor, dass die günstigste Auflösung bei der Auftrennung im Bereich hoher $t_{El.}$-Werte erhalten wird (F 1, 1a und 2). Zwischen F 3–8 ist die Peakfolge bereits so dicht, dass eine vollständige Auflösung der Einzelpeaks nicht mehr erreicht wird. Durch sinnvolle Anpassung der Versuchsbedingungen (Erniedrigung der Säurekonzentration der Elutionslösung: $c_{HCl} = 1 \rightarrow 0.5\ N$) kann eine Verbesserung der Auflösung in diesem Bereich erzielt werden. Die für die beiden Uroporphyrinisomere I und III hierbei resultierenden Elutionszeiten $t_{El.}$ werden in diesem Falle allerdings sehr gross.

Ermittlung des Isomerentyps von Hämpräkursoren durch Überführung in die entsprechenden Coproporphyrinisomere

Die derzeit wohl zuverlässigste Methode zur Identifikation der Porphyrine be-

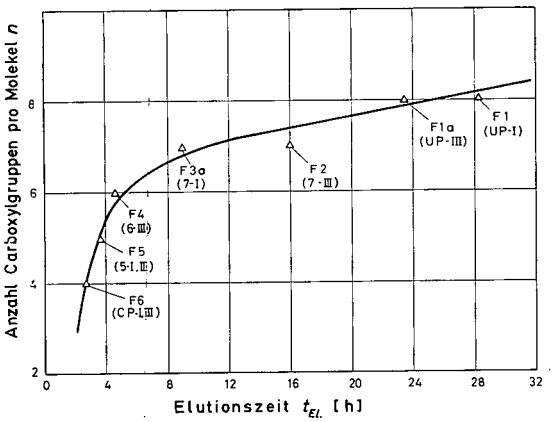

Fig. 4. Auftragung der zwischen der Elutionszeit $t_{El.}$ und Anzahl der Carboxylgruppen n bestehenden Abhängigkeit bei der extraktionschromatographischen Auftrennung der Porphyrine unter den angegebenen Versuchsbedingungen (Fig. 2).

züglich des Isomerentyps besteht in der Überführung in die entsprechenden Coproporphyrinisomere durch Decarboxylierung. Diese können dünnschichtchromatographisch im 2,6-Lutidin/Wasser-System[6] aufgetrennt und anhand der R_F-Werte eindeutig identifiziert werden.

Hierzu werden die um das jeweilige Elutionsmaximum aufgefangenen Fraktionen der einzelnen Peaks vor und nach der Decarboxylierung dünnschichtchromatographisch aufgetrennt. Das Ergebnis dieser Untersuchungen ist in den Fig. 5 und 6 dargestellt.

Während die Fraktionen F 1, 1a, 2, 3a, 4, 5/5a und 6 über die zugehörigen R_F- bzw. λ_p-Werte vor und nach Decarboxylierung identifiziert werden konnten, war

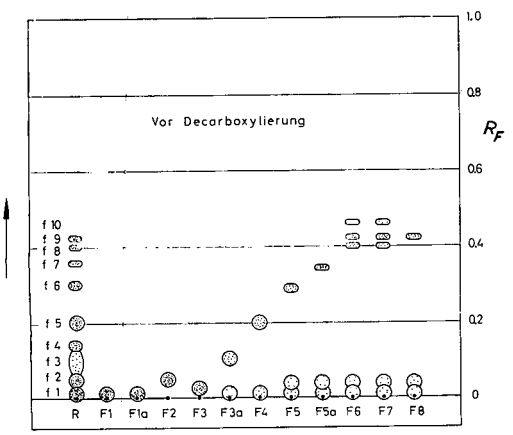

Fig. 5. Dünnschichtchromatographische Auftrennung der extraktionschromatographisch erhaltenen Fraktionen F 1–8 im 2,6-Lutidin/Wasser-System auf aktivierten Kieselgelschichten, vor der Decarboxylierung. Die Punktdichte widerspiegelt annähernd die relative Porphyrinkonzentration der einzelnen Flecken. R ist das zugehörige, aus dem Urin von Patient U. isolierte komplexe Porphyringemisch (Fig. 2, Lit. 8).

die Zuordnung von F 3, 7 und 8 bzw. f 10 bezüglich des vorliegenden Isomerentyps und Einordnung in die homologe Reihe der Hämpräkursoren nicht möglich. Die Gründe hierfür liegen vor allem darin, dass die Peaklagen λ_p der decarboxylierten Reaktionsprodukte deutlich von den für reine Coproporphyrinisomere erwarteten (λ_p = 401.30 nm) abwichen (Tabelle I). So wurde für die Peaklage von F 3, das nach der Decarboxylierung in CP-I-Position auftritt, ein Wert von λ_p = 402.30 nm erhalten (F 7: λ_p = 403.00; F 8: λ_p = 402.20 nm), wodurch die Identität mit einem der Coproporphyrinisomere, die nach der Decarboxylierung an dieser Stelle erwartet werden, ausgeschlossen werden kann. Der R_F-Wert allein kann für die Identität eines Hämpräkursors nicht als ausreichendes Kriterium anerkannt werden.

Bei der Identifikation von F 3a wird angenommen, dass diese Fraktion durch Anteile von F 4 bzw. 1, 1a oder 2 leicht kontaminiert ist, wodurch das Auftreten der Fraktion f 9 (Fig. 6) erklärlich wird. Die übrigen Kenndaten (λ_p-Wert vor und nach Decarboxylierung; R_F-Wert vor Decarboxylierung) sprechen für das Vorliegen eines 7-I-Porphyrins. Die Überlagerung der beiden Fraktionen von F 5 und 5a war so ausgeprägt, dass aus den R_F-Werten die Zuordnung des Isomerentyps nicht vorgenommen werden konnte. Die Einordnung der beiden Fraktionen als Pentacarboxylporphyrine dagegen unterliegt keinem Zweifel.

Bemerkenswert ist weiterhin die Beobachtung, dass bei sämtlichen Fraktionen F 1–8 nach der Decarboxylierung bei der dünnschichtchromatographischen Auftrennung eine Komponente f 10 in CP-II-Position[15] auftritt, die offensichtlich mit dem bei F 7 und 8 auftretenden Hauptbestandteil identisch ist. Im ursprünglichen Porphyringemisch (R, Fig. 5 und 6; Fig. 2, Lit. 8) kann diese Fraktion nicht oder nur in geringer Konzentration nachgewiesen werden. Es wird daher vermutet, dass in dem untersuchten Porphyringemisch (Patient U.) neben den der Isomerenreihen I und III angehörenden Hämpräkursoren noch Porphyrinderivate auftreten, bei denen durch Substituentenaustausch (z.B. Methyl- gegen Äthylrest u.ä.) die beobachtete Ver-

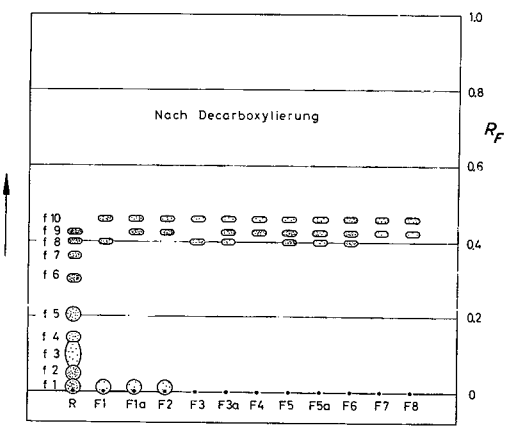

Fig. 6. Dünnschichtchromatographische Auftrennung der extraktionschromatographisch erhaltenen Fraktionen F 1–8 im 2,6-Lutidin/Wasser-System auf aktivierten Kieselgelschichten, nach der Decarboxylierung. Die Punktdichte widerspiegelt annähernd die relative Porphyrinkonzentration der einzelnen Flecken. R ist das zugehörige, aus dem Urin von Patient U. isolierte komplexe Porphyringemisch (Fig. 2, Lit. 8).

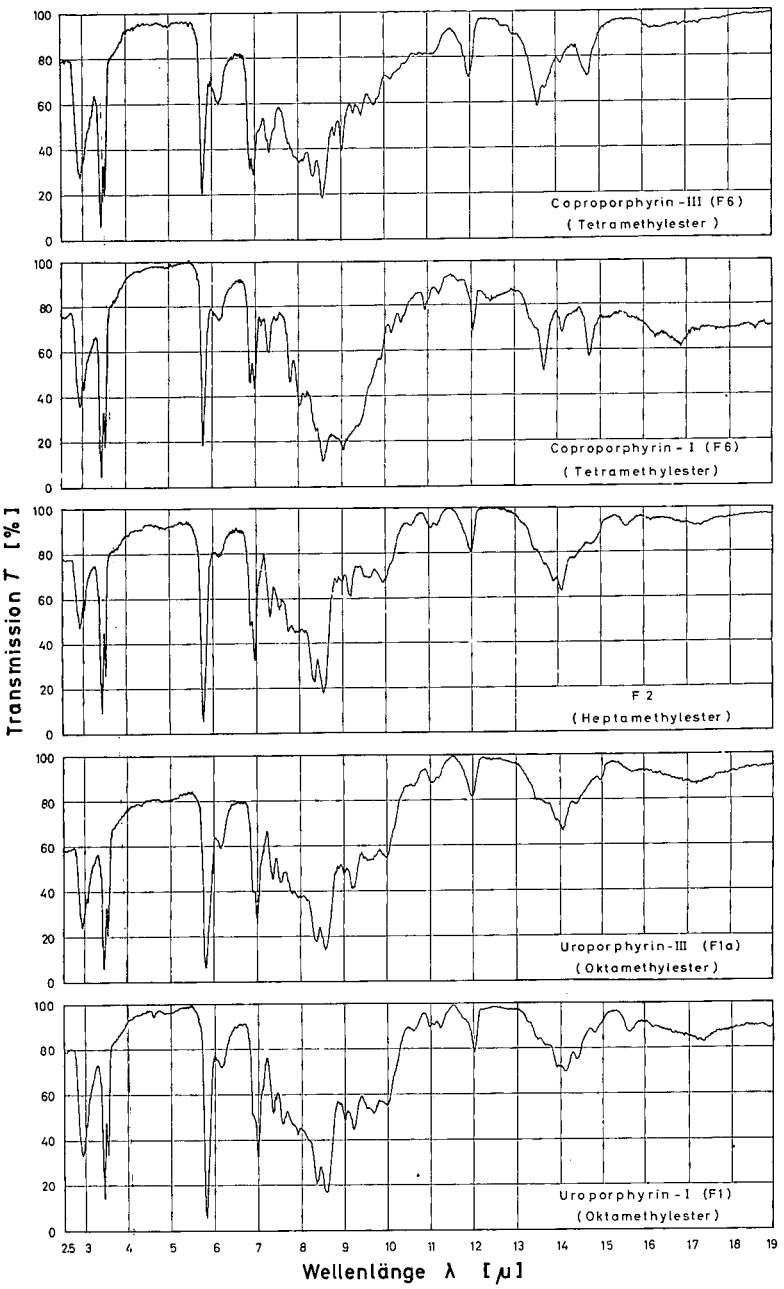

Fig. 7. Zusammenstellung der I.R.-Spektren der in Esterform vorliegenden Porphyrine von UP-I, UP-III, CP-I, CP-III bzw. F 1, 1a, 2 und f 8, 9 (= F 6). Die Spektren wurden mit KBr-Mikropresslingen bei einer Porphyrinkonzentration von 3–5 μg Ester/mg KBr mit einem I.R.-Spektrographen der Fa. E. Leitz, Wetzlar, aufgenommen.

schiebung der Peaklage der Soretbande λ_p verursacht wird. Aus dem Auftreten verschiedener R_F-Werte bei den decarboxylierten Fraktionen von F 3 und 8 (Fig. 6) bei gleichem λ_p-Wert muss geschlossen werden, dass auch die substituierten Porphyrinderivate verschiedenen Isomerenreihen angehören (I und III?). Hierbei scheint der Anteil mit dem grösseren R_F-Wert (III?) zu überwiegen (f 10). Da sämtliche Fraktionen F 1–8 nach der Decarboxylierung bei der dünnschichtchromatographischen Auftrennung neben dem jeweiligen Hauptbestandteil einen Anteil f 10 lieferten, folgt, dass diese Porphyrinderivate ebenfalls in den bei den normalen Porphyrinen auftretenden, durch den Grad der Decarboxylierung gekennzeichneten Zwischenstufen in dem ursprünglichen Porphyringemisch vorliegen. Zur näheren Charakterisierung dieser Verbindungen sind jedoch weitere Untersuchungen erforderlich. Das Vorliegen der den Isomerenreihen II und IV angehörenden Porphyrine kann aus den bereits angedeuteten Gründen ausgeschlossen werden.

Infrarotspektroskopische Identifikation einiger Porphyrine und deren Isomere

Zur weiteren Charakterisierung der aus dem Urin von Patient U. isolierten Fraktionen (F 1, 1a, 2 und f 8, 9) wurden die Infrarotspektren der entsprechenden Ester aufgenommen. Diese wurden den bei reinen Bezugssubstanzen (CP-I, III und UP-I, III) erhaltenen gegenübergestellt. Eine Auswahl der bei diesen Untersuchungen erhaltenen Spektren ist in Fig. 7 zusammengestellt. Hieraus geht hervor, dass die I.R.-Spektren der Fraktionen von F 1, 1a mit den der reinen Bezugssubstanzen UP-I und UP-III übereinstimmen. Die zwischen den I.R.-Spektren von UP-I und UP-III auftretenden geringfügigen Unterschiede reichen nicht aus, um die Isomere anhand ihrer I.R.-Spektren identifizieren zu können. Gleichfalls besteht weitgehende Übereinstimmung der Spektren von F 2 und F 1a (UP-III).

Demgegenüber weisen die I.R.-Spektren von CP-I und CP-III bzw. f 8 und 9 deutlichere Unterschiede auf, die zur Kennzeichnung des Isomerentyps beim Vorliegen reiner Substanzen ausreichen sollten. Eine quantitative Isomerenanalyse aufgrund dieser Verschiedenheiten der I.R.-Spektren, wie an anderer Stelle vorgeschlagen[16], erscheint jedoch kaum möglich.

Zusammenfassend lässt sich feststellen, dass unter den beschriebenen Bedingungen die I.R.-Analyse von Porphyrinester dadurch eingeschränkt wird, dass:

(1) Zur Herstellung der KBr-Mikropresslinge noch relativ grosse Porphyrinmengen benötigt werden (min. 30 μg);

(2) bei der Präparation der Ester extreme Sorgfalt erforderlich ist;

(3) dass die in KBr als Matrix auftretenden Unterschiede des Bandenverlaufes kaum ausreichend sind, um Isomerengemische über eine Auswertung der I.R.-Spektren quantitativ zerlegen zu können.

DANK

Herrn Prof. Dr. H.-J. EICHHOFF und Herrn F. B. WOLF, Institut für Anorganische Chemie und Kernchemie der Johannes Gutenberg-Universität, Mainz, danke ich sehr herzlich für die bei der Aufnahme der I.R.-Spektren gewährte Unterstützung.

ZUSAMMENFASSUNG

Zur Analyse komplexer Porphyringemische wird eine Kombination verschie-

dener Untersuchungsmethoden empfohlen, die eine Identifikation der einzelnen Hämpräkursoren durchzuführen gestattet. Die bei der extraktionschromatographischen Auftrennung erhaltenen Fraktionen können bezüglich des Decarboxylierungsgrades über eine Bestimmung der Peaklage der Soretbande λ_p, die bereits mit Mengen von min. 0.2 μg durchgeführt werden kann, eingeordnet werden. Zur Ermittlung des Isomerentyps werden die Porphyrine in die entsprechenden Coproporphyrinisomere durch Decarboxylierung überführt, die dünnschichtchromatographisch im 2,6-Lutidin/Wasser-System identifiziert werden.

Mit den beschriebenen Verfahren (Extraktionschromatographische Auftrennung der Porphyrine im Verteilungssystem Tri-*n*-butylphosphat/1 *N* Salzsäure; Dünnschichtchromatographische Trennung der Porphyrine im 2,6-Lutidin/Wasser-System; Absorptionsspektrophotometrische Ermittlung der Peaklage der Soretbande λ_p) konnten in einem aus dem Urin eines Patienten (Patient U.) isolierten Porphyringemisch alle die zwischen Uroporphyrin und Coproporphyrin auftretenden Reaktionsstufen der beiden Isomerenreihen I und III, mit Ausnahme des 6-I-Porphyrins, nachgewiesen und z.T. quantitativ bestimmt werden. Darüber hinaus wurden zahlreiche Fraktionen unbekannter Porphyrine aufgefunden, die sich nicht in die normale Hämsynthesekette einordnen liessen. Es wird angenommen, dass es sich hierbei um substituierte Porphyrinderivate der Isomerenreihen I und III handelt. Die Aufklärung der Struktur dieser Verbindungen, insbesondere die Ermittlung der angenommenen Substituenten, muss weiteren Untersuchungen vorbehalten bleiben. Für das Auftreten von Porphyrinen der Isomerenreihen II und IV dagegen ergaben sich keinerlei Anhaltspunkte.

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CHROM. 3742

THE RELATIVE SENSITIVITY OF VARIOUS REAGENTS FOR THE DETECTION AND DIFFERENTIATION OF SUGARS AND SUGAR DERIVATIVES IN GLYCOPROTEINS

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(Received August 14th, 1968)

SUMMARY

Eleven sugars reported to be found in the carbohydrate moiety of different glycoproteins were separated by paper chromatography in *n*-butanol–pyridine–water. A study was made of the relative sensitivity and specificity of twelve colouring reagents for the detection of each sugar and sugar derivative. A distinction between those reagents dependent on the reducing property of the sugar and those involving at one stage or another a condensation reaction is suggested.

INTRODUCTION

Many investigators reported on the composition of the carbohydrate moiety of glycoproteins[1,2] using a variety of techniques for hydrolysis. Others[3,4] demonstrated that not all sugars were released at the same rate and some degraded during hydrolysis. Thus the quantitative results for the carbohydrate composition of glycoproteins may be questioned. As a result, a series of experiments was designed to investigate the effect of hydrolysis on the individual sugars of the glycoproteins. The present paper reports on the sensitivity and specificity of various reagents for visualizing sugars and sugar derivatives separated by paper chromatography.

In choosing from the many colour reagents available, three interrelated factors were considered; sensitivity, specificity and the resolution of the various sugars by the chromatographic system used. The reagent had to be sufficiently sensitive to detect concentrations below that which would overload the chromatographic system. Also the relative sensitivity for each sugar and sugar derivative was of importance in establishing the limits of the reported qualitative and quantitative composition. The fact that certain reagents were specific for an individual sugar or a chemically related group of sugars does not necessarily mean that they were effective at all levels applied.

Of the reagents described in the literature, those with reported sensitivity in the 1–20 μg range were selected[5–13]. Data on the sensitivity of a specific method for a number of sugars and sugar derivatives were found in two of these papers[8,9] but none compared the sensitivity of several methods for a specific sugar. Both aspects were determined in one solvent system for sugars reported to be present in chondroitin sulphate and other polysaccharides associated with proteins.

EXPERIMENTAL

All chemicals were analytical reagents used without further purification. Two standard solutions A and B were made up in distilled water. Each contained a combination of sugars and sugar derivatives, that according to the experimentally established R_g values (the average of 5 measurements), gave the least overlapping of spots (see Table I).

TABLE I

Standard A	*R_g value*	*Standard B*	*R_g value*
D-Glucuronic acid	9 ± 2.0	D(+) Glucosamine·HCl	59 ± 3.4
D(+)Galactosamine·HCl	43 ± 4.0	D(+)Glucose	100
D(+)Galactose	85 ± 4.0	D(+)Mannose	126 ± 1.7
N-Acetyl-D-galactosamine	139 ± 2.6	N-Acetyl-D-glucosamine	150 ± 3.4
D(−)Ribose	185 ± 2.9	D(+)Fucose	175 ± 4.1
D-Glucuronolactone	249 ± 12.0	D-Glucuronolactone	249 ± 12.0

All sugars and derivatives were prepared in 1 μg/μl concentrations. Glucuronolactone, which was less sensitive than all other compounds used, was prepared at the 5 μg/μl concentration in standard solution B.

Sheets of 41.5 × 15 cm Whatman No. 1 paper were used for chromatography. The origin was 7 cm from the short side of the paper. Eight spots were placed 1.5 cm apart, the first 4 spots received 1, 2, 4 and 6 μl of standard A and the next four spots the same volumes of standard B. Descending chromatography was carried out in *n*-butanol–pyridine–water (10 : 3 : 3, v/v) for 25 h at room temperature. After this period of time they were left to dry in the fume hood until only a faint odour of *n*-butanol remained (approximately 4 h).

The sugar and sugar derivatives were then visualized by using one of the following reagents:

(a) The silver nitrate-alkaline reagent according to TREVELYAN *et al.*[10].

(b) 2% *p*-anisidine·HCl in methanol spray[6,7], followed by heating at 100° until spots were developed (2–10 min).

(c) The hexosamine (ELSON–MORGAN) reagent as outlined by SMITH[5]. After the second dipping the spots developed to full intensity within 2 min, then faded.

(d) (e) (f) (g) The aniline–diphenylamine (ADPA), naphthoresorcinol (NR), dinitrosalicylic acid (DNSA) and benzidine reagents were all applied according to SMITH[5].

(h) The aniline hydrogen phthalate (AHPh) reagent was made up and applied according to WILSON[11]. The chromatogram was air dried and then heated at 105° for 2 min to develop the spots.

(i) The periodate–acetylacetone (PAA) reagent was used as described by WEISS AND SMITH[12].

(j) The periodate–permanganate reagent was sprayed as described by LEMIEUX AND BAUER[8] and the chromatogram left to develop for 20 to 30 min at room temperature.

(k) (l) Both the 2,3,5-triphenyl tetrazolium chloride (TTC) and 3,3′-dianisole

TABLE II

DETECTION OF SUGARS AND SUGAR DERIVATIVES ON WHATMAN No. 1 PAPER

Compound	*$AgNO_3$–alkaline*		*p-Anisidine·HCl*		*Elson–Morgan*		*ADPA*		*NR*		*DNSA*		*Benzidine*		*AHPh*		*PAA*		*PP*		*TTC*		*BT*	
	colour	*μg*[a]	*colour*	*μg*	*colour*	*μg*	*colour*	*μg*	*colour*	*μg*	*colour*	*μg*	*colour*	*μg*	*colour*	*μg*	*colour*[b]	*μg*	*colour*	*μg*	*colour*	*μg*	*colour*	*μg*
D(+)Glucose	bn[d]	1	gr-bn	1	b-g	1	b-g	4	—	—	bn	4	bn	1	bn	1	y-gr	1	y	1	r	1	b-v	1
D(+)Galactose	bn	1	gr-bn	1	b-g	6	b-g	6	—	—	bn	6	bn	2	bn	2	y-gr	1	y	2	r	1	b-v	1
D(+)Mannose	bn	1	gr-bn	2	—[c]	—	b-g	6	—	—	bn	6	bn	1	bn	2	y-gr	1	y	1	r	1	b-v	1
D(+)Fucose	bn	1	gr-bn	2	b-g	6	—	—	—	—	bn	6	—	—	bn	2	—	—	y	1	r	1	b-v	1
D(−)Ribose	bn	2	r	1	b-g	6	—	—	—	—	bn	6	bn	2	r	2	y-gr	6	y	4	r	1	b-v	1
D-Glucuronic acid	d-bn	2	o-bn	1	gr-b	2	g	1	b	1	bn	4	g-bn	1	o-bn	1	y-gr	4	y	1	r	1	b-v	1
D-Glucuronolactone	bn	5	bn	20	—	—	b-g	20	b	5	—	—	g-bn	20	o-bn	20	—	—	y	5	r	2	b-v	2
D(+)Glucosamine·HCl	bn	2	gr-bn	6	r	1	—	—	—	—	—	—	l-bn	1	bn	2	y-gr	1	y	2	r	1	b-v	1
D(+)Galactosamine·HCl	bn	2	gr-bn	6	r	1	—	—	—	—	—	—	l-bn	1	bn	2	y-gr	1	y	2	r	1	b-v	1
N-Acetyl-D-Glucosamine	bn	4	gr-bn	4	p	1	—	—	—	—	—	—	—	—	bn	4	y-gr	1	y	2	r	1	b-v	1
N-Acetyl-D-Galactosamine	bn	4	gr-bn	4	p	1	—	—	—	—	—	—	—	—	bn	4	y-gr	1	y	2	r	1	b-v	1

[a] Sensitivity in micrograms.

[b] Colour as seen under U.V.

[c] — = Not detected at levels applied.

[d] Abbreviations used: ADPA = aniline-diphenylamine, NR = naphthoresorcinol, DNSA = dinitrosalicylic acid, AHPh = aniline hydrogen phthalate, PAA = periodate-acetylacetone, PP = periodate-permanganate, TTC = 2,3,5, triphenyl tetrazolium chloride, BT = blue tetrazolium; b = blue, bn = brown, d = dark, g = grey, gr = green, l = light, o = orange, r = red, p = purple, y = yellow.

bis-4,4(3,4-diphenyl)-tetrazolium chloride (or blue tetrazolium = BT) were used according to SZABADOS *et al.*[13] but the heating step was eliminated to avoid excessive background colour. Instead the chromatogram was left to develop for 24 h in the dark.

RESULTS AND DISCUSSION

The relative sensitivity of various reagents for the detection of sugars and sugar derivatives are summarized in Table II. Although in our work the detection of a minimum of 1 μg of an individual sugar was sufficient, the 1 μg levels given in Table II were not necessarily the limit of detection.

The silver nitrate–alkaline test gave distinct brown spots on a light brown background at low levels of all compounds, except N-acetyl-aminosugars and glucuronolactone. The latter formed an elongated spot on Whatman No. 1 paper, which may partially account for its apparent insensitivity in all tests. A similar elongation was reported by WILSON[11] for galacturonolactone using Whatman No. 1 and the same solvent system. The test was not specific for an individual sugar or chemically related group of sugars or sugar derivatives but was one of the more sensitive reagents for detection of well separated sugars. Because of its sensitivity and acceptance by many carbohydrate chemists, this method was compared with other methods.

The dinitrosalicylic acid reagent was insensitive. This may be partly due to the limited temperature range for colour development. Slight overheating of the chromatogram changed the yellow background to brown and thereby masked the similar colour of the spots. The aminosugars, N-acetyl-aminosugars and glucuronolactone did not react at the levels applied. Besides the low sensitivity for those sugars that did react, the test was non-specific.

The *p*-anisidine · HCl was very sensitive except with aminosugars and glucuronolactone. Most compounds gave a green-brown colour with this spray reagent, but glucuronic acid, glucuronolactone and ribose gave an orange-brown, brown and red colour respectively. VEIGA AND CHANDELIER[9] reported other colours for the same compounds under slightly different conditions. This general reagent for sugars gave specific colours in the case of the uronic acids and the single pentose used in this experiment.

The ELSON–MORGAN reagent was more sensitive than silver nitrate–alkaline for aminosugars and N-acetyl-aminosugars. Glucuronolactone and mannose could not be detected at the levels applied, but glucose, galactose, fucose and ribose gave blue-green spots at 6 μg or less. Aminosugars and N-acetyl-aminosugars could be distinguished from other sugars by their red and purple colouration respectively. Glucuronic acid gave a reddish spot after the first heating step but changed to greenish-blue after the second heating. The spots will fade unless heated at a specific stage during the removal of hydrochloric acid. Excess hydrochloric acid resulted in a brownish background upon heating, which masked the red and purple spots. Therefore, judgement was required when to start heating the chromatographic paper. The sensitivity and specificity of this reagent for aminosugars and N-acetyl-aminosugars made it especially useful as a companion to the silver nitrate–alkaline reagent.

It has been reported earlier[5], that the aniline–diphenylamine reagent was difficult to apply, but was useful for colour differentiation. However, it was found to be insensitive to most sugars at the levels considered in this work. The blue-grey back-

ground might have been responsible, since it tended to blend with the similar colours and intensities of the individual sugar spots. Glucuronic acid was the only compound detected at the 1 μg level. SMITH[5] reported the detection of fucose and ribose, but in our laboratory these two sugars failed to show at the levels applied.

The naphthoresorcinol reagent, specific for uronic acids, was more sensitive for glucuronic acid (1 μg) than for glucuronolactone (5 μg). Both of these sugar derivatives gave a very distinct bluish spot on a pink background. Neutral sugars and hexosamines would not react at the levels applied. There is considerable difference in the specific gravities of the chemicals used in this reagent mixture. Care was taken to insure homogeneity, or the test was no longer specific and other sugars also gave a bluish spot.

Aniline hydrogen phthalate had a sensitivity similar to silver nitrate–alkaline for aminosugars, N-acetyl-aminosugars and glucose. Other compounds except uronic acids, were detected at the 2 μg level. Of the uronic acids, glucuronic acid was detected at the 1 μg level, but glucuronolactone was not detected below the 20 μg level. All sugars or sugar derivatives gave a brown coloured spot on the paper chromatogram, except ribose and uronic acids, which were red and orange-brown respectively. Thus in addition to a sensitivity approaching that of silver nitrate–alkaline, aniline hydrogen phthalate reagent gave specific colours for the uronic acids and ribose.

The sensitivity of the benzidine reagent was of the same order as silver nitrate–alkaline for most compounds (1–2 μg). Fucose and N-acetyl-aminosugars were not detected when treated with this reagent.

The recent method (PAA) by WEISS AND SMITH[12] could be used for the detection of most sugars and sugar derivatives in the range of 1 to 6 μg. The spots showed up well under U.V., but the visible yellow colour was rather faint. Fucose and glucuronolactone were not detected using as high as 6 and 30 μg respectively. This method detected hexosamines at the same level (1 μg) as in the ELSON–MORGAN test.

The periodate–permanganate reagent reacted with low concentrations of all compounds, especially the more difficultly detected amino- and N-acetyl-aminosugars. Except for ribose, the reagent was as sensitive as the silver nitrate–alkaline. No colour differentiation was observed.

The data in Table II show, that TTC and BT were sensitive for all sugars and sugar derivatives at the 1 μg level with the notable exception of glucuronolactone, which was detected at the 2 μg level. Both tests, however, were considerably more sensitive for glucuronolactone than any other method used. Although most sugars could be detected after 1 h development in the dark, mannose and glucuronolactone did not reach full intensity before 24 h. All sugars and sugar derivatives used in this study gave a red colour upon development.

Table II illustrates that if N-acetyl-aminosugars or glucuronolactone were present in a hydrolysate, the number of methods for detection at the 1–2 μg level was limited. Hexosamines may be detected at the 1 μg level by ELSON-MORGAN, PAA, TTC and BT reagents. Glucuronolactone was relatively insensitive, although it was detected below the 6 μg level by 5 reagents. The results seem to indicate that redox reactions, such as the ones involving silver nitrate–alkaline[14], periodate–permanganate[8], TTC and BT[15] are more sensitive, but less specific for individual sugars.

Reactions involving at one stage or another a condensation such as the ELSON–MORGAN[16], NR[17] and those using primary aromatic amines[18] appear to be less sensitive, but more specific.

CONCLUSIONS

For the overall detection of sugars and sugar derivatives several reagents may be employed, such as silver nitrate–alkaline, periodate–permanganate, and the triaryl tetrazolium salts. Of these reagents 2,3,5-triphenyl tetrazolium chloride and Blue tetrazolium were the most sensitive. For a combination of sensitivity and specificity the ELSON–MORGAN and naphthoresorcinol tests were the most applicable to amino sugars and uronic acids respectively, whereas the aniline hydrogen phthalate differentiated best between hexoses, uronic acids and the one pentose.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. E. COFFIN for his assistance in the preparation of the manuscript. We also would like to express our appreciation to Dr. JOYCE L. BEARE-ROGERS and Dr. S. GUNNER for their helpful criticisms.

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Notes

CHROM. 3745

Electrodeless discharge as detector in gas chromatography. IV. Comparison with a thermal conductivity detector

The use of an electrodeless discharge as a detector in gas chromatography has been described earlier[1-3]. It consists of two coaxial glass tubes sealed as in a Siemens' type ozonizer through the annulus of which pass the effluents from the gas–solid chromatographic (GSC) or gas–liquid chromatographic (GLC) column. The tube is excited in a low frequency electrodeless discharge at a constant potential and the discharge current, after rectification by a crystal, is measured by a galvanometer, or, a suitable potential drop of the output, after smoothening, is fed to a strip-chart recorder. The background current due to the carrier gas is conveniently brought to zero by a small bias potential and the noise level is satisfactorily reduced. The arrival of each gas or vapour sample into the detector from the column is marked by a signal of a sharp change of discharge current ($\pm \Delta i$) in the galvanometer, or as a peak on the recorder chart. The resolution time is short and no additional amplification is necessary for samples of the order of 0.1 μmole. The signal strength for a given sample, measured as the area under the peak, is controlled by the dimensions of the detector (height, ratio of the diameters of the outer and inner tubes and the annular width), its temperature and the excitation voltage, conditions in regard to the column and the carrier gas being constant. It is easy to find a range of conditions for each GSC or GLC and sample system over which the signal height is linearly related to the quantity of the substance present in the sample injected.

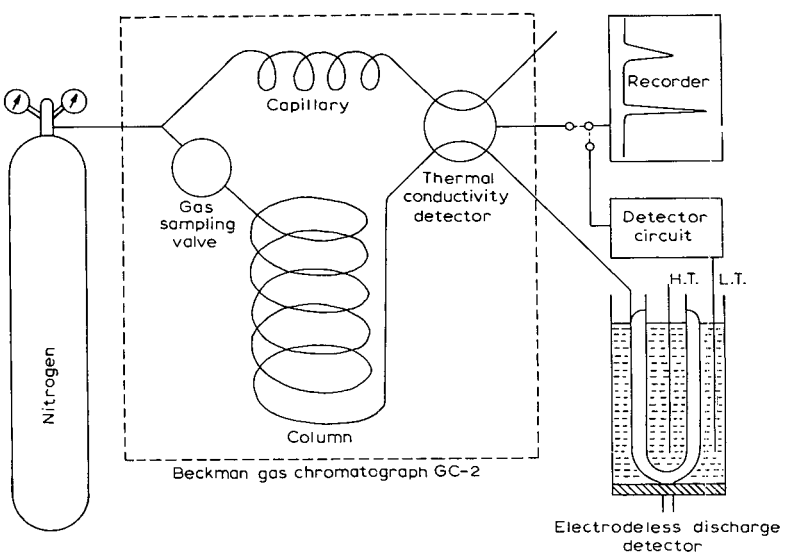

Fig. 1. Schematic diagram of the experimental set up.

Details of the construction and working of the new detector and the results of the separation of a number of inorganic and organic gases and vapours and the factors controlling the detector sensitivity have been published earlier[1-3]. In the present note a comparison has been made between the efficiency of the detector and that of a thermal conductivity detector commonly used in a commercial instrument.

Experimental

In the present work the signals obtained with the electrodeless discharge

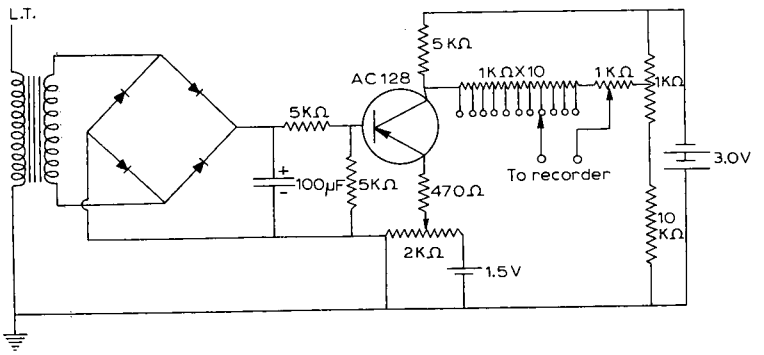

Fig. 2. The detector circuit.

detector for a variety of gases and vapours have been compared with those from the thermal conductivity detector (filament type) of the Beckman gas chromatograph

TABLE I

COMPARISON OF SIGNALS WITH THE THERMAL CONDUCTIVITY DETECTOR AND THE ELECTRODELESS DISCHARGE DETECTOR CONNECTED IN SERIES FOR VARIOUS SUBSTANCES

Carrier gas: nitrogen; column inlet pressure: 2 kg/cm²; column: silicone oil (20%) supported on 40–60 mesh firebrick powder; length, 1.8 m; temperature, 70°. The signal is expressed in number of divisions of the recorder chart paper.

Series No.	*Substance*	*Signal (peak height)*	
		(A) Thermal conductivity detector	*(B) Electrodeless discharge detector**
1	Oxygen	12	>100
2	Carbon dioxide	20	103
3	Chloroform	7	100
4	Carbon tetrachloride	4	88
5	Acetone	8	>100
6	Ethyl methyl ketone	3	48
7	Methyl acetate	3	40
8	Ethyl acetate	6	100
9	'Burshane' (domestic fuel gas, mainly butane)	3 peaks: 1.0, 1.5, 3.5	5 peaks: 78, >100, 85, 36, 22

* Here only a fraction of the signal obtained was fed to the recorder so as to be within the maximum limit of the recorder chart.

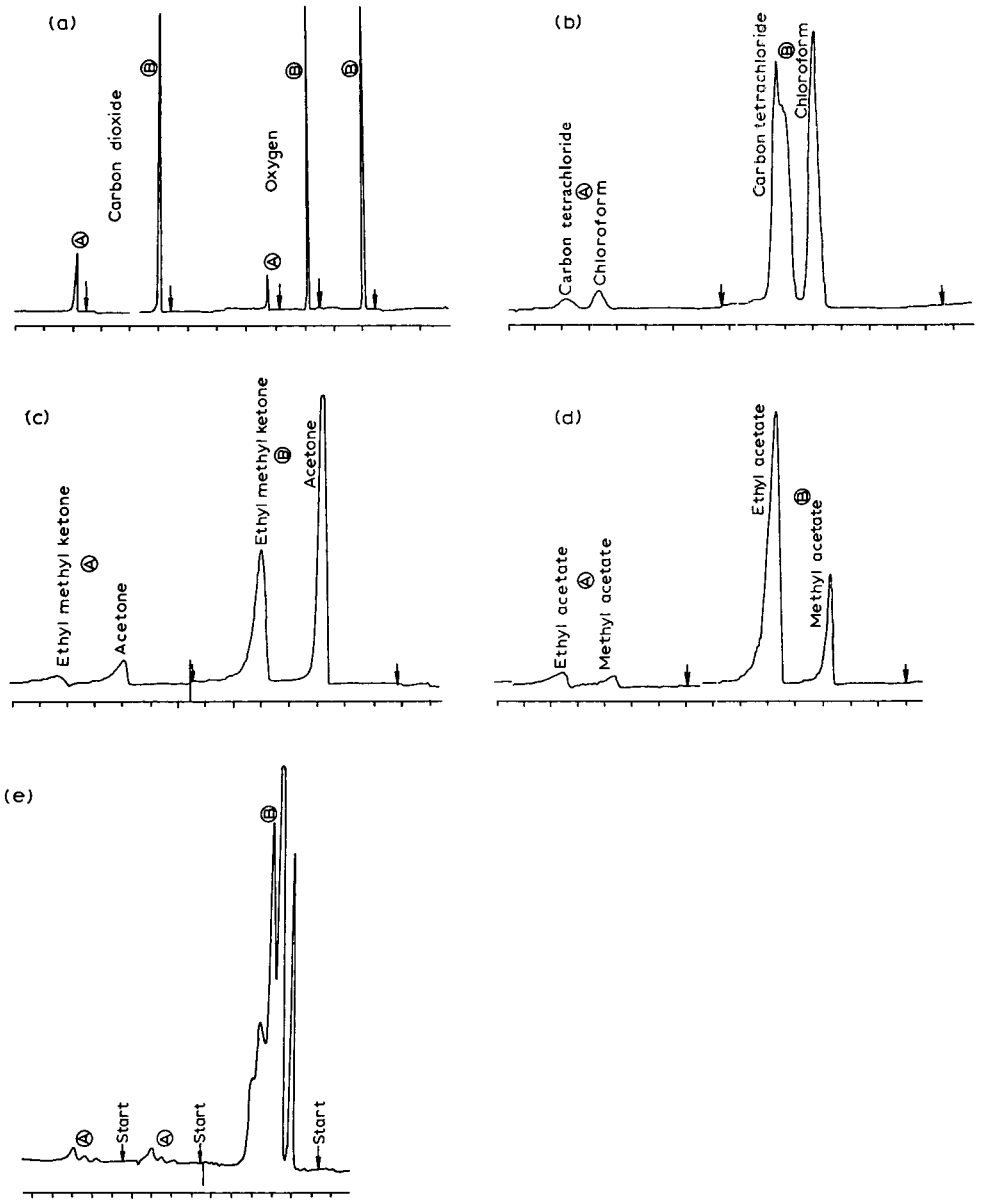

Fig. 3. Comparison of signals with (A) the thermal conductivity detector and (B) the electrodeless discharge detector for various systems: (a) oxygen, carbon dioxide; (b) mixture of chloroform and carbon tetrachloride; (c) mixture of acetone and methyl ethyl ketone; (d) mixture of methyl acetate and ethyl acetate; and (e) domestic fuel gas 'Burshane', mainly butane. Carrier gas: nitrogen.

GC-2. The new detector is connected at the exit of the Beckman gas chromatograph, so that the two detectors (the thermal conductivity and the electrodeless discharge) are in series and are operated simultaneously under identical conditions; the carrier

gas, column, gas sampling valve and injection system for liquids, being common to both. The output of either detector can be fed to the strip-chart recorder (Bristol's Dynamaster Recorder, 1 mV for full-scale deflection). Fig. 1 is a schematic diagram of the experimental set up and Fig. 2 gives the circuit diagram used in conjunction with the new detector. The chromatograms for 5 different systems recorded by the two detectors are shown in Fig. 3 and the corresponding peak heights in Table I.

Results and discussion

Fig. 3 and the results summarized in Table I clearly show that under the same operating conditions the signals from the electrodeless discharge detector are much stronger than those from the thermal conductivity detector. It has been verified that the transistor AC 128 used in the detector circuit causes no amplification of the signal. Thus not only no amplification has been used in the case of the new detector but also the signals often represent only a fraction of the total output which is too high for the recorder. Another advantage of the new detector over the thermal conductivity one is that its sensitivity does not suffer from a rise of temperature; on the contrary, there is a marked increase in the sensitivity at higher temperatures[2].

The absence of metallic electrodes in the new detector distinguishes it from the other discharge detectors with metallic electrodes used by earlier workers[4–7] which are characterised by a steadily decreasing sensitivity and poor reproducibility due to rapid contamination of the electrode surfaces by decomposition products. The electrodeless discharge is free from these limitations and it can be worked under optimum conditions when the signals are reproducible and linearly related to the quantity of the samples of a variety of gases and vapours of varied chemical nature. This high sensitivity and versatility of the electrodeless discharge detector combined with the extreme simplicity of its construction commend its adaption as a cheap and efficient detector in gas chromatography.

Acknowledgement

We thank Mr. S. S. SARDESAI for his help in constructing the detector circuit used in this work.

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Received August 16th, 1968

CHROM. 3748

Separation of some polynuclear aromatic hydrocarbons by gas–solid chromatography on graphitized carbon black

In recent years considerable attention has been given to the separation and identification of polynuclear aromatic hydrocarbons in such complex mixtures as coal-tar pitch[1], cigarette smoke[2], pyrolytic products[3], and air-borne particulate matter[4]. The existing gas chromatographic methods for separating the polynuclear aromatic hydrocarbons are based on gas–liquid chromatography[5–9], whereas gas–solid chromatography has seen limited application[10–12].

In the present paper we have studied the separations of 3- and 4-ring polynuclear aromatic hydrocarbons by gas–solid chromatography using graphitized carbon black. This adsorbent has been used recently in the separation of biphenyl and terphenyls[13].

Experimental

A Varian Aerograph Model 1520-B gas chromatograph with an electron-capture detector (tritium foil) was used for studies ranging to 415°. For higher oven temperatures, a Barber-Colman Model Selectra-System 5000 was used with a flame ionization

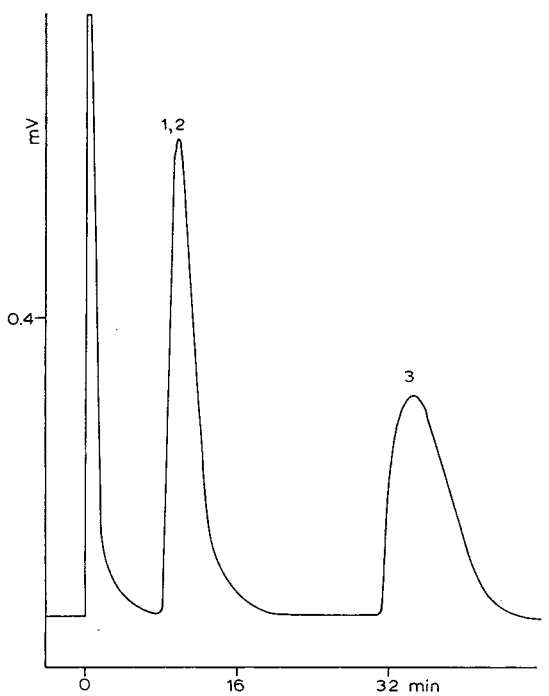

Fig. 1. Chromatogram of a mixture of anthracene (4.8 μg; 1), phenanthrene (2.4 μg; 2), and pyrene (10 μg; 3) on graphitized carbon black at 470°. Attn. 5 × 10^{-11}. Flame ionization detector.

TABLE I

RETENTION TIMES AT 350° FOR POLYNUCLEAR AROMATIC HYDROCARBONS ON GRAPHITIZED CARBON BLACK

80/100 Mesh; carrier gas flow rate of 70 ml/min at 350°.

Hydrocarbon	*Retention time (min)*
Acenaphthene	1.8
Acenaphthylene	1.9
Fluorene	14.0
Phenanthrene	15.0
1-Methylphenanthrene	19.2
Anthracene	20.0
Pyrene	80.0
Fluoranthene	80.0

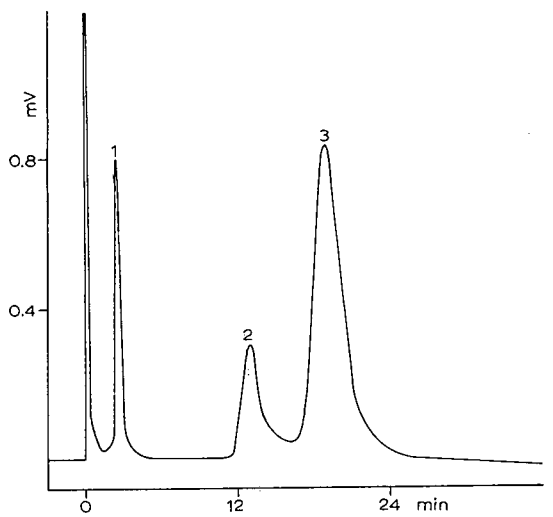

Fig. 2. Chromatogram of a mixture of acenaphthene (0.1 μg; 1), phenanthrene (0.5 μg; 2) and anthracene (0.1 μg; 3) on graphitized carbon black at 415°. Attn. 8. Electron-capture detector.

detector. The chromatographic conditions were: Injector temperature, 320°; detector temperature, 210° (for electron capture) and 340° (for flame ionization); nitrogen carrier gas with an inlet pressure of 5.7 atmospheres; and carrier gas flow rate of 60 ml/min at 30°.

The graphitized carbon black used was a Sterling M.T. FF (D-6) 1009 specimen (Cabot Corp., Boston, Mass., U.S.A.). A stainless-steel tubing (70 cm × 2.3 mm ID) was packed with graphitized carbon black (100/120 mesh) by tapping the sides of the tubing. A second column was loosely packed with the same adsorbent (80/100 mesh). The columns were conditioned in the instrument for two hours at 415° before use.

The sample mixtures in benzene were injected with a Hamilton 10 μl microsyringe. The results of the separations are shown in Figs. 1 and 2 and in Table I.

Discussion

The chromatographic separations of some 3- and 4-ring polynuclear aromatic hydrocarbons on 100/120 mesh graphitized carbon black are shown in Figs. 1 and 2. The retention times obtained when the loosely packed 80/100 mesh material was used are shown in Table I. The larger mesh size permitted operation at oven temperature of 350° instead of 415°, without affecting the chromatographic separations.

Although anthracene and phenanthrene were found to have the same retention times at 470°, these hydrocarbons were separated well at 415° and 350° on 100/120 and 80/100 mesh columns respectively. The separation was superior to those previously reported on potassium antimonate and potassium carbonate–potassium hydroxide columns[12]. Good resolution of anthracene and phenanthrene on gas–liquid packed columns has not been graphically demonstrated[7–9].

In Fig. 2, the poorer electron absorptivity of phenanthrene as compared to that of anthracene accounts for the formation of a smaller peak for phenanthrene on the chromatogram. Electron-capture response *versus* sample size for anthracene and phenanthrene has been demonstrated in an earlier study[14].

We were unable to separate fluoranthene from pyrene, nor acenaphthene from acenaphthylene on these short columns.

At column temperature of 470° injected samples of chrysene and benz(a)-anthracene produced no peaks on the chromatogram after two hours, even when sample sizes of 20 μg were used. It appears that these polynuclear aromatic hydrocarbons were irreversibly adsorbed on the graphitized carbon black.

Conclusions

Gas–solid chromatography using graphitized carbon black was tried on several 3- and 4-ring polynuclear aromatic hydrocarbons. This adsorbent was found to have a unique ability of being able to separate a mixture of anthracene and phenanthrene on a short column.

Acknowledgements

The study was carried out under contract with the Agricultural Research Service, U.S. Department of Agriculture, administered by the Eastern Utilization Research and Development Division, Philadelphia, Pennsylvania.

The author wishes to acknowledge the valuable technical assistance of ESTHER WAN in this work.

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Received August 19th, 1968

J. Chromatog., 38 (1968) 130–133

CHROM. 3756

Gaschromatographische Trennung aller neun struktur- und stereoisomeren Cyclohexantricarbonsäuren

Im Verlauf unserer Arbeiten über Cyclohexanpolycarbonsäuren* konnten wir die drei strukturisomeren Cyclohexantricarbonsäuren gaschromatographisch in die neun möglichen stereoisomeren Formen auftrennen und bestimmen.

In Fig. 1 sind die Strukturformeln aufgezeichnet. Man erkennt, dass es für die Cyclohexantricarbonsäure-1,3,5 zwei, für die Cyclohexantricarbonsäure-1,2,3 drei

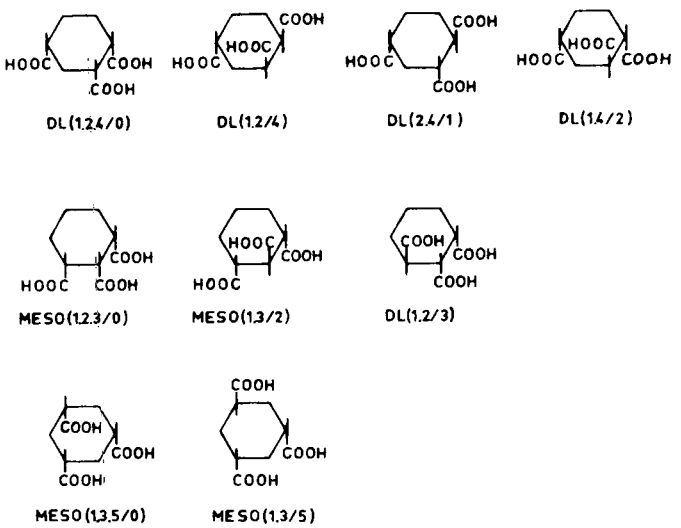

Fig. 1. Strukturformeln der neun isomeren Cyclohexantricarbonsäuren.

und für die Cyclohexantricarbonsäure-1,2,4 vier *cis-trans* isomere Formen gibt. Die Nomenklatur entspricht einem Vorschlag von MCCASLAND[1], nach der die Substituenten in *cis*-Stellung vor und die Substituenten in *trans*-Stellung hinter dem Schrägstrich angegeben werden.

* Veröffentlichung in Vorbereitung.

Fig. 2 zeigt das Gaschromatogramm eines Gemisches (A) aller isomeren Cyclohexantricarbonsäuren, die in Form ihrer Methylester mit einer 100 m Kapillare isotherm analysiert wurden.

Nachfolgend sind die gaschromatographischen Bedingungen aufgeführt:

Gaschromatograph:	WCLID 1680 (Warner-Chilcott).
Trennsäule:	Material Edelstahl (SH 2); Länge 100 m; Innendurchmesser 0.5 mm; stationäre Phase Ucon LB 1715.
Trägergas:	Helium; Durchflussmenge 10 ml/min.
Detektor:	Flammenionisation.
Temperatur: Säule:	170° ± 2%.
Verdampfer:	410° ± 2%.
Detektor:	250° ± 2%.
Einspritzmenge:	1.8 μl; Probenteilung 1:40.
Schreiber:	Honeywell-Brown 1-mV Kompensations-Schreiber; Dämpfung 100.

Die Identifizierung der einzelnen Banden des in Fig. 2 dargestellten Chromatogramms gelang uns nur teilweise. Wir wissen zwar, welche Peaks zu den einzelnen strukturisomeren Cyclohexantricarbonsäuren gehören, können aber bisher nicht mit Sicherheit angeben, welche von diesen den *cis*- und welche den *trans*-Isomeren zuzuordnen sind, da wir über reine *cis*- und *trans*-Testsubstanzen nicht verfügen.

Die Zuordnung der strukturisomeren Cyclohexantricarbonsäuren geschah folgendermassen:

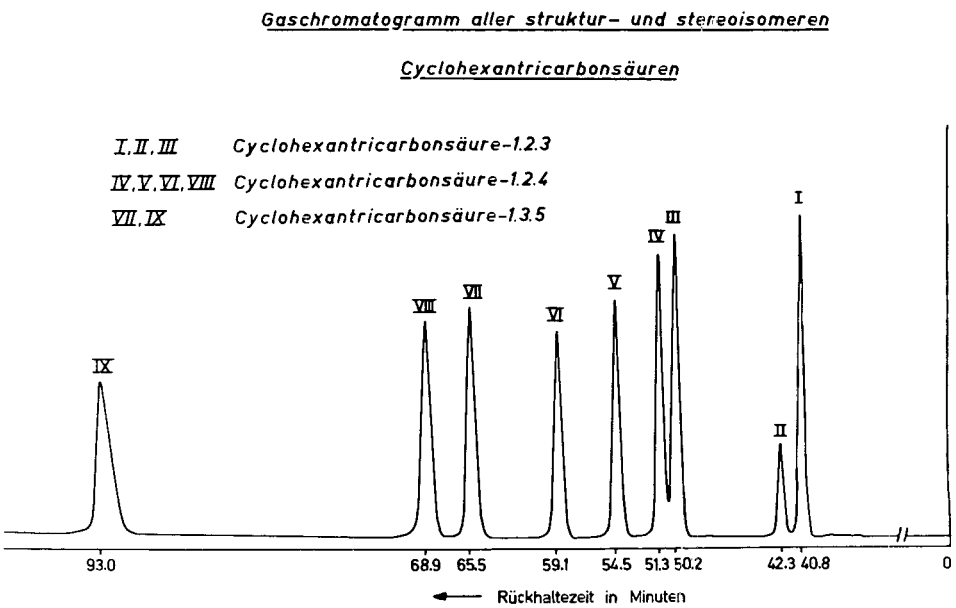

Fig. 2. Gaschromatogramm der neun struktur- und stereoisomeren Cyclohexantricarbonsäuremethylester.

Bei der Untersuchung des Reaktionsproduktes (B) der Hydrocarboxylierung von Δ^4-Tetrahydrophthalsäureanhydrid erhielten wir unter den gleichen gaschromatographischen Bedingungen im Chromatogramm sieben Peaks, die aufgrund der Reaktionsbedingungen nur von den strukturisomeren Cyclohexantricarbonsäuren-1,2,4 bzw. Cyclohexantricarbonsäuren-1,2,3 herrühren konnten. Damit waren zwei von den neun Banden (VII, IX) des Gaschromatogramms (Gemisch A, Fig. 2) der Cyclohexantricarbonsäure-1,3,5 zuzuordnen und die übrigen sieben Peaks den genannten strukturisomeren Säuren.

Zur weiteren Identifizierung wurde die Benzoltricarbonsäure-1,2,3 mit Raney-Nickel hydriert und unter den gleichen Bedingungen wie die Gemische A und B gaschromatographiert. Dabei erhielten wir drei Peaks, deren Retentionszeiten denen der drei ersten Peaks (I, II, III) der obigen Gaschromatogramme entsprachen.

Die Anreicherung des Gemisches A mit dem Hydrierprodukt der Benzoltricarbonsäure-1,2,3 bestätigte die Identität der Verbindungen.

Das Hydrierprodukt der Benzoltricarbonsäure-1,2,4 ergab bei der gaschromatographischen Analyse vier Peaks, die identisch waren mit den restlichen vier Peaks (IV, V, VI, VIII) des Ausgangschromatogramms.

Ausserdem konnten wir bei dieser Säure die *cis-trans*-Isomerenpaare aufgrund folgender Versuche identifizieren:

Die stöchiometrische Normaldruckhydrocarboxylierung von *cis*-Δ^4-Tetrahydrophthalsäureanhydrid ergab ein Reaktionsprodukt, das nur aus zwei der vier stereoisomeren Cyclohexantricarbonsäuren-1,2,4 bestand. Der Schluss lag nahe, dass unter den milden Reaktionsbedingungen die *cis*-Konfiguration der Substituenten in 1,2-Stellung im Ausgangsprodukt erhalten geblieben ist, während der dritte Substituent sowohl in axialer als auch äquatorialer Lage eintreten kann. Somit würden zwei Stereoisomere gebildet, deren Substituenten in 1,2-Stellung *cis*-ständig sind.

Bei der stöchiometrischen Normaldruckhydrocarboxylierung von *trans*-Δ^4-Tetrahydrophthalsäuredimethylester blieb die *trans*-Konfiguration der Substituenten in 1,2-Stellung erhalten und wir erhielten im Gaschromatogramm dieses Reaktionsproduktes die zwei restlichen Peaks der stereoisomeren Cyclohexantricarbonsäuren-1,2,4.

Somit konnten wir den Peaks VI und VIII in Fig. 2 die *cis*-Konfiguration in 1,2-Stellung und den Peaks IV und V die *trans*-Konfiguration in 1,2-Stellung zuordnen.

TABELLE I

Peak-No. (s. Fig. 2)	*I*	*II*	*III*	*IV*	*V*	*VI*	*VII*	*VIII*	*IX*
Retentionsindex	1937	1945	1986	1992	2005	2024	2048	2060	2131

Eine Bestätigung ergab das Produkt der partiellen Hydrierung der Benzoltricarbonsäure-1,2,4 mit Platin, das im Gaschromatogramm ebenfalls nur zwei Peaks ergab, da in 1,2-Stellung nur die *cis*-Konfiguration gebildet wurde[2].

Um ganz sicher zu gehen, dass es sich bei den Peaks VII und IX um die zwei möglichen Isomeren der Cyclohexantricarbonsäuren-1,3,5 handelte, hydrierten wir die entsprechende Benzoltricarbonsäure mit Raney-Nickel und reicherten Gemisch B

mit diesem Hydrierprodukt an. Das Gaschromatogramm zeigte neun Banden und war deckungsgleich mit dem Gaschromatogramm in Fig. 2.

Tabelle I enthält die Retentionsindices der neun isomeren Cyclohexantricarbonsäuren, die nach dem Verfahren von KOVATS[3,4] unter Zuhilfenahme der Retentionszeiten des *n*-Octadecans und des *n*-Eikosans berechnet wurden.

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Eingegangen am 22. August 1968

J. Chromatog., 38 (1968) 133–136

CHROM. 3739

Gas chromatography of cytokinins

We have sought a rapid sensitive means of identifying cytokinins in plant extracts as an adjunct to bioassay methods. The trimethylsilyl (TMS) derivatives of bases and nucleosides have been successfully separated by gas chromatography[1–3]. We therefore examined the possibility of separating the TMS derivatives of isopentenyladenine (2iP), dihydrozeatin, zeatin, isopentenyladenosine (2iPA) and zeatin riboside by this method. Pyrene, kinetin and kinetin riboside were included as standards.

Retention times were determined with a F and M 5750 dual column gas chromatograph fitted with flame ionization detectors. Silanized glass columns (2 m × 6 mm O.D.) were packed with 3% SE 52 (phenyl methyl silicone gum rubber) on 80–100 mesh Diataport S (a silanized diatomaceous earth). The flow-rate of helium was 60 ml/min. The injection port was maintained at 230° and the flame detector at 310°. Retention times were determined both isothermally and by temperature programming at 10°/min from 150° to 300° and then holding at 300° for 5 min.

The free bases and ribosides were dried over P_2O_5 to avoid the formation of small side peaks due to hydrolysis of the TMS derivatives[3]. A mixture of bis(trimethylsilyl) acetamide and methyl cyanide in the ratio 1:2 was added at the rate of 1 μl reagent to 1 μg of sample. The mixture was heated at 60° for 5 min, then centrifuged before sampling for injection into the gas chromatograph. Results are expressed as (a) the absolute retention time in min, (b) the retention time relative to pyrene (R_e Pyrene), and (c) the retention time relative to kinetin (R_e Kinetin). The separation achieved with temperature programming is shown in Table I and isothermally in Table II.

TABLE I

GLC RETENTION TIMES AND RELATIVE RETENTION TIMES OF CYTOKININ BASES AND RIBOSIDES ON 3% SE 52

Compound	*Temperature programmed*			*Retention temperature (°C)*
	Time (min)	R_e *Pyrene*	R_e *Kinetin*	
Pyrene	6.3	1.00	—	213
2iP	6.9	1.09	0.90	219
Kinetin	7.7	1.21	1.00	227
Dihydrozeatin	8.9	1.42	1.16	239
Zeatin	9.7	1.54	1.27	247
2iPA	13.3	2.11	1.74	283
Kinetin riboside	14.0	2.20	1.82	290
Zeatin riboside	15.3	2.42	2.00	300*

* There were no signs of decomposition at 300°.

Using pure compounds, and on the basis of a 1% full scale deflection, the limit of detection was approximately 0.005 μg. Detection of smaller quantities was limited by bleeding from the septa and O rings.

These results show that the three cytokinin bases and the two ribosides can be separated by gas chromatography as the TMS derivative. HASHIZUME AND SASAKI[4] have separated ribonucleotides by gas chromatography. It may also be possible for cytokinin ribonucleotides to be detected by the technique described here.

TABLE II

GLC RETENTION TIMES OF CYTOKININ BASES AND RIBOSIDES ON 3% SE_{52}

Compound	*Isothermal at 210°*			*Isothermal at 250°*		
	Time (min)	R_e *Pyrene*	R_e *Kinetin*	*Time (min)*	R_e *Pyrene*	R_e *Kinetin*
Pyrene	2.5	1.00	—	1.11		
2iP	2.8	1.14	0.77			
Kinetin	3.7	1.47	1.00	1.3	1.18	1.00
Dihydrozeatin	5.7	2.29	1.55			
Zeatin	7.8	3.11	2.11			
2iPA	—			7.1	6.56	5.52
Kinetin riboside	51.9	20.8	14.1	8.8	8.16	6.86
Zeatin riboside	—			15.3	14.1	11.9

The use of combined gas chromatography–mass spectrometry for the identification of gibberellins in plants has been described by MACMILLAN *et al.*[5]. We suggest that it may be possible to couple a mass spectrometer to the gas chromatograph to facilitate the identification of microgram quantities of cytokinins in plant extracts.

Acknowledgements

The cytokinins were given as gifts to Dr. NITSCH from Dr. X. G. MONSEUR (isopentenyladenine), Dr. LETHAM and Dr. SHAW (zeatin), Dr. KOSHIMIZU (dihydro-

zeatin), Dr. HALL (isopentenyladenosine) and Dr. SHAW (zeatin riboside). We wish to thank the donors of the cytokinins and Dr. NITSCH for generously providing samples for our use. One of us (B.H.M.) wishes to acknowledge his appreciation to Dr. NITSCH for facilities and hospitality at Laboratoires de Physiologie Pluricellulaire.

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Received August 12th, 1968

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CHROM. 3741

The selective detection of some steroid acetates using the electron capture detector

Electron capture detectors are used in gas chromatography for the detection of sub microgram quantities of halogen containing molecules and in particular for chlorinated pesticide residues. To measure similar quantities of naturally occurring steroids, which have a low affinity for electrons, halogenated derivatives have been formed, *e.g.* chloroacetates[1,2], heptafluorobutyrates[3,4], chloromethyldimethylsilyl ethers[5], and bromomethyldimethylsilyl ethers[6].

A draw-back to the use of such derivatives for steroids in biological extracts is their lack of selectivity, considerable purification often being necessary prior to gas chromatography.

The electron capture detector is known to give a response with some corticosteroid acetates[7]. In a study of the response of the detector to the acetates of a number of steroids that occur in biological fluids it was found that those with the α-ketol grouping gave a high response, *i.e.* that some selection occurred.

TABLE I

RESPONSE OF THE ELECTRON CAPTURE DETECTOR TO SOME STEROID ACETATES

Steroid	*Response per ng*	*Relative retention time (OV-1)*
1 Cholestane	—	1.00 (4.4 min)
2 Dehydroepiandrosterone chloroacetate	1.00[a]	1.16
3 Dehydroepiandrosterone acetate	—[b]	0.58
4 Androst-5-ene-3β,17β-diacetate	—	0.84
5 3β,16α-Diacetoxyandrost-5-en-17-one	1.50	1.24
6 3α,16α-Diacetoxy-5α-androstan-17-one	1.33	1.13
7 Testosterone chloroacetate	3.98	1.62
8 Androst-4-ene-3,17-dione	0.12	2.22
9 16α-Acetoxyandrost-4-ene-3,17-dione	6.30	1.19
10 3β,21-Diacetoxypregn-5-en-20-one	0.79	2.38
11 21-Acetoxypregn-5-en-3β-ol-20-one	0.61	1.76
12 3β,16α-Diacetoxypregn-5-en-20-one	—	1.89
13 11-Deoxycorticosterone-21-acetate	3.11	2.57
14 Corticosterone-21-acetate	6.26	3.84
15 17α-Acetoxyprogesterone	0.21	1.65
16 3,16α-diacetoxyoestrone	2.58	1.46
17 Oestrone acetate	—	0.67

[a] Absolute response $2.5 \cdot 10^{-10}$ coulombs per ng.
[b] No response with quantities up to 100 ng.

Table I gives the response of the acetates relative to dehydroepiandrosterone chloroacetate and their retention times on a 1% OV-1 column (column 210 × 0.6 cm; support Supasorb 80–100; temp. 230°; nitrogen flow 50 ml per min). The gas chromatograph was a Pye series 104 model 74 with a Nickel-63 source electron capture detector. The detector was operated at 240° without purge or quench gas in the pulse mode.

Pulse amplitude was > 47 V, pulse width 0.75 μS, and pulse space 150 μS. The amplifier was set to give a full scale deflection of 5×10^{-10} A.

The response of the detector to each steroid was obtained from the linear plot of the recorder responses to three or four different quantities injected directly onto the column.

From Table I the selective response is very apparent between steroids 3 and 5, 8 and 9, 16 and 17. The ease with which the acetates are made and their stability to further purification and to gas chromatography makes them preferable in some circumstances to halogenated derivatives—especially when the selective response applies to steroids that are of particular interest. Fig. 1 illustrates this point. It compares the chromatograms of an acetylated extract of pregnancy urine obtained simultaneously with a flame ionisation and electron capture detector.

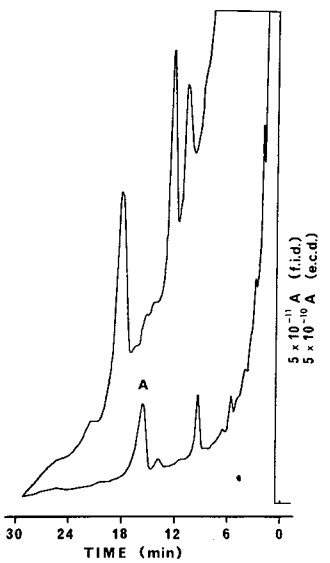

Fig. 1. Chromatograms of an acetylated extract of pregnancy urine. Upper trace, flame ionisation detector (f.i.d.). Lower trace, electron capture detector (e.c.d.). Pye Series 104, Model 84 gas chromatograph. Column 1%OV-17, 150 × 0.6 cm; support Supasorb 100–120; temperature 230°; nitrogen flow 60 ml per min. Effluent split to f.i.d. and e.c.d. in ratio 10:1, respectively. Peak A corresponds to the 3,16-diacetate of 16α-hydroxydehydroepiandrosterone.

Peak A, clearly visible on the electron capture tracing only, is that given by the 3,16-diacetate of 16α-hydroxydehydroepiandrosterone, the estimation of which was the aim in mind.

The selectively high response of the electron capture detector to certain steroid acetates may be of value not only in the quantitative analysis of steroids in biological fluids, but also in qualitative analysis using the technique previously described by LOVELOCK[8].

Acknowledgements

The author wishes to thank Dr. G. W. PENNINGTON for his advice and encourage-

ment in the preparation of this communication. The work was supported by the Medical Research Council of Great Britain.

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Received August 14th, 1968

J. Chromatog., 38 (1968) 139–141

CHROM. 3750

Thin-layer and gas–liquid chromatography of phenolic diterpene acetates

In a current study of extractives of the outer bark of western red cedar (*Thuja plicata* Donn), we have gathered several phenolic diterpenes as reference compounds. Chromatographic data on individual compounds have been published, but there has not been a collection of these data. The phenolic diterpenes were examined as their acetate derivatives because these compounds were more stable than the parent and, also, reference samples most often were donated as the acetates. Although nimbiol is not a diterpene, it is included because of its close structural relationship to the others.

Table I shows the chromatographic behaviour of these compounds. The first four columns of Table I give the thin-layer chromatography data and the last two columns give the gas–liquid chromatography data. The three developing solvents for thin-layer chromatography were petroleum ether (PE) (b.p. 65° to 110°), methylene dichloride (MD) and ether (E). The two columns used for gas–liquid chromatography were one 5 ft. $\times$ $^1/_8$ in. containing silicone gum SE-30 (25%) on Gas Chrom Q (100–200 mesh) and another, same size, containing diethylene glycol succinate (DEGS) (20%) on the same absorbent. A flame ionization detector was used in the gas chromatograph.

Table I shows that these compounds can be divided into groups according to the variety of functional groups present. Thus compounds with only the acetoxy group developed with the least polar PE solvent. Compounds with a carbonyl and acetoxy group were intermediate with the polar E solvent. Similar considerations applied to the gas chromatographic behaviour of the compounds. These data have been used to predict the amount of substitution on unknown compounds isolated from western red cedar bark.

TABLE I

Compound	$R_F \times 100$			$SbCl_5$	Retention time (min)	
	PE[a]	MD[a]	E[b]	colour[c]	SE-30[d]	DEGS[e]
1 Δ^9-Dehydroferruginol acetate	14	68	95	Red-brown	12.8	8.5
2 Δ^6-Dehydrototarol acetate	13	67	89	Olive green	14.2	10.8
3 Ferruginol acetate	17	67	95	Orange brown	13.9	8.7
4 Hinokiol diacetate	0	22	55	Orange red	26.0	22.9
5 Hinokiol monoacetate	0	10	11	Orange red	36.2	38.2
6 Hinokione acetate	0	12	33	Orange	23.8	27.8
7 Nimbiol acetate	0	29	54	Yellow	19.2	26.5
8 Sempervirol acetate	12	64	95	Grey-brown	13.7	8.2
9 Sugiol acetate	0	20	45	Yellow	23.5	21.0
10 Totarol acetate	10	65	95	Brown	17.1	12.0
11 Totarolone acetate	0	18	36	Yellow-orange	28.7	28.4
12 Xanthoperol acetate	0	22	30	Yellow	21.8	26.0

[a] Silica Gel G with 13% binder, E. Merck, Darmstadt, Ger.
[b] Aluminium Oxide G, as before.
[c] On the silica gel plates, colours produced by heating on a hot plate for two min.
[d] Isothermal operation at 215°; N_2 carrier gas at 20 ml/min.
[e] Isothermal operation at 225°; N_2 as before.

We thank Drs. Y. L. CHOW, K. HATA, L. MANGONI, P. SENGUPTA and M. SUMIMOTO for the gifts of compounds used in this study.

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Received August 19th, 1968

CHROM. 3760

Rapid thin-layer chromatographic microassay of ε-aminocaproic acid in urine

A simple method for the estimation of ε-amino-*n*-caproic acid (EACA) became necessary to obtain information about the absorption of this potent inhibitor of plasminogen activation which might be of great interest in dentistry.

Few techniques[1–5] are known to determine the presence of EACA in urine, blood and saliva by means of paper chromatography, column chromatography, high-voltage paper electrophoresis. Moreover they are not easy to deal with and rather time consuming for our purpose. We developed a convenient and simple procedure for the determination of microamounts of EACA using thin-layer chromatography (TLC).

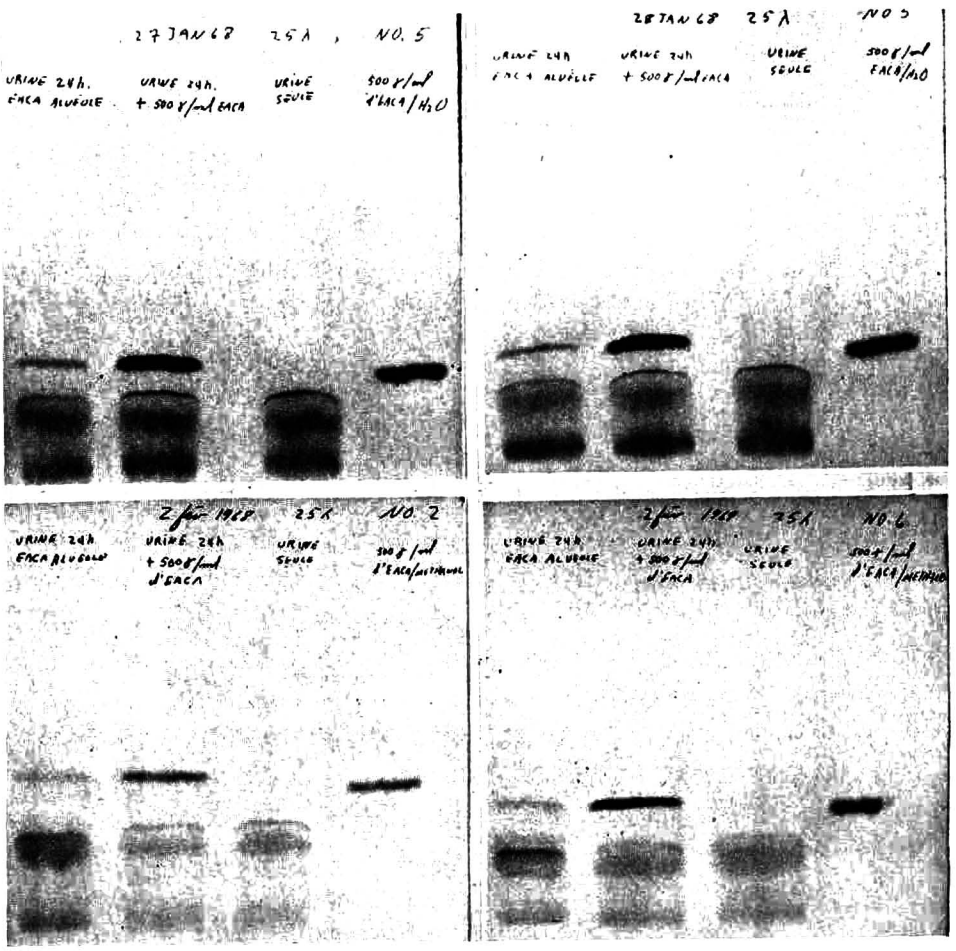

Fig. 1. Chromatographic pictures showing the migration and separation of EACA from the other constituents of urine. From right to left: the control solution (EACA dissolved in methanol or water); urine without EACA; urine in which we added EACA; urine collected from rabbits who received EACA.

Method

EACA is chromatographed on Silica Gel G layers which give better results than Alumina or Kieselgur G. Fisher micropipets were used for spotting. The EACA was supplied by Lederle of Canada. The solvent is a mixture of the following: *n*-butanol–glacial acetic acid–distilled water (8:2:2). Thin-layers, 250 μ thick, are prepared with a Shandon applicator, air-dried for 30 min, heated in an oven at 100° for at least 1 h and allowed to cool before use. The detection reagent applied with chromatosprayer is ninhydrin 0.5% in methanol or water. The chromatograms are photographed with a 35-mm Nikon.

Rabbits (17) weighing from to 2 to 3 kg, are used. After general anesthesia with pentobarbital (Nembutal, Abbott Laboratories, Canada), the lower left central incisor is removed and the socket is packed with an absorbable gelatine sponge (Gelfoam, Upjohn Co., Canada) approx. 20 mm × 20 mm × 7 mm. The Gelfoam has been filled before with EACA at a concentration of 10 mg/kg. The socket is sutured with catgut No. 4. The rabbit is then placed in a metabolism cage and the 24-h urine is collected and filtered. 24-h urine was also collected from rabbits (10) not operated on. For a third group (10) we added in the 24-h urine, 500 μg/ml of EACA. A standard solution was prepared by dissolving 500 μg of EACA per ml of methanol or distilled water. The sample spots are applied, using a volume of 25 λ for each solution, in a straight line parallel to the margin of the layer in order to obtain the cleanest separation. The chromaplates are placed in the chamber for 60 min, removed and dried 10 min with a hot-air blower. Using a Shandon spraygun, the ninhydrin solution is sprayed on in a fume hood. The plate is then heated in an oven at a temperature of 80° during 20 min.

Results

The solvent system butanol–acetic acid–water permits excellent migration and separation. It is the solvent of choice for TLC of small amounts of EACA, the lower detection limit being from 5 to 10 μg. The colour shown by EACA in urine, EACA dissolved in methanol or water is a deep rose (magenta).

The method suggested gives an excellent separation of EACA from normally occuring amino acids in urine as can be seen by the different chromatograms (Fig. 1). These results from 17 rabbits provided with small amounts of EACA in the socket of a lower incisive tooth prove that the compound has been absorbed.

Acknowledgement

The authors are grateful to Dr. C. P. GENDRON, Lederle of Canana Ltd., for the reference substance.

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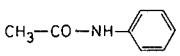
Received August 26th, 1968

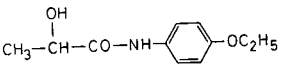
CHROM 3755

Dünnschichtchromatographischer Nachweis von Acetanilid und einigen chemisch verwandten Analgetika

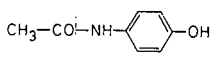
Acetanilid (I), eines der ältesten synthetischen Analgetika, gehört neben Phenacetin (IV) zu den meisterzeugten pharmazeutischen Präparaten; beide werden häufig mit anderen Analgetika und Antipyretika kombiniert. Weniger verbreitet sind Paracetamol (III) und Lactophenin (II); diese letzte Substanz besitzt auch antirheumatische Wirkungen.



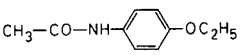
(I) Acetanilid[2].



(II) Lactophenin = DL-Milchsäure-*p*-phenetidid[2].



(III) Paracetamol = *p*-Hydroxyacetanilid[2].



(IV) Phenacetin = *p*-Äthoxyacetanilid[2].

Die Derivate des Acetanilids gehören zu den Stoffen, die bei den chemisch-toxikologischen Untersuchungen aus wässrigen sauren Lösungen mit Äther oder Chloroform extrahiert werden.

Über den dünnschichtchromatographischen Nachweis dieser Substanzen ist schon mehrfach berichtet worden[1,5]. Die bisher angegebenen Nachweismethoden sind jedoch wenig spezifisch. Grössere Spezifität wird der Anfärbung von Phenacetin und einigen chemisch verwandten Substanzen mit Bromdampf bei der Tube-Chromatographie zugeschrieben[4].

Material und Methode

Für die Bestimmung der R_F-Werte benutzten wir Platten von 200 × 200 mm Grösse. Zur Herstellung der Schichten diente die Grundausrüstung nach STAHL[5]. Trägermaterial Kieselgel G (Merck). Entwicklung der Chromatogramme schräg liegend. Laufstrecke 100 mm. Aufgetragene Substanzmenge 30 μg.

Zur Feststellung der Nachweisgrenze wandten wir Platten der Grösse 115 × 80 mm an, die von Hand mit 1 g Kieselgel G und 3 ml dest. Wasser beschichtet worden waren.

Zum Auftragen der Substanzen verwendeten wir eine Spezialpipette der Fa. Desaga von 10 μl. Es wurden Chloroformlösungen benutzt, die 1 μg bzw. 10 μg Substanz pro μl enthielten.

Laufmittel

(I) Chloroform–Aceton (90:10)[5]. Laufzeit etwa 45 Min.

(II) Chloroform–Benzol–Aceton (65:10:25). Laufzeit etwa 50 Min.

Sprühreagenzien

(1) Silbernitrat-Reagens[3]: 10%ige wässrige Silbernitratlösung. Raumtemperatur. Flecke treten nach etwa 10 Min auf.

(2) Eisen(III)-chlorid-Reagens: 5%ige wässrige Eisen(III)-chloridlösung. Das besprühte Chromatogramm wird 10 Min auf 120° erhitzt.

(3) 4-Dimethylaminobenzaldehyd–Salzsäure[5]: 1 g 4-Dimethylaminobenzaldehyd wird in 100 ml eines Gemisches aus 50 ml Äthanol (96%) und 50 ml Salzsäure (32%) gelöst. Das besprühte Chromatogramm wird 5–10 Min auf 100° erhitzt.

TABELLE I

hR_F-WERTE DER VIER ACETANILIDDERIVATE MIT DEN LAUFMITTELN CHLOROFORM–ACETON (90:10) (I) UND CHLOROFORM–BENZOL–ACETON (65:10:25) (II)

Substanz	R_F-*Wert* × 100	
	Laufmittel I	*Laufmittel II*
Acetanilid	49	75
Lactophenin	28	60
Paracetamol	9	33
Phenacetin	43	71

TABELLE II

FARBREAKTIONEN DER VIER ACETANILIDE MIT DEN ANGEWANDTEN SPRÜHMITTELN

Z = Zimmertemperatur; E = Erhitzen.

Substanz	*Sprühreagens*		
	1 (Z)	*2 (E)*	*3 (E)*
Acetanilid	keine Färbung	orange	gelb
Lactophenin	keine Färbung	braun	gelb
Paracetamol	grau-schwarz	grau-blau	gelb
Phenacetin	keine Färbung	braun (rötlich)	gelb

TABELLE III

NACHWEISBARE SUBSTANZMENGE MIT DEN ANGEWANDTEN SPRÜHREAGENZIEN

Substanz	*Sprühreagens*		
	1	*2*	*3*
Acetanilid	—	< 15 μg	1 μg
Lactophenin	—	< 10 μg	1 μg
Paracetamol	< 1 μg	< 5 μg	1 μg
Phenacetin	—	5 μg	1 μg

Ergebnisse

In Tabelle I sind die hR_F-Werte der vier Acetanilidderivate mit den Laufmitteln I und II dargestellt. Die R_F-Werte sollen als Richtwerte betrachtet werden; sie stellen Mittelwerte von zwölf Chromatographien dar.

Das Reagens 4-Dimethylaminobenzaldehyd-Salzsäure[5] färbte alle vier Acetanilidderivate (Tabelle II); es konnten dabei Substanzmengen von 1 μg nachge-

wiesen werden (Tabelle III). Die Spezifität des 4-Dimethylaminobenzaldehyds wurde an 15 Barbituraten, sowie an den Substanzen Adalin, Bromural, Coffein, Noludar und Persedon geprüft; mit diesen Substanzen trat keine Reaktion ein.

Mit Eisen(III)-chlorid und bei Raumtemperatur färbte sich nur Paracetamol (Nachweisbarkeitsgrenze unter 10 μg); die anderen Substanzen erschienen erst nach Erhitzen des besprühten Chromatogramms (Tabelle II und III).

Mit der Silbernitratlösung reagierte nur Paracetamol; mit diesem Reagens konnte weniger als 1 μg Paracetamol nachgewiesen werden.

Den Firmen Heinrich Mack Nachf. (Illertissen/Bayern), Lorenz KG (Essen-Werden) und Stada eGmbH (Dortelweil/Betterau) für die freundliche Überlassung der reinen Substanzen unseren aufrichtigen Dank.

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Eingegangen am 22. August 1968

CHROM. 3730

Densitometric microquantitation of lipid classes separated by thin layer chromatography*

Application of densitometry to quantitation of lipids separated on thin-layer chromatography (TLC) described by BLANK *et al.*[1] became a powerful tool in analysis of both non-polar[2] and polar[3] lipids. By modification of that technique and of the excellent two-step TLC described by FREEMAN AND WEST[4] we were able to quantitate microquantities of lipids of amniotic fluid. We are not aware of another method that will allow such microquantitation with similar speed and relative simplicity. A broad outline of the procedure was reported before[5].

Materials

Reference compounds were obtained as follows: from Hormel Institute (Austin, Minn.): monopalmitin, dipalmitin, tripalmitin, palmitic acid, oleic acid, monoolein, triolein, cholesterol, cholesteryl stearate; from Applied Science Laboratories, State College, Pa.: palmitic acid, stearic acid, myristic acid, methyl palmitate, cholesteryl stearate, *n*-tetracosane, *n*-octadecane; from Supelco Inc, Bellefonte, Pa.: 1,2-dipalmitin and 1,3-dipalmitin. The following compounds were a generous gift of Dr. C. B. BARRETT of Unilever Research Laboratory, The Frythe, Welwyn, Herts., Great Britain: 1-monoolein, 1,3-diolein, 1,3-distearin, 1,3-dipalmitin. Chemical reagents were of analytical grade (Fisher Scientific, New York) but not redistilled.

Methods

Amniotic fluid collected from normal patients in the third trimester of pregnancy by abdominal amniocentesis or in labor by vaginal amniocentesis was centrifuged for 20 min at 3,000 r.p.m. (1500 *g*). The supernatant was kept at −20° until extraction. Fluids were not pooled. Lipids were extracted with 20 volumes of chloroform–ethyl alcohol (2:1, v/v) for 2 h at 40° with shaking. The filtered extract was brought to dryness under nitrogen in a Nutating evaporator (Zymel Corp., Ardsley Corp., New York). Non-lipid contaminants were removed by the method of FOLCH *et al.*[6] or of BIEZENSKI[7]. An aliquot of the extract was taken for lipid P determination[8]. Lipids were concentrated to 10–20 $\mu g/\mu l$. One microliter of the concentrate corresponded to about 0.1 ml of amniotic fluid. Serum lipids were extracted as described before[9].

Procedure

Neutral plates of Silica Gel G, 20 × 20 cm of 0.25 mm thickness were prepared in the usual manner. After drying in air the plate was cleaned by a prerun in Solvent I for 2 h. The plate was then dried in air for 20 min and activated in an oven at 110° for 20 min. Parallel lanes exactly 8 mm wide were drawn with a comblike instrument made with glass rods. Two to four microliters of the extract were applied 3.5 cm from the bottom of the plate with a Hamilton syringe in a series of dots across each lane along a line which fell short by 1 mm from either limiting line. The plate was developed in Solvent I consisting of diethyl ether–benzene–ethyl alcohol–acetic acid (40:50:2:0.2)[4]

* Supported by grants No. FR-05497 and No. HD 00441 from National Institutes of Health, U.S.P.H.S.

for 6.5 cm from the origin. This took approximately 13 min. After drying in air for 20 min the plate was placed in Solvent II consisting of petroleum ether–ethyl ether acetic acid (90:10:1). The plate was developed for 6.5 cm more (13 cm in all from origin) which took 35–45 min. When dry the plate was sprayed lightly with 50% H_2SO_4 and placed on a metal hot plate till fumes disappeared. This took about 8 min. The surface temperature of the hot plate was approximately 260°. The surface temperature of the thin-layer plate was approximately 180°. Under the above conditions the plate burned evenly but plates of thinner glass gave density readings 5–10% higher.

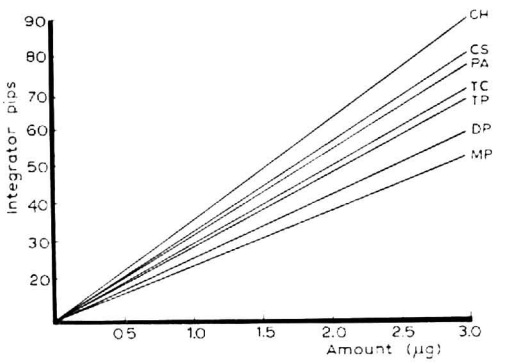


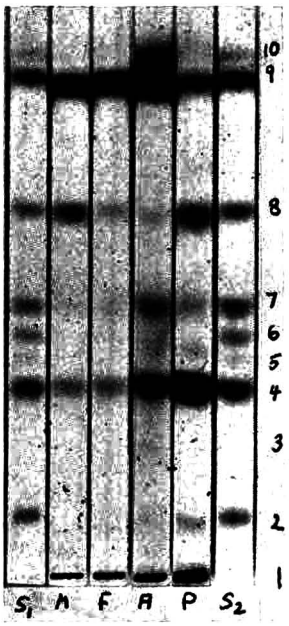
Fig. 1. Relation of amounts of reference compounds to densitometric readings of charred thin-layer plates. CH = Cholesterol; CS = cholesteryl stearate; PA = palmitic acid; TC = *n*-tetracosane; TP = tripalmitin; DP = dipalmitin; MP = monopalmitin.

Comparisons with reference compounds were essential due to specific burning characteristics (Fig. 1). These differences were only partly due to differences in carbon content and cannot be readily explained. Degree of fatty acid unsaturation appeared to be of little significance. The densities were determined essentially as described by BLANK *et al.*[1] by means of a Photovolt Densitometer Model No. 52C equipped with an automatic mobile stage, a Varicord Recorder Model No. 421B and an Electronic Integrator Model No. 49A. The collimating slit measured 0.1 mm × 6.0 mm. No filter was used. The width of the exchangeable apperture disc was adjusted to exactly 5 mm with black tape. Thus the slit was slightly narrower than the width of the streak. The instrument was set for 100% transmission on blank areas. The average number of integrator pips of each spot for the four lanes was converted into micrograms by comparison with reference compounds. Percentages and absolute amounts were then calculated from the microgram values. Density values obtained from phospholipids were not proportional to amounts applied and were disregarded.

Comment

The method as outlined was most suitable for amounts of about 1 µg of lipid in each spot. If any component exceeded grossly that amount it was necessary to run additional plates with diluted samples for better accuracy.

Separation of lipid classes of various tissues was satisfactory (Fig. 2). We found the same method of separation very suitable for preparatory purposes mainly because



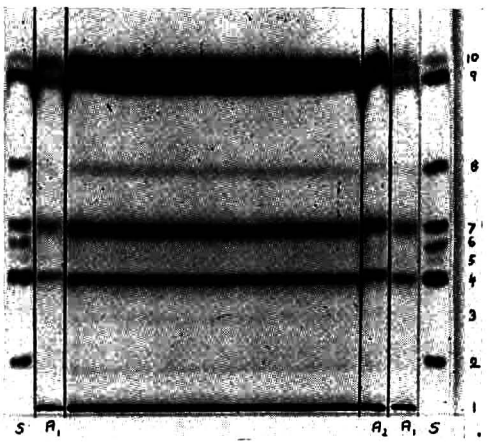


Fig. 2. Separation of lipid classes for densitometric quantitation by two-step thin-layer chromatography. S_1 = Reference compounds (2 μg each); M = pregnant woman (extract from 0.0015 ml serum); F = human cord blood (extract from 0.012 ml serum); A = human amniotic fluid (extract from 0.3 ml); P = human placenta (extract from 15.0 mg fresh tissue); S_2 = reference compounds (4 μg each). (1) Origin and phospholipids; (2) 1- and 2-monoglycerides; (3) unidentified in amniotic fluid; (4) cholesterol; (5) 1,2-diglyceride; (6) 1,3-diglyceride; (7) free fatty acids; (8) triglycerides; (9) cholesteryl esters; (10) hydrocarbons. Charred with 50% H_2SO_4.

Fig. 3. Preparatory thin-layer chromatography of human amniotic fluid. For details of technique see text. Plate thickness: 0.75 mm. Amount of lipid: 900 μg (5.6 ml amniotic fluid). S = Reference compounds (4 μg each); A_1 = extract from 0.225 ml amniotic fluid; A_2 = extract from 0.45 ml amniotic fluid. For identification of compounds see Fig. 2.

TABLE I

HUMAN AMNIOTIC FLUID LIPIDS

Non-polar lipids are determined by quantitative thin-layer densitometry as described in the text. Phospholipids are determined from lipid P × 25.

Class	*mg/100 ml ± 0.38*[a]	*% total lipids*
Phospholipids	3.99 ± 0.38[a]	29.3
Total non-polar	9.65 ± 0.48[b]	70.7
Monoglycerides	0.23 ± 0.02	1.7
Diglycerides	0.89 ± 0.11	6.5
Triglycerides	1.21 ± 0.12	8.9
Free cholesterol	1.40 ± 0.10	10.3
Cholesterol esters	2.00 ± 0.15	14.6
Hydrocarbons	1.75 ± 0.12	12.8
Free fatty acids	2.17 ± 0.13	15.9
Total lipids	13.64	

[a] Average of 43 fluids.
[b] Average of 53 fluids. Represents the sum of components.

each lipid migrated in a straight horizontal line (Fig. 3). Thus staining of outside guides with iodine was sufficient for streak localization.

Human amniotic fluid from patients in the third trimester showed presence of many lipids (Fig. 2). Phospholipids constituted the largest group, monoglycerides the smallest (Table I). Standard errors were occasionally high. This was probably due to the well known natural interfetal differences[10] rather than to methodological variations.

The procedure is particularly useful for microquantitation but we used it to equal advantage in determining the composition of placenta, liver, serum and other tissues.

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Received August 5th, 1968.

CHROM. 3757

The detection of free and N-protected peptides

The detection of cystein peptides[1,2] as well as various lactams[3,4] on thin-layer chromatograms, either by exposure to iodine vapors or by spraying with iodine solution, has been described. Recently, the same method was used for the detection of various peptides[5]. These findings do not give any data about the quantitativity or the limitation of the method.

We would like to report here some of our findings regarding this method. Exposing paper or thin-layer chromatograms of free and protected peptides to iodine vapors results in the formation of yellow-brown spots. These spots disappeared after being in the free atmosphere for some time but reappeared upon a second exposure.

0.1–0.2 μmole of free peptide can be detected on thin-layer chromatograms (using 20 cm $\times$ 5 cm glass plates coated with a 0.25 mm layer of Silica Gel G, Merck, Darmstadt, Germany), while 0.07–0.1 μmole can be detected on paper chromatograms (using Whatman No. 1 paper). The following peptides have been detected: glycylvaline, glycylglycine, alanylalanine, glycylphenylalanine, prolylglycine and phenylalanylalanylglycine, using *n*-butanol–acetic acid–5% NH_4OH–water (6:1:1:2) and butanol–acetic acid–water (4:1:5) as solvent systems. The same sensitivity was found in the case of N-protected peptides. The following derivatives have been detected (using the same solvent systems as above): cyclodiglycyl, cyclophenylalanylglycyl, cycloalanyl-ε-*tert.*-butyloxycarbonyllysyl, benzyloxycarbonylglycylglycine ethyl ester, benzyloxycarbonylserylglycine ethyl ester, benzyloxycarbonyl-β-chloroalanylglycine amide, *tert.*-butyloxycarbonylprolylglycine and *tert.*-butyloxycarbonylalanylglycine. Presence of the hydrazide group increases the sensitivity over 10-fold thus 0.003–0.007 μmole of benzyloxycarbonylglycine hydrazide, benzyloxycarbonylglycylglycine hydrazide and *tert.*-butyloxycarbonyl serine hydrazide were detected.

The sensitivity and simplicity of this detection method enabled us to use it routinely instead of the chlorine method[6].

As other nitrogeneous compounds could be detected by this method (*e.g.* N,N'-dicyclohexylurea, N-acyl-N,N'-dicyclohexylurea, N,N'-dicyclohexylcarbodiimide, N-hydroxysuccinimide) we now use this method to follow up the course of the peptide synthesis reaction as well as to check the purity of complex peptides obtained by the N-hydroxysuccinimide or the N,N'-dicyclohexylcarbodiimide method. This method was adapted to a spot test on paper and is also used by us for the detection of peptides and peptide derivatives eluting from columns.

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Received August 22nd, 1968

CHROM. 3749

Polyamide layer chromatography and electrophoresis of nucleotides

Recently, polyamide-layer chromatography was successfully used for the separation of nucleic acid bases and nucleosides[1]. The sensitivity was high and resolution was good. Its performance was comparable to other thin-layer media, such as ion-exchange cellulose[2]. We would like to describe our further efforts on the separation of nucleotides.

Samples of 1 mg were dissolved in 0.5 ml of distilled water and transferred to a polyamide layer (supplier; Chenchin Trading Co., No. 75, section 1, Hankow St., Taipei, Taiwan). 1 μl of the sample solution was applied for each spot, which is equivalent to 2 μg of sample. The chromatographic method of the previous publication was followed[3] and detection was effected by irradiation of chromatograms with a short-wavelength ultraviolet lamp (2375 Å), spots appearing dark blue on a slightly fluorescent background. The quenched spots were marked by a ball pen, then, the chromatograms were contact-photographed as described before[3].

Table I tabulates the R_F values of 10 nucleotides, in 3 solvent systems. Figs. 1, 2, and 3 are contact photographs of marked chromatograms.

Solvent system II required a 3-h developing time; the spots were circular without any tailing. In Solvent systems I and III, the spots appeared oval.

As Fig. 3 shows, 2'-, and 3'-AMP could be separated by Solvent III. We could not establish which one was the 2' or the 3' nucleotide but the separation is apparent-

Since we could separate 10 common nucleotides, we tried to resolve the hydro.

TABLE I

R_F VALUES OF NUCLEOTIDES ON POLYAMIDE LAYERS

Solvent I: water–glacial acetic acid (20:1, v/v); Solvent II: isopropanol–water–glacial acetic acid (2:2:1, v/v/v); Solvent III: acetone–water–glacial acetic acid (2:2:1, v/v/v). Abbreviations: 5'-ATP, adenosine 5'-triphosphate; 3'(2')-UMP, uridine 3'(2')-phosphoric acid; 3'(2')AMP, adenosine 3'(2')-phosphoric acid; 3'(2')-CMP, cytidine 3'(2')-phosphoric acid; 5'-CMP, cytidine 5'-monophosphoric acid; 5'-GMP, guanosine 5'-monophosphoric acid; 5'-deoxyGMP, deoxyguanosine 5'-phosphoric acid; 5'-deoxyGMP, deoxycytidine 5-monophosphoric acid; 5'-AMP, adenosine 5'-monophosphoric acid.

Sample	*Solvent I*	*Solvent II*	*Solvent III*
3'(2')-UMP**	0.41	0.48	0.36
3'(2')-AMP**	0.82	0.82	0.72
3'(2')-GMP**	0.30	0.51	0.28
3'(2')-CMP**	front	front	front
5'-CMP*	0.97	0.94	front
5'-GMP*	0.50	0.66	0.28
5'-deoxyGMP*	0.51	0.68	0.56
5'-deoxyCMP*	0.97	0.94	front
5'-AMP*	0.50	0.56	0.81
5'-ATP*	0.08	0.1	0.05

* Sigma Chemical Co., St. Louis, Mo., U.S.A.

** Courtesy of Dr. A. T. Tu, Department of Biochemistry, Colorado State University, Fort Collins, Colo., U.S.A.

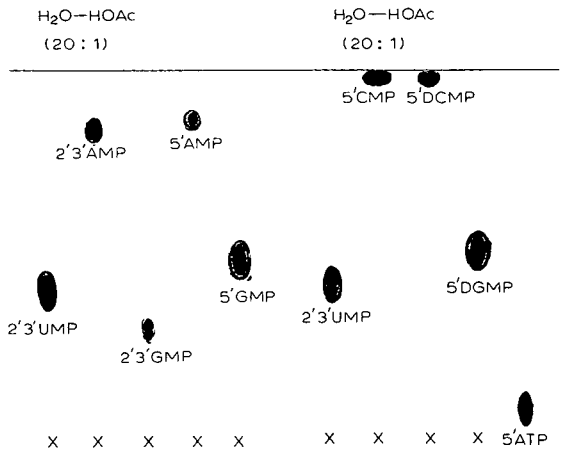

Fig. 1. One-dimensional chromatogram. Solvent: water–glacial acetic acid (20:1, v/v), 50 min for 10-cm development. Crosses show the location of the samples.

lyzate of yeast ribonucleic acid* which was prepared according to JONES AND GERMANN[4]. The result was the same as reported by HIBY AND KROGER[5].

Electrophoresis using polyamide layers exhibits several advantages over other media[6]. We eluted 7 nucleotides at pH 3.4 and 9.3 in a 1-h run with 300 V loaded (20 V/cm) using a home-made electrophoresis cell[7]. The results are fairly good under these conditions (Fig. 4).

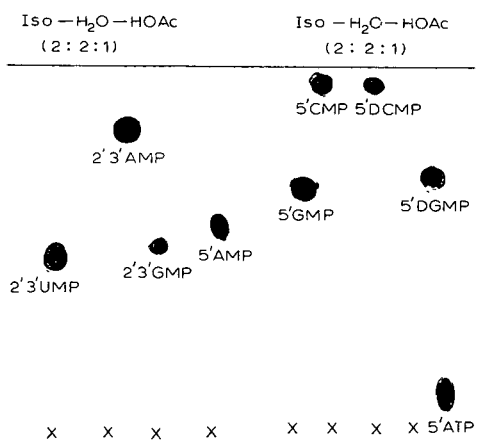

Fig. 2. One-dimensional chromatogram. Solvent: isopropanol–water–glacial acetic acid (2:2:1, v/v/v), 3 h, 10 cm. Spots were complete circles, but 5'-ATP was oval shaped.

* Courtesy of Dr. Y. C. SU, Department of Agricultural Chemistry, National Taiwan University.

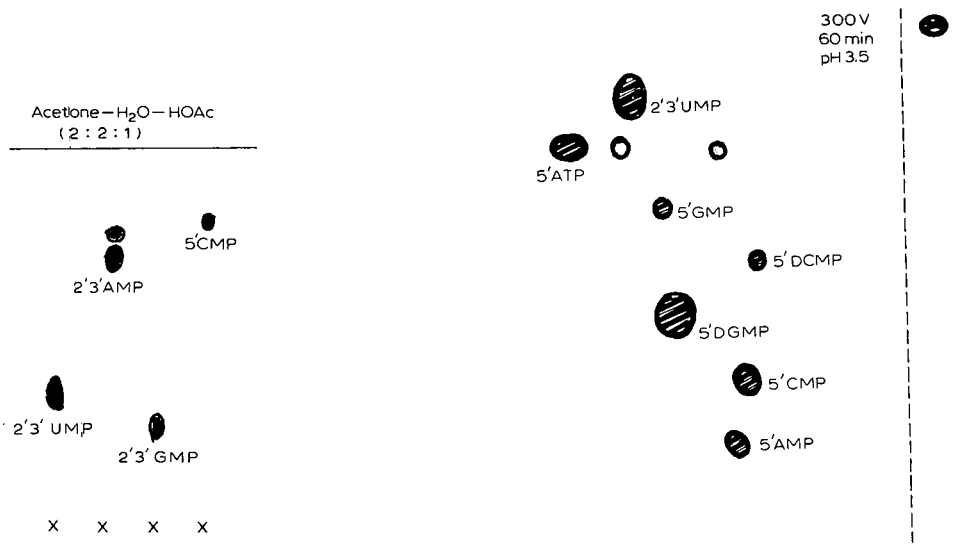

Fig. 3. One-dimensional chromatogram. Solvent: acetone–water–glacial acetic acid (2:2:1, v/v/v), 50 min, 10 cm. In this solvent system, we could separate 3'(2')-AMP which appeared as 2 spots.

Fig. 4. Broken line indicated the location of applied samples. The electrophoresis was run with 300 V loaded, 60 min, with pH 3.5 buffer[8], 7 authentic samples of nucleotides and deoxynucleotides could be separated.

Finally, we would like to point out that the separation pattern of nucleotides by polyamide-layer chromatography was similar to PEI-cellulose thin-layer chromatography of RANDERATH[2]. Further studies on more nucleotides are in progress.

A part of this work was supported by Chemistry Research Center grant (No. CRC 5708) to which thanks are due.

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Received August 19th, 1968

CHROM. 3751

Sorptionsbedingte Fehlerquellen bei der DC-Fluorometrie fluoreszenzmindernder Substanzen (Aminophenazon, Phenyldimethylpyrazolon, *p*-Hydroxybenzoesäureester, Triamcinolonacetonid)

Die quantitative Bestimmung von Arzneistoffen in pharmazeutischen Zubereitungen ist ohne ihre analytische Isolierung in den meisten Fällen nicht durchführbar. Mehrere Autoren berichten über zeitlich aufwendige Trennungen der Be-

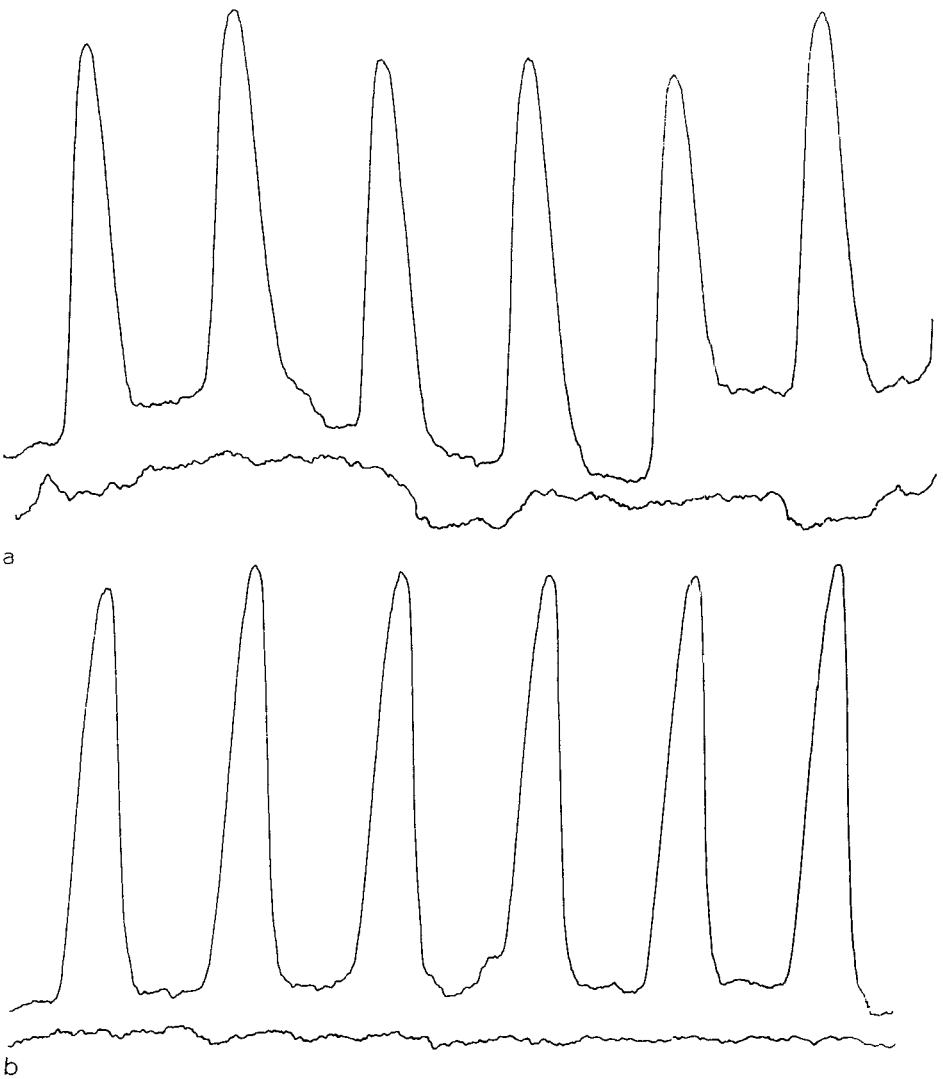

Fig. 1. Registrierte Peaks gleicher Mengen Aminophenazon (je 6 μg) auf einer für quantitative Bestimmungen ungeeigneten (a) und einer brauchbaren (b) DC-Fertigplatte mit Blindmessungen des Plattenuntergrundes.

standteile von Antipyretica und Analgetica[1–3]. Meist ist hier nicht auf chromatographische Methoden zu verzichten. Die eigentlichen Bestimmungen werden anschliessend in der Regel titrimetrisch oder photometrisch durchgeführt. BRODE[4] wies bei der Bearbeitung und Untersuchung Fluocinolonacetonid-haltiger Zubereitungen auf Schwierigkeiten hin und betonte, dass die klassischen Verfahren zur Bestimmung der

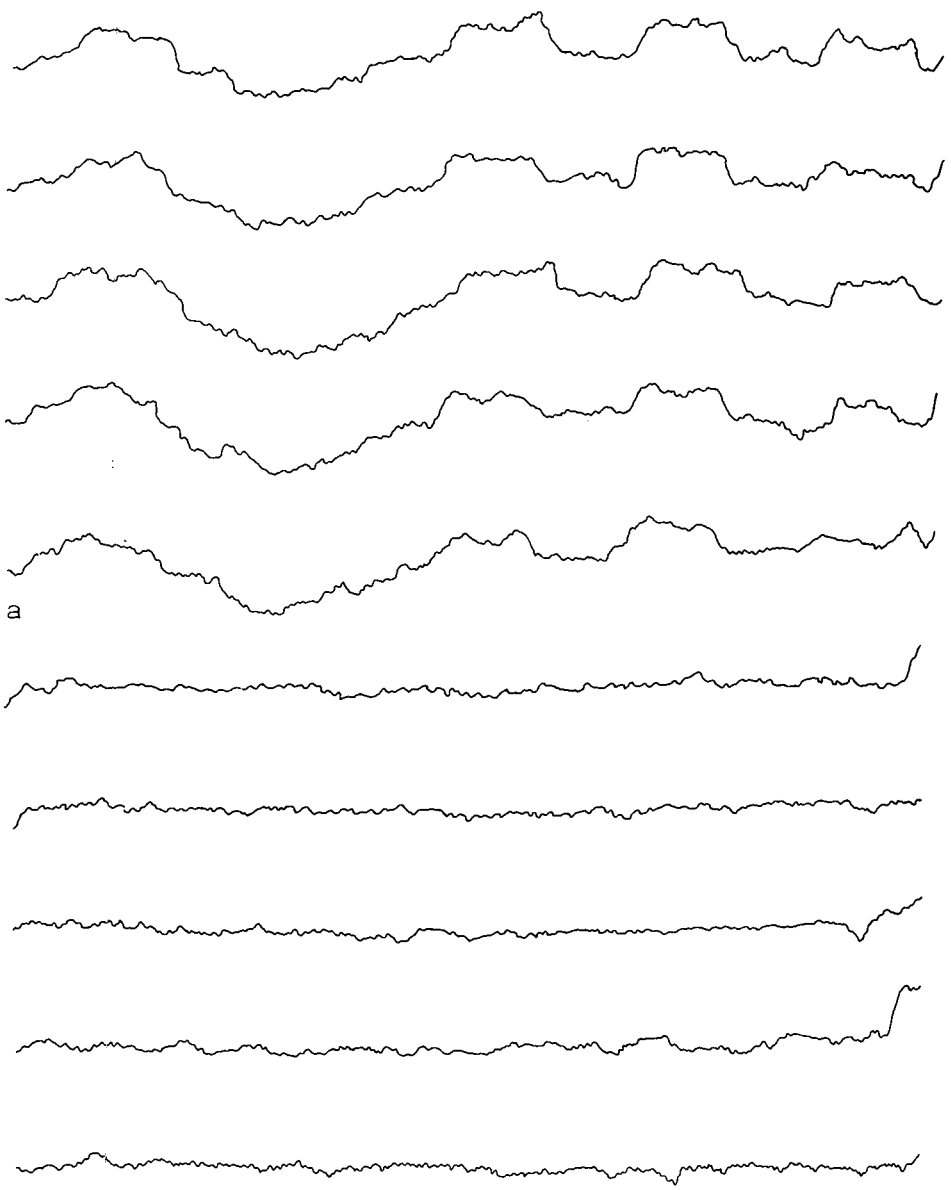

Fig. 2. Messungen des Plattenuntergrundes derselben DC-Fertigplatte in verschiedenen Richtungen (a, b) und mehreren R_F-Bereichen.

Corticosteroide nicht zum Ziel führen. Er empfahl für die Prüfung dieser Präparate Aktivitätsmessungen nach ^{14}C-Markierung der Substanz. Dieses Verfahren ist aber wie die von anderen Autoren beschriebene Methode[5] für Routine-Analysen nicht einsetzbar. Es wurde von uns systematisch versucht, die qualitative Dünnschichtchromatographie für quantitative Aussagen auszubauen. Fluoreszierende Substanzen[6] bieten sich für eine Direktauswertung besonders an. Schwierigkeiten können dann auftreten, wenn fluoreszierende Derivate hergestellt werden müssen[7]. Einen Ausweg bietet in vielen Fällen die quantitative Auswertung nicht fluoreszierender Substanzen auf fluoreszenzhaltigen DC-Platten[8].

Bei der Registrierung der Flecken werden oft Peaks erhalten, die dieses Verfahren für eine quantitative Bestimmung unbrauchbar erscheinen lassen. Wir haben durch Blindmessungen festgestellt, dass plattenbedingte Ungleichmässigkeiten dafür verantwortlich sind (Fig. 1a und b). Weiter konnte durch Untersuchungen automatisch und manuell beschichteter Platten immer wieder festgestellt werden, dass diese Ungleichmässigkeiten bei Messungen quer zur Streichrichtung besonders stark ins Gewicht fallen. Auffallend ist ferner, dass die Schichtbeschaffenheit in weitem Masse R_F-unabhängig ist (Fig. 2). Daraus ergibt sich für die Praxis, dass (1) jede für die quantitative Auswertung einzusetzende fluoreszenzhaltige DC-Platte auf Eignung geprüft werden muss und (2) dass es ausreicht, wenn diese Blindmessung je 1 mal in und quer zur vermutlichen Streichrichtung über die Plattenmitte vorgenommen wird, um gegebenenfalls zu entscheiden, in welcher Richtung die Chromatographie durchgeführt werden kann.

Die erhaltenen Registrierungen des Plattenuntergrundes sind darüber hinaus am besten geeignet, geringfügige Störpegel der Peakregistrierungen zu kompensieren. Für die Ermittlung der Peakflächenwerte scheint uns aus dem gleichen Grunde die Berechnung nach der Methode Höhe $\times$ Halbwertsbreite besonders geeignet.

Die quantitative Bestimmung der im Titel genannten Substanzen erfolgt in üblicher Weise nach DC-Auftrennung (Kieselgel F_{254}) gegen zwei bis drei verschiedene, bekannte Vergleichsmengen, die im Wechsel aufgetragen werden[6]. Die Messungen wurden mit dem Turner-Fluorometer (Camag) und angeschlossenem Kompensationsschreiber (Metrawatt) unter folgenden Bedingungen durchgeführt: Lichtquelle 254 nm; Primärfilter 110–810; Sekundärfilter 110–817, 110–823 (1%); Blendeneinstellung 30 $\times$; Breite des Messspalts, 1 mm.

Aus den Peakflächenwerten der Eichmengen lässt sich eine Eichbeziehung erstellen, mit deren Hilfe der Gehalt in der zu prüfenden Lösung bestimmt werden kann. Die relative, auf 2 Messwerten basierende Standardabweichung lässt sich in allen Fällen unter $\pm$ 5% halten. Einzelheiten der DC-Fluorometrie sind aus Tabelle I ersichtlich.

Zur Abtrennung der genannten Substanzen aus arzneilichen Zubereitungen wurde folgendermassen vorgegangen.

(1) Aminophenazon, Phenyldemithylpyrazolon und Coffein liegen einzeln oder in Kombination meist in Tabletten vor. Zur quantitativen Elution und Abtrennung von Tablettenfüllmitteln eignet sich Methanol. Zum Auffüllen auf ein bestimmtes Volumen (*ca.* 50–100 mg Substanz/100 ml) wird soviel dest. Wasser benutzt, dass eine 80% methanolhaltige Auftraglösung vorliegt.

(2) Die PHB-Ester Nipagin M® und Nipasol M® lassen sich in üblicher Weise nach Stas-Otto[9] aus einem Gemisch isolieren.

TABELLE I

DC-FLUORESZENZDATEN DER MESSSUBSTANZEN

Substanz	ε_{254}	*Messbereich* (μg)	*DC-Laufmittel*	
Phenyldimethylpyrazolon	8 060	5–15	Cyclohexan–Aceton	(4:5)
Aminophenazon	9 670	5–13	Cyclohexan–Aceton	(4:5)
Coffein	6 100	3– 9	Cyclohexan–Aceton	(4:5)
Nipagin M®	16 130	1– 3	Hexan–Essigester–Eisessig	(8:1:1)
Nipasol M®	16 450	1– 3	Hexan–Essigester–Eisessig	(8:1:1)
Triamcinolonacetonid	9 910	4– 8	Dichlormethan–Aceton	(4:1)

(3) Die Verfahren zur Isolierung des Triamcinolonacetonids aus den Zubereitungen (Salbe, Creme, Lotio, Gel) richten sich nach Zusammensetzung und System der entsprechenden Arzneiformen. Nach Abtrennung der eventuell vorhandenen lipophilen Trägerstoffe kann in der Regel im System Wasser–Methanol–Chloroform (1:1:1) eine quantitative Verteilung der Substanz in der Chloroformphase erreicht werden.

In speziellen Fällen muss vor der quantitativen DC noch eine Reinigung mittels präparativer DC eingeschaltet werden, um Störungen auf der Messplatte zu verhindern.

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Eingegangen am 25. Juli 1968

CHROM. 3743

Spectrophotometric determination and thin-layer separation of sulfamethazine and procaine penicillin in medicated feeds

Several reports have appeared concerning the separation and determination of sulfamethazine in combination with other sulfa drugs. A colorimetric method of analysis for sulfamethazine in feeds containing procaine penicillin and chlortetracycline recommended by the manufacturer[1] involves a hot alkaline digestion followed by separation using ion-exchange resin and estimation utilizing the BRATTON–MARSHALL[2] reaction. BERG *et al.*[3] used cold alkali for the extraction of the feed and claimed to have recovered about 94.2% of the sulfa drug. It was reported[4] that from a protein-free extract of the medicated feed, both sulfamethazine and procaine penicillin could be directly converted to the colored dye using the BRATTON–MARSHALL reaction and estimated after separating the procaine azo dye. Although this method offers a simple procedure for the determination of sulfamethazine, it cannot, however, be utilized for the estimation of procaine penicillin, since the dye from the antibiotic could not be recovered quantitatively.

Among the methods available for the determination of procaine penicillin, the microbiological assay procedure of MAYERNIK[5] and the colorimetric method using arsenomolybdic acid method[6] are currently in vogue. These are time-consuming and require elaborate laboratory facilities. The present study relates primarily to the thin-layer chromatographic separation of procaine penicillin and sulfamethazine from chlortetracycline and *p*-arsanilic acid when present as a mixture in medicated feeds. Also, it compares the results obtained by this method with those obtained by the procedure recommended by the manufacturer for sulfamethazine. Procaine penicillin and sulfamethazine are estimated spectrophotometrically after the BRATTON–MARSHALL reaction.

In this procedure an aliquot of protein-free ammoniacal dimethylformamide–chloroform–ethanol extract of the feed sample was spotted and developed on an alumina thin-layer chromatoplate using a double elution method[7]. The procaine penicillin and sulfamethazine regions were then quantitatively extracted and the solutions were subjected to the BRATTON–MARSHALL reaction giving a colored dye. The absorbance of the dyes was measured at 545 mμ. By this method, two samples of commercial medicated swine feed were analyzed.

Experimental

One gram of the medicated feed sample was extracted with refluxing ammoniacal dimethylformamide–chloroform–ethanol for 8 h. The warm extract, after filtration through a celite bed, was concentrated under reduced pressure to about 10 ml. The filtrate was diluted with 15 ml absolute ethanol, evaporated to about 5 ml and the operation was repeated once more. The protein-free extract was then quantitatively transferred to a 10-ml volumetric flask and made to volume with the same solvent.

An aliquot (4 × 250 μl) of this extract was spotted on a processed Camag alumina thin-layer chromatoplate (20 cm × 20 cm)[7]. The alumina plate was first developed for 45 min in benzene–chloroform mixture (60:40) followed by drying in air and redeveloping in the same direction for 25 min in a second solvent system, acetonitrile–benzene–

methanol–25% aq. dimethylamine solution (60:30:7.5:2.5). The regions corresponding to procaine penicillin and sulfamethazine were located using shortwave ultraviolet light (R_F values 0.80–0.85 and 0.38–0.42, respectively). The areas containing the drugs were scraped off the plate and extracted with 12 × 10-ml portions of 1% methanolic hydrochloric acid. The extracts were acidified with 2 ml of 6 *N* HCl, diluted with 8 ml of water and evaporated under reduced pressure to about 15 ml. The concentrates were quantitatively transferred to 50 ml volumetric flasks and diazotized with an excess of 1% aq. sodium nitrite solution. The excess nitrite was then decomposed with an excess of 5% aq. ammonium sulfamate solution and the coupling reaction effected by adding an excess of 0.1% aq. solution of N-(1-naphthyl)-ethylenediamine dihydrochloride to give a red dye. The absorbance was measured at 545 mμ using 1-cm quartz cells and a Beckman Model DU spectrophotometer. The concentration was directly read from the standard curve obtained by plotting concentration *vs.* absorbance.

Results and discussion

In attempting to separate cleanly procaine penicillin and sulfamethazine from other medicaments present in finished feeds, several isolated experiments were carried out with a view to selecting a suitable pH for the thin-layer alumina and the solvent system. It was observed that an alumina with a pH 7.2–7.4 afforded adequate separation of these two drugs from one another and also from other drug ingredients. By this procedure the R_F values of authentic samples of procaine penicillin, sulfamethazine, chlortetracycline and *p*-arsanilic acid were 0.75–0.80, 0.35–0.38, 0.00 and 0.00,

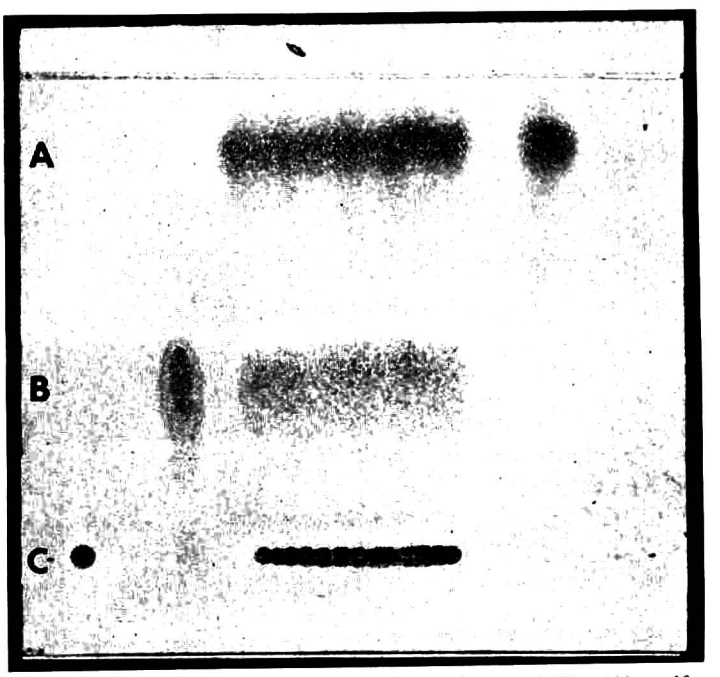

Fig. 1. Chromatogram of a mixture of procaine penicillin (A), sulfamethazine (B), chlortetracycline and *p*-arsanilic acid (C), after the BRATTON–MARSHALL reaction.

TABLE I

ANALYSIS OF FEED SAMPLES FOR PROCAINE PENICILLIN AND SULFAMETHAZINE

Sample	*Sulfamethazine concn. (μg/g)*			*Procaine penicillin concn. (μg/g)*
	Claimed	*This method*	*Manufacturer's method**	*This method*
CF-989	110	107.8	119	132.0
		107.8		118.0
		112.2		113.3
		107.8		126.5
		107.8		135.3
		107.8		126.5
Av.		108.5		125.5
CF-91	110	77.0	77.0	99.0
		71.0		104.5
		82.5		110.0
		79.2		110.0
		73.7		110.0
Av.		76.7		106.7

* We are thankful to Mr. G. D. RITCHIE of this Department for the results.

respectively (Fig. 1). However, the R_F values of the former two drugs when present in feed extracts were slightly altered (0.80–0.85 and 0.38–0.42, respectively). These observed alterations in R_F values were probably due to moisture and other polar compounds extracted from the feed.

Several independent thin-layer chromatographic separations were carried out with known amounts of the drugs which after extraction were estimated as described above. It was observed that the recoveries of procaine penicillin and sulfamethazine were 88–91%.

The results of analysis of the two commercial feed samples after employing an appropriate correction factor (10%) to the observed optical density are presented in Table I.

The values obtained for sulfamethazine in CF-989 indicate that the results are consistent and easily reproducible. By this method, the concentration calculated (108.5 μg/g) is in good agreement with the actual amount (110 μg/g) incorporated in the feed.

On the basis of the above results, it was expected that the values for sulfamethazine estimated by the two methods in CF-91 should be approximately identical. Actually the concentration of sulfamethazine determined by the two methods were 76.7 and 77.0 μg/g of feed. Although the lot of feed represented by sample CF-91 was claimed to contain 110 μg/g of sulfamethazine, it has now been established that the registration information was in error.

In the case of procaine penicillin, the microbiological method which is currently employed, determines the concentration of "active" procaine penicillin at the time of

analysis. This bioassay, however, does not indicate the exact amount of the drug incorporated in the feed because the procaine penicillin is unstable being markedly affected by temperature and the medium. The method described in this communication is developed with a view to determining the amount of the antibiotic which was originally added to the feed.

The samples CF-989 and CF-91 on analysis were found to contain 125.5 and 106.7 μg/g of procaine penicillin, respectively. Since the actual amounts of the drug in these samples were not known, ten standard authentic mixtures containing between 100 and 2000 μg of the drug were estimated by this method. As these results agreed closely with the known concentrations, it may be inferred that the results obtained on Samples CF-989 and CF-91 are substantially correct.

The details of these findings will be reported shortly.

Acknowledgements

We wish to acknowledge our sincere thanks to Drs. K. J. JENKINS and F. B. JOHNSTON of the Research Branch, Canada Department of Agriculture, and Mr. E. J. DOYLE of our Division for their helpful suggestions. Our thanks are due to Miss GAIL STEWART for typing the manuscript.

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Received August 15th, 1968

CHROM. 3747

Spot test for potassium on paper chromatograms

Sodium tetraphenylboron has been shown to be a sensitive reagent for the detection and determination of potassium. The properties and uses of this compound are the subject of a comprehensive review and an extensive bibliography[1,2]. Potassium tetraphenylboron is quite insoluble, with a solubility product of about $2 \cdot 10^{-8}$, but its white color limits its use in the detection of potassium on filter paper chromatograms. However, a sharp color contrast can be produced by treatment of the precipitate with mercuric chloride solution in the presence of bromophenol blue indicator. Mercuric chloride reacts with tetraphenylboron salts to produce hydrochloric acid and boric acid. This test can be used to detect amounts of potassium as small as a few $\mu g/cm^2$ of paper.

Ammonium ion will also be precipitated by tetraphenylboron and can be detected by this method. If ammonium ion interferes with the potassium test, precipitation in a solution made alkaline with NaOH will prevent precipitation of moderate amounts of ammonium[3]. Cesium, rubidium, thallium, and silver ions also precipitate with the reagent and interfere with the test.

Test reagents

(1) A 2% solution of sodium tetraphenylboron in water. (2) A solution of bromophenol blue (0.1%) and mercuric chloride (2.5%) in 80% ethanol. Adjust carefully to the highest acidity which will still produce a blue color when the mixture is sprayed or brushed on dry filter paper.

Procedure

(1) Dip the chromatogram in the tetraphenylboron solution; (2) wash the paper with a stream of water to remove excess reagent; (3) dry the paper, using low heat if desired; (4) brush the dry paper lightly with a soft brush moistened with the mercuric chloride reagent, or spray lightly in a well ventilated hood. (The spray is irritating and toxic.)

Experimental

A 5-μl capillary tube was used to spot solutions of KCl and NH_4Cl on inch-wide strips of Whatman No. 1 paper. Amounts varying from 0.025 to 5.0 μmoles of ion were applied to an area of 1 cm^2 of paper. The paper was then dipped in 2% sodium tetraphenylboron solution. About 200 cm^2 of the paper was then washed uniformly with a stream of water from a wash bottle delivering about 50 ml/min. A total of about 50 ml of wash water was used for each strip, care being taken to wash alternately both sides of the paper. The strip was then dried for a few minutes on a watch glass on a hot plate at a temperature low enough to prevent spattering of water droplets. The mercuric chloride–bromophenol blue solution was then brushed lightly over the paper, producing a blue background with bright yellow spots of the same size as the original spots. For amounts as small as 0.025 μmole, the yellow color appeared slowly on drying and this amount appears to be near the lower limit of detection.

Prevention of ammonium ion precipitation was tested by using a sodium tetra-

phenylboron solution made alkaline by addition of NaOH (3 vol. of the 2% reagent mixed with 1 vol. of 6 *M* NaOH). Strips containing 0.5 μmole or less of ammonium ion produced negative tests when washed and tested as before. Spots containing 0.05 μmole of potassium ion on the same strips produced positive tests. When the amount of ammonium ion is as large as several μmoles/cm^2, a precipitate will form even in alkaline solution. This may be removed by washing with a stream of 1 *M* NaOH solution, followed by the wash with water, but this treatment also removes smaller quantities of potassium ion (0.05 μmole/cm^2).

In testing for small amounts of ions it is necessary to limit the amount of wash water. Washing a sample of 0.025 μmole of potassium with 150 ml of water, instead of 50 ml, usually resulted in a negative test. The minimum amount of water required depends on the area of the paper, efficiency of washing, etc., and should be determined for each operator by running a blank.

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Received August 19th, 1968

News

Meetings

FIFTH INTERNATIONAL SYMPOSIUM ON ADVANCES IN CHROMATOGRAPHY

The Fifth International Symposium on Advances in Chromatography will be held at Caesars Palace in Las Vegas, Nevada on January 20th–23rd, 1969. The meeting will consist of papers by outstanding scientists from the U.S. and abroad. An exhibition of the latest chromatographic instrumentation, ancillary equipment and books will be presented. Details of the meeting and registration forms are available from Prof. A. ZLATKIS, Chemistry Department, University of Houston, Houston, Texas 77004, U.S.A.

PROGRAMME

Monday morning, January 20th, L. S. ETTRE, presiding
Capillary columns and detectors
Preparative scale gas chromatography

Monday afternoon, January 20th, C. G. SCOTT, presiding
Liquid chromatography

Tuesday morning, January 21st, A. KARMEN, presiding
Liquid chromatography

Tuesday afternoon, January 21st.
Informal panels for small discussion groups. Topics to be considered include liquid chromatography, supercritical fluid chromatography, flavors, biomedical applications, pyrolysis, preparative scale chromatography, quantitative analysis, computers and auxiliary techniques.

Wednesday morning, January 22nd, H. H. WOTIZ, presiding
Biomedical applications

Wednesday afternoon, January 22nd, R. E. SIEVERS, presiding
Biomedical, ancillary techniques for chromatography

Thursday morning, January 23rd, C. J. W. BROOKS, presiding
Flavors, pyrolysis, general chromatography

Bibliography Section

Paper Chromatography

1. REVIEWS AND BOOKS

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See also TLC section.

2. FUNDAMENTALS, THEORY AND GENERAL

BAYER, E. *et al.*: Nomenclature in Chromatography. A suggestion. *Chromatografia*, 1 (1968) 153–159 — suggestions for a nomenclature for all types of chromatography.

SOCZEWIŃSKI, E. AND MAŃKO, R.: Some problems of reproducibility of R_F values in liquid–liquid partition chromatography. *J. Chromatog.*, 33 (1968) 40–44 — the influence of pH and volume ratio.

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3. TECHNIQUES I (MATERIAL, SOLVENTS, DEVELOPMENT, DETECTION, QUANTITATIVE ANALYSIS)

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25. ORGANIC PHOSPHORUS COMPOUNDS

See PC section.

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CHROM. 3752

THÉORIE DES VALEURS MOYENNES DE LA VITESSE, DE LA PRESSION ET HEPT EN CHROMATOGRAPHIE EN PHASE GAZEUSE

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SUMMARY

Theory of mean values of velocity, pressure and HETP in gas chromatography

A theory of the mean values of pressure, linear velocity of the carrier gas, and HETP is developed in gas chromatography.

This theory involves two different variables: The length of the column and the flow rate of the gas carrier.

The local values of pressure and linear velocity are defined as functions of the above variables and yield the calculation of the corresponding average local values. These mean values are discussed and two correlations between these values are developed which involve retention time and BOYLE–MARIOTTE's law.

The mean values of HETP expressed in terms of column length and retention time are discussed.

Some applications are developed and some suggested, especially in the case of gas chromatography with variation of the carrier gas linear velocity.

INTRODUCTION

En chromatographie, qu'elle soit en phase liquide ou qu'elle soit en phase gazeuse, la propagation de la phase mobile est assurée par le maintien d'un gradient de pression le long de la colonne. Cependant dans le cas de la chromatographie en phase gazeuse, la phase mobile est compressible selon une loi de variation que l'on peut admettre correspondant à la loi de BOYLE–MARIOTTE, et les valeurs de la pression et de la vitesse linéaire du gaz varient le long de la colonne, ainsi d'ailleurs que l'efficacité de la séparation exprimée par la hauteur équivalente à un plateau théorique.

Il n'est possible de mesurer la valeur de la pression, ou de la vitesse linéaire du gaz qu'à l'entrée et à la sortie de la colonne mais si l'on connaît la loi de variation de ces grandeurs, on peut en calculer la valeur moyenne. Ainsi on trouve dans la littérature la valeur moyenne de la pression[1,2], cette valeur moyenne étant calculée par rapport à la longueur de la colonne. De même, la variation de la hauteur équivalente à un plateau théorique avec la pression du gaz a été examinée[3–5], et des valeurs moyennes de cette hauteur en fonction de la pression et de la vitesse linéaire du gaz ont été proposées[6–9].

Cependant, pour étudier la propagation d'un soluté dans une colonne, il faut considérer le fait qu'il existe deux variables: l'espace que parcourt le soluté, et le temps nécessaire au soluté pour parcourir cette longueur.

Dans l'élaboration de notre théorie, nous déterminerons la loi de variation de la pression et de la vitesse linéaire du gaz par rapport à chacune de ces deux variables, et nous calculerons la valeur moyenne de ces grandeurs dans chaque cas. Il sera alors possible de trouver des relations entre ces différentes valeurs moyennes, et nous pourrons préciser la valeur moyenne de la hauteur équivalente à un plateau théorique en fonction des différentes variables que sont la vitesse linéaire du gaz, la longueur de la colonne, et le temps.

PARTIE THÉORIQUE

Les deux variables considérées étant la longueur de la colonne parcourue par le soluté, et le temps qu'il met à la parcourir, nous définirons successivement la valeur de la pression et de la vitesse linéaire du gaz par rapport à ces deux variables.

Les deux équations fondamentales (1 et 2) représentent la loi de propagation de la phase mobile, et la loi de BOYLE–MARIOTTE des gaz parfaits.

$$U_{(x)} = -\frac{K}{n}\cdot\frac{\mathrm{d}p}{\mathrm{d}x} \tag{1}$$

$$U\cdot p = U_e\cdot p_e = U_s\cdot p_s \tag{2}$$

Variation de la pression en fonction de la longueur

En transformant l'équation (1) à l'aide de l'équation (2), on obtient aisément la relation différentielle (3) entre la pression et la longueur.

$$p\cdot\mathrm{d}p = -\frac{n}{K}\cdot U_s\cdot p_s\cdot\mathrm{d}x \tag{3}$$

En intégrant cette relation différentielle d'une part entre 0 et L pour la longueur et entre p_e et p_s pour la pression, et d'autre part entre 0 et x et entre p_e et p_x, on obtient les équations (4 et 5):

$$\frac{1}{2}(p_s^2 - p_e^2) = -\frac{n}{K}\cdot U_s\cdot p_s\cdot L \tag{4}$$

$$\frac{1}{2}(p_x^2 - p_e^2) = -\frac{n}{K}\cdot U_s\cdot p_s\cdot x \tag{5}$$

Le rapport des deux équations (4) et (5) devient:

$$\frac{p_x^2 - p_e^2}{p_s^2 - p_e^2} = \frac{x}{L} \tag{6}$$

et l'on peut obtenir la relation entre la pression et la longueur:

$$p_x = \left[p_e^2 - \frac{x}{L}(p_e^2 - p_s^2)\right]^{\frac{1}{2}} \tag{7}$$

L'équation (7) est très importante comme nous l'avions montré précédemment[10]. Elle permet de calculer la valeur de la pente de la courbe représentant la variation de la pression avec la longueur relative de la colonne, et notamment lorsque cette longueur relative est égale à l'unité:

$$\frac{\mathrm{d}(p_x)}{\mathrm{d}\left(\frac{x}{L}\right)} = -\frac{1}{2}\cdot\frac{p_e^2 - p_s^2}{p_s} \text{ pour } \frac{x}{L} = 1 \tag{8}$$

D'autre part, elle permet de calculer la valeur de la pression moyenne par rapport à la longueur. En effet, la pression moyenne est définie par la relation (9):

$$\bar{p}_{(x)} = \frac{\int_0^L p_x \cdot \mathrm{d}x}{\int_0^L \mathrm{d}x} \tag{9}$$

et en y remplaçant la pression par sa valeur définie dans l'équation (7), et en intégrant, on obtient la pression moyenne:

$$\bar{p}_{(x)} = \frac{2}{3}\cdot p_s \cdot \frac{P^3 - 1}{P^2 - 1} \tag{10}$$

en fonction de la pression relative P:

$$P = \frac{p_e}{p_s} = \frac{U_s}{U_e} \tag{11}$$

Variation de la pression en fonction du temps

Le temps étant la variable choisie, l'équation (1) doit être transformée pour la faire apparaître:

$$U = -\frac{K}{n}\cdot\frac{\mathrm{d}p}{\mathrm{d}t}\cdot\frac{\mathrm{d}t}{\mathrm{d}x} \tag{12}$$

En tenant compte de la définition de la vitesse linéaire:

$$U = \frac{\mathrm{d}x}{\mathrm{d}t} \tag{13}$$

L'équation (12) s'écrit alors:

$$U_{(t)}^2 = -\frac{K}{n}\cdot\frac{\mathrm{d}p}{\mathrm{d}t} \tag{14}$$

En remplaçant dans l'équation (14) la valeur de la vitesse linéaire du gaz en fonction de la pression définie dans la relation (2), on obtient l'équation différentielle fondamentale:

$$p^2 \cdot \mathrm{d}p = -\frac{n}{K}(U_s \cdot p_s)^2 \cdot \mathrm{d}t \tag{15}$$

L'équation (15) intégrée d'une part entre o et T entre p_e et p_s, et d'autre part entre o et t et entre p_e et p_t, permet d'obtenir les deux relations (16) et (17):

$$\frac{1}{3}(p_s^3 - p_e^3) = -\frac{n}{K}(U_s \cdot p_s)^2 \cdot T \tag{16}$$

$$\frac{1}{3}(p_t^3 - p_e^3) = -\frac{n}{K}(U_s \cdot p_s)^2 \cdot t \tag{17}$$

Le rapport de ces deux relations peut s'écrire:

$$\frac{p_t^3 - p_e^3}{p_s^3 - p_e^3} = \frac{t}{T} \tag{18}$$

et l'équation (18) permet de calculer la valeur de la pression en fonction du temps relatif t/T (éqn. 19):

$$p_t = \left[p_e^3 - \frac{t}{T}(p_e^3 - p_s^3)\right]^{\frac{1}{3}} \tag{19}$$

Comme dans le cas précédent, l'équation (19) permet de calculer la valeur de la pente de la courbe représentant la variation de la pression avec le temps relatif, lorsque ce temps relatif est égal à l'unité:

$$\frac{\mathrm{d}(p_t)}{\mathrm{d}\left(\frac{t}{T}\right)} = -\frac{1}{3} \cdot \frac{p_e^3 - p_s^3}{p_s^2} \tag{20}$$

De même, la valeur de la pression moyenne par rapport au temps, défini par la relation (21):

$$\bar{p}_{(t)} = \frac{\int_0^T p_t \cdot \mathrm{d}t}{\int_0^T \mathrm{d}t} \tag{21}$$

peut être calculée en y remplaçant la pression par sa valeur présentée dans l'équation (19):

$$\bar{p}_{(t)} = \frac{3}{4} \cdot p_s \cdot \frac{P^4 - 1}{P^3 - 1} \tag{22}$$

On considère aisément que les équations représentant la valeur de la pression diffèrent notablement lorsque l'on choisit comme variable l'espace relatif (éqn. 7) ou le temps relatif (éqn. 19).

En ce sens qu'il se trouve une puissance 1/2 dans le premier cas, alors qu'il apparaît une puissance 1/3 dans le second. Les valeurs moyennes de la pression ont été calculées pour différentes valeurs de la pression relative, et réunies dans le Tableau I.

TABLEAU I

VALEURS DES PRESSIONS MOYENNES PAR RAPPORT À LA LONGUEUR ET PAR RAPPORT AU TEMPS

P	1	1.1	1.2	1.3	1.4	1.5	1.6	1.7	1.8	1.9	2	5
$\frac{\bar{p}_{(x)}}{\bar{p}_s}$	1	1.047	1.105	1.16	1.212	1.267	1.325	1.38	1.44	1.52	1.555	3.445
$\frac{\bar{p}_{(t)}}{\bar{p}_s}$	1	1.05	1.108	1.167	1.223	1.286	1.348	1.41	1.47	1.55	1.606	3.77

Variation de la vitesse linéaire en fonction de la longueur

En différentiant l'équation (2), on obtient la différentielle dp:

$$\mathrm{d}p = -U_s \cdot p_s \cdot \frac{\mathrm{d}u}{u^2} \tag{23}$$

et en remplaçant dp par sa valeur dans l'équation (1), on obtient l'équation différentielle reliant la vitesse linéaire à la longueur de la colonne:

$$u^{-3} \cdot \mathrm{d}u = \frac{n}{K \cdot U_s \cdot P_s} \cdot \mathrm{d}x \tag{24}$$

L'équation (24) intégrée successivement entre les limites o et L, et o et x permet d'obtenir deux équations dont le rapport s'écrit:

$$\frac{U_e^{-2} - U_x^{-2}}{U_e^{-2} - U_s^{-2}} = \frac{x}{L} \tag{25}$$

De l'équation (25), il est possible de tirer la valeur de la vitesse linéaire à l'abscisse x:

$$U_x = \left[U_e^{-2} - \frac{x}{L}\left(U_e^{-2} - U_s^{-2}\right)\right]^{-\frac{1}{2}} \tag{26}$$

ou encore

$$\frac{U_x}{U_s} = p_s \left[p_e^2 - \frac{x}{L}\left(p_e^2 - p_s^2\right)\right]^{-\frac{1}{2}} \tag{26 bis}$$

Nous avons représenté sur la figure 1 la variation de la vitesse linéaire relative du gaz le long de la colonne, pour différentes valeurs de la pression du gaz à l'entrée de la colonne. L'équation (26 bis) permet de calculer la pente de cette courbe à la sortie de la colonne:

$$\frac{\mathrm{d}\left(\frac{U_x}{U_s}\right)}{\mathrm{d}\left(\frac{x}{L}\right)} = \frac{1}{2} \cdot \frac{p_e^2 - p_s^2}{p_s^2} \text{ pour } \frac{x}{L} = 1 \tag{27}$$

TABLEAU II

PENTES DES COURBES 1 ET 2 POUR L'ABSCISSE ÉGALE À L'UNITÉ

P	1	1.1	1.2	1.3	1.4	1.5	1.6	1.7	1.8	1.9	2	5
$\frac{d\left(\frac{U_x}{U_s}\right)}{d\left(\frac{x}{L}\right)}$	0	0.105	0.22	0.345	0.48	0.625	0.78	0.945	1.12	1.305	1.50	12.0
$\frac{d\left(\frac{U_t}{U_s}\right)}{d\left(\frac{t}{T}\right)}$	0	0.11	0.243	0.399	0.58	0.791	1.035	1.304	1.61	1.95	2.33	41.3

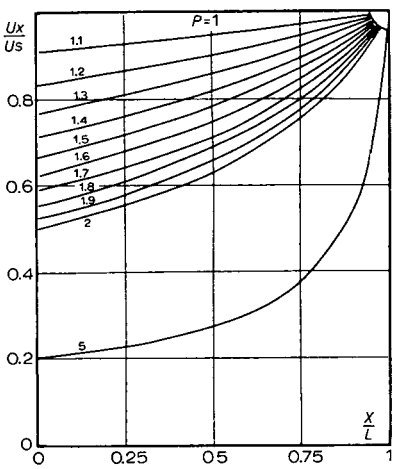

Fig. 1. Variation de la vitesse linéaire relative du gaz vecteur en fonction de l'abscisse relative dans la colonne, pour différentes valeurs du rapport des pressions d'entrée et de sortie.

Les valeurs de la pente ont été regroupées dans le Tableau II (deuxième ligne). Il faut remarquer que ces valeurs sont égales et de signe opposé au valeurs correspondantes des pentes des courbes représentant la variation de la pression le long de la colonne.

L'équation (26 bis) permet aussi de calculer la valeur de la vitesse linéaire moyenne par rapport à la longueur définie par la relation (28):

$$\bar{U}_{(x)} = \frac{\int_0^L U_x \cdot dx}{\int_0^L dx} \tag{28}$$

$$\bar{U}_x = 2 \cdot U_s \cdot \frac{P - 1}{P^2 - 1} \tag{29}$$

Variation de la vitesse linéaire en fonction du temps

L'équation différentielle (30) reliant la vitesse linéaire du gaz au temps est obtenue aisément en remplaçant dans l'équation (14) la différentielle dp par sa valeur représentée dans l'équation (23):

$$U^{-4} \cdot \mathrm{d}u = \frac{n}{K \cdot U_s \cdot p_s} \cdot \mathrm{d}t \tag{30}$$

En intégrant cette équation (30) entre les limites o et T d'une part, et o et t d'autre part, on obtient deux équations dont le rapport s'écrit:

$$\frac{U_t^{-3} - U_e^{-3}}{U_s^{-3} - U_e^{-3}} = \frac{t}{T} \tag{31}$$

L'équation (31) permet de tirer la valeur de la vitesse linéaire au temps t:

$$U_t = \left[U_e^{-3} - \frac{t}{T}\left(U_e^{-3} - U_s^{-3}\right)\right]^{-\frac{1}{3}} \tag{32}$$

qui s'écrit aussi:

$$\frac{U_t}{U_s} = p_s \left[p_e^3 - \frac{t}{T}\left(p_e^3 - p_s^3\right)\right]^{-\frac{1}{3}} \tag{32 bis}$$

Sur la figure 2 est représentée la variation de la vitesse linéaire relative en fonction du temps relatif. La pente de cette courbe au moment où le temps est égal à l'unité est définie par l'équation (33) obtenue en dérivant l'équation (32 bis).

$$\frac{\mathrm{d}\left(\frac{U_t}{U_s}\right)}{\mathrm{d}\left(\frac{t}{T}\right)} = \frac{1}{3}\,\frac{p_e^3 - p_s^3}{p_s^3} \text{ pour } \frac{t}{T} = 1 \tag{33}$$

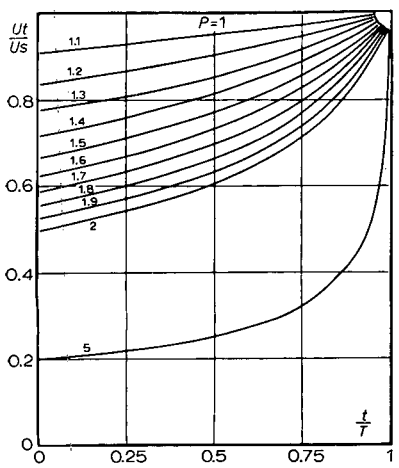

Fig. 2. Variation de la vitesse linéaire relative du gaz vecteur en fonction du temps relatif, le temps de traversée de la colonne par le gaz vecteur étant choisi comme unité de temps, pour différentes valeurs du rapport des pressions d'entrée et de sortie.

Les valeurs de la pente, regroupées dans le Tableau II, permettent de constater qu'à la sortie de la colonne la variation de la vitesse linéaire du gaz est bien plus élevée lorsque la variable est le temps.

La vitesse linéaire moyenne par rapport au temps, définie par l'équation (34):

$$\bar{U}_{(t)} = \frac{\int_0^T U_t \cdot \mathrm{d}t}{\int_0^T \mathrm{d}t} \tag{34}$$

peut être calculée en utilisant l'équation (32 bis)

$$\bar{U}_t = \frac{3}{2} \cdot U_s \cdot \frac{P^2 - 1}{P^3 - 1} \tag{35}$$

TABLEAU III

VALEURS DES VITESSES MOYENNES PAR RAPPORT À LA LONGUEUR ET PAR RAPPORT AU TEMPS

P	1	1.1	1.2	1.3	1.4	1.5	1.6	1.7	1.8	1.9	2	5
$\frac{\bar{U}_{(x)}}{\bar{U}_s}$	1	0.955	0.91	0.87	0.833	0.80	0.77	0.741	0.715	0.69	0.667	0.333
$\frac{\bar{U}_{(t)}}{\bar{U}_s}$	1	0.955	0.905	0.864	0.825	0.79	0.756	0.725	0.695	0.659	0.644	0.290

Comme dans le cas des pressions moyennes, on constate (Tableau III) que les valeurs des vitesses linéaires moyennes diffèrent quelque peu lorsque l'on choisit comme variable la longueur ou le temps, et cette différence est d'autant plus importante que la valeur de la pression relative est élevée. Ainsi, la vitesse linéaire moyenne par rapport au temps est toujours inférieure à la vitesse linéaire moyenne par rapport à la longueur. Ce fait pouvait d'ailleurs être prévu, tout du moins de façon qualitative, en considérant les équations (26 bis) et (32 bis). Dans le première, représentant la valeur de la vitesse par rapport à la longueur, l'exposant est égal à $-1/2$, alors que l'exposant de l'équation représentant la valeur de la vitesse par rapport au temps est égal à $-1/3$.

Calcul de HEPT moyenne

La valeur de HEPT est reliée à la valeur de la vitesse linéaire du gaz par l'équation de VAN DEEMTER:

$$H = A + \frac{B}{u} + C \cdot u \tag{36}$$

Il faut remarquer que certains auteurs, considérant le fait que le coefficient B est sensiblement inversement proportionnel à la pression, admettent que H est une

fonction linéaire de la vitesse du gaz. Nous ne prendrons pas position sur ce point, et nous conserverons l'équation de VAN DEEMTER originelle, car les équations que nous obtiendrons peuvent être transformées aisément si l'on veut tenir compte de la remarque précédente.

La vitesse linéaire du gaz variant avec la longueur de la colonne et avec le temps, la valeur instantanée de H varie avec chacun de ces paramètres, et nous choisirons la longueur ou le temps comme variable.

HEPT moyenne par rapport à la longueur. La valeur moyenne de HEPT par rapport à la longueur est définie par la relation (37):

$$\bar{H}_{(x)} = \frac{1}{L}\int_0^L H \cdot \mathrm{d}x \tag{37}$$

L'équation de VAN DEEMTER représente la variation H_u de HEPT avec la vitesse linéaire; en remplaçant dans cette équation la vitesse linéaire par sa valeur en fonction de la longueur (éqn. 26 bis), on obtient H_x dont la variable est x. En intégrant par rapport à x l'équation (37), on obtient la valeur moyenne de HEPT par rapport à la longueur:

$$\bar{H}_{(x)} = A + \frac{B}{U_s}\cdot\frac{2}{3}\cdot\frac{P^3-1}{P^2-1} + C\cdot U_s\cdot\frac{2}{P+1} \tag{38}$$

Cette équation (38) peut être transformée, en remplaçant dans le second membre la pression relative P par sa valeur en fonction de la vitesse linéaire et de la pression du gaz à la sortie de la colonne. Pour cela, il suffit de déterminer la valeur de la pression relative dans l'équation (4)

$$P = (K'\cdot U_s\cdot p_s^{-1} + 1)^{\frac{1}{2}} \tag{39}$$

avec

$$K' = \frac{2\cdot n\cdot L}{K} \tag{40}$$

et de remplacer P par cette valeur dans l'équation (38):

$$\bar{H}_{(x)} = A + \frac{2B}{3U_s}\cdot\frac{2 + K'\cdot U_s\cdot p_s^{-1} + (1 + K'\cdot U_s\cdot p_s^{-1})^{\frac{1}{2}}}{1 + (1 + K'\cdot U_s\cdot p_s^{-1})^{\frac{1}{2}}} + \frac{2CU_s}{1 + (1 + K'U_s\cdot p_s^{-1})^{\frac{1}{2}}} \tag{41}$$

HEPT moyenne par rapport au temps. Le temps de rétention du soluté peut être une variable intéressante et la valeur moyenne de HEPT par rapport au temps est définie par la relation (42):

$$\bar{H}_{(t)} = \frac{1}{T}\int_0^T H_t \cdot \mathrm{d}t \tag{42}$$

En remplaçant dans l'équation (37) la vitesse linéaire par sa valeur en fonction du temps (éqn. 32 bis), et en intégrant par rapport au temps, l'équation (42) devient:

$$\bar{H}_{(t)} = A + \frac{3}{4}\cdot\frac{B}{U_s}\cdot\frac{P^4-1}{P^3-1} + C\cdot U_s\frac{3(P^2-1)}{2(P^3-1)} \tag{43}$$

DISCUSSION ET CONCLUSIONS

Importance des variables longueur et temps

Nous nous sommes efforcé dans cette étude de présenter les deux variables que sont la longueur de la colonne et le temps de rétention, et de faire une distinction très nette entre ces deux notions. Ainsi les expressions représentant la pression et la vitesse linéaire du gaz, ou HEPT, sont très différentes lorsque l'on choisit l'une ou l'autre variable. De même les valeurs moyennes de ces trois grandeurs diffèrent lorsqu'elle sont calculées par rapport à la longueur ou par rapport au temps. Il est intéressant de remarquer que la loi de BOYLE–MARIOTTE s'étend aux valeurs moyennes, lorsque l'on considère la valeur moyenne de la pression $\bar{p}_{(x)}$ considérée comme fonction de la longueur et la valeur moyenne de la vitesse linéaire, $\bar{U}_{(t)}$, considérée comme fonction du temps:

$$U_e \cdot p_e = U_s \cdot p_s = \bar{U}_{(t)} \cdot \bar{p}_{(x)} \tag{44}$$

On s'aperçoit aisément que le temps de traversée T de la colonne par une molécule de gaz vecteur est relié d'une façon simple à la vitesse linéaire moyenne par rapport au temps, d'après la définition de cette vitesse moyenne donnée dans l'équation (34):

$$\bar{U}_{(t)} = \frac{L}{T} \tag{45}$$

Valeur moyenne de HEPT

Comme dans le cas des autres valeurs moyennes, l'expression de la valeur moyenne de HEPT diffère lorsque l'on choisit comme variable la longueur ou le temps. Il faut remarquer que la valeur de $\bar{\bar{H}}_{(x)}$ représentée dans l'équation (40) ressemble quelque peu à l'équation (8), proposée récemment de façon empirique par KAMBARA[8].

$$\bar{\bar{H}} = \frac{B}{\bar{U}_s} + C\sqrt{\bar{U}_s} \tag{46}$$

lorsque la valeur de la vitesse linéaire du gaz n'est pas très faible. Et effectivement, nous avons observé lors de l'élution d'alcanes une allure parabolique de la variation de la vitesse linéaire avec la valeur de $\bar{\bar{H}}_{(x)}$ mesurée[9]. Dans le cas des faibles vitesses linéaires, les deux valeurs de $\bar{\bar{H}}_{(x)}$ diffèrent, mais malgré tout assez peu.

Choix des deux variables

On comprend que selon les cas, il soit plus intéressant de choisir une variable plutôt que l'autre. Cependant, on peut dire que lorsque l'on utilise la vitesse linéaire du gaz, la variable temps apparaît être préférable. De même, nous constatons dans une étude en cours concernant la chromatographie avec changement de débit précédemment décrite[10,11] que pour les applications de la notion de HEPT moyenne, la variable temps est plus commode.

SYMBOLES UTILISÉS

A,B,C : Constantes caractérisant l'équation de VAN DEEMTER;
H : hauteur équivalente à un plateau théorique;
n : viscosité dynamique du gaz vecteur;
K : perméabilité de la colonne;
L : longueur de la colonne;
p : pression du gaz vecteur;
p_e,p_s : pression du gaz vecteur à l'entrée et à la sortie de la colonne;
$\bar{p}_{(x)}$: pression moyenne du gaz vecteur par rapport à la longueur de la colonne;
$\bar{p}_{(t)}$: pression moyenne du gaz vecteur par rapport au temps de traversée de la colonne T;
U : vitesse linéaire du gaz vecteur;
U_e,U_s : vitesse linéaire à l'entrée et à la sortie de la colonne;
$\bar{U}_{(x)}$: vitesse linéaire moyenne du gaz vecteur par rapport à la longueur de la colonne;
$\bar{U}_{(t)}$: vitesse linéaire moyenne du gaz vecteur par rapport au temps de traversée de la colonne T;
T : temps de traversée de la colonne par une molécule de gaz vecteur.

REMERCIEMENT

Nous remercions Monsieur GAUME, Professeur de Physique expérimentale à la Faculté des Sciences de Lyon, pour ses conseils fort précieux ayant trait notamment au concept des deux variables temps et espace.

RÉSUMÉ

Une théorie des valeurs moyennes de la pression et de la vitesse linéaire du gaz vecteur, ainsi que de la HEPT, est élaborée en chromatographie en phase gazeuse. Cette théorie tient compte du fait qu'il existe deux variables distinctes au cours de l'élution d'un soluté: la longueur de la colonne, et le temps de séjour d'une molécule de gaz vecteur dans la colonne. Les valeurs instantanées de la pression et de la vitesse linéaire ont été définies par rapport à chacune de ces deux variables, et ces valeurs ont permis de calculer les valeurs moyennes correspondantes. Après une discussion concernant ces valeurs moyennes deux relations sont présentées entre ces valeurs, qui ont trait au temps de rétention et à la loi de BOYLE–MARIOTTE. Les valeurs moyennes d' HEPT par rapport à la longueur de la colonne, et par rapport au temps d'écoulement sont discutées et des applications sont présentées et suggérées notamment dans le cas de la chromatographie avec variation de la vitesse linéaire du gaz vecteur.

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CHROM. 3746

MICRO INFRA-RED SPECTROSCOPY OF GAS CHROMATOGRAPHIC FRACTIONS

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(Received August 19th, 1968)

SUMMARY

A simple and effective method of obtaining infra-red spectra of gas chromatographic fractions has been developed making possible rigorous identification with microgram quantities.

After gas–liquid chromatographic injection, the separated components which emerge from the column are passed through a heated line via a stream splitter and condensed independently on a glass surface. The isolated component is then transferred to KBr for microdisc preparation. The method of sample transfer described gives a high recovery, eliminates many possible sources of contamination and yields high quality infra-red spectra for 1–10 μg samples.

The results of the technique are illustrated with a number of typical spectra.

INTRODUCTION

The introduction of gas chromatography and its rapid development has provided the forensic scientist with a convenient method of separating microgram quantities of complex organic mixtures into their different components. The technique, however, is essentially one of separation and supporting evidence is usually required to identify the separated components of the mixture. Comparison of retention times of the unknown components and simple derivatives of these unknowns with those of standards using two dissimilar stationary phases is frequently used to establish identity[1–3]. Alternatively, the column effluents may be collected and the pure components subsequently analysed by instrumental techniques such as infra-red and ultra-violet spectrophotometry, nuclear magnetic resonance spectroscopy and mass spectrometry. The measurement of other physical properties such as mobility in thin layer and paper chromatographic systems[4] has also provided supporting evidence for identification.

In many problems involving the use of gas chromatography, the component of interest is frequently a trace constituent of the sample under investigation. The injection of larger volumes into the gas chromatograph is not possible if sample economy is a necessity and is not desirable as this results in a reduced separating efficiency of the column. Consequently it is necessary to use a spectroscopic method

of analysis which is sensitive at the microgram level in combination with chromatographic separation. Organic mass spectrometry is able to analyse sub-microgram quantities of material but requires considerable expertise and the maximum amount of information is only deduced after lengthy interpretation. For many laboratories the cost of this type of instrumentation is prohibitive. I.R. spectroscopy has proved a useful and relatively inexpensive method of analysis, particularly when the spectra have been recorded in standard collections of reference spectra[5]. The technique, however, is usually employed for the analysis of sub-milligram amounts of material, but with special micro-sampling techniques and instrumental methods yields high quality spectra with as little as 1 μg of component[6,7]. GLC–I.R. combination systems in which the gas chromatograph is coupled directly to a fast scanning I.R. spectrophotometer and the separated components analysed in the gas phase as they emerge from the column is, because of the very different sampling conditions required for the two techniques, only sensitive for components in excess of 100 μg[8–10]. It is, therefore, necessary to employ an intermediate trapping system to collect microgram components for subsequent I.R. analysis.

Many methods have been reported for trapping gas chromatographic fractions for further I.R. examination[11–17]. The majority of these methods are only applicable for components in the sub-milligram range, others require elaborate and time consuming techniques or are too specific in their applications. This paper reports a simple and effective method of GLC–I.R. microanalysis which has produced rigorous identification of microgram components with only minor modifications to existing equipment. The analytical technique is of particular value in toxicological investigations in forensic science laboratories.

EQUIPMENT AND MATERIALS

The gas chromatograph employed is a Pye 104/84 fitted with a flame ionisation detector. The gas chromatograph is equipped with a stainless steel stream splitter linked to a Research and Industrial Instruments heated line. The arrangement of the equipment is shown in Fig. 1. The flow splitter, inserted between the end of the column and the flame ionisation detector, gives a split ratio of approximately 90:10. The smaller part of the effluent is allowed to enter the flame ionisation detector for monitoring the separation of the mixture on a Perkin-Elmer Hitachi 159 flat bed recorder. The larger part of the effluent gases is passed through a stainless steel capillary tube to an electrically heated exhaust assembly on the side of the chromatograph. To the stainless steel exhaust capillary is silver soldered a capillary gas line which terminates in a needle. This needle is used for piercing the silicone rubber septa enclosing the glass vessels in which the components are to be trapped. The line and needle are both resistively heated along their entire length from a low voltage transformer and variac, thus preventing premature condensation of the components and the possibility of contamination. To prevent blowback of carrier gas through the detector or ejection of the trap by the build up of gas pressure during trapping, the needle incorporates a concentric outer shell linked to an exhaust tube.

"Analar" grade chloroform (British Drug Houses Ltd.) employed in the transfer of condensed components is initially dried over anhydrous calcium chloride and distilled prior to use. Evaporation of this solvent on KBr powder (50 μl/0.5 mg) and

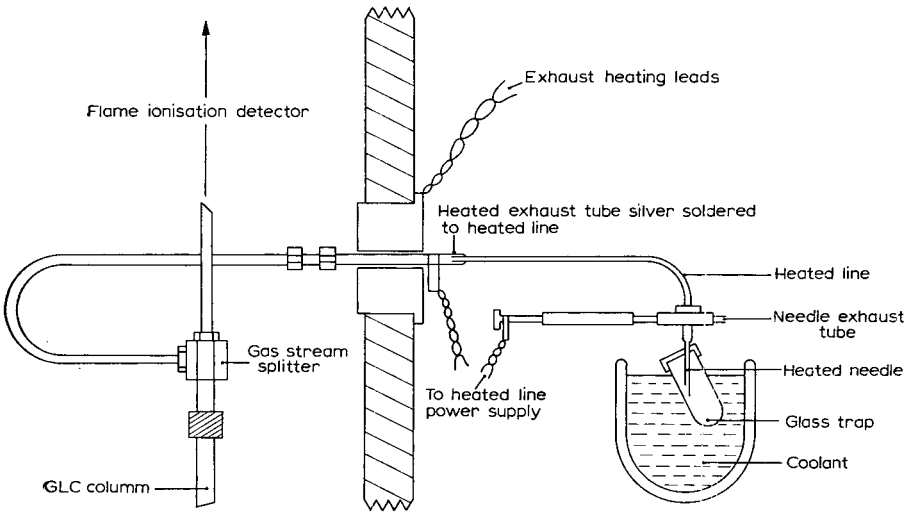


Fig. 1. Diagram indicating arrangement of trapping modifications on the Pye 104/84 gas chromatograph.

examination by I.R. spectroscopy indicated no detectable non-volatile organic residues present in the solvent. Optical grade potassium bromide (Alpha Inorganics Ltd.) is used for microdisc preparation. The potassium bromide is stored in an oven at 150° when not in use.

The I.R. spectra were recorded on a Perkin-Elmer 225 Spectrophotometer using a slit programme of 6–7 and a scan rate of 1.5 min/micron. To illustrate that satisfactory spectra can equally well be obtained from less sophisticated instrumentation, the spectra of the isolated components were subsequently recorded on the Unicam SP200 I.R. spectrophotometer. In all cases the spectra of microdiscs were recorded using a Research and Industrial Instruments 4× KBr lens refracting beam condenser to increase sample transmission. Energy balance between the reference and sample beams of the spectrophotometers was obtained with a Research and Industrial Instruments AT02 reference beam attenuator. In the majority of cases, ordinate scale expansion was found to be unnecessary for recording good spectra of 1 μg components trapped in the manner described in this communication.

EXPERIMENTAL

Choice of split ratio

The ability of the detector to resolve and monitor the separated components emerging from the column is dependent on the design of the chromatograph and the flow rate of the effluent gas entering the flame ionisation detector. High split ratio's (*e.g.* 95:5, 99:1) give rise to a substantial reduction in gas flow to the detector and result in broadening and distortion of the peaks. This phenomenon can to a certain extent be overcome by the injection of more concentrated solutions onto the column. This is not always possible in forensic science investigations. Attempts to remove these dead volume effects of the detector by the addition of a purge gas (oxygen free

nitrogen) between the detector and the splitter were unsuccessful. Low split ratios (*e.g.* 50:50, 75:25) give the required sensitivity but reduce the proportion of component available for I.R. examination.

The minimum quantities of a mixture of five barbiturates necessary for a satisfactory separation to be monitored with different stream splitters are indicated in Table I.

It can be seen that in order to trap 1 μg of one of these barbiturates and also have the separation simultaneously monitored on a recorder, a split ratio of 90:10 is best employed.

TABLE I

MINIMUM QUANTITIES OF 5 BARBITURATES (BARBITONE, BUTOBARBITONE, PENTOBARBITONE, AMYLOBARBITONE AND SECOBARBITONE) WHICH WERE READILY RESOLVED BY THE FLAME IONISATION DETECTOR FOR DIFFERENT STREAM SPLITTERS*

Stream splitter approximate split ratio	*Minimum quantity of each barbiturate in the mixture required for a satisfactory separation to be monitored* (μg)	*Quantity of each barbiturate passing through the FID* (μg)
No splitter	0.02	0.02
90:10	0.4	0.04
95:5	1.8	0.09
99:1	50	0.50

* A Pye 10% SE30 column was used for this study.

Collection of GLC effluents

Two main methods have been described for trapping gas chromatographic effluents. These are (i) the trapping of the fraction in a tube containing normal gas chromatography column packing[12], and (ii) allowing the components to condense in glass or teflon capillary tubing or in glass trapping vessels[18,19]. Trapping efficiencies approaching 100% are obtainable by trapping on column packing but to date, the trapped component has to be subsequently concentrated, transferred and converted to a form suitable for I.R. examination. This is achieved either by subliming the component directly on to a KBr microdisc[13] or eluting the chromatographic fraction into silver chloride I.R. solution cells cooled in liquid nitrogen[14]. We found that the procedure of trapping on column packing and transferring the trapped components by these two methods was relatively unsuccessful for 1–10 μg quantities of barbiturates and alkaloids.

The trapping efficiency of a simple condensing system depends upon a number of factors. These include the temperature and geometry of the trapping vessel, the flow rate of the effluent gases emerging from the chromatograph trapping capillary, the time the sample resides in the trap and the vapour pressure of the sample under the trapping conditions employed. Some materials present the additional problem that they form aerosols as they emerge from the exhaust of the gas chromatograph, particularly when drastic cooling conditions are employed. For this reason condensing in glass or teflon capillary tubing is inefficient for many components. We obtained trapping efficiencies at the 1–10 μg level of 20–35% for barbiturates and 5–10% for

amphetamine and nicotine using this method of trapping. However, by employing a carefully aligned glass trap in the manner described in this communication with moderate cooling conditions (cold water) trapping efficiencies up to 70% for barbiturates were consistently recorded. For components with high vapour pressures such as nicotine and amphetamine, drastic cooling with liquid nitrogen or a solid CO_2–acetone mixture was found to be essential for recoveries in excess of 50%.

Micro I.R. spectrophotometry

To achieve maximum sensitivity in the I.R. examination of microgram quantities of material it is necessary to confine the component to the smallest cross sectional area. For this reason, micro KBr pellet techniques proved to be more sensitive than solution or gas cell methods with the presently available equipment[20]. Further advantages of the micro pellet technique include high optical transmission by the KBr matrix and good heat dissipation, thereby lessening the likelihood of sample deterioration. Attenuated total reflectance techniques also proved to be less sensitive than the KBr pellet methods.

The difficulty with the KBr method of sample presentation is the transfer of minute amounts of material to a small quantity of KBr with a minimum of manipulative losses. Grinding techniques on such small samples proved to be most unsatisfactory. Addition of the solution containing the component to KBr in a mortar and lightly grinding after the solvent has evaporated resulted in partition between the mortar and KBr and generally introduces impurities that far exceed the small amount of sample present. Similarly, although the addition of the solution to finely ground powder in the die was simpler, the sample loss was very great. The lyophilization technique[21,22] gave essentially quantitative sample transfer but was difficult with small amounts of KBr, proved time consuming and because of large contributions by the lyophilization blank made identification of the spectra difficult. The syringe technique described in this communication resulted in substantially quantitative recovery of high boiling components. Incorporation of components with high vapour pressures in KBr by all the methods described resulted in a very substantial loss of sample. We found that for amphetamine or nicotine, an injection of 10 μg of these compounds on the column was necessary to obtain acceptable I.R. spectra. An alternative procedure for positively identifying these amines is to convert the bases into simple less volatile derivatives (*i.e.* Schiff's bases for primary amines) either before injection or on the gas chromatographic column itself, when 1 μg will suffice if the Hamilton syringe technique is used.

METHOD OF TRAPPING GAS CHROMATOGRAPHIC COMPONENTS FOR I.R. EXAMINATION

The gas chromatograph is adjusted to give maximum recorder response for an injection of 1μg of a material similar to the components under investigation. For the Pye 104/84 gas chromatograph the optimum operating conditions for the identification of barbiturates were:

(a) 5 ft. × ¼ in. O.D. glass column packed with 10% SE30 on silanized Chromosorb W, 100/120 mesh, maintained at 175°.
(b) Carrier gas — oxygen free nitrogen at 50 ml/min.
(c) Hydrogen flow rate to the detector was 5 ml/min (adjusted to give maximum

recorder response on injection of 1 μg of barbitone). Air flow was approximately 120 ml/min.

(d) The injection and detector temperatures exceeded the oven temperature by 20°.

For trapping gas chromatographic fractions, the variac is adjusted to give a heated line temperature approximating to that of the chromatographic oven. All chromatographic columns employed must be well aged before use to minimise column bleed.

In the system described, the sample, in this case a mixture of four barbiturates in ethanol, is injected into the gas chromatograph in the orthodox manner and the components of the mixture separated on the column. As each component emerges it is simultaneously recorded and passed along the heated line via the flow splitter to the heated needle and condensed on a glass surface.

The trapping cell is connected to the heated effluent outlet by piercing the serum cap with the heated needle. For high trapping efficiencies it is important to align the trap so that the point of the heated needle is very close to and at an angle of 30–45° to the sides of the trapping vessel (Fig. 1). On emergence of the component from the column, the trap is immediately immersed in a suitable coolant while the peak is being eluted. When the recorder has indicated that the component of interest has been completely eluted from the column, the trap is quickly removed and a second assembly attached to the needle to trap the next peak as required. This removal and replacement of glass traps takes less than 30 sec.

To obtain an I.R. spectrum of the isolated component, the condensate on the sides of the trap is immediately dissolved in 25 μl of chloroform; care being taken to completely wash the internal walls of the trap with the solvent. This solution is then removed from the trap with a 25 μl Hamilton syringe equipped with a multiple repeating dispenser and 0.5 μl of solution "dispensed" to the tip of the needle. A small quantity (approximately 0.5 mg) of KBr can then be taken up by dipping the needle into finely ground KBr powder. On removal of the needle a further 0.5 μl of solution is dispensed to the KBr residing at the needle point and the chloroform allowed to evaporate under a table lamp (Fig. 2). The procedure of releasing 0.5 μl volumes of the solution from the syringe to the KBr powder and allowing the solvent to evaporate is continued until the Hamilton syringe is empty and the chromatographic component completely transferred to the 0.5 mg of KBr at the point of the needle. The dry powder obtained is transferred to a Perkin-Elmer KBr Ultra Micro Die and a 0.5 mm disc pressed by applying vacuum ($\leq$ 10 mm Hg) for a few minutes. The spectrum is recorded using a beam condenser and a reference beam attenuator. To correct for impurities present in the KBr matrix and possible effects of column bleed, the procedure is repeated without sample injection on the gas chromatographic column and a blank KBr disc prepared.

RESULTS AND DISCUSSION

The majority of GLC–I.R. systems of microanalysis have many disadvantages. Poor trapping efficiencies and additional manipulative losses give rise to low recoveries and necessitate quantities well in excess of 1 μg for identification. A further disadvantage frequently results from the introduction of extraneous materials in the

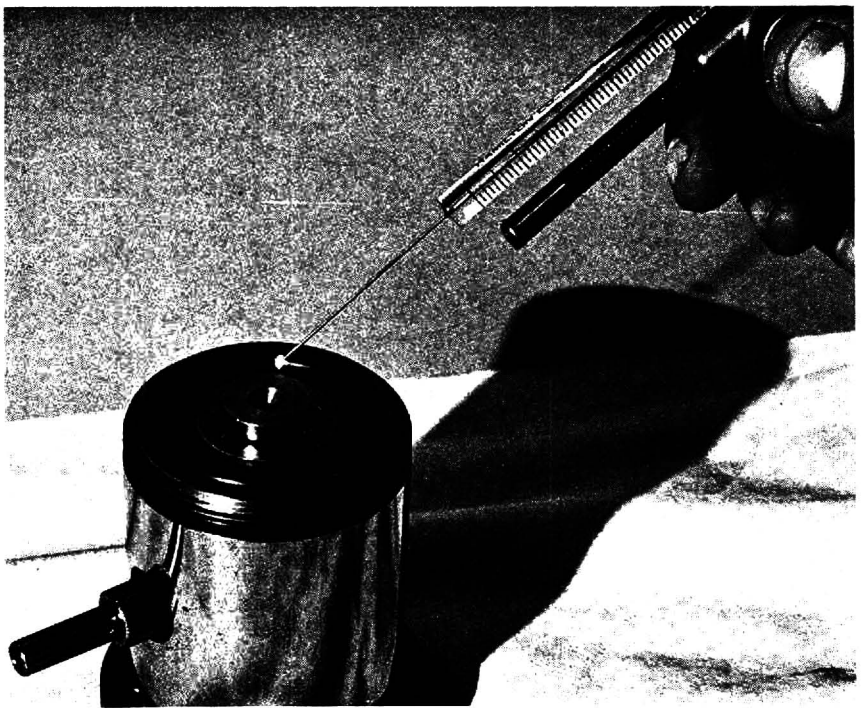


Fig. 2. Syringe technique for transferring microgram components to KBr powder.

analytical procedure and gives rise to large blanks which may obscure the sample spectrum and make identification difficult.

The GLC–I.R. method of microanalysis described in this paper yields good quality spectra for 1 μg of high boiling components. For liquid compounds with high vapour pressures such as nicotine and amphetamine, quantities of up to 10 μg are necessary to produce satisfactory spectra. Provided well aged gas chromatography columns with substrates of low vapour pressure such as SE30 are used, small blanks are obtained (Fig. 4) and unequivocal identification of gas chromatographic fractions

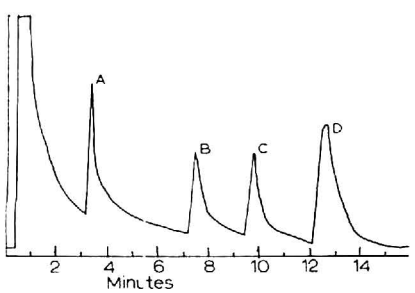


Fig. 3. Gas chromatogram obtained for the micro-analysis of 1 μl of an ethanol solution containing (A) barbitone (1 μg), (B) amylobarbitone (1 μg), (C) quinalbarbitone (1 μg) and (D) hexobarbitone (1 μg).

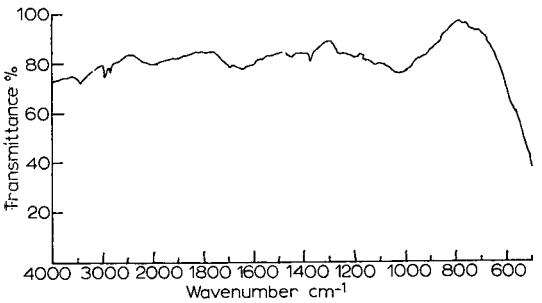
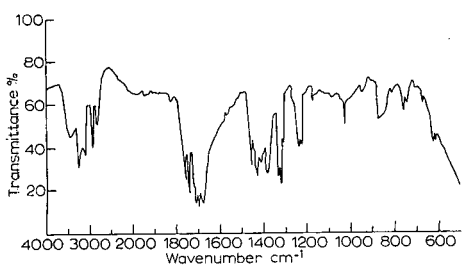


Fig. 4. Typical I.R. blank obtained using a 10% SE30 column operating at 175°.



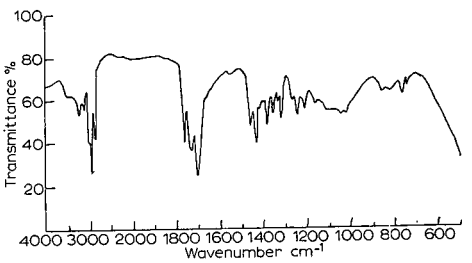


Fig. 5. I.R. spectrum of barbitone recovered from the gas chromatographic effluent of peak A.

Fig. 6. I.R. spectrum of amylobarbitone recovered from peak B.

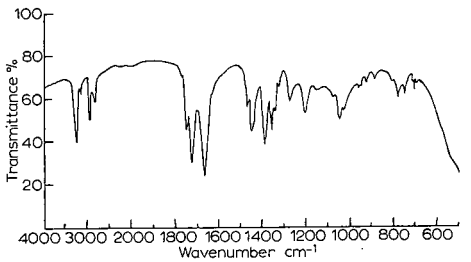


Fig. 7. I.R. spectrum of quinalbarbitone recovered from peak C.

Fig. 8. I.R. spectrum of hexobarbitone recovered from peak D.

is possible. The use of this GLC–I.R. procedure may be best illustrated by the analysis of a synthetic mixture of barbiturates. Samples of the peaks of the chromatogram (Fig. 3) were collected using the procedure described and the spectra recorded. These spectra are indicated in Figs. 5–8. Using this technique we have found no evidence of cross contamination between peaks in close proximity to each other. However, the use of interrupted elution chromatography[23] would be advantageous for trapping peaks following closely on each other.

Each of the spectra indicated in Figs. 5–8 represent 0.5–0.6 μg of barbiturate

in the sample beam of the spectrophotometer; full scale spectra being obtained for less than 1 μg of these compounds in the KBr microdisc. The use of ordinate scale expansion would enable the sample in the KBr microdisc to be further reduced probably to about 0.1 μg. The utilisation of computer addition techniques could further extend the detection limit to approximately 0.01 μg[24]. However, with these further increases in sensitivity an appreciable KBr impurity problem arises.

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LIQUID ADSORPTION CHROMATOGRAPHY IN COLUMNS AND ON THIN LAYERS

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(Received July 17th, 1968)

SUMMARY

The work described in this paper provides basic practical detail for the construction of liquid chromatographic systems superior in performance to existing column and thin-layer techniques.

Practical detail, both of components and materials, is given and the performance of high efficiency columns compared with existing techniques. Whilst the new column systems are shown to be capable of much better performance, nevertheless because of its simplicity and cheapness thin-layer chromatography will remain the method of choice for multi-sample routine analysis not requiring the highest resolving power.

INTRODUCTION

Liquid chromatography in columns (LCC) has been applied to the analysis of petroleum products for over 70 years. One of the first to make use of it was DAVID DAY who, in 1897 said, "I believe that we will be able to obtain complete resolutions... Imagine how valuable it would be to characterise a lubricant oil by the correct percentages of the component hydrocarbons instead of densities, inflammation points and viscosities"[1]. This prediction of complete resolution remains unfulfilled for the high-boiling fractions of petroleum although gas chromatography (GC) has been used to characterise the more volatile fractions by complete resolution of the components present. The analysis of the high-boiling petroleum fractions and of additive concentrates and finished lubricants, new and used, their decomposition products, and related materials remains a major problem.

Thin-layer chromatography (TLC) has proved of greatest value in separating the high-boiling mixtures not amenable to GC. TLC has been shown to be capable of resolving mixtures of great complexity with speed and convenience. Used on a preparative scale, chromatography on layers (PLC) has been used to prepare pure samples of components of complex blends for further examination by spectroscopic and related methods.

LCC has been used in these laboratories to separate larger samples (1–10 g) where more material was required or where PLC failed to separate a trace component in sufficient quantity. As used in the immediate past, however, LCC has been a crude

and inefficient process. The classical technology has changed very little over 50 years of accumulated experience. A number of guidelines have emerged for the choice of column configuration, desirable sample size, adsorbent properties and solvent elution strengths.

It has recently been realised that LCC is capable of a performance comparable to that routinely expected of GC and that the tedium associated with LCC because of the slow flow rates of solvents is not a necessary limitation of the process. Theoretical[2,3] and practical studies[4–6] have begun to appear in the literature and suitable instrumentation for the re-creation of LCC as a major separations tool has been invented and described[7–9]. The studies so far made have indicated the criteria for increased speed of analysis, optimum separation efficiency and the more important problems and weaknesses that remain.

In order to take advantage of this work it has been necessary to assemble a suitable apparatus capable of meeting the requirements of the revised approach to LCC. It has also been necessary to supplement, and in many instances anticipate, published work of a fundamental nature in order to establish the best conditions for column performance. The interim conclusions we have reached on the criteria for high efficiency LCC have also been subjected to a limited comparison with the established techniques of TLC and PLC. An attempt has been made to indicate the future role of LC in columns and on layers and the strengths and weaknesses of the methods have been critically examined.

EXPERIMENTAL

Equipment and the preparation of stationary phase

Columns. Conventional LCC requires a column, usually of glass, with average dimensions of 1–3 cm i.d. and 30–100 cm length, fitted with a stop-cock and packed with a suitable adsorbent, *e.g.* alumina or silica gel. Experience has shown that narrow-bore columns give better separations than wide ones. Studies in GC have established the same principle. Columns were constructed for preliminary experiments from medium wall glass tubing (1.5–2.0 mm wall) with 2, 4 and 6.5 mm i.d. Contrary to one report in the literature[10] that glass columns can only be used to ~ 10 p.s.i.g. we found these able to withstand solvent pressures up to 300 p.s.i.g. For more advanced work at higher pressures stainless steel columns were used. These were 0.25 in. o.d., 0.18 in. i.d. (4.65 mm). It was found inconvenient to pack columns in lengths greater than 1 m. Columns were therefore made up in 75 cm lengths when made of glass and 1 m lengths when of stainless steel.

Column connections. In order to make up columns in lengths greater than 1 m it was necessary to connect a number of metre lengths in series. Remixing of solutes in connections between column lengths is to be avoided and connectors having a very low volume were constructed from 0.020 in. i.d. × 1/16 in. o.d. stainless steel tubing and ¼ in. × 1/16 in. "Swagelok" couplings specially constructed to minimise dead volume. The adsorbent packing was retained in the column by means of a disc of 0.4 μ pore size "Gelman" filter pressed into the Swagelok coupling. Extra-column connections (*e.g.* to the detector or fraction collector) were made with 0.020 in. i.d. × 1/16 in. o.d. tubing of minimum length.

Introduction of solute. Recently, reports of solute introduction into LC columns

by means of valves[6] and injection devices[4] have appeared. The primary objective of this investigation was a small-scale efficient separation of a variety of petroleum products. Sample sizes not greater than 100 mg (100 μl) were envisaged. Solvent switching valves capable of handling samples smaller than 100 μl are not readily available and the further requirement of operational efficiency at pressures up to 2000 p.s.i.g. was an additional discouragement. It was decided to attempt the construction of an injection device. Experiments with coloured solutes injected into glass columns showed that disturbance of the upper surface of the column packing during solute injection produced very broad bands of irregular distribution across the column cross-section. SCOTT *et al.*[4] have described an injection port giving greatly reduced band widths on injection. This design incorporates a needle guide which ensures axial injection into 8–10 cm of ballotini glass beads (150–170 mesh) at the top of the column. The combined effect of the axial injection and glass beads is to give even distribution of solute across the cross-section of the column, at least for samples of small enough volume (< 100 μl).

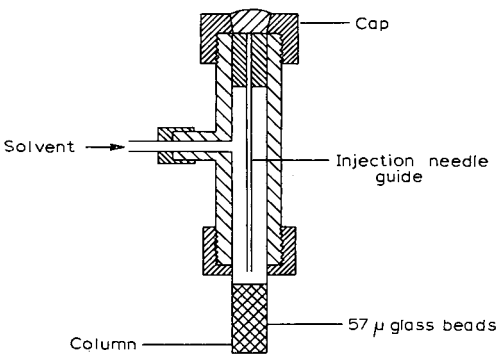


Fig. 1. Injection port constructed from "Swagelok Tee" based on SCOTT's design.

An injection port based on design by SCOTT *et al.* was constructed from a "Swagelok Tee", as illustrated in Fig. 1. With a 4 mm i.d. column, packed with silica gel and with 7 cm of glass beads (60 μ, Perkin-Elmer Co. Ltd., Beaconsfield) injection through this port gave band widths of 0.2–0.5 cm. These were measured with a coloured solute (azulene) at the top of the silica gel. It was observed that the solute band was sharpened up on passing from the beads to the silica gel giving a further improvement in band width at this point. Injection was made by removing the metal cap from the injection port with the solvent pump switched off. The cap was replaced while care was taken to exclude air and the solvent pump restarted. Silicone caps were found to contaminate the solvents with silicone oil, so that injection through a silicone septum, as in GC, was not possible. It was also observed by SCOTT *et al.* that the septa normally used in GC are extruded through even 1 mm orifices at high pressure. This was confirmed in our work.

Solvent pump. Solvents for LCC using the conventional, inefficient systems in common use are either allowed to percolate the column under gravity or are placed under slight (0–10 p.s.i.g.) pressure of nitrogen. With coarse particles of adsorbent (*e.g.* 100–200 mesh) and wide-bore columns this gives satisfactory flow judged by convenience and simplicity.

With the introduction of adsorbents of optimum particle diameter (see below) in tightly packed beds and narrow-bore columns a solvent pump has become necessary. Pumps based on displacement from a solvent reservoir have been widely used[7]. When gas pressure is used to displace solvent into the column a diaphragm or interfacial liquid is necessary to prevent the solvent becoming saturated with the gas which appears as bubbles in the column and ruins its efficiency. More satisfactory is a pump of the plunger type. This suffers from the disadvantage of giving rise to pulsating flow but this can be fairly effectively smoothed out[5]. For preliminary experiments, a DCL Micro-Pump (F. A. Hughes Ltd., Epsom) with a stainless steel plunger head assembly was found to be fairly satisfactory at pressures up to 200 p.s.i.g. Random pressure fluctuations were observed, probably due to poor seating of the non-return valves. This pump is capable of delivering a nominal flow of up to 12 ml/min, continuously adjustable from zero, and was useful for determining the effect of solvent velocity on column performance. For more advanced experiments and subsequent analytical evaluation a more sophisticated pump capable of delivering against higher pressure was required. An Orlita Diaphragm Dosing Pump Type DMP-1515 (Orlita KG, Giessen, Lahn) capable of delivering up to 10.5 ml/min against up to 4000 p.s.i.g. has been used. This pump is completely adjustable by micrometer variation of plunger stroke length over the range from maximum to zero flow. After the replacement of faulty ball valves this pump has given excellent performance.

Pressure protection switch. In the event of a blockage or restriction upstream of the Orlita pump a catastrophic rise in solvent pressure between the blockage and pump could occur. To prevent this happening a pressure switch (K.D.G. Instruments Ltd., Crawley) has been incorporated in the solvent flow line immediately after the pump. This switch is adjustable over the range 0–3000 p.s.i.g. and is set to trip the power supply to the pump in the event of a pressure rise in excess of safe operation.

Pressure gauge. Although the actual solvent pressure is of minimal interest in LCC, the solvent velocity being the important related parameter, it is nevertheless necessary to have an indication of the pressure for safe operation. For low pressures up to 300 p.s.i.g. a 0–300 p.s.i.g. Budenberg (Altrincham, Cheshire) direct reading gauge with 4-in. dial was used. For high pressures up to 3000 p.s.i.g. an Ashcroft-Durrance 0–6000 p.s.i.g. direct reading gauge (Dresser Europe S.A., London) with a 6-in. dial was fitted. For work at intermediate pressures an Ashcroft-Durrance 0–1000 p.s.i.g. direct reading gauge with 6 in. dial was more accurate.

Preparation of stationary phase. Silica gel has been firmly established as the most important general purpose adsorbent for LCC of petroleum products. The separations made by TLC in these laboratories are made almost exclusively on silica gel. One of the major objectives of this work was to compare separation of petroleum products by TLC and LCC and silica gel was selected as most suitable for this purpose. The principles established are without doubt applicable to other adsorbents and to liquid–liquid chromatography (LLC).

Experience in GC has shown the importance of narrow-range particle sizes in making columns of high performance. The existence of an optimum mean particle size for a given column length etc. has also been demonstrated in theory and in practice. Theoretical work[11] has shown that for LLC the smallest possible mean particle diameter, ideally zero, would give most rapid equilibrium between stationary and moving phases and maximum efficiency. The practical limitations are in preparation

of the material and in the increasing flow resistance of beds of fine particles. HUBER[12] finds particles of less than 25 μ difficult to pack and GIDDINGS[13] has discussed the effect of formation of aggregates of fine particles into macro particles in the packed bed.

For this study silica gel was prepared in narrow ranges from 28–36 μ up to 100–125 μ.

To prepare this material it was necessary to use a wet sieving technique. (Elutriation was also considered and might be used with advantage.) The Fritsch Pulverisette 3 (A. Fritsch OHG, Idar Oberstein) sieve shaker fitted with wet-sieving head was used. The charge of 100 g wide particle range material was first sieved on the finest mesh (28 μ) to remove fines and the retained material (*ca.* 50 g) was re-sieved on a nest of sieves to give the required size ranges. Table I gives the yield of various sizes obtained from silica gel for TLC (E. Merck A.G., Darmstadt). For preparation of silica gel from other sources preliminary grinding was required. This was conveniently carried out with the Pulverisette 2 Automatic Mortar Grinder (A. Fritsch OHG). The yield of a given size range was necessarily very low and the process of grinding and sieving is very wasteful of material. Table II gives typical recovery from grinding of "Davison 923" silica gel (74–149 μ). The final preparation of the silica gel included drying at 160° and deactivation with water, usually 4 % w/w to increase the linear capacity, according to SNYDER[14].

TABLE I

YIELDS OF SIZE GRADED MATERIAL FROM SILICA GEL FOR TLC (E. Merck A.G., Darmstadt)

Size range (μ)	*Weight* (%)
< 28	62.0
28–36	9.2
36–44	13.2
44–56	14.8
> 56	nil

TABLE II

YIELDS OF SIZE GRADED MATERIAL FROM GRINDING "DAVISON 923" SILICA GEL (74–149 μ) FOR 1 MIN

Size range (μ)	*Weight* (%)
< 28	34.3
28– 36	4.3
36– 63	9.0
63– 80	12.3
80–100	19.0
100–125	11.6
> 125	9.3

Solute detection. Three methods of solute detection were used in the course of the present work. For the first experiments a Barber-Colman (Rockford, Ill.) flame-ionisation detector, with a chain transport mechanism for eluate from the column, through a solvent evaporator to the ionisation detector, was used.

This detector, as received, could not be used at a sensitivity higher than a nominal 10^{-9} A FSD ($\times$ 1K). Much of the background noise was traced to the two "Teflon" pulleys used to support the transport chain. These were either contaminating the chain or causing production of static electricity. As one pulley was stationary and the chain slid around it, static might well have been produced. These "Teflon" pulleys were replaced with pulleys of aluminium and both were made to revolve. The noise level was reduced approximately 50-fold and a stable base line was obtained at a sensitivity of 10^{-10} A FSD in the absence of solvent. In the presence of solvent residual noise was traced to particles of silica gel adhering to the chain. It has not proved

possible to achieve a noise-free performance from the detector and it has been used most effectively for detection of milligram amounts of non-volatile solutes. A detector similar in principle to the Barber Colman based on a design by SCOTT[15] and marketed by W. G. Pye, (Cambridge) has been evaluated. This detector exhibited a stable sensitivity one hundred-fold better than the Barber Colman and would be more suitable for LCC.

In view of the difficulties with the Barber-Colman instrument column performance was roughly evaluated with coloured solutes and direct measurement of the solute band width. For accurate measurement of column efficiency fractions were collected and the concentration of solute determined by U.V. absorption at 294 nm using a Unicam SP 600 and 0.5 mm cells. The concentration in absorbance units was plotted against solvent volume and the efficiency calculated in the usual way.

RESULTS

Criteria for an efficient LC column–preliminary studies

Packing technique. The packing of the column in LCC has received little attention. The usual procedure is to fill the column with dry adsorbent whilst tapping the side of the column to induce local vibration. After this consolidation the dry bed is wetted with solvent. The retention of air between and within the particles leads to channelling and an improved technique is often used in which the adsorbent is added to the column in the form of a slurry in the solvent. Trapped air is excluded and the settling out of the adsorbent from the slurry leads to a more regular packing. However, in columns < 5 mm i.d. settling of slurries of the very fine particles used in this work is impracticably slow. HOWARD AND MARTIN[16] introduced a tamping device which consists of a rod with a perforated disc at the end. The disc just fits into the internal diameter of the column and increments of packing can be tamped down while solvent flows up through the holes in the disc. A more recent study of packing methods, including loose filling, tamping, tapping and wet filling, concludes that tapping and bouncing is most convenient and gives the best results with particles of 76 μ average diameter[6].

For the initial experiment in the current study glass columns were packed by tapping. Adsorbent was added in small increments. The efficiency was roughly estimated by direct observation of the broadening of a band of coloured solute progressing through the column. Tapping and bouncing the column during packing was found to be ineffective for the finer adsorbents, *e.g.* 28–36 μ. Experiments with a combination of tapping and bouncing and gentle tamping with a close fitting rod were much more promising and a 3-fold increase in efficiency for 28–36 μ particles resulted. The most effective packing method was found to be incremental addition of dry adsorbent to fill about 10 cm column length, gentle tapping and periodic bouncing and tamping by allowing the weight of a glass or metal rod to rest on the top of the packing while tapping continued.

Column diameter. Columns of internal diameter close to 4 mm were found to pack best. Columns of 6.5 mm I.D. gave about 4-fold increase in band width compared with a similar length of 4 mm I.D. column. Columns 2 mm I.D. gave band widths similar to the 4 mm columns but were much more difficult to pack. Unless the increments of adsorbent in packing were very small (< 1.5 cm) the packing tended to break

up into zones with spaces between. For other studies of the effect of column diameter on efficiency see refs. 4–6.

Sample size. With the SCOTT design of injection port described earlier, experiments with coloured solutes showed that samples up to 100 μl were evenly distributed, provided the sample viscosity was not too high. For higher viscosity samples, *e.g.* lubricants, a preliminary dilution with solvent was necessary. Dilution with an equal volume of solvent was adequate for most samples of this class. An increase in sample band width was observed with increase in sample size from 2 μl to 100 μl of from *ca.* 0.2 cm to *ca.* 1 cm. VAN DEEMTER *et al.*[17] have studied the contribution of sample volume to peak variance in GC and the injection band widths in the current LC study do not make an excessive contribution to the final band variance. A study of the effect of solute/adsorbent weight ratio on efficiency[6] concludes that 0.5–1 mg/g should not be exceeded. In general, samples which exceed 100 μl in volume and 2 mg/g solute/adsorbent weight ratio are likely to nullify the benefits of a well-packed column.

Particle size. Studies with coloured solutes showed that the advantage predicted by theory for reduction in mean particle diameters in LC were significant down to about 40 μ. Particles in the range 28–36 μ gave slightly less efficient columns than those of 44–56 μ, and an increase in particle diameter beyond 56 μ also resulted in some loss of efficiency. For the most exacting work requiring the highest efficiency particles in the 44–56 μ range are about optimum. For many applications the conclusion[6] that 74–125 μ (120–200 mesh) may represent a good compromise is quite valid.

Criteria for an efficient LC column–final evaluation

When the above preliminary studies were completed a series of experiments was conducted designed to lay down operational requirements for LC at the highest practically achievable efficiency likely to be useful in petroleum product separations. For this work stainless steel columns were used, 4.65 mm I.D. and 1 m length. The Orlita pump, 0–6000 p.s.i. range pressure gauge, pressure safety switch and the SCOTT injection port were all utilised. The layout of the final apparatus is illustrated schematically in Fig. 2. Fractions were collected and solute concentration determined by U.V. absorption measurement and efficiencies were calculated in the usual way.

The beneficial effect of tamping the packing revealed by the preliminary work

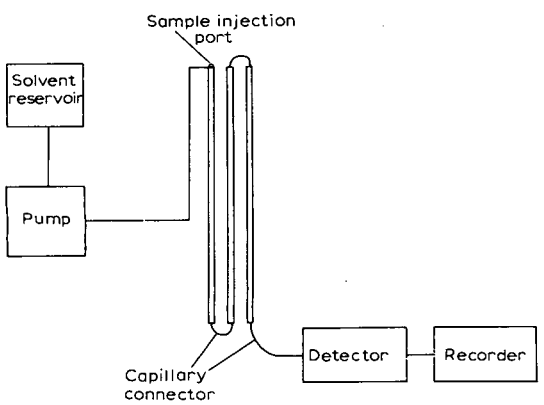


Fig. 2. Layout of liquid chromatograph.

TABLE III

VARIATION IN EFFICIENCY/VELOCITY RELATIONSHIP WITH PARTICLE DIAMETER AND PACKING TECHNIQUE

Column: 1 m; solvent: *n*-heptane; solute: azulene; adsorbent: silica gel (4 % w/w water). Sample: 10 μl 5 % w/w solution.

Particle size (μ)	*Packing technique*	*Solvent velocity* (*cm/sec*)	*Solvent pressure* (*p.s.i.g.*)	*HETP* (*mm*)
28–36	Tapping and bouncing	0.9	630	9.8
		0.082	56	3.5
	Tapping, tamping and bouncing	1.21	700	3.3
		1.15	650	3.5
		0.83	600	3.4
		0.10	85	1.8
		0.10	75	1.8
100–125	Tapping and bouncing	0.9	85	12.5
		0.075	8	3.9
	Tapping, tamping and bouncing	0.85	85	9.3
		0.45	42	7.2
		0.16	20	4.3
		0.092	10	1.8

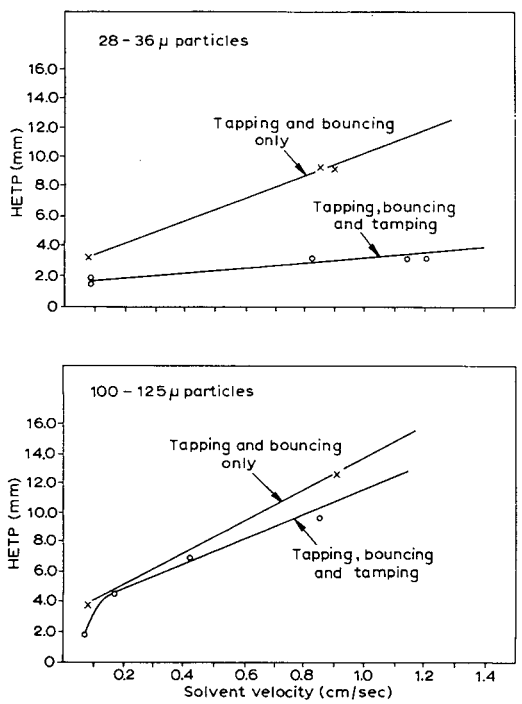


Fig. 3. Variation in efficiency/velocity relationship with particle diameter and packing technique.

was confirmed quantitatively. Efficiency determinations for 28–36 μ silica gel and 100–125 μ material (the extremes of the ranges previously evaluated) were made and the results are given in Table III and graphically in Fig. 3. The increase in efficiency with the 28–36 μ is more marked but some effect persists with the 100–125 μ range. The efficiencies obtained with the 100–125 μ range material were poor even when the tamping technique was used.

The optimum range of particle size was confirmed to be about 44–56 μ. Table IV lists the efficiencies obtained with 28–36 μ, 44–56 μ and 100–125 μ particles at a range of solvent velocities. The comparison is presented graphically in Fig. 4. More detailed study of ranges between 56 μ and 125 μ might be worth while. For a limited study of material in this range see ref. 6.

TABLE IV

EFFICIENCY/SOLVENT VELOCITY RELATIONSHIP WITH DIFFERENT PARTICLE DIAMETER RANGES

Columns: 1 m; solvent: *n*-heptane; solute: azulene; packing: tamping, tapping and bouncing; sample: 10 μl 5% w/w solution.

Particle diameter 28–36 μ		*Particle diameter 44–56 μ*		*Particle diameter 100–125 μ*	
Solvent velocity (cm/sec)	*HETP (mm)*	*Solvent velocity (cm/sec)*	*HETP (mm)*	*Solvent velocity (cm/sec)*	*HETP (mm) %*
1.21	3.3	—	—	—	—
1.15	3.5	—	—	—	—
0.83	3.4	0.86	2.4	0.85	9.3
—	—	0.44	1.7	0.45	7.2
—	—	0.22	1.5	0.16	4.3
0.10	1.8	—	—	0.092	1.8

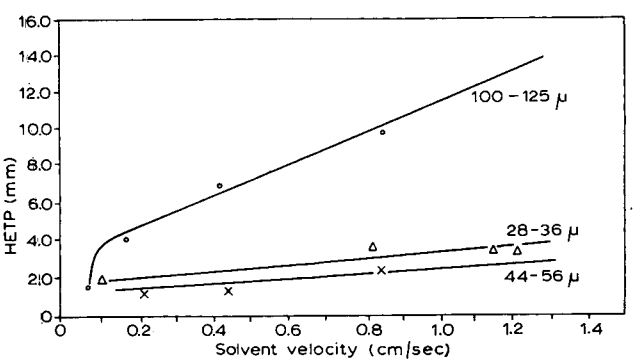


Fig. 4. Efficiency/solvent velocity relationship with different particle diameters.

A final experiment on column efficiency was performed with 44–56 μ particle size silica gel and *n*-pentane eluent. This allows a comparison with a similar column prepared by SNYDER[6]. Detailed experimental conditions are compared in Table V. Results of efficiency measurements at various solvent velocities are listed in Table VI and graphical comparison is made in Fig. 5. The combination of improved apparatus and packing technique has resulted in a 2-fold improvement in efficiency by comparison

TABLE V

EXPERIMENTAL CONDITIONS FOR FINAL EVALUATION OF COLUMN EFFICIENCY AND COMPARISON WITH SNYDER'S RESULTS

Variable	*This work*	*Snyder's work*[6]
Column length	1 m	122 cm (4 ft.)
Column diameter	0.46 cm	0.46 cm
Sample introduction	Injection (10 μl)	Switching valves (0.3 ml)
Adsorbent	Silica gel	Silica gel
Deactivation	4 % w/w water	4 % w/w water
Size range	44–56 μ	57 $\mu \pm$ 20 %
Solvent	*n*-Pentane	*n*-Pentane
Solute	Azulene	Dibenzyl
Solute retention, $R°$	3.8 ml/g	4.4 ml/g
Packing	Tamping, tapping, bouncing	Tapping and bouncing
(HETP at solvent velocity 0.5 cm/sec)	(1.2 mm)	(2.2 mm)

TABLE VI

HETP VALUES AT VARIOUS SOLVENT VELOCITIES FOR (A) COLUMN PACKED WITH 44–56 μ SILICA GEL AND (B) SIMILAR COLUMN EVALUATED BY SNYDER[6]

44–56 μ packing		*Snyder's column*	
Solvent velocity (cm/sec)	*HETP (mm)*	*Solvent velocity (cm/sec)*	*HETP (mm)*
0.86	1.7	1.0	2.8
0.4	1.1	0.55	2.4
0.205	0.88	0.45	2.2
0.055	0.46	—	—

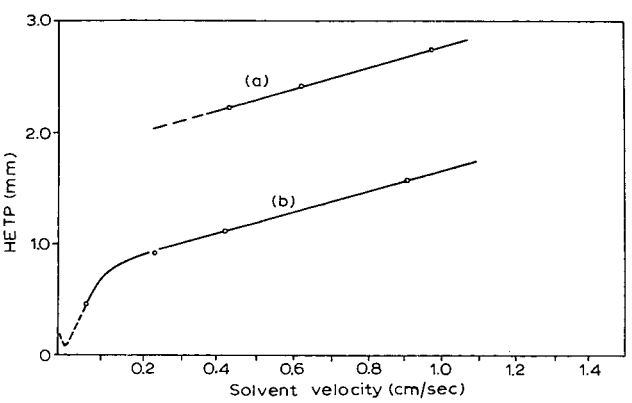


Fig. 5. Column efficiences. (a) Upper curve, published by SNYDER; (b) lower curve, 44–56 μ silica gel, 1 m column.

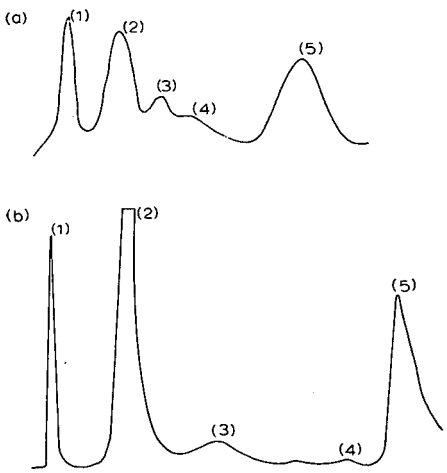
with SNYDER's results. No other values are available for further comparison but for related studies of the effects of solvent velocity and particle size on efficiency in LC see the work of HORVATH, PREISS AND LIPSKY[18] on ion-exchange resins and also refs. 4 and 5. The broken trace on the lower left of Fig. 5 shows the general shape of the

efficiency/solvent velocity curve at low values of solvent velocity. Efficiencies of $>$ 4000 plates/m are quite possible but as the rate of plate generation is only 30/min separations are very time consuming.

Comparison of LC in columns with TLC

Three separation problems, typical of petroleum product analysis, were tackled simultaneously by efficiency optimised LC and by TLC. These separations, which form a basis for evaluation of the relative merits of the techniques, were of (a) a mixture of phenol alkylation products, (b) a mixture of polynuclear hydrocarbons, and (c) a simulated lubricating oil. This last separation was made on a small-scale (mg) preparative basis. The following detailed observations were made:

Phenol alkylation product. The separation of the mixture of nonylphenols by TLC and LC as illustrated in Fig. 6a is of the densitometer trace of the TLC separation and shows peaks as follows: (1) impurities; (2, 3 and 4) dialkylphenols; and (5) monoalkylphenol. Fig. 6b is of the recorder trace of the separation of the mixture on a 1 m column with a gradient of ether in hexane as solvent. The column separation compares with that of the TLC plate in all respects. The LC separation took 30 min and the TLC separation 45 min. The resolution could be increased by using a longer column or a lower solvent velocity. Only in initial cost of equipment has TLC an advantage for separation of a single sample.



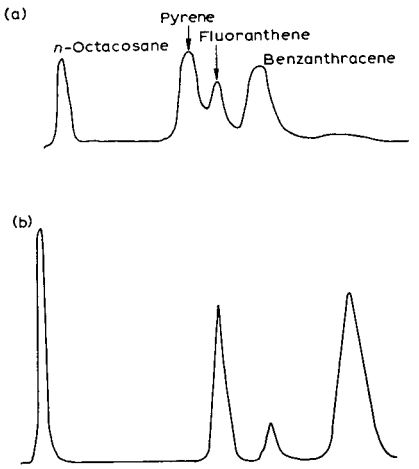


Fig. 6. Separation of alkylphenols by (a) TLC (toluene 45 min) and (b) 1 m column (hexane → ether 30 min).

Fig. 7. Separation of polynuclear hydrocarbons by (a) TLC (*n*-pentane double development 45 min), and (b) 1 m column (*n*-heptane 30 min).

Polynuclear hydrocarbons. The separation of *n*-octacosane, pyrene, fluoranthene and 1,2-benzanthrene was made by TLC in 45 min by double development of the plate with *n*-pentane. The densitometer trace of this separation is shown in Fig. 7a. The same mixture was separated by LCC in 30 min on a 1 m column with *n*-heptane as eluent (Fig. 7b). The column could separate the mixture as well as the plate in as little as 5 min and repeat analyses could be made continuously as in GC. The column length

could be increased by at least 10-fold to make much more difficult separations of polynuclear materials possible. For a further discussion see refs. 2 and 6.

Lubricating oil. The components of the simulated lubricating oil are: base oil, di-*tert.*-butylmethylphenol, *n*-octylphenol, zinc dialkyl dithiophosphate, and calcium sulphonate. The presence of calcium sulphonate in the mixture makes the separation difficult. Conventional wide-bore columns with the high sample loading usually employed are not very effective due to poor resolution and micelle formation. This type of mixture has been separated by preparative-layer chromatography (PLC) with some success. A separation by PLC was made and compared with a separation by LCC. The solvents used for step-wise elution of the components were *n*-hexane, toluene, 10 % acetic acid–hexane and 20 % aqueous ammonia (0.88)–isopropyl alcohol in that order. For the column separation a 1 m column was used with a 100 mg sample and solvent velocity of 0.5 cm/sec. Fractions were collected in combination with the Barber-Colman detector which revealed the presence of solutes in the eluant. Infrared spectra of the separated solutes were recorded and compared with the pure component to give a measure of effectiveness of separation under conditions which would apply in practical analysis. For samples of less than 2 mg the I.R. spectra were obtained either on thin-films or by multiple internal reflectance.

Recovery of solutes from the column was over 90 % with the exception of calcium sulphonate which was irreversible adsorbed. Recovery of solutes from PLC was poor and contamination with silica gel fines was a problem.

Fig. 8 shows (a) the I.R. spectrum of the sample of simulated lubricant and (b) the base mineral oil separated on the column. The absence of peaks due to additives

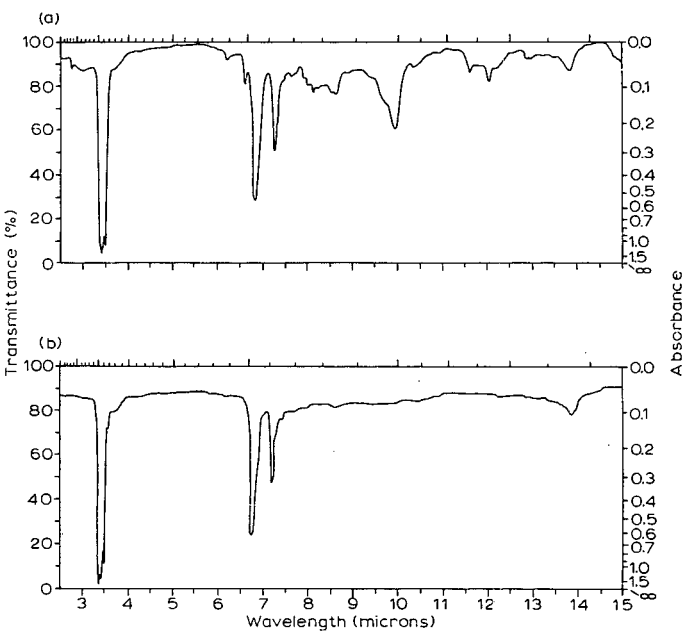


Fig. 8. Column separation of base oil from lubricant. (a) Lubricant blend; (b) base oil separated on column.

is noteworthy. The efficient column, with sample loadings up to 10 mg/g adsorbent, has avoided micelle formation and cleanly separated the additives from the oil.

Fig. 9a shows the I.R. spectrum of *n*-octylphenol, (b) the spectrum of compound separated by LCC from the lubricant, and (c) the spectrum of the component separated by PLC. A similar set of spectra were obtained for the ZDDP component.

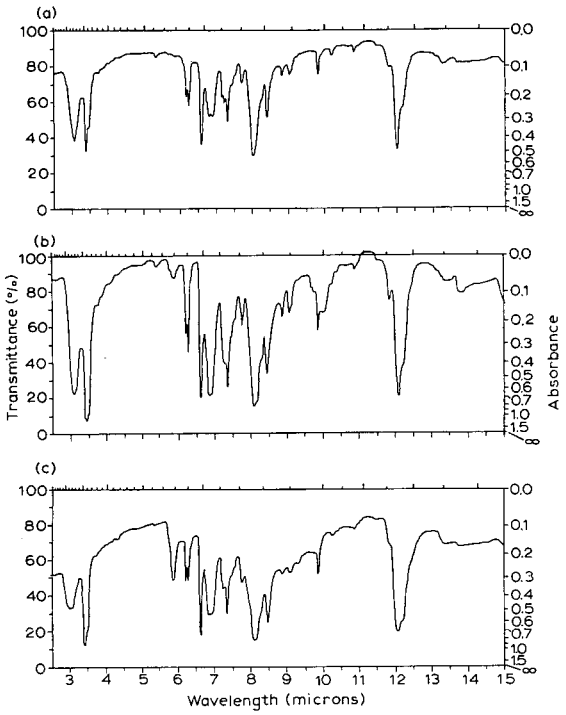


Fig. 9. Separation of *n*-octylphenol by column and TLC from lubricant blend. (a) *n*-Octylphenol; (b) separated by column; (c) separated by TLC.

The major points of comparison which emerged from these experiments were: (1) The techniques of PLC and LCC were equally effective. (2) Fraction collection was much simpler with LCC.

Fraction collection was simpler with LCC because as each solute emerged from the column it could be collected in a tared vessel, the solvent evaporated, the recovery measured and the I.R. spectra recorded with no intervening steps. With PLC even after development of the plate the steps of revealing the solute bands, scraping off solute and associated adsorbent, desorption, filtration (partially effective) and evaporation of desorbing solvent all remained to be carried out. The time spent on these exercises was much greater than that required for LCC separations. The time required to prepare a good column is not much greater than that required to prepare a plate for PLC.

Mention should be made of a technique for recovering material from TLC plates in amounts of about 50 μg. This technique described only recently[19] shows promise of eliminating the need for PLC with milligram amounts. In the new micro-

method the material on the TLC plate is revealed by complexing with iodine, a reversible process. A region of silica gel is drawn out around the spot in the shape of a "tear drop" and the solute is "chased' from this into 2–3 mg of dry potassium bromide. A disc is prepared from this and its I.R. spectrum recorded. Wherever a larger amount (mg) is required LCC would offer advantages for its separation and recovery.

DISCUSSION AND CONCLUSIONS

A number of principles for the practical application of LCC have emerged from this work. It should now be possible to choose the technique and conditions for an LC separation most appropriate to the problem. For many analytical separations TLC will not be replaced by LCC. TLC has the advantages of simplicity and cheapness. The wide range of specific revealing agents can afford important information about the presence of functional groups, elements and structural features in addition to visualising the material on the TLC plate. The ready accessibility of the whole plate avoids part of the sample being overlooked. This could happen in column work when a portion of the sample is not eluted. The possibility of making simultaneous analyses of many samples on one plate is also advantageous in TLC. Rapid screening of samples for a single component *e.g.* an additive can often be conveniently carried out.

The optimised LCC which has been described will greatly extend the range of TLC to more difficult and faster separations. Many separations which cannot be carried out by TLC could be made quite easily and rapidly on a good column. Such columns are not unduly difficult to make although care and attention to detail is essential in their preparation. The equipment required although more elaborate than the conventional crude systems hitherto used in LC is not unduly expensive or exotic. Adequate equipment was used in this work at a cost of under £ 1,500 including the Barber Colman detector, a cost comparable with a modern gas chromatograph.

The major advantage of optimised LCC lies in its flexibility. This is limited only by the practical limitations of increasing pressure requirements with increasing column length. Separations of complex mixtures of polynuclear hydrocarbons, potential carcinogens, can be envisaged and any separation currently being made could be improved by optimisation of operating conditions along the lines described. With the less complex separations involving a single non-polar solvent *e.g.* pentane or hexane a fully automated system could be planned and constructed for evaluation with no further fundamental experimentation.

For preparative separations it has been demonstrated that a good column can give good recovery and has marked advantages over PLC in convenience of solute detection and collection. The separation efficiency of both optimised LCC and PLC are markedly superior to the conventional wide-bore column; in addition optimised LCC is much faster than either PLC or conventional LCC.

Some of the references to related work which have been given, *e.g.* refs. 2 and 5, refer to studies in liquid–liquid (partition) chromatography (LLC). Some experiments have been made with LLC and will be described in a subsequent paper. LOCKE has reviewed much of the work done in LLC and made a number of contributions to the theory[20,21]. LLC has advantages of selectivity over adsorption LC and the additional advantage that columns should be reusable indefinitely. Most, if not all, the results of

the present work which has concentrated on silica gel adsorbent stationary phase, should apply to LLC with equal validity in making an efficient column system.

The judicious choice of the appropriate system, adsorption or partition, TLC or LCC, should lead to the most effective separation of most mixtures of organic and organo-metallic components with which we are faced. The next stage is the rapid accumulation of practical experience, in the application of the techniques now developed to real analytical problems.

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CHROM. 3669

PEAK BROADENING IN PAPER CHROMATOGRAPHY AND RELATED TECHNIQUES

VI. THE EFFICIENCY OF VARIOUS KINDS OF CHROMATOGRAPHY PAPER AND THIN-LAYER CELLULOSE POWDER FOR THE SEPARATION OF AMINO ACIDS

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SUMMARY

The efficiency of several chromatography papers and thin-layer cellulose powders for the separation of amino acids is investigated, using the minimum elution time for a given resolution as the criterion.

INTRODUCTION

Recently, DE LIGNY AND REMIJNSE[1] compared the efficiencies of ten kinds of Whatman paper and six kinds of thin-layer cellulose powder for the separation of amino acids by means of a 4:1:5 butanol–acetic acid–water mixture. In this study, they used the following criterion for the efficiency:

That paper or powder, which gives the best resolution under standard conditions (*i.e.*, standard positions of starting point and solvent front**) is the most effective one.

However, another quite different criterion is possible, namely:

That paper or powder, which gives some desired resolution in the shortest elution time is the most effective one.

As time plays a very important role in many separation problems this factor should not be neglected, so the second criterion will be the better one.

It is to be expected, that the order of efficiency by the first criterion will be different from the order of efficiency by the second. In general, a paper or powder having a high eluent flow velocity will get a better classification by the second criterion than by the first.

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** The distances of starting point and solvent front from the surface of the eluent in the tank were 1 and 11 cm, respectively, in thin-layer chromatography and 5–6, and 25–30 cm, respectively, in paper chromatography.

TABLE I

EFFICIENCY OF CELLULOSE POWDERS. $R = 4$[a]

Cellulose powder	R_F (1)	R_F (3)	$B \times 10^6$	C_M	k[b]	l_f	l_0	$t_{elution}$
M & N 300	0.21	0.30	3.07	1.27	0.017	13	0.9	9 600
Camag D	0.21	0.20						very long
Whatman CC41	0.13	0.20	3.56	4.67	0.029	28	3.0	28 000
S & S 144	0.22	0.32	3.01	3.24	0.037	19	3.0	9 300
S & S 142 dg	0.17	0.34	2.96	1.61	0.039	8	1.7	1 500
S & S 140 dg	0.24	0.41	2.81	3.10	0.069	15	3.5	3 400
	R_F (5)	R_F (6)						
M & N 300	0.36	0.48	2.71	0.70	0.017	8	0.8	3 600
Camag D	0.37	0.48	2.71	0.92	0.018	11	0.9	6 300
Whatman CC41	0.26	0.35	2.93	3.08	0.029	22	2.8	16 000
S & S 144	0.38	0.51	2.67	1.68	0.037	13	2.3	4 500
S & S 142 dg	0.40	0.53	2.65	0.82	0.039	9	1.7	2 300
S & S 140 dg	0.48	0.60	2.58	1.42	0.069	18	3.5	4 500
	R_F (6)	R_F (9)						
M & N 300	0.48	0.63	2.56	0.37	0.017	5	0.6	1 200
Camag D	0.48	0.64	2.53	0.44	0.018	5	0.6	1 100
Whatman CC41	0.35	0.50	2.68	1.82	0.029	10	1.9	3 600
S & S 144	0.51	0.64	2.53	0.91	0.037	11	1.8	3 200
S & S 142 dg	0.53	0.64	2.53	0.48	0.039	10	1.4	2 600
S & S 140 dg	0.60	0.72	2.50	0.70	0.069	14	2.8	3 000

[a] B in [cm^2·sec^{-1}]; B accounts for peak broadening by diffusion.
C_M in [sec]; C_M accounts for peak broadening by resistance to mass transfer in the mobile phase.
k in [cm^2·sec^{-1}]; k governs the eluent flow rate.
l_f in [cm]; l_f is the distance from the surface of the eluent in the tank to the solvent front.
l_0 in [cm]; l_0 is the distance from the surface of the eluent in the tank to the starting point.
$t_{elution}$ in [sec].
[b] Upward flow.

In the following, the efficiencies of the same six powders and ten papers are compared once more, now observing the second criterion.

For this purpose we can use the procedure described earlier[2] for calculating the minimum separation time for some desired resolution.

In this method the R_F values of the compounds to be separated, and data on peak broadening and eluent flow rate for the paper or thin-layer cellulose powder to be used are required. The calculations yield, for some specified value of the peak resolution*, the minimum elution time, the corresponding optimum position of the starting point, and the necessary distance of travel of the eluent.

Following this procedure the minimum separation times for the six powders and ten papers were calculated for the following combinations of amino acids**: L-α,γ-diaminobutyric acid (1) and L-aspartic acid (3); L-threonine (5) and L-α-aminobutyric acid (6); L-α-aminobutyric acid (6) and L-norvaline (9).

The peak resolution R was taken to be 4 for thin-layer chromatography and 3 for paper chromatography.

* The peak resolution R is defined as follows: $R = (l_A - l_B)/(\sigma_A + \sigma_B)$; where l = distance, travelled by the solute; σ = standard deviation of the solute distribution; A and B = faster and slower moving solute, respectively.

** For numbering of amino acids see ref. 4.

The R_F values were taken from the work of DE LIGNY AND REMIJNSE[3,4], the other data from DE LIGNY AND KOK[2]. Data for the papers W3 and W7 were determined by the present authors.

RESULTS

The results of the calculations are summarized in Tables I and II.

TABLE II

EFFICIENCY OF CHROMATOGRAPHY PAPERS. $R = 3^*$

Chromatography paper	R_F (1)	R_F (3)	$B \times 10^6$	C_M	k^{**}	l_f	l_0	$t_{elution}$
W1	0.09	0.14	5.60	15.5	0.032	67	3.6	142 000
W2	0.08	0.14	5.48	15.5	0.028	45	3.5	71 800
W3	0.14	0.22	2.38	12.8	0.031	32	4.2	32 200
W3 MM	0.10	0.17	4.12	14.4	0.040	38	5.1	36 700
W4	0.13	0.21	3.37	13.1	0.060	39	6.7	25 400
W7	0.19	0.26	3.90	11.5	0.036	49	4.4	65 800
W17	0.15	0.25	3.70	11.8	0.069	33	6.4	15 900
W20	0.07	0.13	4.83	15.9	0.016	43	1.9	116 000
W31 ET	0.20	0.33	3.17	9.4	0.106	30	6.6	8 700
W54	0.11	0.20	3.71	13.4	0.071	35	6.7	17 300
	R_F (5)	R_F (6)						
W1	0.20	0.33	4.40	9.4	0.032	20	3.0	12 600
W2	0.20	0.31	4.34	9.8	0.028	24	2.9	21 000
W3	0.21	0.33	1.34	9.4	0.031	21	3.3	13 900
W3 MM	0.23	0.35	3.24	8.9	0.040	24	3.7	14 200
W4	0.27	0.43	2.66	6.8	0.060	19	3.7	6 100
W7	0.27	0.41	3.49	7.3	0.036	19	2.9	10 400
W17	0.31	0.45	3.18	6.3	0.069	24	4.3	8 300
W20	0.18	0.31	3.53	9.8	0.016	16	1.8	16 000
W31 ET	0.39	0.51	2.86	5.0	0.106	34	5.9	10 600
W54	0.25	0.35	2.75	8.9	0.071	36	6.3	18 600
	R_F (6)	R_F (9)						
W1	0.33	0.49	4.11	5.5	0.032	16	2.1	8 000
W2	0.31	0.49	3.99	5.5	0.028	13	1.9	6 000
W3	0.33	0.48	1.67	5.7	0.031	16	2.5	8 500
W3 MM	0.35	0.50	2.99	5.2	0.040	18	2.7	8 200
W4	0.43	0.54	2.52	4.4	0.060	27	3.8	12 500
W7	0.41	0.54	3.32	4.4	0.036	22	2.4	13 400
W17	0.45	0.54	3.07	4.4	0.069	45	4.7	28 700
W20	0.31	0.45	3.24	6.3	0.016	16	1.4	16 700
W31 ET	0.51	0.64	2.74	2.7	0.106	28	4.2	7 200
W54	0.35	0.51	2.49	5.0	0.071	20	3.8	5 600

* For explanation of symbols see footnote to Table I.
** Downward flow.

DISCUSSION

It follows from Tables I and II that the elution times in paper chromatography are as a rule much longer than in thin-layer chromatography, even in spite of the fact that the resolution in paper chromatography is taken to be less complete than in thin-layer chromatography.

The efficiency varies appreciably with the pair of amino acids to be separated.

The orders of efficiency of the various powders and papers are given in Tables III and IV.

By summing the order numbers for the three pairs of amino acids we obtain a rough assessment of the efficiency of the various powders and papers. The order of efficiency in thin-layer chromatography is:

S & S 142 dg > M & N 300 > S & S 140 dg > S & S 144 ≈ Camag D > Whatman CC41;

and in paper chromatography:

W31 ET > W4 > W54 > W17 > W3 > W3 MM ≈ W7 > W1 > W2 > W20.

As expected, the orders of efficiency are different from those, found previously under standard conditions (namely: Camag D ≈ M & N 300 > Whatman CC41 and W3 > W7 ≈ W20 > W2). The main cause for this discrepancy is that in the present comparison of the efficiencies the flow rate of the eluent plays a large role, whereas it did not enter into the previous comparison.

In the case of paper chromatography the optimum values of l_0 generally do not differ much from the values used in practice (≈ 5 cm). If they do, the optimum values are so small that it is difficult to realize them experimentally.

In thin-layer chromatography, however, the optimum values of l_0 are often appreciably larger than the usual one (≈ 1 cm).

TABLE III

ORDER OF EFFICIENCY OF CELLULOSE POWDERS

Cellulose powder	*Order of efficiency*			*Sum of orders*
	1 + 3	*5 + 6*	*6 + 9*	
M & N 300	4	2	2	8
Camag D	6	5	1	12
Whatman CC41	5	6	6	17
S & S 144	3	4	5	12
S & S 142 dg	1	1	3	5
S & S 140 dg	2	3	4	9

TABLE IV

ORDER OF EFFICIENCY OF CHROMATOGRAPHY PAPERS

Chromatography paper	*Order of efficiency*			*Sum of orders*
	1 + 3	*5 + 6*	*6 + 9*	
W1	10	5	4	19
W2	8	10	2	20
W3	5	6	6	17
W3 MM	6	7	5	18
W4	4	1	7	12
W7	7	3	8	18
W17	2	2	10	14
W20	9	8	9	26
W31 ET	1	4	3	8
W54	3	9	1	13

The influence of the value of l_0 upon resolution in thin-layer chromatography, for a constant value of l_f, is shown in Fig. 1*.

All the graphs have a distinct maximum near $l_0 = 2.0$ cm. As this maximum was calculated to be at 2.3 cm for the acids 5 and 6, $l_f = 13$ cm and at 1.8 cm for the acids 6 and 9, $l_f = 11$ cm (see Table I) the agreement between theory and experiment is good.

The calculated optimum values of l_0 do not depend strongly upon the pair of

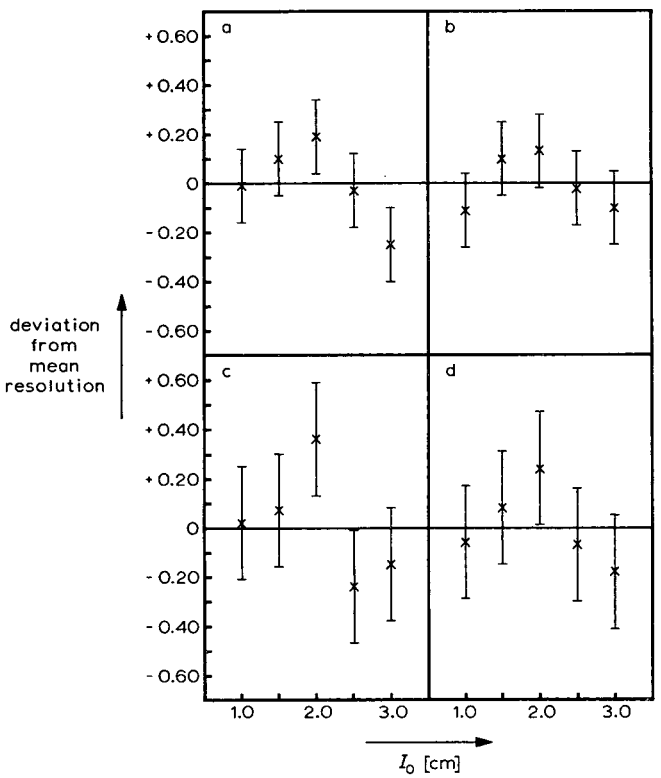

Fig. 1. (a) Resolution of amino acids 5 and 6 as a function of l_0, $l_f = 11$ cm; (b) resolution of amino acids 5 and 6 as a function of l_0, $l_f = 13$ cm; (c) resolution of amino acids 6 and 9 as a function of l_0, $l_f = 11$ cm; (d) resolution of amino acids 6 and 9 as a function of l_0, $l_f = 13$ cm. The results shown are mean values of 4 to 6 replicate experiments. The bars represent 90% probability intervals.

* The following procedure was used to obtain these data: After applying 1 μl of an aqueous solution, containing 1 μg of each of the amino acids 5, 6 and 9 to the ground edge of a glass plate (20 × 1 mm) this edge was then pressed on a thin-layer of S & S 144 cellulose powder. The mixture was applied five times to each thin-layer plate, the distances from the end of the plate varying from 1.0 up to 3.0 cm.

After equilibration with the vapour of the lower layer of a 4:1:5 butanol–acetic acid–water mixture, for at least 18 h at 21.5°, the upper layer was poured into the tank and the solvent was allowed to travel for a distance of 11 or 13 cm.

Then the plates were dried and stained with a ninhydrin solution and the densitograms obtained. From the peak width at half height and the distance between the peak maxima the resolution R was calculated.

amino acids to be separated and probably hold approximately for a much wider range of separations. Therefore, the values of l_0 given in Table I can serve as a rough guide for any separation problem in thin-layer chromatography.

CONCLUSIONS

The time needed to obtain a given resolution is much longer in paper chromatography than in thin-layer chromatography.

The efficiency of a paper or powder varies appreciably with the pair of amino acids to be separated. On the average, the order of efficiency by the criterion of minimum elution time for a given resolution is:

S & S 142 dg > M & N 300 > S & S 140 dg > S & S 144 ≈ Camag D > Whatman CC41 for thin-layer chromatography;

and

W31 ET > W4 > W54 > W17 > W3 > W3 MM ≈ W7 > W1 > W2 > W20 for paper chromatography.

If optimum separation in thin-layer chromatography is desired it is advisable to choose the following values for l_0, the distance between the starting point and the eluent in the tank:

M & N 300 ≈ 1.0 cm; Camag D ≈ 1.0 cm; Whatman CC41 ≈ 2.5 cm; S & S 144 ≈ 2.5 cm; S & S 142 dg ≈ 1.5 cm; S & S 140 dg ≈ 3.0 cm, instead of the usual value l_0 ≈ 1 cm.

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DETERMINATION OF THE DISTRIBUTION OF THE ALIPHATIC GROUPS OF GLYCERYL ETHERS BY GAS–LIQUID CHROMATOGRAPHY OF THE DIACETYL DERIVATIVES

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(Received July 17th, 1968)

SUMMARY

The alk-1-enyl and alkyl glyceryl ethers produced by hydrogenolysis of the parent lipids were separated from each other and from other hydrogenolysis products by silicic acid chromatography. The isolated ethers were acetylated and the diacetates fractionated by gas–liquid chromatography on the basis of the different aliphatic groups. The free and acetylated alk-1-enyl glyceryl ethers were characterized by thin-layer chromatography, chemical analysis and infrared spectroscopy in addition to gas–liquid chromatography. The feasibility of the method for the determination of the distribution of the aliphatic groups in the alk-1-enyl glyceryl ethers of rat brain and of the alk-1-enyl and alkyl glyceryl ethers in ox brain ethanolamine phosphoglycerides was demonstrated.

INTRODUCTION

The distribution of the types of aliphatic groups found in the aldehydogenic moiety of plasmalogens has been determined in the past by gas–liquid chromatography of the dimethyl acetals[1–6], alcohols[2], acetylated alcohols[2,7], cyclic acetals of diols[8], and fatty acid methyl esters[2,9] derived from this portion of the plasmalogens. The most frequently used derivative, the dimethyl acetal, has the advantage of being simply prepared but there has been a recurring question in regard to the occurrence of artefacts[2,5,6,10]. They also have the disadvantage that fatty acids are methylated under the same conditions used in their preparation, and the fatty acid methyl esters are poorly resolved in most chromatographic systems. The preparation of the other derivatives cited employs more complicated preparative procedures but otherwise apparently gives satisfactory data except that the oxidation of double bonds in the preparation of fatty acid derivatives prevents the analysis of unsaturated aliphatic groups[2]. We present here another method of analysis in which the vinyl or alk-1-enyl glyceryl ethers derived from plasmalogens by hydrogenolysis are separated as the diacetates. The gas–liquid chromatographic separation of the diacetates of synthetic *cis* and *trans* hexadec-1-enyl glyceryl ethers has been described[11].

METHODS

Hydrogenolysis of lipids

Hydrogenolysis was carried out by a modification of the method described by THOMPSON[12]. A sample with the equivalent of up to 40 μmoles of lipid phosphorus (approximately 50 mg of lipid) was dissolved in 3 ml of dry diethyl ether and cooled in an ice bath. Twenty milligrams of $LiAlH_4$ was added, the reaction mixture was brought up to room temperature and then refluxed for 30 min. The mixture was returned to the ice bath and excess $LiAlH_4$ decomposed by the addition of 0.5 ml of ice cold water. The salts formed were centrifuged down and the ether supernatant solution decanted. The precipitate was washed in succession once with 2 ml of acetone and twice with 2 ml of diethyl ether. The hydrogenolysis products have been reported to be difficult to recover from the precipitate[12] and we are indebted to Dr. M. A. WELLS, University of Arizona, for the details of this quantitative extraction procedure. The washes were combined with the original ether solution and the mixture dried under reduced pressure. This solution contained sterols, long-chain alcohols derived from the fatty acids of the original lipids, alk-1-enyl ethers derived from the plasmalogens and the alkyl glyceryl ethers derived from both simple and complex parent lipids.

Column chromatographic fractionation of hydrogenolysis products

The hydrogenolysis products were separated into three major fractions by chromatography on silicic acid. The dried products were dissolved in hexane and chromatographed on a silicic acid column (Mallinkrodt, 100 mesh) made up in hexane. A loading of up to 20 mg of lipid per g of silicic acid was used. Sterols and fatty alcohols were eluted with 40 ml of 20 % ether in hexane. Alk-1-enyl glyceryl ethers were eluted with 15 ml of 45 % ether in hexane and a final fraction which was primarily alkyl glyceryl ethers was eluted with 20 ml of ether saturated with water. The volumes of solvents given are for a one-gram column. The "activity" of the silicic acid is important and for reproducible results it was heated at 100° overnight before use. We found it advisable to routinely monitor the fractionation by chromatographing samples of each fraction on silica gel thin-layer plates with solvent 3 (Table III).

While the alkenyl ethers obtained by silicic acid fractionation were satisfactory for gas–liquid chromatographic analysis of the diacetate derivatives, for purposes of characterization some preparations were subjected to further purification after conversion to the diacetates. The diacetate derivatives prepared as described below were taken to dryness under reduced pressure, dissolved in hexane and chromatographed on Florisil (60/100 mesh from Varian Aerograph, Walnut Creek, Calif.) columns prepared in hexane and eluted in succession with 15 ml of 5 % ether in hexane and 15 ml of 20 % ether in hexane. The Florisil was deactivated before use by mixing with 7 % its weight of water and allowing it to stand for several days in a closed container. The volumes given were for a one-gram column, and a loading of 10 to 20 mg of lipid per gram of adsorbent was used. The diacetyl alk-1-enyl glyceryl ethers were recovered with the second solvent.

Acetylation of hydrogenolysis products

Up to 100 mg of the lipid to be acetylated was taken to dryness under reduced

pressure, 1 ml of pyridine and 0.4 ml of acetic anhydride were added and the mixture left to stand overnight at room temperature in the dark. Two milliliters of water were added and the lipid extracted with two 3 ml aliquots of petroleum ether. The petroleum ether phase was washed once with 0.1 M Na_2CO_3, once with water and taken to dryness under reduced pressure. A similar procedure was used for the preparation of the dipalmityl alk-1-enyl ethers except the reaction was run in CCl_4–pyridine with palmitoyl chloride. We are indebted to Dr. M. A. WELLS for details of the latter preparation not yet published.

Conversion of the vinyl ethers to long-chain alcohols

An aliquot of approximately 2 μmoles of the alk-1-enyl glyceryl ethers was treated by a modification of the procedure described by FARQUHAR[2] for conversion of dimethyl acetals. The lipid was dried under reduced pressure in a screw-capped tube and hydrolized for 3 h at 50° in 5 ml of 90 % acetic acid to which 0.1 ml of saturated mercuric chloride solution was added. The aldehydes were isolated by pouring the reaction mixture into 25 ml of hexane, adding 25 ml of water and removing the upper hexane phase. This phase was washed twice with 25 ml of water and taken to dryness under reduced pressure. Alkaline conditions were avoided in order to guard against polymerization of the aldehydes. The isolated aldehydes were reduced by dissolving in 10 ml of dry diethyl ether and then adding dropwise to 15 ml of a 7 mg/ml suspension of $LiAlH_4$ in ether. From this point the mixture was handled as described in the hydrogenolysis procedure given above.

Gas–liquid chromatography

The acetyl derivatives were chromatographed in a Barber-Colman series 5000 apparatus with an argon ionization detector on a 122 × 0.5 cm glass column packed with 3 % SE-30 on 80–100 mesh acid-washed Chromosorb W (Applied Sciences Laboratory, College Station, Pa.) treated with hexamethyldisilazane. Runs were made with the column at 230°, the injection port at 260° and the detector at 275° with a flow rate of 60 ml of argon/min. Samples were injected dissolved in hexane. Acetates of fatty alcohols were chromatographed on a 0.5 × 180 cm column of 10 % ethylene glycol adipate on 80–100 mesh acid-washed Chromosorb W with a column temperature of 185°. The sample injection port and detector were maintained at 260 and 270°, respectively. A flow rate of 60 ml/min was used.

Materials

An ether-soluble fraction was prepared from a chloroform–methanol extract of beef brain and an ethanolamine glycerophosphatide fraction was prepared from it by chromatography on Florisil followed by further purification on alumina[13]. The ethanolamine phosphoglycerides were eluted from Florisil with chloroform–methanol–water (10:5:1.2) and from alumina with chloroform–methanol–water (30:20:2.5). The product appeared homogeneous on silica gel thin-layer plates[14], and analysis by differential hydrolysis[15] showed it to consist of 48 % plasmalogen and 6 % alkyl glyceryl ether. Rat brain lipid extracts were obtained as described elsewhere[15].

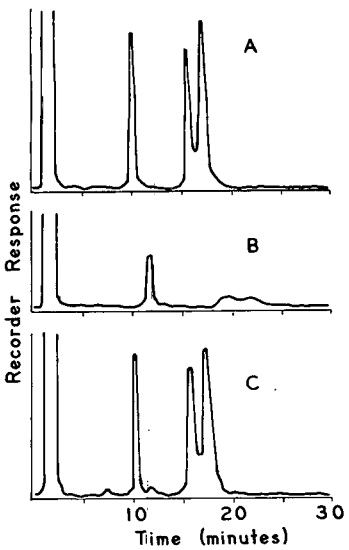
All solvents and other chemicals were analytical grade and were used without further purification except the hexane was redistilled from $KMnO_4$ and the fraction boiling between 67.5 and 68.5 was used. Diethyl ether was dried over Al_2O_3 before

use. Most standards for gas–liquid and thin-layer chromatography were either commercially available products or, as in the case of acetate derivatives and alcohols, were prepared from commercially available material by the methods indicated above. The exceptions are the palmital cyclic acetal of glycerol, which was a gift from Dr. D. N. RHODES, and the dimethyl acetal of palmitaldehyde, which was prepared from commercially available palmitaldehyde bisulfite[16].

RESULTS

Gas–liquid chromatography of the diacetyl derivatives of alkyl and alk-1-enyl glyceryl ethers

The glyceryl ethers prepared by hydrogenolysis of 180 mg samples of rat brain lipids were separated by silicic acid fractionation, acetylated and used to establish the conditions for gas–liquid chromatography described above (Fig. 1 and Table I). Diacetyl alkyl glyceryl ethers prepared from commercially available ethers were used



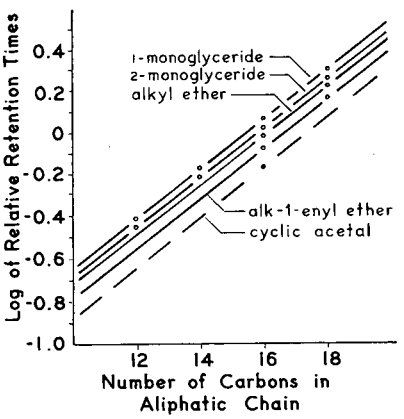


Fig. 1. Gas–liquid chromatograms on 3% SE-30 columns of the diacetate derivatives of (A) the alk-1-enyl, (B) the alkyl, and (C) the alk-1-enyl and alkyl glyceryl ethers prepared from rat brain lipids.

Fig. 2. Resolution on SE-30 columns of various acetylated lipid derivatives. The relative retention times were calculated on the basis of diacetyl chimyl alcohol equal to one.

to establish the identity of these compounds derived from brain lipids, and the identities of the diacetyl derivatives of the alk-1-enyl ethers were established by independent means described below. The acetate derivatives of α- and β-monoglycerides and the acetyl derivative of palmital cyclic acetal of glycerol and fatty alcohol acetates were also chromatographed. All of these compounds were potential contaminants of the fractions chromatographed. Fig. 2 shows the resolution obtained with the various compounds, except the alcohol acetates, which were eluted with the solvent front when they contained up to 18 carbon atoms.

TABLE I

RELATIVE RETENTION TIMES AND DISTRIBUTION OF THE DIACETATES OF ALK-1-ENYL AND ALKYL GLYCERYL ETHERS DERIVED FROM RAT BRAIN LIPIDS

Structure of aliphatic moiety[a]	*Relative retention times*[b]	*% distribution*	
		Sample 1	*Sample 2*
Alk-1-enyl ether			
16:0	0.87	24.2	24.3
18:0	1.56	39.7	41.1
18:1	1.45	36.0	34.4
Alkyl ethers			
16:0	1.00	39.2	37.5
18:0	1.80	26.4	27.0
18:1	1.64	34.4	35.3

[a] The first number indicates the number of carbons in the aliphatic chain and the second the number of double bonds exclusive of the vinyl ether.

[b] Calculated relative to the retention time of chimyl diacetate.

Identification of the diacetyl alk-1-enyl glyceryl ethers

Since standards of individual alk-1-enyl glyceryl ethers were not available to us, it was necessary to establish the identity of the peaks obtained by gas–liquid chromatography by converting to a derivative for which standards were available. Fatty alcohols corresponding to the aliphatic chain of the ethers were prepared by hydrolyzing the vinyl ethers to the aldehyde and reducing the aldehyde. For this purpose the alk-1-enyl glyceryl ether fraction from ox brain ethanolamine glycerophosphatides was used. Prior to reduction, the fraction was shown to be free of alcohols by thin-layer chromatography as described below. The alcohols were acetylated by the method described for glyceryl ethers and the retention times of the acetates were compared with standard fatty alcohol acetates prepared by reducing the corresponding fatty acid methyl esters with $LiAlH_4$ and acetylating. Table II gives the retention times of the alcohol acetates derived from ox brain ethanolamine phosphoglycerides and the relative distribution of the alcohol acetates and the alk-1-enyl glyceryl ether diacetates from the same source. On the basis of these data an

TABLE II

CHROMATOGRAPHIC CHARACTERIZATION OF THE ALCOHOL ACETATES PREPARED FROM THE ALK-1-ENYL GLYCERYL ETHERS DERIVED FROM OX BRAIN ETHANOLAMINE PLASMALOGENS

Structure of aliphatic chain	*Relative retention times of alcohol acetates*	*% distribution*	
		Alcohols derived from ethers[a]	*Alk-1-enyl ethers as diacetates*
16:0	1.0	34.0	33.0
18:0	1.89	26.8	28.2
18:1	2.12	38.0	38.6

[a] Traces of several components were observed and identified on the basis of retention times as most probably 12:0, 14:0, 16:1, 17:0 and 18:2.

TABLE III

THIN-LAYER CHROMATOGRAPHY OF GLYCERYL ETHERS, ACYL DERIVATIVES AND RELATED STANDARDS

Chromatograms were run on 0.5 mm Silica Gel G plates with the following solvent systems: (1) *n*-heptane–ethyl acetate (20:3); (2) *n*-hexane–diethyl ether–methanol (90:20:4); (3) chloroform–methanol (95:5); (4) benzene–chloroform (1:3).

Compound	R_F *value*			
	Solvent 1	*Solvent 2*	*Solvent 3*	*Solvent 4*
Heptacos-13-ene	0.97	0.98	0.98	0.90
Cholesterol			0.81	0.13
Cholesterol stearate	0.91	0.96	0.98	0.97
Methyl palmitate	0.85	0.96	0.98	0.77
12-Tricosanone	0.81	0.95	0.98	0.89
Chimyl dipalmitate	0.61	0.94	0.96	0.57
Alk-1-enyl glyceryl ether dipalmitate (brain)	0.61	0.93	0.95	0.33
Tripalmitin	0.52	0.89	0.97	0.46
Tricosan-12-ol	0.41	0.58	0.91	0.57
Behenyl alcohol	0.21	0.33	0.69	0.23
Behenyl acetate			0.70	0.79
Oleyl alcohol	0.18	0.31	0.67	0.25
Oleyl acetate			0.70	0.78
1,3-Dipalmitin	0.13	0.31	0.87	0.08
1,2-Dipalmitin	0.07	0.24	0.87	0.08
Palmital cyclic glyceryl acetal	0.06	0.20	0.63	0.73
Palmital cyclic glyceryl acetal acetate				0.24
Monopalmitin	0.00	0.05	0.30	0.00
Stearic acid	0.00	0.00	0.15	0.06
Alk-1-enyl glyceryl ether	0.00	0.12	0.37	0.32
Alk-1-enyl glyceryl ether diacetate			0.32	0.25
Batyl alcohol	0.00	0.07	0.27	0.02
Batyl diacetate			0.92	0.23
Chimyl diacetate			0.92	0.23

assignment of the structures indicated in Tables I and II was made for the peaks observed in the chromatography of the diacetate derivatives of the alk-1-enyl ethers.

Thin-layer chromatography of glyceryl ethers, their acyl derivatives and associated compounds

The mobilities of a number of compounds on Silica Gel G (Merck) thin-layer plates developed in four different solvent systems are given in Table III. The standards were selected primarily because they could appear as lipid-soluble products after hydrogenolysis of lipids. The compounds were detected by spraying in succession with acidic dinitrophenylhydrazine (saturated 2 *N* HCl solution) and Rhodamine 6G (0.005 % aqueous solution) as carbonyl specific and general reagents, respectively. The freshly prepared free alk-1-enyl ethers and their diacetates showed only one spot which reacted strongly with dinitrophenylhydrazine. Both the free ether and diacetates showed additional spots after storage for a few days. The alkyl ethers from brain lipids generally appeared homogeneous and chromatographed similarly to standards. In some preparations of alkyl ethers from total rat brain, a second minor component was detected with solvent 3 (Table III), which had a slower mobility than the main component. This compound was not identified.

Vinyl ether and aldehyde content of the alk-1-enyl glyceryl ethers and derivatives

The alk-1-enyl ethers isolated from ox brain ethanolamine phosphoglycerides and their acyl derivatives were used for these analyses. The vinyl ether content of the free ethers, the diacetyl ethers and dipalmitoyl ethers was analyzed by the specific iodination assay of GOTTFRIED AND RAPPORT[17] and the values obtained were compared with the total aldehyde content as determined in the free ethers by the dinitrophenylhydrazine method of SCHWARTZ *et al.*[18] and in the diacetate by the *p*-nitrophenylhydrazine method of PRIES AND BÖTTCHER[19]. With the latter assays, the ratio of moles of aldehyde determined/moles of aldehyde calculated from mass was found to be 0.98 for both the free ether and dipalmitoyl derivatives and 1.0 for the diacetate derivative. The iodination assay gave somewhat equivocal data. The free ethers reproducibly gave values of only 17 % that expected from the aldehyde content, and the dipalmitin derivatives would not take up any iodine under the usual assay conditions. When the reaction with the dipalmitin derivatives was run by adding 1 ml of 6×10^{-4} *N* methanolic I_2 to 1 ml of a chloroform–methanol (2:1) solution of the compound followed by the addition of 0.5 ml of 3 % aqueous KI, an iodine uptake equivalent to 80 % of the aldehyde content was found. Unlike the free ethers and dipalmityl derivatives, the diacetates reacted to give an iodine uptake with the standard assay procedure which was 92 % of the theoretical uptake based on aldehyde content. This is in good agreement with the uptake found with intact phospholipids with this method.

While these experiments suggest that the isolated free ethers and dipalmitate derivatives may not be entirely in the vinyl ether form, we believe that the discrepancies are due to limitations of the iodination assay system. The differences observed in reactivity of plasmalogen preparations from different sources[20] support this. The possibility that the free ether preparation is in fact the cyclic acetal was ruled out on the basis of gas–liquid and thin-layer chromatographic analyses and, of course, this structure is impossible with the dipalmitate derivative. The possibility that the vinyl ether may be in equilibrium with the hemiacetal and that this structure is favored in the free ether and dipalmitate cannot be entirely ruled out.

Infrared analyses

Spectra of thin films dried on NaCl plates were obtained with a Perkin Elmer Model 21 Spectrometer. Reference spectra were run on chimyl alcohol, chimyl diacetate, palmital cyclic glyceryl acetal and its acetate, palmitaldehyde, tripalmitin and oleyl acetate. The spectra of the free alk-1-enyl ethers, the diacetates and dipalmitates were very similar to the spectra of chimyl alcohol and its diacetates except the vinyl ethers showed a strong peak at 6.0 μ and a peak at 13.55 μ which are probably due to the *cis* double bond and =C–H, respectively[21]. The free ethers showed a stronger band at 8.95 μ which is presumably evidence for a predominance of the ether linkage on the primary hydroxyl group[22].

Hydrogenation of the alk-1-enyl ethers

Reduction of the alk-1-enyl ethers to the alkyl analogues would provide a convenient route to the identification of the original compounds. Numerous reports of the hydrogenation of vinyl ethers have appeared. The conditions used for reduction have varied from hydrogen at atmospheric pressure with platinum oxide as

the catalyst, under which conditions 96.6 % conversion in 20 min was reported[23], to the use of 4.0 atm of pressure for 3 h with the same catalyst[24]. We were limited to working at or near atmospheric pressure and under such conditions attempted to reduce ox brain ethanolamine phosphatides, the free alk-1-enyl glyceryl ethers and their diacetates with platinum oxide, palladium on charcoal or palladium on $BaSO_4$ as catalyst. Gas–liquid chromatography of the acetylated products failed to show any saturated ethers except, as in the case of the ethanolamine phosphatides, those present in the unreduced lipids. Free aldehydes and fatty alcohols were observed by infrared spectroscopy and gas–liquid chromatography, respectively, and the former predominated with short reaction times. We are unable to account for our failure to obtain conversion under essentially the same conditions as used by SCHOGT *et al.*[23] but point out that because of reduction to the alcohol, the disappearance of dinitrophenylhydrazine-reactive material as used by these workers as a criterion for conversion to the saturated ether is not reliable.

Periodate oxidation of the alk-1-enyl glyceryl ethers

One goal of preparing the saturated analogues of the vinyl ethers was so that periodate oxidation, selective for vicinal hydroxy groups, could be used to determine the proportion of 1- (3-) and 2- ethers. Having failed in the preparation of the alkyl ethers, periodate oxidation was carried out on the vinyl ethers directly at pH 7 (ref. 25). An aliquot of the alk-1-enyl ether fraction from ox brain ethanolamine phosphoglycerides which contained the equivalent of 1.16 μmoles of aldehyde was taken to dryness and dissolved in 1 ml of 95 % ethanol. One ml of 0.1 *M*, pH 7.0 acetate buffer and 0.5 ml of 0.1 *M* $NaIO_4$ were added and the mixture was incubated in the dark at 25° until no further decrease in absorption at 300 mμ could be detected over a 15 min interval. The total reaction time was 3 h. One half milliliter of 10 % aqueous $NaHSO_3$ was added and 1 ml aliquots were taken for the analysis of formaldehyde[26]. The equivalent of 1.19 μmoles of formaldehyde was formed or 103 % of the yield expected if all of the alk-1-enyl ethers were linked with the primary hydroxyl group. A sample of chimyl alcohol, 0.235 μmoles, yielded exactly 0.235 μmoles of formaldehyde under the same conditions of oxidation.

Quantitative recovery and precision of the method

Samples of ox brain ethanolamine phosphoglyceride and rat brain total lipid were assayed for total aldehyde content by Schiff's assay[16], and it was shown by the same assay that from 94 to 96 % of the total aldehyde was recovered after hydrogenolysis and of that recovered from 90 to 95 % was recovered in the alk-1-enyl glyceryl ether fraction isolated from silicic acid. When lipid fractions which had been stored for several weeks were assayed, the aldehyde content as determined by Schiff's assay was as much as two times the values consistent with the plasmalogen content determined by selective hydrolysis[15]. The aldehyde values were restored to a consistent value when the lipid was chromatographed on a silicic acid column eluted in succession with 20 ml/g of chloroform and 10 ml/g of methanol. The phospholipids were eluted with the methanol. Aldehyde assays on aged samples after hydrogenolysis also were consistent with the plasmalogen values of the original extract which indicates that whatever gives the high aldehyde values with Schiff's reagent is lost during the hydrogenolysis or subsequent isolation of the products.

TABLE IV

REPRODUCIBILITY OF THE ASSAY FOR DISTRIBUTION OF BRAIN GLYCERYL ETHERS

Sample	*% distribution ± S. D.*[a]		
	16:0	*18:0*	*18:1*
Rat brain lipid			
alk-1-enyl ether	24.1 ± 0.6(6)	39.2 ± 1.4(6)	36.2 ± 1.3(6)
Ox brain ethanolamine phosphoglycerides			
alk-1-enyl ether	30.8 ± 1.9(4)	31.7 ± 1.8 (4)	36.7 ± 1.4(4)
alkyl ether	41.3 ± 1.7(3)	19.7 ± 0.5(3)	37.7 ± 1.2(3)

[a] The designation of the aliphatic group is the same as used in Table I. The number in parentheses after the standard deviation indicates the number of samples assayed.

The precision of the method was tested by assaying the distribution of aliphatic groups in the alk-1-enyl glyceryl ether fraction from 25 mg samples of rat brain lipids and from samples of ox brain ethanolamine phosphoglycerides that contained 40 μmoles of phosphorus. The alkyl ethers from the latter samples were also assayed. At least five times the quantity of rat brain lipid would have been needed to obtain satisfactory data on the alkyl ethers. As can be seen from the data in Table IV, the method is generally reproducible within approximately 5 %.

DISCUSSION

The conversion of plasmalogens and glyceryl ether derivatives to the alk-1-enyl and alkyl glyceryl ethers by hydrogenolysis was used by THOMPSON[12,27] to measure tissue content and the extent of incorporation of various labeled precursors into the parent lipids. More recently, WOOD AND SNYDER[22] have used densitometry of thin-layer chromatograms of the alkyl and alk-1-enyl glyceryl ethers derived from lipids by hydrogenolysis as a measure of the tissue content of the parent lipids. We have extended these applications by use of a simple silicic acid fractionation and acetylation of the ethers to permit the gas–liquid chromatographic analysis of the distribution of the various types of aliphatic groups. We have affirmed the identification and extended the characterization of the hydrogenolysis products.

The feasibility of using the diacetates to characterize the ether fractions by gas–liquid chromatography has been demonstrated by a check on the recovery of the total aldehydogenic material in lipid extracts in the appropriate fractions before and after acetylation and by a combination of chromatographic, chemical and infrared spectroscopic characterization of the isolated diacetate derivatives. As applied to rat brain lipids and ox brain ethanolamine phosphoglycerides, the method gives reproducible results and the identification of the major aliphatic groups in the alkyl ethers of rat brain lipids is in agreement with the identification made on the basis of the isopropylidine derivatives[15]. No previous information is available on the distribution of aliphatic groups in the alk-1-enyl ethers of rat brain, but the similarity to the aliphatic groups of the alkyl ethers is consistent with the metabolic interrelationship of alkyl and alk-1-enyl ethers in rat brain proposed by HORROCK AND ANSELL[28] on the basis of ethanolamine incorporation into the parent compounds.

Studies on the interrelationship of these two classes of lipids and those studies concerned with the origin of the aliphatic groups[12] can be made more definitive by the isolation and examination of the individual ethers by the method described here.

ACKNOWLEDGEMENTS

This work was supported by a U. S. Public Health Service Research Grant, AM-09766 and a Public Health Predoctoral Fellowship (PWA), GM 35,152. The technical assistance of Miss CHARLOTTE BROWN is gratefully acknowledged.

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CHROM. 3670

CHROMATOGRAPHIC ANALYSIS OF METHYL(PROPYL)DIMETHYL-CYCLOSILOXANES

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(Received June 29th, 1968)

SUMMARY

For some cyclosiloxanes it was found that a relationship exists between retention behaviour in gas chromatography and structure. A nomogram is proposed, based on data for some standard compounds, by means of which other compounds can be identified.

In order to determine the effect of substituent groups on the reactivity of the siloxane bond, we have synthesized a number of new compounds hitherto not described in the literature: cyclotrisiloxanes and cyclotetrasiloxanes containing various numbers of dimethyl- and methyl(propyl)siloxane groups in the cyclic structure.

In an earlier work[1,2] we carried out the synthesis and chromatographic analysis of the mixture of 3,3,3-trifluoropropyl(methyl)dimethylsiloxanes having the general formula F_mD_n, where F = the 3,3,3-trifluoropropylmethylsiloxane group; D = the dimethylsiloxane group; m = 0–5, n = 0–5, and $(m + n)$ = 3–6. Further analysis of the experimental chromatographic data for this mixture from the separation on methyltrifluoropropylsiloxane resin as the stationary phase showed that the logarithms of the retention volumes of cyclosiloxanes of the same homologous series were directly proportional to the number of groups D or F in the ring. Furthermore, a linear change in the logarithm of the retention volumes of cyclosilioxanes which were not part of a homologous series, was also observed for cyclotrisiloxanes: D_3, FD_2, F_2D, and F_3; cyclotetrasiloxanes: FD_3, F_2D, F_3D, F_4; cyclopentasiloxanes, FD_4, F_2D_3, F_3D_2, F_4D; and cyclohexasiloxanes, FD_5, F_2D_4, F_3D_3, F_4D_2, F_5D.

Fig. 1 shows the dependence of the logarithm of the retention volumes of F_mD_n cyclosiloxanes on the magnitude of $n(m)$. Each point lying at the intersection of three straight lines corresponds to an individual cyclosiloxane, the exception being cyclosiloxanes containing only dimethylsiloxane or only 3,3,3-trifluoropropyl-methyl siloxane groups: D_4, D_5, F_4, F_5 (these cyclosiloxanes correspond to points lying at the intersection of two straight lines). These differ qualitatively from mixed cyclosiloxanes. The homocyclics D_3 and F_3 behave analogously to mixed cyclosiloxanes, owing apparently to their good solubility in the stationary phase.

Fig. 2 shows a chromatogram obtained from a mixture of methyl(propyl)-dimethylcyclosiloxanes having the general formula P_mD_n, taken on the same sta-

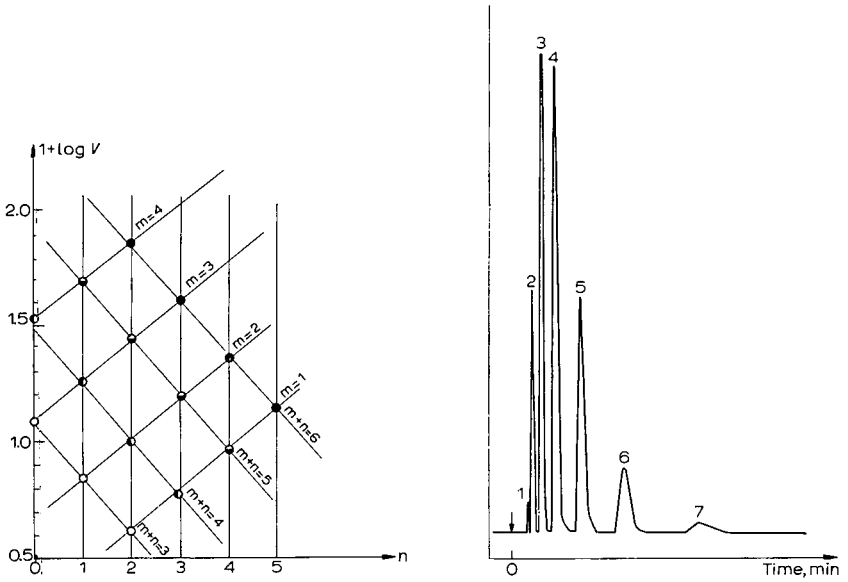

Fig. 1. Dependence of the logarithm of the retention volume on n for mixture P_mD_n. ○ = Cyclotrisiloxanes; ◐ = cyclotetrasiloxanes; ◒ = cyclopentasiloxanes; ● = cyclohexasiloxanes.

Fig. 2. Chromatogram for mixture P_mD_n. Chromatograph "Griffin"; katharometer; SF = 20% SKTFT-50 on Celite-545; $l_{column.} = 1.87$ m, $t = 193°$.

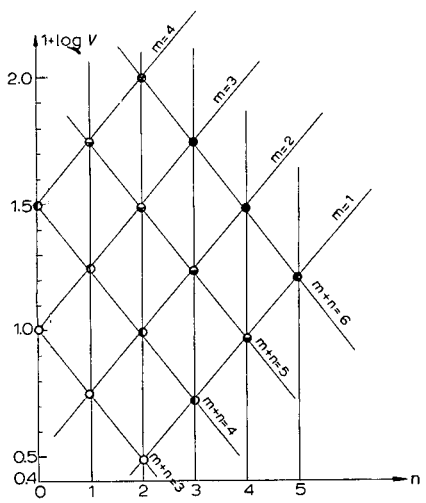

Fig. 3. Dependence of the logarithm of the retention volume on n for mixture P_mD_n.

tionary phase. The P_mD_n mixture was obtained by catalytic destruction of the cohydrolysis products of propylmethyldichlorosilane and dimethyldichlorosilane. By plotting the logarithm of the retention volume for these cyclosiloxanes as the function of n (Fig. 3) in a similar way to that for the above-mentioned dependence for the F_mD_n mixture, we were able to calculate the retention volume for cyclopentasiloxanes and cyclohexasiloxanes (Table I).

By comparing the data shown in Table I and Fig. 2 it is possible to assume that peak I is due to pure compound D_3; peak II—to compound PD_2 containing a trace of D_4; peak III—to the main component P_2D containing some D_5 and PD_3; peak IV—to the main component P_2D_2 admixed with PD_4 in front and P_3 behind; peak V—to the main compound P_3D and an admixture of PD_5 and P_2D_3 in front, which do not become separated; peak VI—to the simultaneous appearance of P_2D_4, P_3D_3 and P_4; peak VII—to overlapping of P_4D and P_3D_3 components.

TABLE I

THE RELATIVE RETENTION VOLUME FOR COMPONENTS OF THE P_mD_n MIXTURE

Component	*V_R relative**	*Component*	*V_R relative***
D_3	0.0785	D_5	0.511
PD_2	0.306	PD_4	0.934
P_2D	0.574	P_2D_3	1.718
P_3	1.083	P_3D_2	3.09
D_4	0.259	P_4D	5.51
PD_3	0.527	PD_5	1.585
P_2D_2	1.0	P_2D_4	3.08
P_3D	1.753	P_3D_3	5.47
P_4	3.04		

* Values determined from chromatogram (Fig. 2).
** Values calculated from the graph (Fig. 3).

As may be seen from Fig. 2, the P_mD_n mixture does not become completely separated on SKTFT-50 phase; however, this does not make it impossible to calculate from the plot the retention volume for all the components of the mixture. The most complete separation of this mixture was obtained with diphenylene oxide siloxane resin as the stationary phase (Fig. 4).

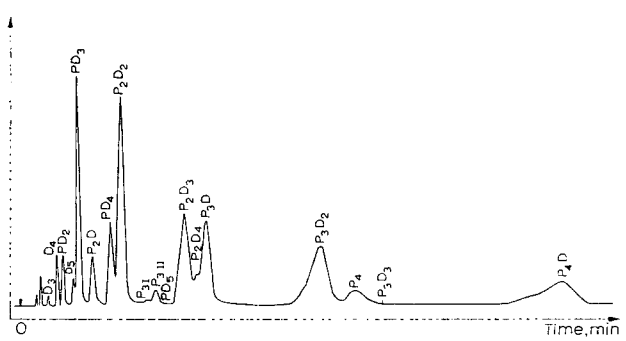

Fig. 4. Chromatogram for mixture P_mD_n. SF = siloxane resin containing diphenylene oxide group; $t = 190°$.

The analysis of F_mD_n and P_mD_n cyclosiloxanes was carried out on the "Griffin" chromatograph utilizing a detector of the heat-conductivity type. Various siloxane resins were used as the stationary phases. The amount of the phase was 20% of the weight of the solid support (Celite-545). Helium was used as the carrier gas ($v = 30$ cm^3/min).

In gas chromatographic analysis of F_mD_n and P_mD_n cyclosiloxanes using siloxane phases of different polarities[3], we observed the same regularities in the changes in the retention volume of these compounds.

The established regularity is apparently common for cyclosiloxanes having different siloxane groups in the ring, since it was observed both for polar (F_mD_n) and nonpolar (P_mD_n) compounds when the polarities of the siloxanes were different.

It is known[4-6] that in the cohydrolysis or catalytic destruction of cohydrolysis products of diorganodichlorosilanes, mixed cyclosiloxanes are formed having different siloxane groups in the ring. As a rule, such a reaction mixture contains a large amount of closely boiling components that are difficult to separate by distillation. The proposed method makes it possible, on the basis of several known reference cyclosiloxanes, to identify all the components of such mixtures, even when they are not well separated.

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CHROM. 3671

CERTAIN REGULARITIES IN THE RETENTION OF CHLORINE-CONTAINING INORGANIC AND ORGANOMETALLIC COMPOUNDS

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SUMMARY

It was found that for some inorganic compounds there is a certain regularity in their retention behaviour when chromatographed on different stationary phases.

An investigation was made of the separation of volatile metal chlorides and some organometallic compounds having boiling points up to 250°. Based on the retention characteristics for a series of stationary phases, certain regularities have been analyzed which show a relationship between the retention volume and the physicochemical properties of the stationary phases and the analyzed compounds.

Despite progress made in arriving at a theory of separation, separation conditions are often selected experimentally, the selection sometimes requiring a great number of experiments. In the case of halogen-containing inorganic and organometallic compounds, the difficulties arise from the lack of systematic investigations on the composition of the sample *versus* output characteristics, such as has been carried out to a considerable extent for hydrocarbons. Thus it is of interest to establish the relationship between the retention characteristics and the properties of the halogen-containing inorganic and organometallic compounds.

The separation of the investigated compounds was carried out on a chromatograph with a hot wire detector equipped with Teflon-coated tungsten coils and a pneumatic device for the injection of hydrolyzable samples[1], and a Teflon column 4 mm in diameter and 1.8 m long.

A type of Teflon, "Polychrom" (ref. 2), served as support. Based on known data[3,4] we used the following stationary phases: siloxane elastomer E 301 (E^* = 2.9); Apiezon-N (E = 2.65); *n*-octadecane (E = 2.15); polyorganosiloxane liquid VKZh-94 (E = 2.5); liquid polytrifluorochloroethylene of the Kel F-10 type (E = 2.6); Kel F-3 and $AlBr_3$ (E = 3.88).

The results were analyzed according to the generally accepted concepts regarding specific retention volume, height equivalent of the theoretical plate, selectivity coefficient, and separation coefficient. The best results were obtained in the case of stationary phases consisting of polytrifluorochloroethylene, Kel F-3 and *n*-octadecane

* E = Dielectric constant of the stationary phase.

(for example, the height equivalent of the theoretical plate for $GeCl_4$ on *n*-octadecane was 1.3 mm).

A plot was obtained for the dependence of the logarithm of the retention volume of silicon tetrachloride, germanium tetrachloride, tin tetrachloride, and titanium tetrachloride for a stationary phase (polytrifluorochloroethylene) *versus* the logarithm of the retention volumes of the same compounds on other stationary phases. The dependences obtained in Fig. 1 can be used in identifying inorganic chlorides according to known retention volumes on two or more stationary phases.

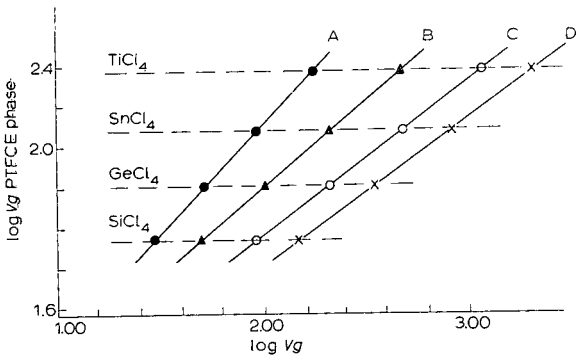

Fig. 1. Dependence of the retention volume of silicon tetrachloride, germanium tetrachloride, titanium tetrachloride *versus* the logarithm of the retention volume of the same compounds separated on other types of stationary phase. A = $AlBr_3$ melt; B = VKZh-94; C = Kel F-3; D = *n*-octadecane. The Teflon column was 1.8 m long and 4 mm in diameter using polytrifluorochloroethylene (PTFCE) as the stationary phase (5 %) on a Teflon support. Temperature of the column: 80°. Carrier gas: He; flow rate of carrier gas: 26.6 cm^3/min; detector: katharometer.

According to theory, the dependence of the logarithm of the retention volume on the reciprocal of the dielectric constant of the stationary phase must be linear, while the slope of the straight lines depends on the nature of the substance being analyzed[5]. The relationships shown in Fig. 2 indicate that one can determine with sufficient precision the linear relation between the logarithm of the retention volume for some metal chlorides and the reciprocal of the dielectric permeability of the phases, the slope of the straight lines varying with the change in the polarity of the substances being analyzed (*e.g.*, in the case of $SiCl_4$ and PCl_3). Since the dielelectric constant is not directly related to the parameters of the chromatographic experiment, the plotting of such relationships makes it possible to predict to a certain degree the magnitude of retention for the investigated compounds on new stationary phases.

The plot of the logarithm of the retention volume *versus* the boiling point of the compounds separated on polychlorotrifluoroethylene substrate also gives linear dependences (Fig. 3).

Since the straight lines have different slopes, characterizing different groups of compounds, it is clear that the dependence is not limited to the boiling points of the compounds, but includes the number of halogen atoms and the presence of other atoms (*e.g.*, O and S) attached to the metal. In this case a significant effect is also exerted by the different polarities of these compounds (the dipole moments of silicon tetrachloride, phosphorus trichloride and phosphorus oxychloride being 0, 0.78, 2.40

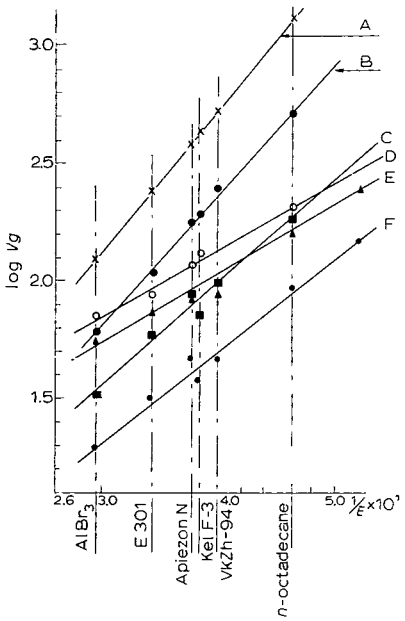

Fig. 2. Dependence of the retention volume of chlorine-containing compounds on the reciprocal of the dielectric permeability for some stationary phases. Experimental conditions as described in Fig. 1. A = $TiCl_4$; B = $SnCl_4$; C = $GeCl_4$; D = $POCl_3$; E = PCl_3; F = $SiCl_4$.

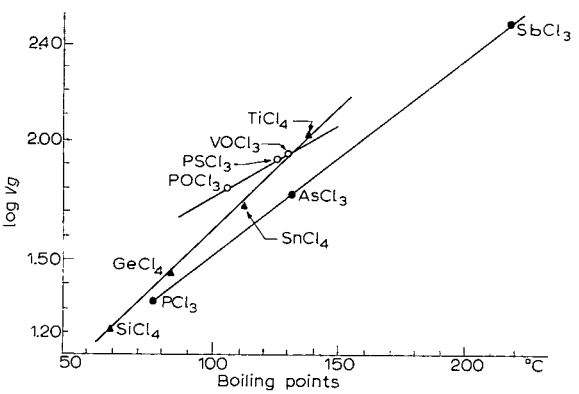

Fig. 3. Dependence of the logarithm of the retention volume on the boiling point of the metal chlorides. Experimental conditions as described in Fig. 1.

Debye units respectively). Owing certain differences in retention volumes, the investigated inorganic and organometallic compounds can be divided into three groups: nonpolar compounds ($SiCl_4$), compounds having a medium polarity (PCl_3), and polar compounds ($POCl_3$).

As is known, the solubility in nonpolar solvents (*i.e.*, in the stationary phase) is determined by the dispersion forces which depend on the polarizability (α) or molecular

refraction (R). It has already been shown[6] that for a homologous series of hydrocarbons there is a linear dependence of the logarithm of retention volume on the molecular refraction of the analyzed compounds in the case of nonpolar solvents. In the case of nonpolar molecules, such as the compounds of the first group (MR_4): $\alpha_{atomic} = 0$ and $\alpha_{orientational} = 0$ (here, M = metal); the molar refraction (R_D) here is an approximate indication of the electron polarizability of the molecule.

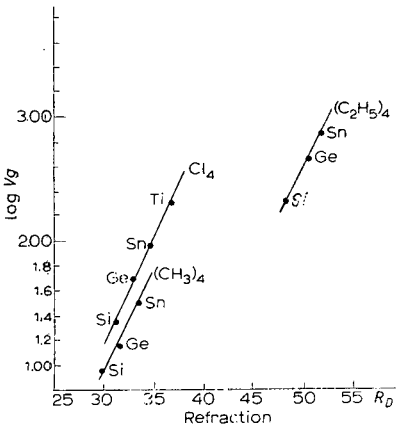

Fig. 4. Dependence of the logarithm of retention volume of some of the investigated compounds on the molar refraction of the same compounds using polytrifluorochloroethylene as the stationary phase (5%) on a Teflon support at 80°. The remaining experimental conditions as described in Fig. 1.

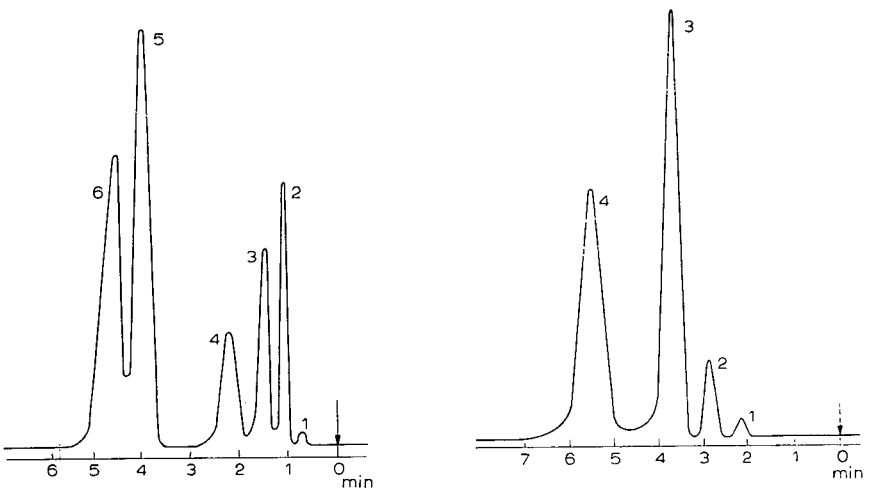

Fig. 5. Chromatogram obtained during the separation of titanium tetrachloride from vanadium oxychloride using polytrifluorochloroethylene as the stationary phase (5%) on a Teflon support at 80°. 1 = HCl; 2 = $SiCl_4$; 3 = $GeCl_4$; 4 = $SnCl_4$; 5 = $VOCl_3$; 6 = $TiCl_4$.

Fig. 6. Chromatogram of the reaction products of germanium tetrachloride and tetramethyltin using n-octadecane as the stationary phase (20%) on a Teflon support ("Polychrom") at 120°. 1 = $(CH_3)_4Sn$; 2 = $GeCl_4$; 3 = $(CH_3)GeCl_3$; 4 = $(CH_3)_3SnCl$.

The plot of the logarithm of the retention volume *versus* their molar refraction (Fig. 5) for the compounds being separated on *n*-octadecane as the stationary phase is linear in nature. The relationships obtained were used in the analysis of mixtures of chlorine-containing inorganic and organometallic compounds.

One of the most undesirable impurities contained in technical grade titanium tetrachloride is vanadium oxychloride, which has approximately the same boiling point as titanium chloride. Owing to a small partition coefficient difference it is impossible to achieve the complete separation of $VOCl_3$ from $TiCl_4$ by means of distillation. A chromatogram (Fig. 5) shows the presence of impurities in $TiCl_4$.

The study of the synthesis of chlorine derivatives of tin and organogermanium compounds by the reaction of tin tetraalkyls with germanium tetrachloride in the presence of γ-radiation from a ^{60}Co source[7] showed (Fig. 6) that the reaction proceeds according to the scheme:

$$(CH_3)_4Sn + GeCl_4 \xrightarrow{\gamma} (CH_3)_3SnCl + CH_3GeCl_3$$

The yield of methyltrichlorogermanium in this reaction based on chromatographic analysis is 96%. The relationships between the logarithm of the retention volume and the boiling point of the corresponding compounds is used for the identification of the compounds. The mixed chloro- and alkyl-derivatives show a linear dependence having a different slope from that for tetrachloro- and tetraalkyl-derivatives (Fig. 7).

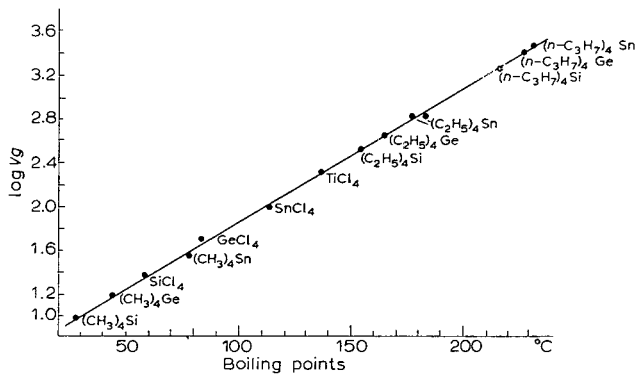

Fig. 7. Dependence of the logarithm of the retention volume on the boiling point of the investigated compounds. Column length: 2 m; column diameter: 3 mm; column temperature: 120°. The column is made of Teflon and filled with 20% of *n*-octadecane on a Teflon support "Polychrom".

The results obtained indicate that the retention data for this group of compounds conform to the same regularities as those for hydrocarbons[6]. The data obtained for the retention volumes for different stationary phases make it possible to predict the conditions for the separation of other mixtures containing the investigated compounds. The dependences obtained should only be considered as a first approximation towards the solution of the problem under discussion, which nevertheless is useful in the analysis of retention data and in designing separation schemes for the analysis of volatile metal compounds.

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CHROM. 3758

EFFECT OF pH AND COLUMN SIZE ON CM-CELLULOSE CHROMATOGRAPHY OF BOVINE MYOGLOBIN

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(Received August 26th, 1968)

SUMMARY

The several fractions obtained by chromatography of bovine myoglobin on CM-cellulose can be explained partially in terms of a pH anomaly. Adsorption of myoglobin on CM-cellulose causes the pH to rise, and unadsorbed myoglobin moves down the column with the solvent front. This fast-moving fraction (FMF) contains the several fractions that make myoglobin microheterogeneous, and the FMF is gradually adsorbed as it moves down the column. Subsequent elution of myoglobin leads to distinct fractions, which are heterogeneous on gel electrophoresis. This explanation of the chromatographic behavior of myoglobin, along with a suggested nomenclature for the chromatographic fractions, should remove some of the confusion concerning the microheterogeneity of myoglobin.

INTRODUCTION

Several people[1-4] have observed the microheterogeneity of myoglobin on CM-cellulose chromatography, but the pattern and number of fractions seem to differ with each observer. Furthermore, the discrete fractions obtained from CM-cellulose columns are frequently heterogeneous upon rechromatography or by gel electrophoresis. While investigating the microheterogeneity, we have made observations of bovine myoglobin on CM-cellulose and have discovered some characteristics of the chromatography that can account for the different patterns and numbers of fractions. In this paper, we give an explanation for these differences and suggest a nomenclature for the fractions, which should help to avoid confusion. The actual cause of the microheterogeneity of myoglobin remains a challenging problem.

MATERIALS AND METHODS

Myoglobin was isolated from bovine leg muscles (semitendinosus, semimembranosus and biceps femoris) and purified by following the procedure of SNYDER AND

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AYRES[5]. Only fractions precipitated after 90% saturation with ammonium sulfate were used. Myoglobin was oxidized to MetMb before adsorption on the columns.

All CM-cellulose used in this study was from the same batch (Sigma Chemical Co.) and was of medium mesh with a capacity of 0.63 mequiv./g. New cellulose (not regenerated) was used for all experiments. Before use, cellulose was suspended in equal volumes of 0.5 *M* NaCl and 0.5 *M* NaOH overnight. It was then washed with deionized water until neutral, and all fines not settling in 15 min were decanted. The final step was equilibration of the cellulose with starting buffers.

Columns were gravity packed. Eluting buffers were composed of 0.01 *M* phosphate and pH gradients for elution were linear. The eluting buffer was pumped through the columns at a rate of 1 ml/min, and 7–8 ml fractions were collected. Absorbance of the fractions was measured at 280 and 409 nm, and the relative amounts of myoglobin were estimated by triangulation of the area under the 280 nm curves.

The numbering of myoglobin fractions eluted from CM-cellulose columns is an important feature of the microheterogeneity problem. A rational method for numbering and referring to the fractions would be most useful in trying to understand and explain the various elution patterns appearing in the literature. We propose that the largest and most positive charged myoglobin component (usually the last component eluted from a CM-cellulose column) be designated fraction I. This fraction is normally so much larger than the others that it is readily identified. We propose that adjacent fractions be numbered II, III, IV, etc., in sequence from fraction I. Usually, the minor components are present in decreasing concentration in the same order as II, III, IV, etc.

There is a myoglobin fraction that can be readily observed but does not fit the numbering system just given. We have named it FMF (fast-moving fraction) for reasons which will be obvious from the results section. Nonheme proteins normally do not present any difficulties or confusion in interpreting elution patterns of myoglobin and do not require a special numbering system.

RESULTS AND DISCUSSION

The frequent separation of MetMb fractions on CM-cellulose columns led us to observe closely what was happening as the colored protein was applied to the column. Invariably, a MetMb fraction moved quickly away from the main MetMb fraction adsorbed at the top of the column. Fig. 1 shows a sequence of photographs depicting this separation of FMF (fast-moving fraction). The FMF decreases in quantity as it moves down the column.

It is possible to elute and collect FMF as a fraction distinct from the main MetMb fraction on a short (5-cm) column. Fig. 2 shows that two distinct fractions are formed on short columns, and the FMF is not due simply to overloading the column. If the column length is extended to 17.5 cm, an elution pattern such as that of Fig. 3 is obtained. The MetMb fractions are numbered according to the system explained in the methods section. Note that the amount of FMF in relation to the other fractions is much less than in Fig. 2. By extending the column length to 60 cm, the FMF is lost entirely as shown in Fig. 4. Although a small colorless protein peak is evident at fraction No. 40, no MetMb peak corresponding to the rate of movement of FMF can be found.

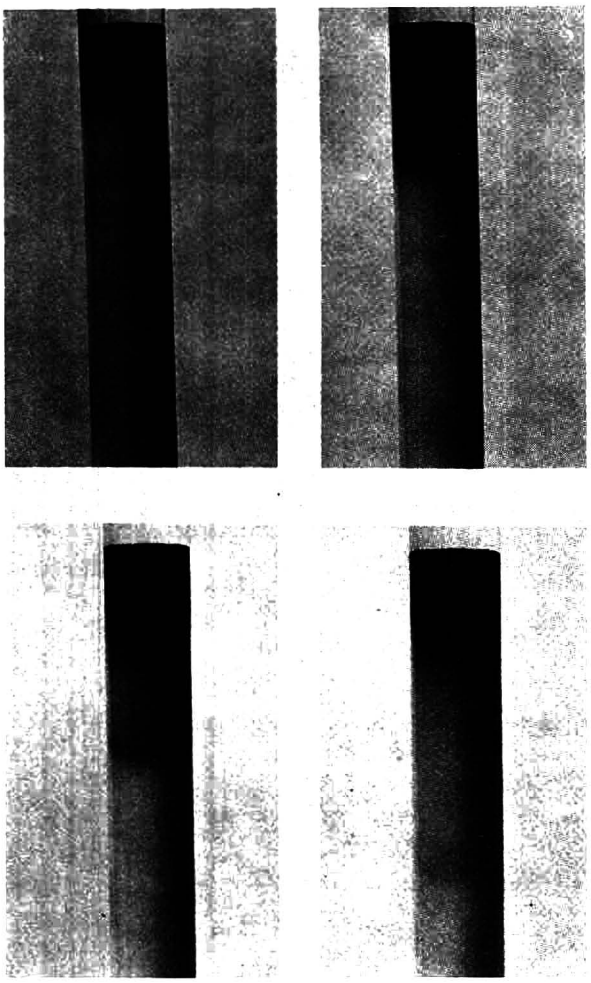
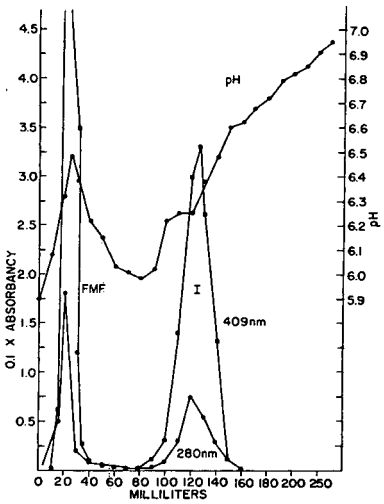


Fig. 1. Formation and re-equilibration of the FMF. Photographs taken at approximately 5 min intervals starting with upper left, then upper right, lower left, and lower right.

Further study of the conditions affecting the size of FMF showed that the volume of the column bed was important in relation to the total amount of MetMb applied to the column. Table I summarizes the results from several columns. If the amount of MetMb per cc of column material is fairly large, as in columns F, G, and J, then the amount of FMF is large. If the amount of MetMb per cc of CM-cellulose is small, as in columns A, B, C, D, and K, then the FMF is small or nonexistent. These results are contrary to the expected behavior of a protein on CM-cellulose. One would not expect to increase the proportion of a separate protein fraction by putting more of the original protein mixture on the column. Nor, would it be expected that the proportion of one of the fractions could be changed by changing the size of the column.



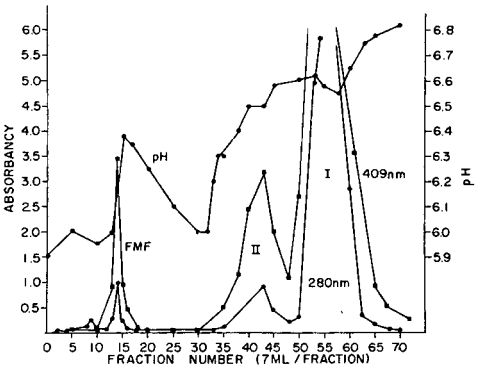


Fig. 2. CM-cellulose chromatogram of bovine metmyoglobin on a 2.5 × 5 cm column. Buffer gradient started at pH 6, and 200 mg of myoglobin was applied.

Fig. 3. CM-cellulose chromatogram of bovine metmyoglobin on a 2.5 × 17.5 cm column. Buffer gradient started at pH 6.0, and 280 mg of myoglobin was applied.

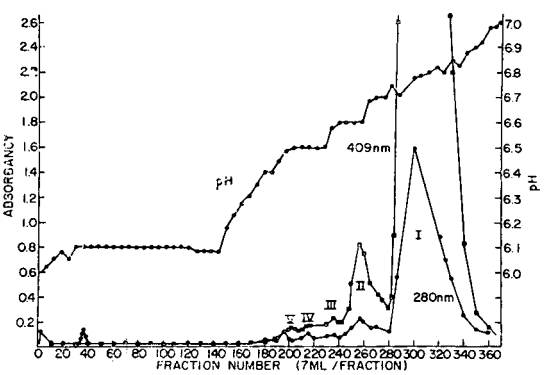


Fig. 4. CM-cellulose chromatogram of bovine metmyoglobin on a 2.5 × 60 cm column. Buffer gradient started at pH 6.0, and 300 mg of myoglobin was applied.

In our study of the relative size of the FMF, we found that it could be influenced by the initial pH of the column. These data are also shown in Table I. If the initial pH was 5.5, even a large proportion of MetMb to CM-cellulose gave a small FMF; columns H and I are examples. In contrast, column E, with a starting pH of 6.8, has a relatively large proportion of FMF even though the amount of MetMb per cc of CM-cellulose is low.

The observation that starting pH had an effect on the relative amounts of FMF caused us to measure pH in the effluent fractions. The results are plotted with

TABLE I

RELATIONSHIP OF EQUILIBRATING pH AND COLUMN CAPACITY TO MYOGLOBIN FRACTIONS ELUTED FROM CM-CELLULOSE COLUMNS

Column designation	*Equilibrating pH*	*Column capacity in cc*	*mg Mb chromato-graphed*	*mg Mb per cc CM-cellulose*	*Total number fractions eluted*	*Per cent FMF (estimate)*
A	5.9	40.7	125	3.1	3	4%
B	5.9	85.9	280	3.3	3	4%
C	6.0	295	300	1.0	4 or 5	None
D	6.0	43.4	23	0.53	3 or more	None
E	6.8	44.3	22	0.49	2	7%
F	6.0	24.6	250	10.2	2	24%
G	6.0	27.0	206	7.7	2	17%
H	5.5	88.4	437	4.9	4	0.5%
I	5.5	83.5	704	8.4	4	0.3%
J	6.0	39.3	252	6.4	2	8%
K	6.0	39.3	174	4.4	2	2%

the leution patterns in Figs. 2, 3, and 4. We found a sharp increase in pH associated with the FMF, followed by a pH decrease. The increase in pH was most pronounced in very short (5-cm) columns.

Since the columns were being used in a pH region in which the carboxyl groups of the CM-cellulose were not completely ionized, the pH anomaly may have been due to interaction of buffer with CM-cellulose. Hence, we examined the effluent pH from two columns that had no MetMb adsorbed but were treated with a pH gradient. One column was started at pH 5.6, and the second was started at pH 6.0. The pH measurements on effluent fractions are shown in Fig. 5. Both columns showed some buffering activity with an abrupt increase in pH at fraction XXVII for the pH 6.0 column and fraction XXXVI for the pH 5.6 column. These pH changes could not explain the abrupt increase and decrease in pH associated with the FMF. It is surprising that the pH of the column effluent changed as readily as it did. The buffer was only 0.01 *M*, and we know from experience that far more than 600 ml of 0.01 *M* phosphate buffer is required to re-equilibrate a CM-cellulose column from pH 5.5 or 6.0 to pH 7.0. Evidently, only some superficial carboxyls are being titrated, and with sufficient time, the pH of the column effluent would decrease.

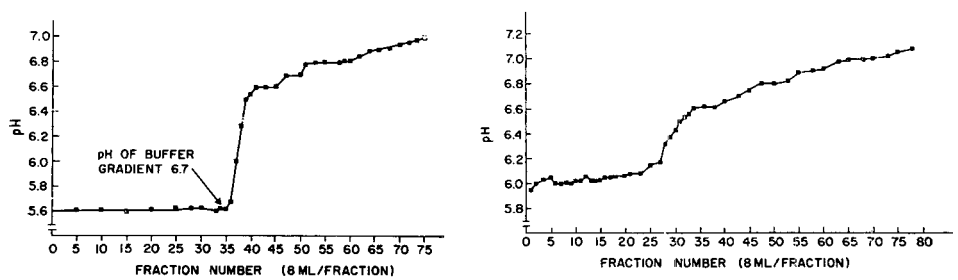


Fig. 5. Titration curves for 2.5 × 18 cm CM-cellulose columns using a linear buffer gradient starting at pH 5.6 for (a) and pH 6.0 for (b). No myoglobin was applied to these columns.

Because of the enhanced pH change with short columns and the failure to find a pH change when myoglobin was not placed on the columns, we concluded that the abrupt pH increase and decrease was probably associated with the initial adsorption of MetMb to the CM-cellulose. CLAYTON AND BUSHUK[6] predicted that such a pH change should take place if the eluting solvent were insufficiently buffered and if the cations replaced by the protein were more basic than the cation groups on the protein.

Such an explanation provides some understanding of the origin of the FMF and of the relationship between the proportion of MetMb per cc of CM-cellulose and the size of the FMF. If the pH increases abruptly as MetMb is bound to CM-cellulose, then MetMb in buffer at pH 6.3 or 6.4 will not be adsorbed and will begin to move down the column. The more MetMb applied to the column originally, the larger will be the MetMb fraction (which would be the FMF) moving down the column. For a short column, the FMF is eluted in the presence of the buffer with higher pH. If the column is long, there is increased opportunity for the buffer to interact with CM-cellulose and gradually decrease in pH. As the buffer decreases in pH, the MetMb being carried by the buffer will begin to interact with the CM-cellulose and be slowly adsorbed as it moves down the column. With a long column, the FMF will be completely adsorbed, and the initial pH increase will not be evident as in Fig. 4.

This explanation of the FMF is helpful in understanding another anomaly of separating MetMb fractions on CM-cellulose. Except for fraction I, the well-defined MetMb fractions that can be eluted from CM-cellulose are far from pure as analyzed by gel electrophoresis. Although fraction I is more pure than the MetMb before chromatography, fraction II and FMF are still heterogeneous. Attempts to minimize the heterogeneity by extruding the columns before elution of fractions and by cutting the columns in sections, from which fractions could be eluted, were not successful in decreasing the heterogeneity.

If the increased pH is responsible for the FMF and if the FMF is gradually readsorbed as it moves down the column, then the origin of the heterogeneity in all but fraction I is not difficult to understand. With MetMb spread over a large section of the column, it would be extremely difficult to get elution of homogeneous MetMb fractions. That distinct (but heterogeneous) MetMb fractions are eluted from CM-cellulose columns may be due to a poor pH gradient. Even though a linear pH gradient was attempt, the pH measurements in Figs. 2, 3, and 4 show some distinct pH steps, with an actual decrease during elution of fraction I. The decrease in pH during elution of fraction I is presumably due to the reverse of the process that causes the rise in pH when fraction I was adsorbed. The origin of the other discontinuities in pH is not known, but abrupt pH changes could cause the appearance of artifactual fractions. An abrupt change in pH after elution of the main MetMb fraction I is sufficient to cause the appearance of a new MetMb fraction following I, but with a pH gradient, no fractions have been observed following fraction I.

With MetMb spread over the column, any stepped elution of the column would be certain to elute MetMb fractions that would be heterogeneous upon rechromatography. The 11 fractions of sperm whale MetMb found by ATASSI[1] may be in this category. ATASSI[1] interpreted the heterogeneity upon rechromatography as evidence for an interconversion of the fractions, but the same result would be obtained if the original fractions were heterogeneous.

We are uncertain about the applicability of these results to other proteins, but,

in any chromatography experiment in which discrete but heterogeneous fractions are obtained from CM-cellulose, the possibility of a pH change in the buffer causing the anomalous results should be explored.

Our results do not help to explain the actual reasons for microheterogeneity in purified myoglobin samples. The fractions obtained from CM-cellulose, however, have been difficult to understand in relation to the fractions obtained by gel electrophoresis. Our results are helpful in understanding why the CM-cellulose fractions are heterogeneous on gel electrophoresis.

ACKNOWLEDGEMENTS

Journal Paper No. J-6038 of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa, Project No. 1549; Center for Agriculture and Economic Development cooperating. This investigation was supported in part by research grant UI-00272-06 and training grant UI-01043-02 from the National Center for Urban and Industrial Health, U.S. Public Health Service.

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CHROM. 3727

THE IDENTIFICATION OF THE FREE AMINO ACIDS PRESENT IN SOME GRASS POLLENS BY THIN-LAYER CHROMATOGRAPHY*

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(Received August 5th, 1968)

SUMMARY

The free amino acid content of aqueous extracts (preserved in 50 % glycerol) of eleven grass pollens (*Festuca pratensis* Huds., *Lolium perenne* L., *Poa trivialis* L., *Dactylis glomerata* L., *Cynosurus cristatus* L., *Anthoxanthum odoratum* L., *Arrhenatherum elatius* (L.) J. & C. Presl, *Holcus lanatus* L., *Agrostis tenuis* Sibth., *Phleum pratense* L., *Alopecurus pratensis* L.) has been investigated using a thin-layer chromatographic technique. α-Alanine, γ-amino-*n*-butyric acid, glycine, DL-leucine, L-lysine, L-proline and DL-serine were identified. No species differences were detected and there was no difference between the viable and non-viable pollen extracts examined.

INTRODUCTION

All the common amino acids have been reported in pollen either bound to the protein or free[1]. While there are numerous references to the amino acid content of dicotyledonous[1–5], gymnospermous[6] and monocotyledonous[7–10] pollens there are few specific references to grass pollens.

AUGUSTIN[11] examined the amino acid pattern of whole extracts of *Phleum pratense* L. and *Dactylis glomerata* L. pollens and ultra-filtrates (using collodion membranes); both were reported to give identical amino acid patterns but it is difficult to interpret the chromatograms presented. The eluted base line fraction (allergenically active) on hydrolysis yielded cystine, lysine or arginine, histidine, aspartic acid, glycine or serine, threonine or glutamic acid, alanine, proline, tyrosine, valine, methionine, tryptophan, phenylalanine and the leucine group.

Four grass pollens (*Festuca rubra* L. subsp. *commutata* Gaud., *Dactylis glomerata* L., *Lolium perenne* L. and *Phleum pratense* L.) assayed microbiologically for their free or bound amino acids showed wide variation[12], the free amino acids identified being proline, alanine, glycine, serine and valine. However, SYNGE[13] reports that it is

* This work forms part of a thesis submitted by G. H. J. for a Ph. D. degree of the University of London, May, 1967.

unwise to use a microbiological assay with unhydrolysed plant extracts since the bound amino acids can influence the behaviour of the micro-organism used.

EXPERIMENTAL

Pollens from the following tribes of the family *Gramineae* were investigated:

Festuceae:	*Festuca pratensis* Huds.	(Meadow fescue)
	Lolium perenne L.	(Perennial rye-grass)
	Poa trivialis L.	(Rough meadow-grass)
	Dactylis glomerata L.	(Cocksfoot)
	Cynosurus cristatus L.	(Crested dog's-tail)
Aveneae:	*Arrhenatherum elatius* (L.) J. & C. Presl	(Tall or false oat grass)
	Holcus lanatus L.	(Yorkshire fog)
	Anthoxanthum odoratum L.	(Sweet vernal-grass)
Agrostideae:	*Agrostis tenuis* Sibth.	(Common bent or brown top)
	Phleum pratense L.	(Timothy)
	Alopecurus pratensis L.	(Meadow or common fox-tail)

The maturing culms were collected and, as soon as possible, the cut ends were placed in long metal troughs containing water. The troughs had sloping sides to allow the flowering heads to hang beyond the edge of the trough and drop their pollen on strips of black, glazed paper. The pollens were shed about 8.30–9.30 a.m. with the exception of *H. lanatus*, which shed its pollen about 4 p.m.

Extraction procedure

The pollen sample (2.0 g) was extracted with successive quantities of petroleum ether (60/80) until the solvent was colourless, filtered, the residue extracted with 50 % glycerol (25 ml) by shaking, at room temperature, on a mechanical shaker for 4 h and filtered.

Viable pollen. The pollens were extracted within 2 h[14] of being shed from the rips anthers.

Non-viable pollen. The pollens were stored (two–four days) at room temperature over silica gel and extracted when no tube growth was observed in the germination test[14].

Thin-layer chromatography

Essential details are given in Table I.

RESULTS AND DISCUSSION

The difficulties encountered in the resolution of amino acids in pollen extracts preserved in 50 % glycerol and methods for the identification of amino acids in the presence of glycerol based on the patterns obtained by plotting hR_F values as a function of the pH of the layer or as a function of the sequence of the solvent system used have been reported[16,17]. The latter method has been used in the identification of the free amino acids present in grass pollen extracts. hR_F values of the ninhydrin-

TABLE I

SUMMARY OF EXPERIMENTAL PROCEDURE FOR THIN-LAYER CHROMATOGRAPHY

Adsorbent:	Silica Gel G (Merck), 250 μ, air dried overnight.
Solvent systems:	(I) 96% Ethanol–water (70:30, v/v). (II) Phenol–water (75:25, w/w; 20 mg NaCN added per 100 g mixture). (III) *n*-Butanol–glacial acetic acid–water (80:20:20, v/v). (IV) 96% Ethanol–water–diethylamine (70:29:1, v/v).
Method:	Ascending, in saturated chamber; 20–22°; 15 cm.
Load:	Pollen extracts (preserved in 50% glycerol), 2 μl. Reference amino acids, 2 μl (1% solutions of the reference compounds dissolved in 50% glycerol).
Detection:	The plates were dried at 110° for 10 min and sprayed with modified ninhydrin reagent[15]. After spraying, the plates were further heated to give optimum colour development of the amino acid spots. The position of the glycerol was readily distinguished as a whitish zone on a pinkish-buff background on prolonged heating of the sprayed plate at 110°.

reacting components resolved in solvent systems I–IV are given in Table II and specimen chromatograms are illustrated in Fig. 1.

Examination of the chromatograms shown in Fig. 1 shows that there are seven ninhydrin-reacting spots resolved in solvent systems I and IV while six are apparently resolved in solvent systems II and III. However, the pink spot with an hR_F value of 23 and 22 in solvent systems II and III respectively, in some instances showed a decided "waist" and these spots were subsequently each resolved into two. Since the free amino acids were similar in all the species examined, both in the viable and non-viable samples, the extracts from *Dactylis glomerata* L. and *Phleum pratense* L. pollens were selected for further investigation in order to identify the amino acids present.

Using the mean hR_F values (shown in Table II) obtained in the four solvent systems the patterns for the unknown amino acids were built up (Fig. 2). After plotting the hR_F values against the solvent system sequence there was no difficulty in joining up the appropriate points for the orange, yellow and pinkish-purple spots. The diagrams so obtained were then traced on tracing paper, using the same scale as for the known amino acids (patterns for 24 amino acids previously published[17]) and then fitted into these patterns. The orange spot corresponded with the shape for glycine, the yellow spot with L-proline and the slow moving pinkish-purple spot with L-lysine monohydrochloride (Fig. 3). Some difficulty, however, was experienced in joining up the points obtained for the four pink coloured spots. By plotting these points on tracing paper and superimposing on the patterns obtained for the known amino acids[17] the following was deduced:

α-Alanine corresponded with points (2) or (3), (2) or (3), (1) or (2) and (2) or (3) in solvent systems I, II, III and IV, respectively.

γ-Amino-*n*-butyric acid corresponded with points (1), (2) or (3), (2) or (3) and (1) in solvent systems I, II, III and IV, respectively.

DL-Serine corresponded with points (2) or (3), (1), (1) or (2) and (2) or (3) in solvent systems I, II, III and IV, respectively.

TABLE II

MEAN hR_F VALUES OF THE NINHYDRIN-REACTING COMPONENTS RESOLVED IN AQUEOUS EXTRACTS OF SOME GRASS POLLENS PRESERVED IN 50% GLYCEROL

V = Extract from viable pollen; N = extract from non-viable pollen.

Pollen extract		*Mean[a] hR_F values in solvent system I*						
		Yellow[b]	*Orange[b]*	*Pinkish-purple[b]*	*Pink[b]—in order of increasing hR_F value*			
					1	*2*	*3*	*4*
Anthoxanthum odoratum L.	V	30	37	3	25	41	48	53
	N	27	34	3	25	40	46	52
Poa trivialis L.	V	29	35	3	27	40	45	50
	N	30	34	2	26	41	45	49
Dactylis glomerata L.	V	30	35	2	26	39	46	49
	N	29	35	3	26	39	44	54
Lolium perenne L.	V	29	34	3	25	40	47	52
	N	28	35	3	25	41	45	50
Alopecurus pratensis L.	V	30	36	2	29	40	44	51
	N	31	34	3	29	42	44	50
Festuca pratensis Huds.	V	31	37	2	29	43	44	51
	N	30	39	2	28	40	45	54
Cynosurus cristatus L.	V	29	36	2	29	42	44	50
	N	30	36	2	28	39	47	49
Arrhenatherum elatius	V	32	34	3	28	40	47	49
(L.) J. & C. Presl	N	30	35	2	27	42	44	52
Holcus lanatus L.	V	31	37	2	28	43	43	49
	N	30	37	2	29	42	43	50
Agrostis tenuis Sibth.	V	32	36	2	29	42	43	50
	N	32	35	2	29	43	44	51
Phleum pratense L.	V	37	34	2	27	42	45	54
	N	35	36	2	29	42	45	52
Mean hR_F values		31	36	2	27	41	45	51

Pollen extract		*Mean[a] hR_F values in solvent system II*						
		Yellow[b]	*Orange[b]*	*Pinkish-purple[b]*	*Pink[b]—in order of increasing hR_F value*			
					1	*2*	*3*	*4*
Anthoxanthum odoratum L.	V	42	21	7	16	25	27	52
	N	42	21	7	16	25	27	51
Poa trivialis L.	V	40	20	7	16	24	26	51
	N	43	21	7	16	25	26	52
Dactylis glomerata L.	V	42	21	8	15	25	26	50
	N	42	20	8	16	26	26	51
Lolium perenne L.	V	42	20	7	15	24	26	52
	N	42	20	7	15	25	26	52
Alopecurus pratensis L.	V	44	21	7	15	24	26	54
	N	42	21	7	15	25	25	54
Festuca pratensis Huds.	V	43	21	7	16	25	25	53
	N	42	20	7	16	25	25	52
Cynosurus cristatus L.	V	42	20	8	17	25	25	53
	N	42	20	8	15	26	26	53
Arrhenatherum elatius	V	42	20	7	17	24	25	52
(L.) J. & C. Presl	N	42	20	7	16	24	25	52
Holcus lanatus L.	V	40	20	7	16	25	26	51
	N	40	21	7	16	25	27	50
Agrostis tenuis Sibth.	V	42	20	7	16	25	27	52
	N	42	20	7	16	25	27	53
Phleum pratense L.	V	43	20	8	15	25	27	54
	N	43	20	8	15	26	27	52
Mean hR_F values		42	20	7	16	25	26	52

(*continued on p. 261*)

TABLE II (*continued*)

Pollen extract		*Mean*[a] *hR_F values in solvent system III*						
		Yellow[b]	*Orange*[b]	*Pinkish-purple*[b]	*Pink*[b]*—in order of increasing hR_F value*			
					1	*2*	*3*	*4*
Anthoxanthum odoratum L.	V	12	17	3	18	20	24	40
	N	13	17	3	19	22	23	40
Poa trivialis L.	V	13	17	3	19	22	24	41
	N	13	18	4	19	23	24	40
Dactylis glomerata L.	V	14	18	4	19	22	24	42
	N	14	17	3	18	22	24	40
Lolium perenne L.	V	13	18	3	18	21	25	42
	N	13	18	3	18	21	24	42
Alopecurus pratensis L.	V	12	17	3	18	21	23	42
	N	13	19	3	18	21	24	41
Festuca pratensis Huds.	V	14	19	4	19	21	24	42
	N	14	18	4	19	21	24	41
Cynosurus cristatus L.	V	14	19	4	19	22	24	41
	N	13	19	4	19	21	24	40
Arrhenatherum elatius	V	12	17	4	18	22	24	42
(L.) J. & C. Presl	N	13	18	4	18	22	25	42
Holcus lanatus L.	V	13	18	4	17	22	25	41
	N	13	18	4	18	21	23	40
Agrostis tenuis Sibth.	V	12	18	4	18	21	24	40
	N	13	17	4	17	21	24	41
Phleum pratense L.	V	13	18	4	18	21	24	40
	N	14	18	4	18	21	24	40
Mean hR_F values		13	18	4	18	21	24	41

Pollen extract		*Mean*[a] *hR_F values in solvent system IV*						
		Yellow[b]	*Orange*[b]	*Pinkish-purple*[b]	*Pink*[b]*—in order of increasing hR_F value*			
					1	*2*	*3*	*4*
Anthoxanthum odoratum L.	V	25	30	6	23	38	42	68
	N	25	30	7	24	28	42	70
Poa trivialis L.	V	24	30	7	24	39	41	69
	N	23	30	7	23	38	42	68
Dactylis glomerata L.	V	25	32	7	24	40	43	68
	N	25	33	6	24	40	44	68
Lolium perenne L.	V	24	32	7	24	38	44	68
	N	24	32	7	23	38	44	69
Alopecurus pratensis L.	V	24	31	7	24	39	44	69
	N	24	30	7	24	39	43	69
Festuca pratensis Huds.	V	23	31	7	24	39	44	68
	N	24	31	6	23	39	43	70
Cynosurus cristatus L.	V	24	30	7	23	39	43	70
	N	24	30	7	24	38	43	68
Arrhenatherum elatius	V	25	30	7	23	39	43	68
(L.) J. & C. Presl	N	24	31	7	22	38	42	69
Holcus lanatus L.	V	25	30	6	23	38	42	69
	N	25	30	6	23	38	41	69
Agrostis tenuis Sibth.	V	23	31	7	22	38	41	68
	N	23	31	7	22	38	41	68
Phleum pratense L.	V	24	32	7	21	39	42	70
	N	24	31	7	22	38	44	70
Mean hR_F values		24	31	7	23	39	43	69

[a] These figures represent the mean of four replicates with the exception of *Dactylis glomerata* L. (viable and non-viable) and *Phleum pratense* L. (viable and non-viable) extracts on which twenty-five replicates were performed.

[b] Colour of spot after heating.

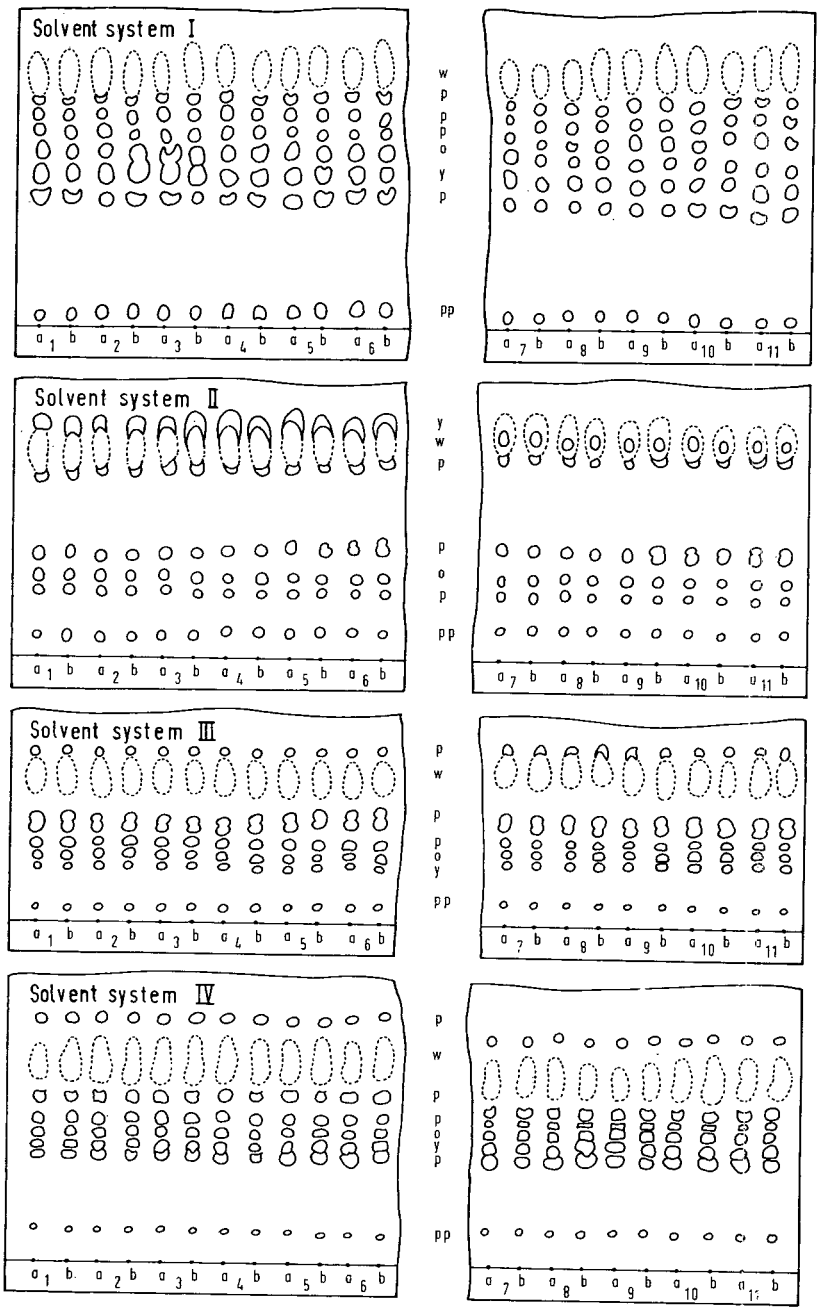

Fig. 1. Tracings of chromatograms of pollen extracts (preserved in 50% glycerol). Layer: silica gel, air dried. Distance: 15 cm. (– – – –) Glycerol; (——) amino acid. The colours obtained on heating the plate, after spraying with ninhydrin reagent[15] are the following: o = orange; p = pink; pp = pinkish-purple; w = white (glycerol); y = yellow. 1 = *Anthoxanthum odoratum* L.; 2 = *Poa trivialis* L.; 3 = *Dactylis glomerata* L.; 4 = *Lolium perenne* L.; 5 = *Alopecurus pratensis* L.; 6 = *Festuca pratensis* Huds.; 7 = *Cynosurus cristatus* L.; 8 = *Arrhenatherum elatus* (L.) J. & C. Presl; 9 = *Holcus lanatus* L.; 10 = *Agrostis tenuis* Sibth.; 11 = *Phleum pratense* L. a = Extract from viable pollen; b = extract from non-viable pollen. Solvent systems: (I) 96% ethanol–water (70:30, v/v); (II) phenol–water (75:25, w/w; 20 mg NaCN added per 100 g mixture); (III) *n*-butanol–glacial acetic acid–water (80:20:20, v/v); (IV) 96% ethanol–water–diethylamine (70:29:1, v/v).

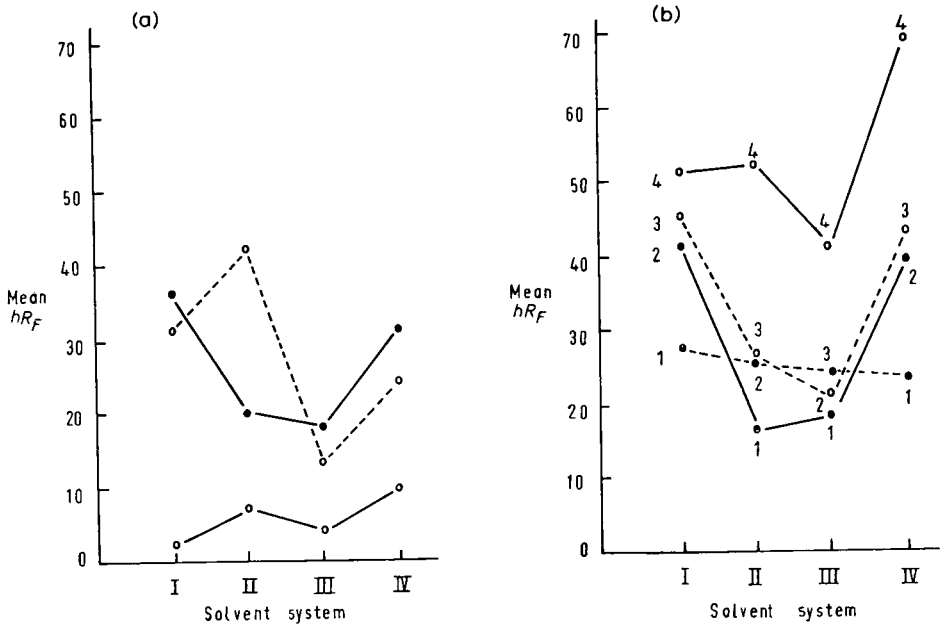

Fig. 2. Mean hR_F values, plotted as a function of the solvent system sequence, for the ninhydrin-reacting components resolved in aqueous extracts of some grass pollens preserved in 50% glycerol. Layer: silica gel, air dried. Distance: 15 cm. (a) Components yielding orange (●——●), pinkish-purple (○——○), and yellow (○- - - -○) spots. (b) Components yeilding pink spots: (○——○) pattern for DL-leucine, (○- - - -○) possible pattern for α-alanine, (●——●) possible pattern for DL-serine, (●- - - -●) possible pattern for γ-amino-*n*-butyric acid. Solvent systems: (I) 96% ethanol–water (70:30, v/v); (II) phenol–water (75:25, w/w; 20 mg NaCN added per 100 g mixture; (III) *n*-butanol–glacial acetic acid–water (80:20:20, v/v); (IV) 96% ethanol–water–diethylamine (70:29:1, v/v).

DL-Leucine corresponded with points (4), (4), (4) and (4) in solvent systems I, II, III and IV, respectively.

(The numbers in parentheses appear against the appropriate point in Fig. 2.)

From this information it was concluded that DL-leucine was almost certainly one of the components in the grass pollen extracts and that α-alanine, γ-amino-*n*-butyric acid and DL-serine accounted for the remaining three (Fig. 3), although with the latter it was not possible to decide precisely which points had to be joined up to get the true pattern. However, by running the *Dactylis* and *Phleum* viable and non-viable pollen extracts, a known mixture (prepared in 50% glycerol) containing glycine, L-proline, L-lysine monohydrochloride, α-alanine, γ-amino-*n*-butyric acid, DL-serine and DL-leucine and the individual amino acids in the mixture, also dissolved in 50% glycerol, on the same plate in the four solvent systems a clear picture was obtained. Fig. 4 shows that the position of the various spots in the grass pollen extracts corresponded both in rate of movement and colour on heating, after spraying with the ninhydrin reagent, with those of the single amino acids mentioned and when they were in admixture. It was concluded, therefore, that the free amino acids present in both viable and non-viable extracts of the pollen of all the species examined were α-alanine, γ-amino-*n*-butyric acid, glycine, DL-leucine, L-lysine, L-proline and DL-serine.

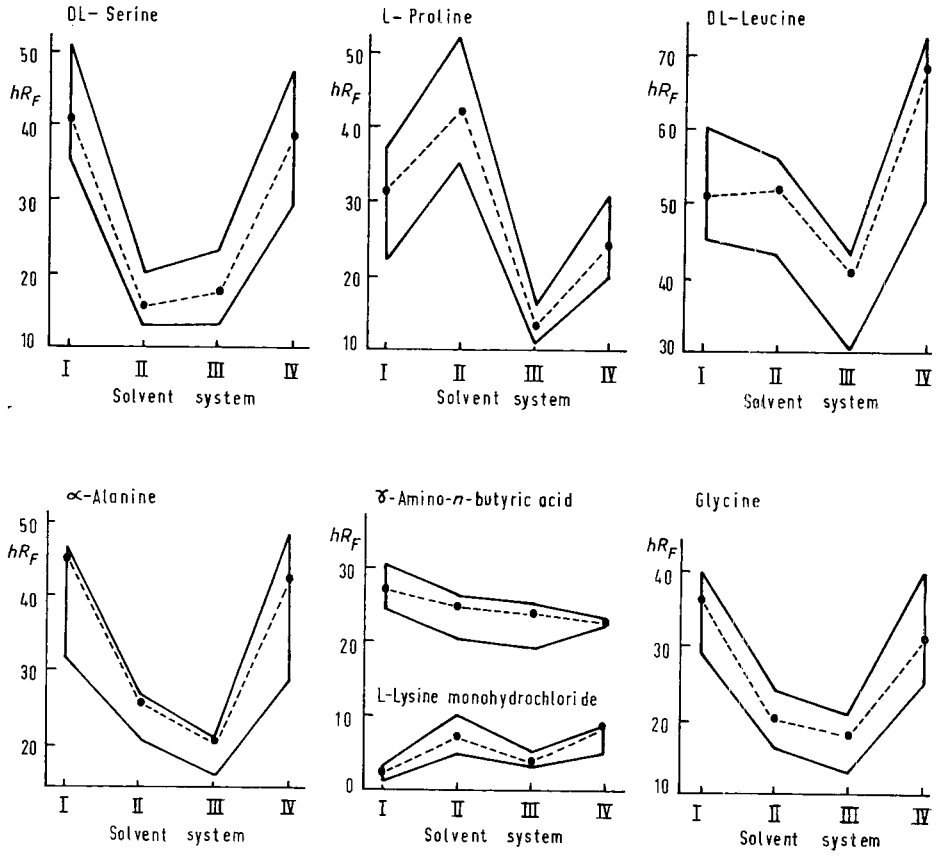

Fig. 3. hR_F patterns for the amino acids resolved in aqueous extracts of some grass pollens. Solvent systems: (I) 96% ethanol–water (70:30, v/v); (II) phenol–water (75:25, w/w; 20 mg NaCN added per 100 g mixture); (III) *n*-butanol–glacial acetic acid–water (80:20:20, v/v); (IV) 96% ethanol–water–diethylamine (70:29:1, v/v).

The presence of γ-amino-*n*-butyric acid is of great interest since it has not previously been reported in any pollen. However, since SYNGE[13] observed that γ-amino-*n*-butyric acid had been found chromatographically in nearly all plant tissues and often represented a substantial fraction of the non-protein nitrogen, the presence of this amino acid in grass pollen is not surprising. Although BATHURST[12], using a microbiological assay technique, reported free valine in four grass pollens, no trace of valine was found in the extracts examined in the course of this work.

With respect to the free amino acid content of the grass pollens examined no species differences were detected and there was no difference between viable and non-viable pollen extracts.

ACKNOWLEDGEMENT

Grateful thanks are expressed to Dr. F. H. MILNER of Beecham Research Laboratories, Allergy Division, for his generous gift of pollens and grasses.

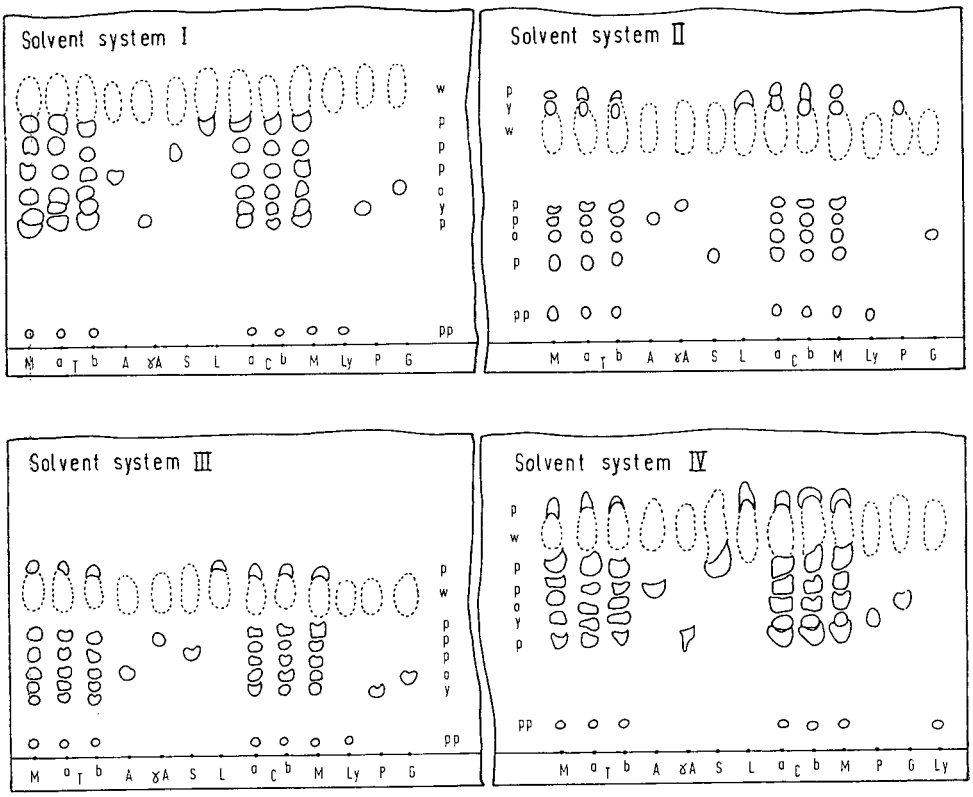

Fig. 4. Tracings of chromatograms of some amino acids and grass pollen extracts preserved in 50% glycerol. Layer: silica gel, air dried. Distance: 15 cm. (– – –) Glycerol; (——) amino acid. The colours obtained on heating the plate, after spraying with ninhydrin reagent[15] are the following: o = orange; p = pink; pp = pinkish-purple; w = white (glycerol); y = yellow. A = α-Alanine; γA = γ-amino-*n*-butyric acid; S = DL-serine; L = DL-leucine; Ly = L-lysine monohydrochloride; P = L-proline; G = glycine; M = mixture of the reference amino acids. All the amino acids were dissolved in 50% glycerol. T = *Phleum pratense* L.; C = *Dactylis glomerata* L. a = Extract from viable pollen; b = extract from non-viable pollen. Solvent systems: (I) 96% ethanol–water (70:30, v/v); (II) phenol–water (75:25, w/w, 20 mg NaCN added per 100 g mixture); (III) *n*-butanol–glacial acetic acid–water (80:20:20, v/v); (IV) 96% ethanol–water–diethylamine (70:29:1, v/v).

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CHROM. 3761

TWO-DIMENSIONAL REACTION THIN-LAYER CHROMATOGRAPHY IN THE ANALYSIS OF MIXTURES OF ALKENYL ACYL-, ALKYL ACYL- AND DIACYL CHOLINE PHOSPHATIDES

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(Received August 26th, 1968)

SUMMARY

Micro-samples of mixtures of alkenyl acyl-, alkyl acyl- and diacyl choline phosphatides, after their conversion into three families of diglyceride acetates by subjecting them to phospholipase C action followed by acetylation, were analyzed by a two-dimensional reaction thin-layer chromatographic procedure which permitted a quantitative determination of the three analogs, their individual fatty acid compositions and fatty aldehyde composition of the alkenyl acyl analog.

INTRODUCTION

Because alkenyl acyl-[1] and alkyl acyl phosphatides[2] accompany the diacyl phosphatides[3] ubiquitously in nature, the problem of their separation from one another has plagued researchers[4]. Even now, a quantitative separation of these analogs from one another in *Native Form* has not been described. However, their quantitative conversion to diglyceride acetates by enzymatic cleavage with phospholipase C followed by acetylation with pyridine and acetic anhydride[5] has enabled us today at least to separate them quantitatively in a non-polar derivatized form from one another. Thus the lipophilic components of the individual phosphatide analogs can be characterized by their suitable derivatization followed by gas chromatographic analysis[6]. However, the present available methods require larger quantities of samples, more time and more chemical steps before the final characterization of the individual phosphatide analogs is achieved. This paper describes a two-dimensional reaction thin-layer chromatographic procedure by which a microsample of a mixture of three families of diglyceride acetates derived from alkenyl acyl-, alkyl acyl-, and diacyl choline phosphatides has been analyzed for the contents of individual analogs, the fatty acid compositions of individual analogs, and the fatty aldehyde composition of the alkency acyl analog.

EXPERIMENTAL

The isolation of choline phosphatides from beef heart[7], preparation of Silica

Gel G plates[8], and the gas–liquid chromatographic analysis of fatty acid methyl esters and fatty aldehydes[9] was carried out as described previously[7–9]. A 2 *N*-sodium methoxide reagent in absolute methanol[10] was prepared by dissolving 4.6 g of clean metallic sodium in 100 ml of absolute methanol. All solvents used were of reagent grade.quality.

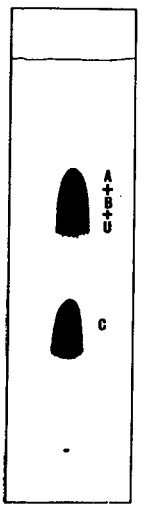

Fig. 1. Thin-layer chromatography of diglyceride acetates derived from choline phosphatide analogs of beef heart. Adsorbent: Silica Gel G. Activation: 1 h at 110°. Solvent system: Skelly F–diethyl ether (88:12). Spray reagent: Aqueous sulfuric acid (50%), plate charred at 160° for 10 min. Spots identification: (A) = Alkenyl acyl glyceryl acetate; (B) = alkyl acyl glyceryl acetate; (U) = unknown compound; (C) = diacyl glyceryl acetate.

The quantitative conversion of choline phosphatides from beef heart to diglyceride acetates (alkenyl acyl-, alkyl acyl- and diacyl glyceryl acetates) was carried out by enzymatic cleavage of the phosphatides with phospholipase C followed by acetylation with pyridine and acetic anhydride[5].

The separation of alkenyl acyl- (A) and alkyl acyl glyceryl acetates (B) with an unknown compound (U) as a group from the accompanying diacyl glyceryl acetates (C) was achieved on layers of Silica Gel G with Skelly F–diethyl ether (88:12) as the developing solvent (Fig. 1). The same system was useful in separating alkyl acyl glyceryl acetate (B), diacyl glyceryl acetates (C), 2-acyl, 3-acetyl glycerol (A′), free aldehyde (D), (the latter two products are formed during acid hydrolysis of alkenyl acyl glyceryl acetate) and an unknown compound (U) from one another (Fig. 2).

Two-dimensional thin-layer chromatography was carried out as follows: A microsample (250–1000 μg) of a mixture of diglyceride acetates derived from beef heart choline phosphatides was spotted as a chloroform-solution at A′, the lower left hand corner of a thin-layer plate (Fig. 3). The same amount was also spotted at the lower right hand corner of the same thin-layer plate. After removing the chloroform from these spots with a stream of dry nitrogen, the spots were exposed to fumes of concentrated hydrochloric acid for 2.5 min[8]. The excess of hydrochloric acid was removed from the plate by blowing with a stream of dry nitrogen and the plate was then developed with Skelly F–diethyl ether (88:12) to a height of 14 cm. The plate was

removed from the solvent and dried with a stream of dry nitrogen. A strip 4 cm wide at the right side of the plate (reference strip) was sprayed with a 5 % solution of iodine in chloroform which exhibited the positions of the liberated aldehydes (D), alkyl acyl glyceryl acetate (B), diacyl glyceryl acetate (C) and 2-acyl, 3-acetyl glycerol (A′) and the unknown compound (U). The position corresponding to the aldehydes (D) liberated from diglyceride acetates that were spotted at the lower left hand corner of the plate was scraped off with a razor blade, extracted with diethyl ether, and analyzed by gas chromatography[8]. A strip 4 cm wide at the left side of the plate was then spray-

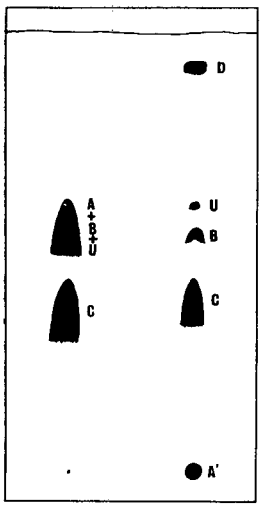

Fig. 2. Thin-layer chromatographic separation of diglyceride acetates derived from beef heart choline phosphatides (left) and their acid hydrolysis products (right). Adsorbent: Silica Gel G. Activation: 1 h at 110°. Solvent system: Skelly F–diethyl ether (88:12). Spray reagent: Aqueous sulfuric acid (50 %). Plate charred at 160° for 10 min. Spots identification: (D) = Fatty aldehydes; (U) = Unknown compound; (B) = akyl acyl glyceryl acetate; (C) = diacyl glyceryl acetate; (A′) = 2-Acyl, 3-acetyl glycerol.

ed with a solution of 2 *N* sodium methoxide in absolute methanol which almost instantaneously interesterified the fatty acid esters of alkyl acyl glyceryl acetates, diacyl glyceryl acetates and 2-acyl, 3-acetyl glycerol. The sprayed strip was dried with a stream of dry nitrogen for 10 min, after which appropriate quantities of an internal standard (methyl heneicosanoate) were spotted at locations corresponding to alkyl acyl glyceryl acetate, diacyl glyceryl acetate and 2-acyl, 3-acyl glycerol in the left hand strip. As a reference standard, methyl linoleate was spotted at the upper left hand corner of the plate (E). The plate was then turned counterclockwise through 90° and developed with toluene.

When the solvent front rose to a height of 14 cm, the plate was removed, dried with a stream of dry nitrogen and then a strip 4 cm wide on the left side of the plate (second dimension) was sprayed with 5 % iodine solution in chloroform. Thus having located the position of the reference methyl ester (ML_R) and reference alkyl acyl glyceryl acetate (B), diacyl glyceryl acetate (C) and 2-acyl, 3-acetyl glycerol (A′), the methyl esters (together with their internal standards) liberated by the latter com-

pounds from the left hand strip (first dimension) were located, scraped off, extracted with diethyl ether and analyzed by gas chromatography.

RESULTS AND DISCUSSION

The gas chromatographic analysis of methyl esters obtained from alkenyl acyl-, alkyl acyl- and diacyl phosphatides and the aldehydes obtained from alkenyl acyl phosphatides by the previous method[8] and the present method are given in Table I. The previous method did not separate the alkyl acyl- and diacyl phosphatides from

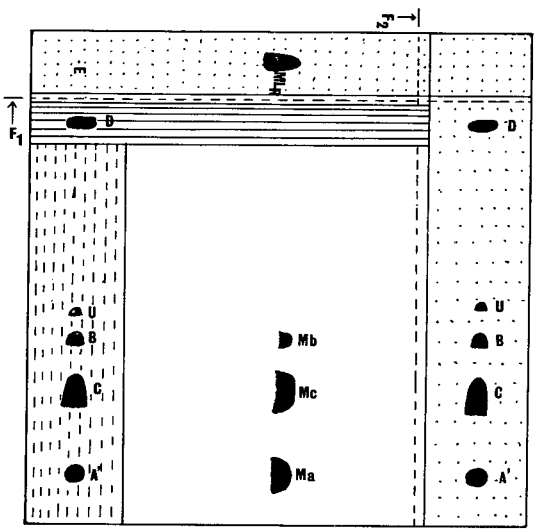

Fig. 3. Two-dimensional thin-layer chromatographic analysis of mixtures of alkenyl acyl-, alkyl acyl- and diacyl glyceryl acetates. Adsorbent: Silica Gel G. Activation: 1 h at 110°. Solvent system: 1st dimension, Skelly F–diethyl ether (88:12); 2nd dimension, toluene. Spray reagent: Aqueous sulfuric acid (50%), plate charred at 160° for 10 min. Spots identification: (A') = The location of original samples where the 2-acyl, 3-acetyl glycerol remains held after acid treatment of the diglyceride acetates followed by development with Skelly F–diethyl ether (88:12); (C) = diacyl glyceryl acetate; (B) = alkyl acyl glyceryl acetate; (U) unknown compound; (D) = aldehyde; (E) = location for spotting reference methyl linoleate. Abbreviations: Mb, Mc and Ma, fatty acid methyl esters derived from alkyl acyl-, diacyl- and 2-acyl glyceryl acetate, respectively; ML_R = reference methyl linoleate; F_1 = first solvent front; F_2 = second solvent front. ▒▒▒ = Zone of Silica Gel G scraped off for recovering the liberated aldehydes from alkenyl acyl glyceryl acetate; :::: = area sprayed with 5% iodine solution in chloroform; ||||||| = area sprayed with a 2 *N* solution of sodium methoxide in absolute methanol.

one another because of their stability toward fumes of concentrated hydrochloric acid under the experimental conditions. In the present method, the diglyceride acetates derived from diacyl phosphatides are separated as a class from the diglyceride acetates derived from alkenyl acyl- and alkyl acyl phosphatides which move together in the investigated system. But because of the susceptibility of alkenyl acyl glyceryl acetate alone to fumes of concentrated hydrochloric acid, these two could be separated from one another and also from diglyceride acetates derived from diacyl phosphatides. Thus the present method gives an added advantage over the previous method[8]. In this method also a prior isolation of individual phosphatide analogs as

diglyceride acetates is avoided, thus improving a method devised in another laboratory[6].

The results in Table I demonstrate a good agreement in the fatty acid composition of the alkenyl acyl phosphatides by the previous method[8] as well as by the present method. The same is true in the case of diacyl phosphatides because only

TABLE I

COMPOSITION OF LIPOPHILIC COMPONENTS OF ALKENYL ACYL-, ALKYL ACYL- AND DIACYL CHOLINE PHOSPHATIDES FROM BEEF HEART

Fatty acid composition of alkenyl acyl choline phosphatides (% of total fatty acids)		*Fatty acid composition of alkyl acyl choline phosphatides (% of total fatty acids)*		*Fatty acid composition of diacyl choline phosphatides (% of total fatty acids)*		*Fatty aldehyde composition of alkenyl acyl choline phosphatides (% of total fatty aldehydes)*	
	Previous method[8]	*Present method*	*Previous method*[8]	*Present method*	*Previous method*[8] (*includes the values of alkyl acyl analogs*)	*Present method*	*Previous method*[8]	*Present method*
C14:0	—	—	[a]	—	—	—	2.1	2.1
C15:0	—	—		—	—	—	2.1	3.5
C15:?	—	—		2.1	—	—	2.2	2.0
C16:0	1.6	1.8		4.7	32.4	33.3	64.9	65.0
C16:1	tr.	0.3		0.5	1.8	2.1	2.7	3.3
C17:?	—	—		—	—	—	5.4	5.0
C17:0	—	—		1.7	—	0.7	2.6	3.1
C18:0	1.2	0.9		3.7	10.8	12.2	13.5	12.2
C18:1	12.7	11.8		13.7	18.5	18.3	4.5	3.8
C18:2	58.5	59.3		50.0	30.2	27.1		
C18:3	1.2	1.3		2.6 (18:3 ?)	—	0.8		
C20:3	8.6	8.7		4.8	2.8	2.0		
C20:4	16.2	15.9		9.9	3.5	3.5		
C20:5				6.3	—	—		
C22:5				—				
C22:?				tr.				

[a] These cannot be determined separately. The values included with diacyl analogs.

4.25 % of alkyl acyl choline phosphatide occur in the original sample.

The figures in Table II show good agreement in the plasmalogen content of the original sample as determined by the previous method as well as by the present method. The slightly higher value obtained for the content of diacyl phosphatide by the previous method is explained by the inability of that method to distinguish between the diacyl- and alkyl acyl phosphatides. The content of the alkyl acyl phosphatide as determined by the fatty acid internal standard method (4.25) in the present studies is higher than that obtained by subtracting the diacyl values obtained by the two methods ($0.5(47.3 + 46.9) - 44.0 = 3.1$). This difference is understandable because of the low content of alkyl acyl phosphatides as well as the approximations used in these calculations.

The present technique of separating the diglyceride acetates derived from alkenyl acyl phosphatides from the diglyceride acetates derived from alkyl acyl phosphatides has advantages over the double development technique described by RENKONEN[7]. The present method requires only one development for separating these

analogs from one another and also liberates the aldehyde from the alkenyl acyl analog which also is separated from the rest of the compounds in the mixture. This separation aids easy characterization of the homologs and vinylogs of aldehydes.

In the previous method[8] from this laboratory the phosphatides were interesterified with the methanolic KOH reagent of KAUFMANN *et al.*[11]. This reagent could not

TABLE II

ALKENYL ACYL-, ALKYL ACYL- AND DIACYL CHOLINE PHOSPHATIDE CONTENTS OF BEEF HEART CHOLINE PHOSPHATIDE

Alkenyl acyl choline phosphatide content of beef heart choline phosphatide (%)			*Alkyl acyl choline phosphatide content of beef heart choline phosphatide* (%)		*Diacyl choline phosphatide content of beef heart choline phosphatide* (%)		
Previous method[8]		*Present method*	*Previous method*[8]	*Present method*	*Previous method*[8]		*Present method*
Fatty acid internal standard method	*Phosphorous determination method*	*Fatty acid internal standard method*		*Fatty acid internal standard method*	*Fatty acid internal standard method*	*Phosphorous determination method*	*Fatty acid internal standard method*
52.7	53.1	51.7	[a]	4.25	47.3[b]	46.9[b]	44.0

[a] This cannot be determined by this method. It is included in the value of diacyl analog.
[b] Includes the value of alkyl acyl analogs.

be used in the present method because of its inability to interesterify the triglyceride fatty acids. The recently reported method of OETTE AND DOSS[10] in which a 2 *N* solution of sodium methoxide in absolute methanol was found to interesterify directly the fatty acids of triglycerides on thin-layer plates was successfully used as described for interesterifying the fatty acids of alkenyl acyl-, alkyl acyl- and diacyl glyceryl acetates. The present method reported here fails only to convert microquantities of residual glyceryl ethers on the plate to suitable derivatives for gas chromatographic analysis of their molecular species, and as only about 20 μg of residual glyceryl ethers can be derived from 1000 μg of diglyceride acetates from the plate, their conversion to suitable derivatives by techniques other than reaction chromatography may very likely introduce too many uncertain factors to make the method dependable.

The availability of the unknown compound (U) in very small amounts precluded its further investigation.

Although the new method has been used in analyzing mixtures of analogs of choline phosphatides, its application to other phosphatides (ethanolamine-, serine- and inositol phosphatides) is possible, since they all can be converted to diglyceride acetates.

ACKNOWLEDGEMENTS

The authors are grateful to Mr. S. P. HOEVET for preparing chromatographically pure choline phosphatides from beef heart.

This investigation was supported in part by PHS Grant No. HE-02772 from the National Institutes of Health, Public Health Service.

NOTE ADDED IN PROOF

The unknown compound (U) has been tentatively identified as dialkyl glyceryl acetate on the basis of its chromatographic characteristics and non-liberation of long-chain fatty acid methyl esters or aldehydes on the TLC plate: This indicates indirectly the presence of dialkyl choline phosphatides in the original choline phosphatides of beef-heart.

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DÜNNSCHICHTCHROMATOGRAPHIE ISOMERER TRITERPENE AN ANASIL B UND KIESELGEL/$AgNO_3$

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(Eingegangen am 12. Juli 1968)

SUMMARY

Thin-layer chromatography of isomeric triterpenes on Anasil B and silica gel/$AgNO_3$

Application of the continuous development technique allows the separation of some isomeric pentacyclic triterpenes as their esters on Anasil B or silver nitrate impregnated silica gel thin layers. By chromatography on Anasil/$AgNO_3$ the most can be made of the advantages of both methods.

EINLEITUNG

In den vergangenen Jahren wurden die chromatographischen Trennmöglichkeiten von Sterinen und Triterpenen systematisch untersucht. Da sich beide Substanzklassen sehr ähnlich verhalten, konnten viele bei den Steroiden gewonnene Erkenntnisse direkt auf Trennprobleme der Triterpenoide übertragen werden. So erwies sich bei der Papierchromatographie der Sterine die "reversed-phase"-Technik als vorteilhaft[1,2]. NISHIOKA[3] und HASHIMOTO UND CHATANI[4] erreichten mit dieser Technik auch bei den Triterpenen gewisse Trennungen. Eine direkte Papierchromatographie von Triterpenoidsäuren wurde ebenfalls beschrieben[5]. Allerdings gelang es mit keiner dieser Methoden, Gemische von isomeren oder nahe verwandten Triterpenoiden zu trennen[6]. Systematische Untersuchungen von SNATZKE und Mitarbeitern über die Dünnschichtchromatographie an Kieselgel[6] und an mit Kieselgel oder Aluminiumoxid pigmentierten Papieren[7] ergaben eine bessere Trennung vieler Triterpenoide. Die Auftrennung von isomeren Triterpencarbonsäuren gelang an Ionenaustauscherpapieren[6] und an Kieselgur G[8]. Isomere tetracyclische Triterpene liessen sich teilweise als Epoxidacetate an Kieselgel-Dünnschichten trennen[9]. Ebenso wurde über die Trennung einiger tetracyclischer Triterpene an Kieselgel/$AgNO_3$ berichtet[10,11]. Viele Autoren geben an, dass sich isomere pentacyclische Triterpene nicht trennen liessen[5,6,12–15]; lediglich die Auftrennung der epimeren 3-Hydroxy-triterpene wurde bisher beschrieben[12].

Wir fanden, dass sich die Methode von SCHREIBER *et al.*[16–18] ebenfalls nicht zur

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Trennung von 4,4-Dimethylsterinen oder Triterpenen eignet: Cycloartenol und alle nachfolgend beschriebenen Triterpene hatten einen konstanten Platz im Chromatogramm. Die R_F-Werte lagen zwischen 0.25 und 0.34.

Obgleich dieses Problem durch die Möglichkeiten der Gaschromatographie weitgehend gelöst ist[19–22], besteht weiterhin das Bedürfnis einer dünnschichtchromatographischen Trennung solcher Substanzen.

EXPERIMENTELLER TEIL

*Anasil B-Schichten**

Ein Gemisch von 1 Teil Anasil B und 1.3 Teilen destilliertem Wasser wurde durch 10 min langes Schütteln suspendiert und mittels eines Giessgerätes (VEB Labortechnik Ilmenau) Glasplatten 20 × 20 cm oder 10 × 20 cm beschichtet. Die Schichtdicke betrug *ca.* 0.3 mm. Die Platten wurden über Nacht an der Luft getrocknet. Das Auftragen der Substanzen erfolgte aus 1 %-iger benzolischer Lösung mittels Glaskapillaren. (Bei den Sterinen verursachte eine geringe Überdosierung bereits einen weitgehenden Verlust der Trennschärfe; dies war bei der Chromatographie der Triterpenester weit weniger der Fall, so dass diese Aufgabetechnik völlig ausreichend war.) Am anderen Ende wurde ein Zellstoffpolster aufgebunden, dessen Ende man in die Atmosphäre führte. Als Entwicklergemisch wurden 97 ml Hexan mit 3 ml Diäthyläther gemischt und mit zwei Tropfen Wasser durchgeschüttelt. Mit der vorsichtig abgegossenen organischen Phase wurde der Tank vor der Chromatographie zwei Stunden gesättigt.

Kieselgel/$AgNO_3$-Schichten

Als Kieselgel wurde mit gleichem Erfolg Kieselgel D (VEB Chemiewerk Greiz-Dölau) und in einer Kugelmühle gemahlenes Kieselgel (VEB Laborchemie Apolda) benutzt. Bei letzterem wurden die durch ein Sieb 0.16 mm gehenden Anteile mit 8 % gebranntem Gips vermischt. Kieselgel und $AgNO_3$ wurden im Verhältnis 1:9 gewogen und 1 Teil dieses Gemisches in 1.7 Teilen Wasser suspendiert. Die Platten wurden ebenfalls mit einer Schichtdicke von 0.3 mm gegossen.

Als Lösungsmittel wurden bei der Chromatographie der Acetate Hexan–Benzol (2:1) und Benzoate Hexan–Benzol (4:1) gemischt und der Tank damit vorher zwei Stunden gesättigt.

Anasil B/$AgNO_3$-Schichten

Anasil B und $AgNO_3$ wurden im Verhältnis 99:1 eingewogen und 1 Teil dieses Gemisches in 1.3 Teilen destilliertem Wasser suspendiert. Es wurden ebenfalls Schichtdicken von 0.3 mm benutzt.

Detektion

Zur Detektion wurden die Platten auf 110° erwärmt und heiss mit einem Gemisch H_3PO_4–H_2SO_4 (1:1, v/v), welches 10 % Wasser und 2 % Molybdatophosphorsäure enthielt, besprüht und die Farbentwicklung beobachtet. Danach wurde 5 min auf 110° erhitzt.

* Anasil B ist ein grobkörniges, gebrannten Gips enthaltendes Kieselgel der Analytical Engineering Laboratories, Hamden, Conn., U.S.A.[33].

DÜNNSCHICHTCHROMATOGRAPHIE AN ANASIL B

Die hohe Selektivität der Anasil-Schichten, die die Trennung der Acetate von Stigmasterin, Cholesterin und β-Sitosterin[23,24] einerseits und die Trennung der Benzoate von β-Sitosterin, Campesterin, Cholesterin und Stigmasterin[25] andererseits ermöglicht, erwies sich auch bei der Chromatographie der im cubanischen Zuckerrohrwachs enthaltenen Triterpene als vorteilhaft[26].

Wir benutzten die von BENNETT UND HEFTMANN[23,24] angegebene Technik, wobei der Durchlaufeffekt durch ein am oberen Ende der Platte aufgebundenes Zellstoffpolster erreicht wurde, dessen Fortsetzung aus dem weitgehend abgeschlossenen Chromatographietank in die Atmosphäre reichte. Beim Einsatz der Triterpenacetate konnte das Lösungsmittelgemisch Hexan–Diäthyläther (97:3, v/v; wassergesättigt) verwendet werden[23,24,26]. Wegen der grösseren Wanderungsgeschwindigkeit der Triterpenbenzoate musste man bei deren Chromatographie den polaren Anteil des Systems weiter verringern. (Es erwies sich ein wassergesättigtes System Hexan plus 1 % Dioxan oder Äthylacetat als brauchbar.) Allerdings bietet der Einsatz der Triterpenbenzoate — im Gegensatz zu den Sterinen, bei denen erst durch den Einsatz der Benzoate die Trennung von β-Sitosterin und Campesterin möglich wurde — in der Regel keine qualitativ neuen Trennmöglichkeiten, so dass man sich auf die besser zugänglichen Acetate beschränken kann.

Da die Anasil-Schichten sehr schnellaufend sind, ist eine Durchlaufzeit von zwei bis drei Stunden ausreichend. Bekanntlich lässt sich die absolute Laufhöhe der Substanzen bei der Anwendung der Durchflusstechnik schlecht reproduzieren, da man die Vielzahl der Einflussgrössen über längere Zeiträume nicht genügend konstant halten kann. Man lässt deshalb stets einen bekannten Triterpenester mitlaufen. Zur zahlenmässigen Erfassung gibt man dann das Verhältnis der auf *einer* Platte gemessenen Laufstrecke zur Laufstrecke des entsprechenden β-Amyrinesters an (R_S-Wert). Es sei bemerkt, dass β-Amyrinacetat und β-Sitosterinbenzoat stets gleiche Laufhöhe zeigten, also den R_S-Wert 1.00 hatten (Fig. 1b).

Durch Zusammenfassung mehrerer Versuchsergebnisse, die unter unterschiedlichen, im Labor auftretenden Bedingungen (Temperatur, Laufzeit, Lösungsmittelsättigungen usw.) erhalten wurden, kann man die Reproduzierbarkeit der R_S-Werte mit 0.10 bis 0.15 angeben. Die Trennschärfe zur Bestimmung der Einheitlichkeit von Triterpen-Proben ist aber weitaus grösser; eine wirksame Unterstützung erfährt diese Prüfung durch die Unterschiedlichkeit in der Farbentwicklung, wenn man die heisse Platte mit dem Detektionsreagenz besprüht.

Diese Methode kann selbstverständlich auch zur Identifizierung benutzt werden. Wenn die Beschichtung der Platte genügend homogen war, wurden stets übereinstimmende R_S-Werte erhalten.

Wie aus der Tabelle I ersichtlich ist, sind mit dieser Methode Trennungen möglich, die man mit keiner der bisher in der Literatur beschriebenen Methoden erreichen kann. Sogar die homologen Verbindungen Gramisterin (I)* und Citrostadienol (II)* sind als Benzoate sicher trennbar. Andererseits lassen sich nicht alle untersuchten Triterpene auftrennen. So sind die Acetate von Cycloartenol (III), Simiarenol (IV) und

* Wir benutzten das aus *Saccharum officinarum* isolierte Gemisch von 24-Methylen-lophenol und 24-Äthyliden-lophenol; die Identität mit Gramisterin bzw. Citrostadienol (vergl. Lit. 32) war nicht bewiesen.

Taraxerol (V) praktisch ebensowenig trennbar wie die Triterpenmethyläther Arundoin (VI) und Crusgallin (VII).

Die Anwendung von Kieselgel D statt Anasil B führt zum vollständigen Verlust der Trennmöglichkeiten.

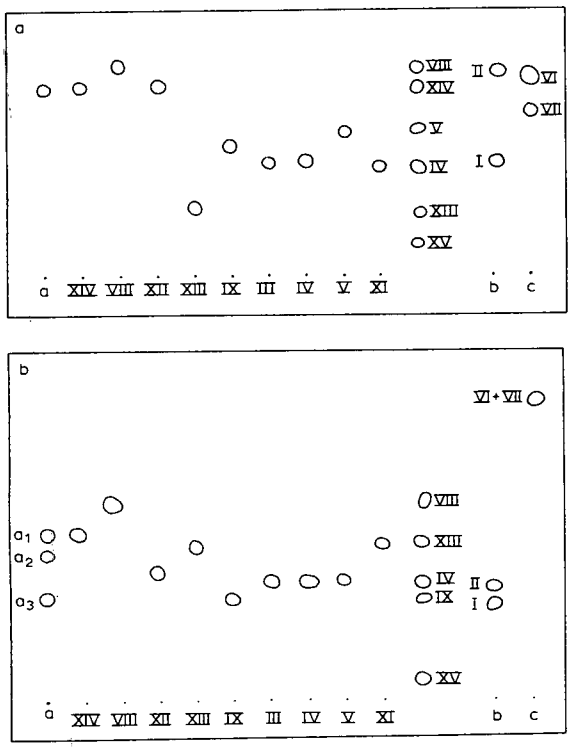


Fig. 1. (a) Dünnschichtchromatographie an Kieselgel/10% $AgNO_3$. Durchlauf: 2–3 Std.; Entwicklung mit Hexan–Benzol (2:1) (Acetate) oder 4:1 Hexan–Benzol (Benzoate). (b) Dünnschichtchromatographie an Anasil B. Durchlauf: 2–3 Std.; Entwicklung mit Hexan–Diäthyläther (97:3) (Acetate) oder Hexan–Dioxan (99:1) (Benzoate). a = Phytosterinbenzoat-Gemisch aus *Saccharum officinarum*[25]; a_1 = β-Sitosterylbenzoat; a_2 = Campesterylbenzoat; a_3 = Stigmasterylbenzoat; b = 4α-Methylsterinbenzoat-Gemisch aus *Saccharum officinarum*[26]; c = Triterpenmethyläther aus *Saccharum officinarum*[26]. Für weitere Bezeichnungen, siehe Tabelle I.

DÜNNSCHICHTCHROMATOGRAPHIE AN KIESELGEL/$AgNO_3$

Wir hatten bereits an anderer Stelle[6] die erfolgreiche Trennung von Gramisterin/Citrostadienol und Arundoin/Crusgallin an mit Silbernitrat imprägnierten Kieselgel-Schichten kurz erwähnt. Auch die Trennung der aus Zuckerrohrwachs isolierten Triterpene Simiarenol (IV), Taraxerol (V) und Fernenol (VIII) war möglich.

Eine systematische Untersuchung dieser Methode unter Einbeziehung weiterer Triterpenester sollte die Frage nach ihrer eventuell erweiterten Brauchbarkeit beantworten. Die Dünnschichtchromatographie an mit Silbernitrat imprägnierten Kieselgel-Schichten ist in den letzten Jahren intensiv bearbeitet worden[27–29]. Sie hat zur Lösung vieler Trennprobleme beigetragen.

TABELLE I

DÜNNSCHICHTCHROMATOGRAPHISCHES VERHALTEN EINIGER TRITERPENESTER (SOWIE METHYLÄTHER UND FRIEDELIN) RELATIV ZUM ENTSPRECHENDEN β-AMYRINESTER (R_S-WERTE)

	Anasil B		*Kieselgel/$AgNO_3$*	
	Acetate	*Benzoate*	*Acetate*	*Benzoate*
β-Amyrin (XIV)[a]	= 1	= 1	= 1	= 1
Gramisterin (I)	—	0.48	—	0.10
Citrostadienol (II)	—	0.66	—	0.36
Cycloartenol (III)[b]	0.94	1.08	0.66	0.55
Simiarenol (IV)	0.90	1.04	0.60	0.57
Taraxerol (V)	0.94	0.99	0.75	0.68
Fernenol (VIII)	1.56	1.56	1.07	1.02
Isoarborinol (IX)[c]	0.78	0.89	0.72	0.68
Taraxasterol (XI)[a]	1.29	1.14	0.60	0.46
Bauerenol (XII)[d]	1.39	1.33	1.00	0.91
Lupeol (XIII)[a]	1.26	1.17	0.45	0.38
Arundoin (VI)	1.98	1.89	1.94	1.05
Crusgallin (VII)	1.98	1.89	1.70	0.91
Friedelin (XV)[a]	0.18	0.21	0.18	0.13

[a] Erhalten von Dr. S. HUNECK.
[b] Erhalten von Prof. Dr. K. SCHREIBER.
[c] Erhalten von Miss Dr. W. H. HUI.
[d] Erhalten von Prof. Dr. J. HEROUT.

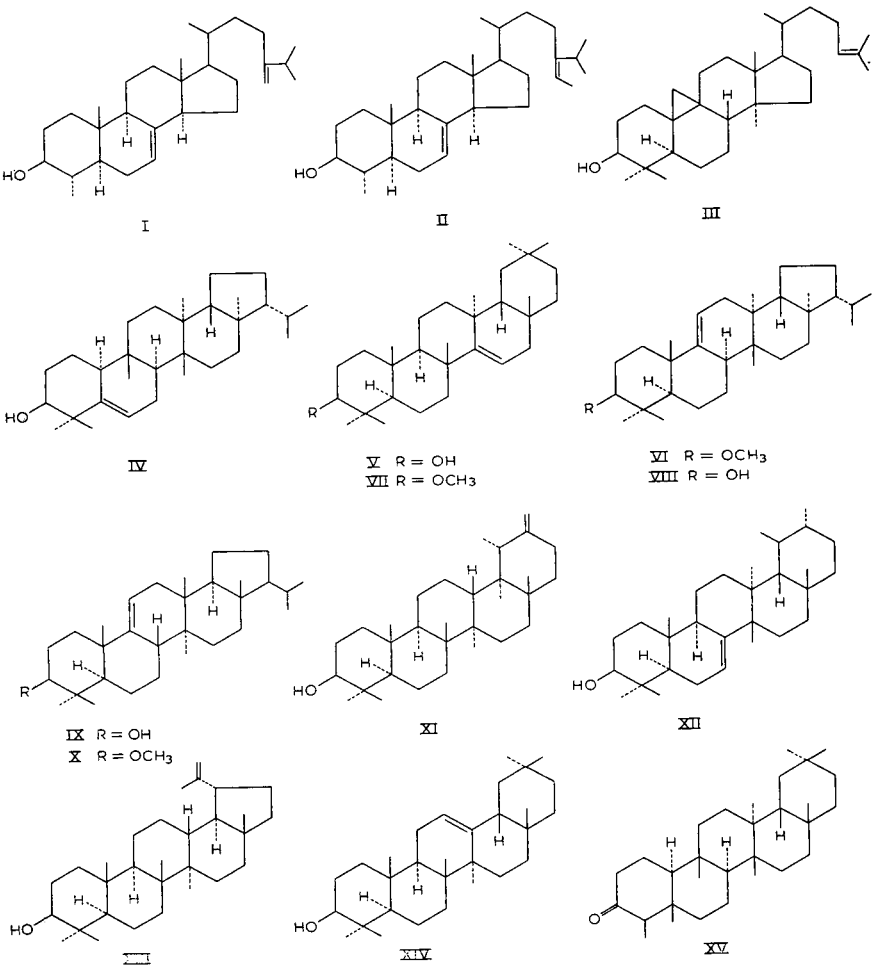
Das Prinzip beruht auf der Bildung von π-Komplexen des Silberions mit Doppelbindungen, wobei sich die Substanzen nach der Anzahl der Doppelbindungen oder nach der sterischen Anordnung der Umgebung derselben auf dem Chromatogramm anordnen. Sogar *cis/trans*-Isomere sind manchmal trennbar[30,31]. Diese Methode wurde bereits mit Erfolg zur Auftrennung einiger tetracyclischer Triterpene[10,11] angewandt. OHMOTO[32] berichtete über die Chromatographie von Fernenol (VIII)/Isoarborinol (IX) und deren Methyläther Arundoin (VI) und Cylindrin (X). Bei der Nacharbeitung ergab sich allerdings, dass die Flecken sehr nahe beieinander lagen, so dass eine Trennung nicht immer sicher erkannt werden konnte.

Wir zogen es deshalb vor, die Ester der Triterpene zu chromatographieren. Erste Versuche an Kieselgel/10 % $AgNO_3$ mit Hexan–Benzol-Gemischen verschiedener Zusammensetzung ergaben teilweise sehr gute Trennungen. Allerdings waren die Flecken sehr langgezogen. Diese "Schwänze" liessen sich weder durch Uneinheitlichkeit der aufgegebenen Substanzproben erklären, noch gelang ihre Unterdrückung, wenn man die aufgetragene Menge bis zur Grenze ihrer Nachweisbarkeit verringerte.

Eine Unterdrückung dieses Effekts konnte durch die Anwendung der Durchflusstechnik erreicht werden: Bei kaum erhöhter Wanderung wurden die Flecken klein und rund. Beim Einsatz der Acetate und Entwicklung mit Hexan–Benzol (2:1) genügten ebenfalls Durchlaufzeiten von zwei bis drei Stunden. Zur Chromatographie der Benzoate wurde ein System Hexan–Benzol (4:1) benutzt. Aber auch hier ergaben sich aus dem Einsatz der Triterpenbenzoate keine qualitativ neuen Trennmöglichkeiten.

In der Tabelle I sind die gefundenen Verhältnisse — wieder als R_S-Werte, bezogen auf den jeweiligen β-Amyrinester — aufgeführt.

Bei der Anwendung von Schichten, die nur 3 % $AgNO_3$ enthielten, liess die



I Gramisterinbenzoat
II Citrostadienolbenzoat
III Cycloartenolester
IV Simiarenolester
V Taraxerolester
VI Arundoin
VII Crusgallin
VIII Fernenolester
IX Isoarborinolester
X Cylindrin
XI Taraxasterolester
XII Bauerenolester
XIII Lupeolester
XIV β-Amyrinester
XV Friedelin

Trennschärfe nach: Eine Auftrennung der Acetate von β-Amyrin (XIV) und Taraxerol (V) war nicht mehr möglich.

Aus dem Vergleich beider Methoden kann man einige Erkenntnisse ableiten: Cycloartenol (III) und Simiarenol (IV) bilden auch hier ein kritisches Paar, von dem sich aber jetzt das Taraxasterol (XI) nicht mehr abtrennen lässt. Ebenfalls ist die Trennung von Fernenol (VIII) und Bauerenol (XII) nicht mehr sicher. Besonders gross ist die Differenz der Laufhöhe an den beiden Schichten bei Substanzen, die Methylengruppen enthalten (Lupeol (XIII), Taraxasterol (XI) und Gramisterin (I)). Die Chromatographie an Anasil B und Kieselgel/$AgNO_3$ kann damit Hinweise auf dieses Strukturelement liefern. Da die Anasil B-Schichten besonders auf den räum-

lichen Bau des Gesamtmoleküls ansprechen, bei der Chromatographie an Kieselgel/$AgNO_3$ dagegen geringe Polaritätsunterschiede, die sich aus der unterschiedlichen Lage der Doppelbindung ergeben, bestimmende Faktoren sind, wurde versucht, durch direkte Kombination aus der Überlagerung beider Effekte eine Erweiterung der Trennmöglichkeiten zu erreichen. Anasil B/10 % $AgNO_3$ verhielt sich wie Kieselgel/10 % $AgNO_3$, das heisst die Selektivität des Adsorbens war bereits verlorengegangen. Wenn man dem Anasil 1 % $AgNO_3$ beimischt und mit dem für Anasil angegebenen Lösungsmittelgemisch arbeitet, kann eine gewisse Überlagerung der oben angeführten Effekte erreicht werden. Diese Überlagerung führt aber dazu, dass die Flecken von Substanzen, deren Wanderungsgeschwindigkeit in beiden Systemen sehr unterschiedlich ist (Taraxasterol (XI), Lupeol (XIII)) sehr langgezogen sind. Immerhin war eine Auftrennung von Fernenol (VIII), Bauerenol (XII), β-Amyrin (XIV), Taraxerol (V) oder Simiarenol (IV), Isoarborinol (IX), Cycloartenol (III) und Friedelin (XV) möglich (siehe Fig. 2). Auch die Trennmöglichkeit der Benzoate von β-Sitosterin, Campesterin und Stigmasterin blieb erhalten.

Es muss erwähnt werden, dass diese Phytosterinbenzoate an Kieselgel/10 % $AgNO_3$ nicht auftrennbar waren. Sie zeigten dieselbe Laufhöhe wie der entsprechende β-Amyrinester im jeweiligen Lösungsmittelsystem.

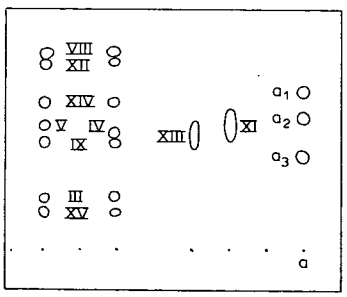


Fig. 2. Dünnschichtchromatographie der Triterpenacetate und Phytosterinbenzoate an Anasil B/1 % $AgNO_3$; Durchlauf: 3 Std.; Entwicklung mit Hexan–Diäthyläther (97:3). Bezeichnungen, siehe Fig. 1 und Tabelle I.

DANK

Den Herren Prof. Dr. E. HEFTMANN (Bethesda, Md.) und Prof. Dr. R. D. BENNETT (Pasadena, Calif.) sind wir für die freundliche Überlassung von Anasil B sehr verbunden.

Die in der Tabelle I bezeichneten Substanzen erhielten wir von Miss Dr. W. H. HUI (Hongkong), Prof. Dr. J. HEROUT (Prag), Prof. Dr. K. SCHREIBER (Gatersleben) und Dr. S. HUNECK (Tharandt), wofür wir an dieser Stelle bestens danken.

ZUSAMMENFASSUNG

Durch Anwendung der Durchflusstechnik gelingt es, eine Reihe isomerer pentacyclischer Triterpene als Ester an mit Anasil B bzw. mit Kieselgel/$AgNO_3$ beschichteten Platten zu trennen. Die Chromatographie an Anasil/$AgNO_3$ erbrachte eine teilweise Kombination der Vorteile beider Methoden.

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Short Communication

CHROM. 3791

Sandwiched capillary columns for gas chromatography

We use the term "sandwiched capillary columns" to describe columns made of any material, in which the fractionating medium is a thread inserted inside the capillary. Any material which is available in thread form may be used for this purpose; however, this research deals with glass capillaries in which carbon yarns have been sandwiched and with the evaluation of this new type of gas chromatographic column[1].

Experimental

A glass-drawing apparatus similar to the one developed by DESTY *et al.*[2] was modified to obtain glass capillaries of different internal diameters. A piece of carbon yarn was inserted into a length of glass tubing (2.2 mm I.D. and 2.6 mm O.D.); one end of the tube was heated until it was soft and pulled out to obtain an internal diameter of 0.4–0.5 mm, leaving the yarn hanging out. The stretched glass tubing was set into the apparatus and drawn out as usual (Fig. 1). While the spool of carbon yarn unwound continuously, the thread was sandwiched inside the glass capillary tubing. Columns of any length, in the form of spirals (diameter, 12 cm), may be obtained quite easily; their preparation is simple, and they give highly reproducible results, provided the surface of the yarn is not scratched when the glass tubing is drawn out.

Union Carbide carbon and graphite yarns designated WYB, WYD and VYB were used. WYB and WYD are two-ply yarns; they were washed several times with isopropyl alcohol to remove the lubricant and dried in an oven at 300°; they were unwound to obtain the one-ply yarn used in the preparation of these columns.

VYB, a one-ply yarn, was treated in the same way. The characteristics of these yarns, which consist of a bundle of filaments, are given in Table I.

The sandwiched columns were evaluated on a commercial apparatus (Fractovap., C. Erba, Milan), equipped with a flame ionization detector, by injecting a mixture of *n*-pentane, isopentane, and methane, using nitrogen as the elution gas; the volume of nitrogen used to elute methane was taken as the dead volume of the column.

Usually tailed peaks were obtained. However, if the eluting gas contained

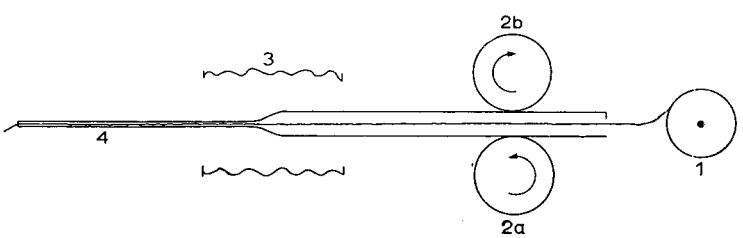

Fig. 1. Apparatus for the production of sandwiched columns. 1 = Spool of carbon yarn; 2 = feed rollers; 3 = electric furnace; 4 = carbon yarn sandwiched into glass capillary tubing.

TABLE I

CHARACTERISTICS OF CARBON AND GRAPHITE YARNS

Brand	*Type*	*Surface area*		*Filament size μ*
		m^2/g	m^2/m	
WYB	graphitized carbon black (2800°)	0.45	0.027	10
WYD	graphitized carbon black (2800°)	0.75	0.028	7.5
VYB	carbon fibres (1300°)	154	12.8	7.5

traces of compounds such as water or carbon disulphide, an improvement in the shape of the peaks was observed. As it has been found that also formic acid acts as an effective peak-tailing reducer, the elution gas was split into two streams, one of which was passed through a trap containing formic acid; the two streams were then combined. The temperature of the trap was changed in order to obtain symmetrical peaks; it was kept at 0° for a column temperature of 40°.

To obtain information on the behaviour of sandwiched columns, plots of the height equivalent to a theoretical plate *versus* velocity of the carrier gas were made for various hydrocarbons (Fig. 2). The minimum was obtained for quite a low velocity of the elution gas, and the efficiency of the column decreases with increasing flow rate. The values of the VAN DEEMTER equation of Fig. 2 are unsatisfactory, and occasionally negative values were obtained for the A term. The B and C values collected in Table II were obtained from the plot hU *versus* U^2 (Fig. 3), according to the equation:

$$h = \frac{B}{U} + CU$$

In the same table, experimental and calculated values for the minimum plate height, h, are reported.

Sandwiched columns differ from conventionally packed columns, where the adsorbing material is randomly distributed, and from porous layer columns, where the adsorption takes place on the walls. In sandwiched columns the adsorbing medium is inserted in the columns in an orderly fashion, and since it is made of a bundle of filaments, a sandwiched capillary column may be regarded as a set of equal micro-capillaries, so that the multiple path term of the VAN DEEMTER equation is missing.

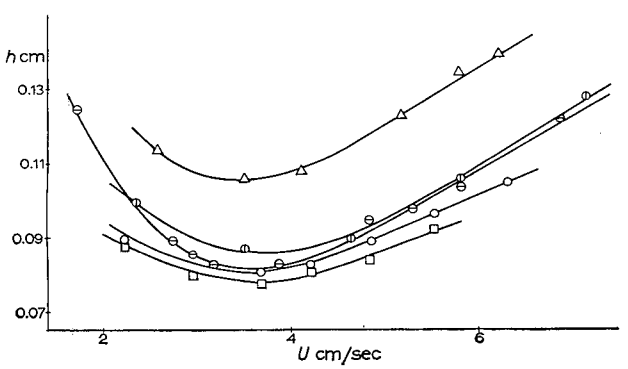

Fig. 2. Plots of HETP *vs.* linear velocity for various hydrocarbons on sandwiched columns of different internal diameters. □ = Isopentane, on 0.41 mm; ○ = pentane, on 0.41 mm; ⓘ = pentane, on 0.48 mm; ⊖ = pentane, on 0.44 mm; △ = heptane, on 0.41 mm.

TABLE II

VAN DEEMTER EQUATION CONSTANTS AND MINIMUM EXPERIMENTAL AND CALCULATED PLATE HEIGHT ON A WYD SANDWICHED COLUMN

	t (°C)	$I.D.$ (mm)	B (cm^2/sec)	C (sec)	h min (cm)	
					exptl.	calc.
Isopentane	40	0.41	0.145	0.0095	0.078	0.0075
Pentane	40	0.41	0.130	0.0123	0.080	0.080
Pentane	40	0.44	0.130	0.0154	0.082	0.088
Pentane	40	0.48	0.130	0.0145	0.086	0.087
Heptane	60	0.41	0.120	0.0199	0.105	0.098

The B term has twice the value of the interdiffusion coefficient in the mobile phase, D_g, reported in the literature, as in an open tubular column, the labyrinth factor is equal to unity.

An examination of the VAN DEEMTER constants calculated for columns which had a different diameter shows that a better performance is obtained when the internal diameter is smaller. The same conclusion is reached by determining the pressure drop, Δp, necessary to obtain a specific separation within a certain time. As may be seen by considering the separation of isopentane–pentane shown in Fig. 3, Δp was plotted *versus* the time of analysis to obtain a resolution equal to 1.5. A smaller pressure drop and a shorter analysis time were found for the column with a smaller diameter.

Other data (capacity ratio, K'; performance index, P.I.; permeability, P) obtained for the separation of isopentane–pentane in sandwiched columns of various diameters are collected in Table III.

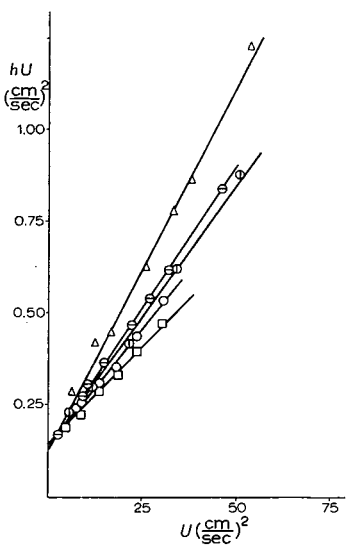

Fig. 3. Plot of hU *vs.* U^2 for the data of Fig. 2.

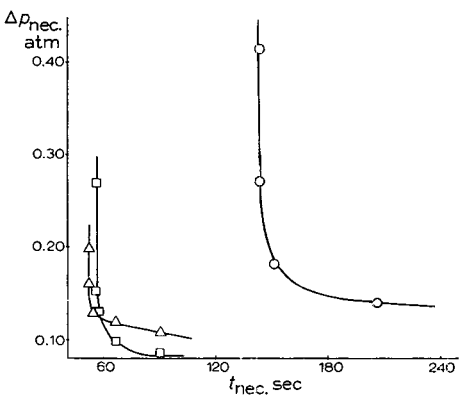

Fig. 4. Pressure drop *vs.* analysis time necessary to obtain complete resolution (R = 1.5) for a mixture of isopentane–pentane on columns having different internal diameters. ○ = 0.48 mm I.D.; □ = 0.44 mm I.D.; △ = 0.41 mm I.D.

TABLE III

COLUMN DATA AND PERFORMANCE INDICES AT 50°

Column		$\bar{K}$		*U min.* ($cm \cdot sec^{-1}$)	$P \cdot 10^7$ (cm^2)	*P.I.* (*poises*)
I.D. (*mm*)	*Length* (*m*)	*iso*-C_5	*n*-C_5			
0.48	6.0	0.21	0.3	3.6	27.9	55.4
0.44	4.3	0.49	0.8	3.2	10.5	88
0.41	4.8	0.51	0.83	3.5	8.3	91

TABLE IV

OPTIMAL COLUMN DATA AND PERFORMANCE PARAMETER

Length (*m*)	$Time_{nec.}$ (*sec*)	$\Delta p_{nec.}$ (*atm*)	*P.P.* (*atm/sec*)
6.4	143	0.271	38.7
1.5	57	0.133	7.55
1.2	54	0.129	6.9

The optimum values necessary to carry out the above separation ($R = 1.5$) are given in Table IV with the values of performance parameter, P.P., calculated according to the method of HALASZ[3].

These data clearly show that more satisfactory results in terms of column length, pressure drop and analysis time are obtained with the column of smaller diameter.

Specific gas permeability and other operational chromatographic parameters of sandwiched columns compare favourably with those of packed capillary columns.

Analytical applications

Although sandwiched columns are limited in use to materials available in

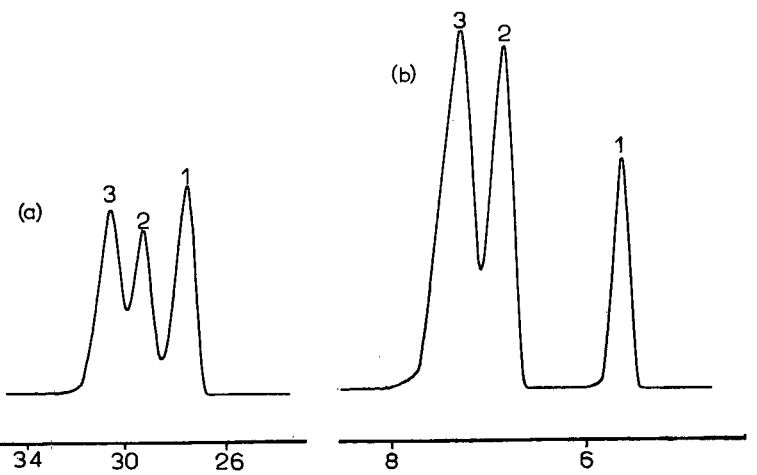

Fig. 5. Chromatogram of geometric isomers on a 10 m WYB sandwich column. (a) 1 = *m*-Xylene; 2 = *p*-xylene; 3 = *o*-xylene. Temperature = 124°; P_{N_2} = 0.8 atm. (b) 1 = *o*-Cresol; 2 = *m*-cresol; 3 = *p*-cresol. Temperature = 164°; P_{N_2} = 0.8 atm.

thread form, a large number of analytical applications may be expected, according to the specific nature of the adsorption material selected. The use of inorganic threads permits, one to operate at fairly high temperatures, and different results may be obtained if the surface area of the medium is modified. For example, gaseous hydrocarbons are separated above 100° on carbon yarn with a large surface area, whereas when the surface is small, separation occurs below room temperature.

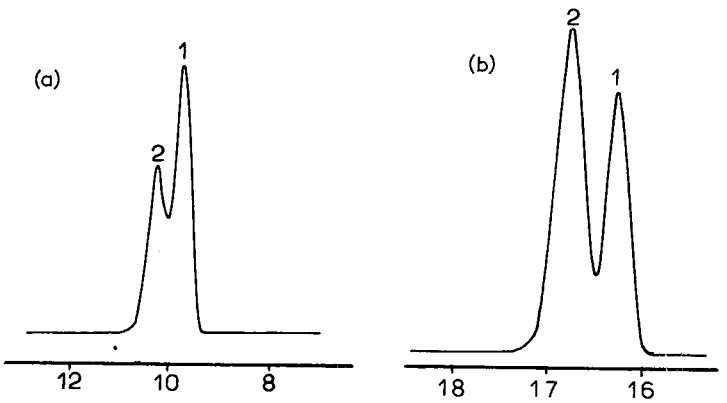

Fig. 6. Chromatogram of isotopic molecules. (a) 1 = d_6-Ethane; 2 = ethane. 10 m VYB sandwich column. Temperature = 115°; P_{N_2} = 1 atm. (b) 1 = d-cyclohexane; 2 = cyclohexane. 10 m WYB sandwich column; Temperature = 20°; P_{N_2} = 0.5 atm.

This research was limited to the use of graphitized carbon yarn and to carbon yarn, and examples of separations are described. The former is specifically used for the separation of polar compounds and geometric isomers, the latter, because of its high surface area, for the separation of permanent gases and volatile hydrocarbons at a fairly high temperature. Fig. 5 reports the separation of a mixture of geometric isomers on a WYB sandwich column. Fig. 6 shows the separation of isotopic pairs consisting of hydrocarbons and their deuterated homologues: ethane–deuteroethane and cyclohexane–deuterocyclohexane; the former is separated on a VYB (154 m^2/g) at 115°, and the latter on a WYB column (0.45 m^2/g) at 20°. One of the more interesting features of sandwiched columns is the reproducibility of results when columns of the same geometric properties are used.

Acknowledgement

The authors are indebted to the CNR (Consiglio Nazionale delle Recerche) for providing funds for this investigation.

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Received September 19th, 1968

Notes

CHROM. 3672

Improvement of the properties of the Teflon support

The effectiveness of a Teflon support is usually not lower than that of diatomite supports, but its mechanical properties are worse[1]. The properties of the proposed support "Polychrom"[2] made from polytetrafluoroethylene type 4D[3] (surface area 10 m^2/g, bulk weight 0.57 g/cm^3) and its effectiveness (height equivalent of the theoretical plate 1.5–2.5 mm) approach those of the Chromosorb T[4].

We have investigated two methods of improving the mechanical properties of the Teflon support without causing a decrease in the effectiveness of separation. The first method consists in carrying out an additional thermal treatment of the Teflon support; the second in joining the particles of the polytetrafluoroethylene by means of soluble fluorinated copolymers.

The study of the support was made on a chromatographic apparatus with a hot wire detector and U-type stainless steel columns 1.8 m long and 4 mm in inner diameter. The temperature of the column and the detector was maintained at 70°. The space velocity of the helium carrier gas was approximately 30 ml/min.

The thermal treatment of the support was carried out at various temperatures from 280 to 390° and with various heating periods from 5 to 30 min. After the thermal treatment the support readily turned into a powder without additional grinding and was then passed through a sieve. Fig. 1 shows the dependence of the height equivalent of the theoretical plate on the duration of the thermal treatment of the support at 300°. When the thermal treatment period was 10 min, the hardness of the support considerably improved[5]. After treatment, the specific surface area of the support was somewhat decreased while the bulk weight was increased. Apparently it took the first 7 min to heat the polymer through, and after this (from 10 to 15 min) the thermal treatment itself took place. The polytetrafluoroethylene particles were photographed under an electron microscope after various periods of thermal treatment. As may be seen from Fig. 2, the pores of randomly selected particles of polytetrafluoroethylene (size 1–2 microns) are mostly 0.1–0.2 microns in diameter (1000–2000 Å); this agrees well with the pore size determined by other methods. The particles of the Teflon support (25×10^5–50×10^5 Å) apparently consist of a large number of single particles joined together by the action of electrostatic forces and form large aggregates. As a result of the thermal treatment of the support, the single particles join together. In the process the number of pores decreases as Fig. 2 shows, whereupon the surface area of the support decreases while its bulk weight increases. The size of the pores remains practically unchanged (Fig. 2C).

The mechanical strength of the support is also increased when the particles of polytetrafluoroethylene are joined together by impregnating the support with an acetone solution of tetrafluoroethylene–vinylidene fluoride copolymer. The copolymer is deposited on the untreated Teflon powder by a method similar to impregnation of

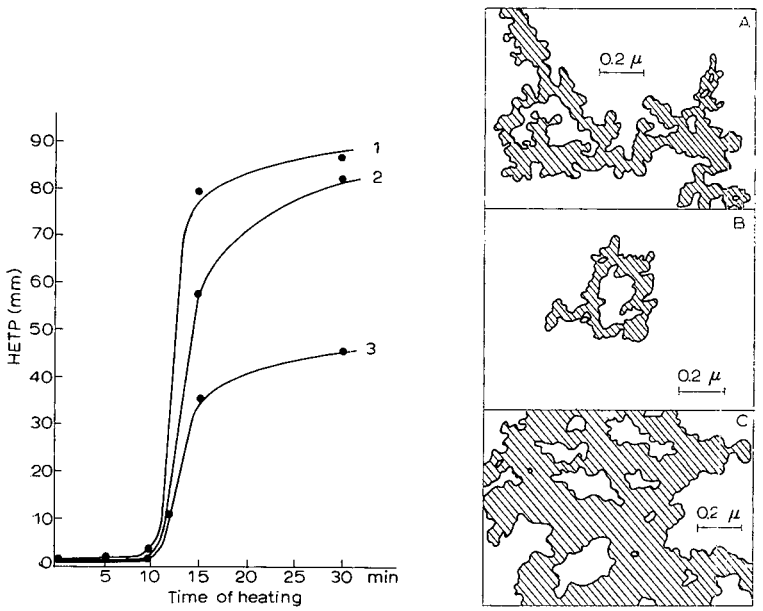

Fig. 1. Dependence of the height equivalent of the theoretical plate on the period of thermal treatment of the Teflon support at 300°. 1 = Benzene; 2 = toluene; 3 = chlorobenzene.

Fig. 2. Electron microscope photographs of Teflon particles of "Polychrom" type support at 300° and after different periods of thermal treatment. Magnification: 100,000 ×. (A) = 8 min; (B) = 10 min; (C) = 15 min.

the support with a stationary phase. The amounts of the deposited copolymer (based on weight % of the support) were 0.1, 0.5, 1, 3 and 5 %. The dependence of the specific surface area of the support on the amount of the copolymer deposited on the support is shown in Fig. 3. In the case of small amounts of copolymer the surface area of the Teflon support decreases more rapidly owing to preferential filling-in of the small pores[6]. The mechanical strength of the support after deposition of up to 1 % of the

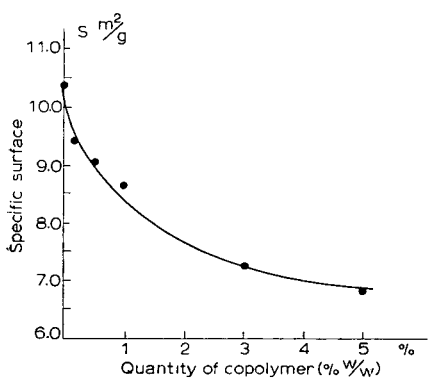

Fig. 3. Dependence of the specific surface area of the Teflon support on the amount of deposited copolymer (based on the weight % of the support).

copolymer remains practically unchanged, but it improves considerably when the amount of the copolymer exceeds 3 %.

Fig. 4 shows the differential dependence of the volume of the pores according to the *radii* after impregnation with the copolymer in amounts of 1 and 5 %. The total volume of the pores of the support decreases from 0.370 cm^3/g for 1 % of the copolymer to 0.243 cm^3/g for 5 % of the copolymer. Most pores of the support are from 500–2000 Å in dimension, the number of small pores (with diameters less than 300 Å) being few. As a result of filling of small pores with the acetone solution of the copolymer and subsequent formation of a copolymer film following the evaporation of the solvent, the single particles of the support are joined together, and their mechanical strength improves. With small amounts of the copolymer, the height equivalent of the theoretical plate first somewhat increases (for example, in the case of propanol, from 1.4 to 2 mm); at a concentration of 1–5 % it remains practically independent of the amount of the copolymer deposited on the support.

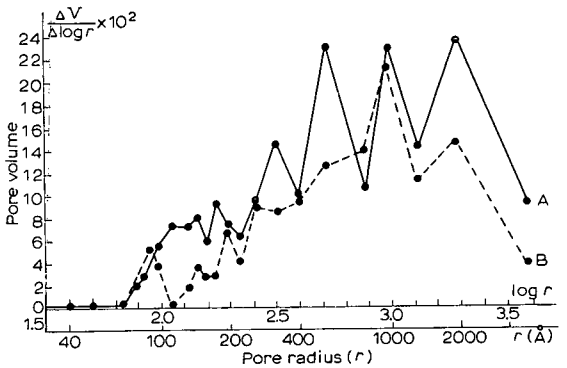

Fig. 4. Differential dependence on the distribution of the volume of the pores according to the *radii* for Teflon supports with 1 % (A) and 5 % (B) of copolymer.

The method described makes it possible to prepare Teflon supports of sufficiently high chromatographic effectiveness with mechanical strength approaching that of the usual diatomite supports of the Chromosorb W type.

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Received June 29th, 1968

CHROM. 3720

Comportement en chromatographie en phase gazeuse de quelques β-D glucopyranuronosides (tri-O-acétylés esters méthyliques) d'hydroxystéroïdes

Les stéroïdes éliminés dans les urines humaines le sont sous forme hydrosoluble, conjugués aux acides sulfurique et D-glucuronique sous forme d'hémi-ester sulfurique et de β-D glucopyranuronoside.

Jusqu'alors leur analyse et leur dosage ont été faits après coupure de la copule sulfurique ou glucuronique par procédé chimique ou enzymatique.

Les premières tentatives de dosage de ces entités conjuguées l'ont été par JAAKOMAKI *et al.*[1], VANDENHEUVEL[2] and HORNING *et al.*[3].

Cependant, les dérivés utilisés, esters méthyliques-triméthylsilyl éthers, ont été préparés ex-temporanément et ne sont pas décrits.

Nous nous sommes adressés aux β-D glucopyranuronosides (2,3,4-tri-O-acétylés, esters méthyliques) qui peuvent être préparés aisément par acétylation et méthylation des β-D glucuronosides libres[4], procédé que nous avons amélioré. Ces composés sont d'autre part des intermédiaires dans la synthèse organique.

La présente note rapporte leur comportement en chromatographie en phase gazeuse sur trois types de colonnes W98, OV.1, OV.17 à une température de 310°.

L'appareil utilisé est un Gas-Chromatograph F et M Scientific Hewlett-Packard, modèle 402.

Le détecteur est à ionisation de flamme, il a été maintenu à 320° durant les manipulations (chambre d'injection également à 320°). L'échelle de sensibilité adoptée 2 × 10 correspond à un courant de 8.10^{-11} A. La pression du gaz vecteur azote a été ajustée à 4 bars, son débit fixé à 46 ml/min.

Les trois colonnes de verre (en forme de U, préalablement traitées par le diméthyldichlorosilane) furent ainsi constituées:

(a) Phase stationnaire à 3.8 % de W98 (silicone) sur un support de Diatoport S 80–100 mesh. Longueur 1.2 m; diamètre intérieur 4 mm; environ 3000 plateaux théoriques.

(b) Phase stationaire à 4 % d'OV1 (polymère de méthylsiloxane) sur Aeropak 100–120 mesh. Longueur 1.8 m; diamètre intérieur 4 mm; environ 4000 plateaux théoriques.

(c) Phase stationnaire à 4 % d'OV.17 (polymère de méthylphénylsiloxane) sur Gas Chrom Q 100–120 mesh. Longueur 1.2 m; diamètre intérieur 4 mm; environ 2000 plateaux théoriques.

Les composés suivants ont été étudiés et sont affectés d'un chiffre, volontairement choisi en fonction de leur ordre d'élution:

(1) Oxo-17 androstane-5αH yl-3α (β-D tri-O-acétyl 2,3,4-glucopyranosiduronate de méthyle) "glucuronide d'androstérone Ac_3Me"; F = 176°–178°.

(2) Oxo-17 androstane-5βH yl-3α (β-D tri-O-acétyl 2,3,4-glucopyranosiduronate de méthyle) "glucuronide de 5β androstérone Ac_3Me"; F = 172°–175°.

(3) Oxo-17 androstane-5βH yl-3β(β-D tri-O-acétyl 2,3,4-glucopyranosiduronate de méthyle) "glucuronide de 5β épiandrostérone Ac_3Me"; F = 158°–160°.

(4) Oxo-17 androstane-5αH yl-3β(β-D tri-O-acétyl 2,3,4-glucopyranosiduronate de méthyle) "glucuronide d'épiandrostérone Ac_3Me"; F = 175°–178°.

(5) Oxo-17 androstène-5 yl-3β(β-D tri-O-acétyl 2,3,4-glucopyranosiduronate de méthyle) "glucuronide de déhydroépiandrostérone Ac_3Me"; F = 194–195°.

(6) Oxo-3 androstène-4 yl-17β(β-D tri-O-acétyl 2,3,4-glucopyranosiduronate de méthyle) "glucuronide de testostérone Ac_3Me"; F = 186°–188°.

(7) Acetoxy-3 oestra 1-3-5(10) triène yl-17β (β-D tri-O-acétyl 2,3,4-glucopyranosiduronate de méthyle) "glucuronide (17) d'oestradiol Ac_4Me"; F = 194°–196°.

Les six premiers composés de la série de l'androstane ont éte décrits par l'un de nous[5]; le septième, unique représentant oestrogène le sera prochainement[6].

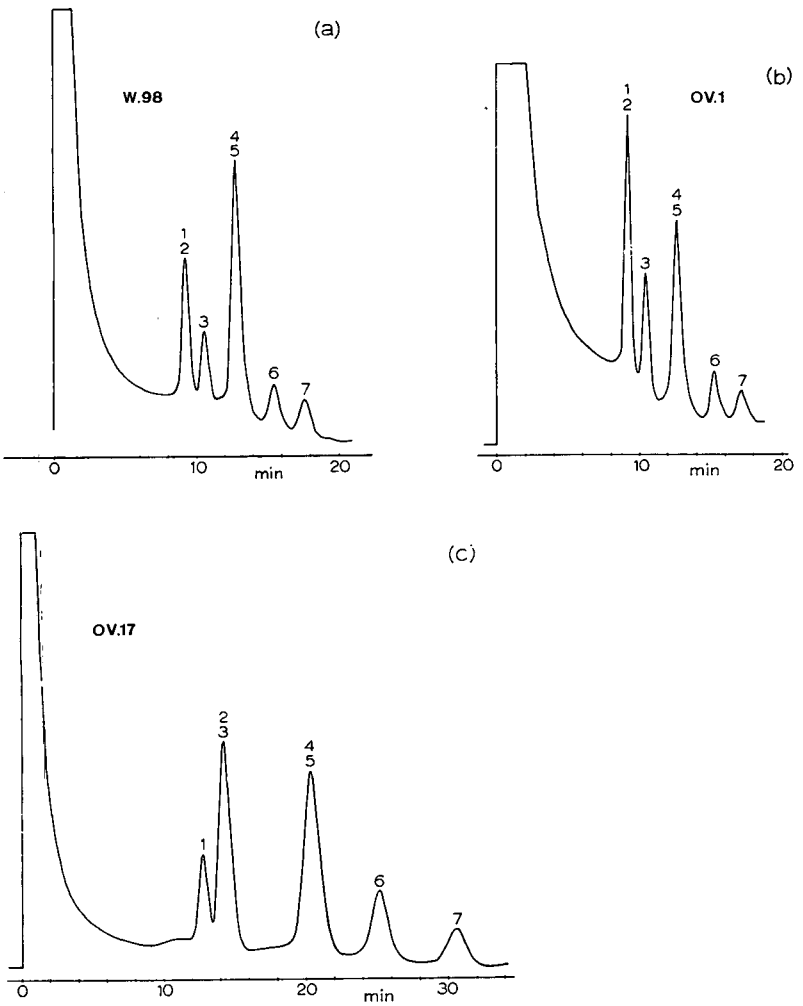

Fig. 1. Chromatogrammes d'un mélange de sept stéroïdes conjugués sur colonnes de W.98 (a) OV.1 (b) et OV.17 (c).

TABLEAU I

TEMPS DE RÉTENTION DES β-D GLUCURONOSIDES (TRI-O-ACÉTYLÉS, ESTERS MÉTHYLIQUES) D'HYDROXYSTÉROÏDES

Composée	*Colonne*					
	W.98		*OV.1*		*OV.17*	
	t	*s*	*t*	*s*	*t*	*s*
1	10	0.25	9.20	0.12	12.40	0.05
2	10	0.25	9.20	0.25	14.10	0.06
3	11	0.50	10.40	0.50	15.30	0.25
4	13.10	0.50	12.50	0.50	19.30	0.13
5	13.10	0.25	12.50	0.25	19.30	0.13
6	16	0.50	15.30	0.50	24.30	0.25
7	17.30	0.50	17.30	0.50	30.20	0.25

t = Temps de rétention en minutes secondes; s = quantité minimum dosée en μg.

Le Tableau I résume les résultats obtenus: mis à part les deux dérivés de l'épiandrostérone et de la déhydroépiandrostérone, on voit que ces composés présentent des temps de rétention différents.

Nous avons ensuite injecté le mélange de ces sept composés. Les chromatogrammes obtenus sont reproduits dans les clichés ci-contre où chaque pic a été identifié par les temps de rétention précédemment établis.

Aucune décomposition de ces produits n'a été observée; pour trois d'entre eux une opération conduite avec piégeage au sortir de la colonne a permis de les retrouver inaltérés, le contrôle étant effectué par chromatoplaque.

Cette étude a été entreprise dans un but de trouver une méthode d'analyse des stéroïdes conjugués qui éviterait l'hydrolyse préalable, hydrolyse qui peut n'être pas toujours reproductible.

Nous nous proposons d'évaluer l'intérêt de cette méthode dans l'analyse des stéroïdes urinaires.

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Reçu le 26 juillet, 1968.

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CHROM. 3766

A modified two-column procedure for the analysis of the basic amino acids found in elastin, collagen and antibiotics

The analysis of most protein hydrolysates can be accomplished satisfactorily using the accelerated two-column system[1,2]. However, resolution of the amino acids from hydrolysates of elastin, collagen, and various antibiotics, with their unusual complement of basic amino acids, cannot be achieved by this procedure. A modified system[3] devised for the analysis of amino acids found in plant hydrolysates likewise will not resolve all of the basic amino acids found in elastin and collagen. This system also suffers from the disadvantage of requiring a column and buffer other than those used in routine analysis. It was our intent to devise a rapid and simple method that could be used when necessary to resolve these unusual amino acids, and at the same time would utilize the same column and buffers as the standard procedure.

Methods

A Beckman model 116 amino acid analyser equipped with an automatic buffer-change module for both the long (acidic and neutral) and the short (basic) column was used for these experiments. The short column, 0.9 × 23 cm, was packed with PA-35 resin to a column height of 11 cm. Flow rates were 50 ml/h for the buffer and 20 ml/h for the ninhydrin reagent. Water jacket temperature was maintained at 55° throughout the run.

All reagents and buffers were prepared as described in the Beckman manual. Distilled water used in the preparation of the buffers was redistilled over sulfuric acid to remove ammonia. The short column was equilibrated with 0.20 *M* sodium

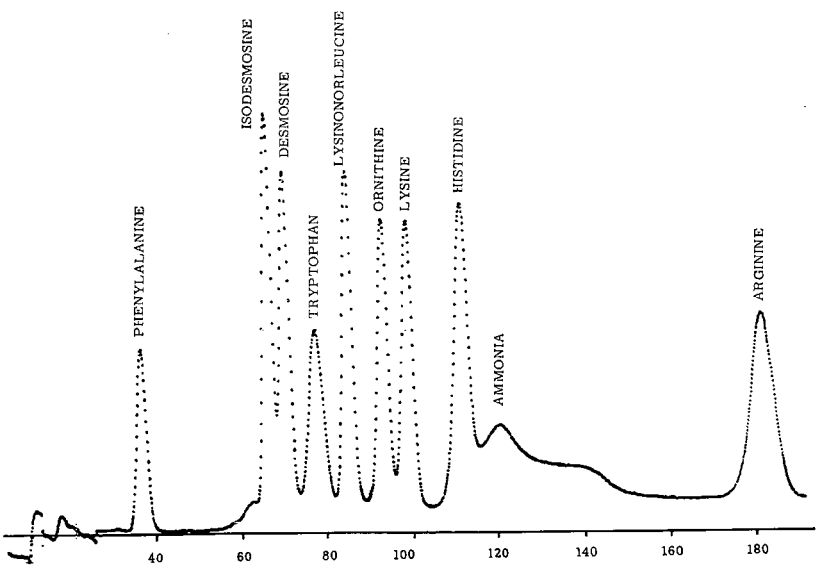

Fig. 1. Chromatogram of a synthetic mixture of basic amino acids using the procedure as described in the text.

citrate, pH 4.30, prior to the start of the run. Elution was started with this buffer and switched after 25 min to 0.35 *M* sodium citrate buffer, pH 5.25.

Results and discussion

Fig. 1 shows a chromatogram of a synthetic mixture of amino acids. Retention times for these and for other amino acids not shown on the chromatogram are listed in Table I. Although some of the amino acids listed in Table I have the same retention

TABLE I

PEAK ELUTION TIME OF BASIC AMINO ACIDS FROM A 0.9 × 11 CM PA-35 RESIN COLUMN

Amino acid	*Retention time (min)*
Tyrosine	33
Phenylalanine	37
Isodesmosine	66
γ-Amino butyric acid	66
Desmosine	70
Tryptophan	77
Merodesmosine	78
Hydroxylysine	83
Lysinonorleucine	84
Ornithine	93
Diamino butyric acid	98
Lysine	99
Diaminopropionic acid	107
Histidine	111
Ammonia	118
Arginine	182

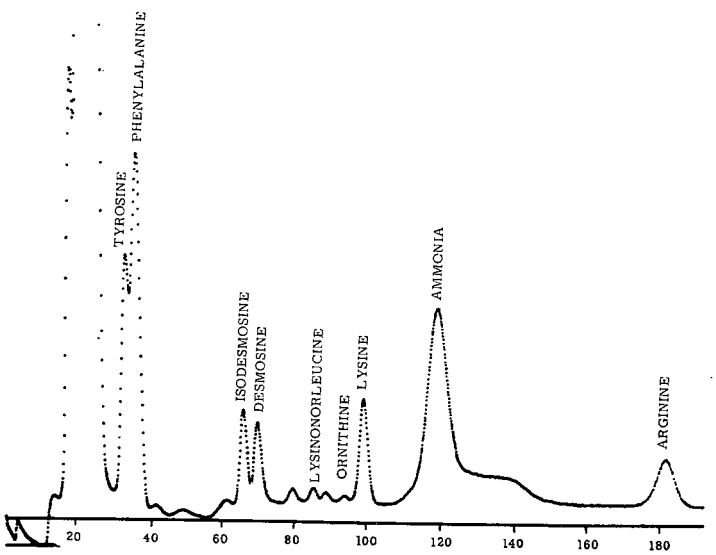

Fig. 2. Chromatogram of a 24-h hydrolysate of aortic elastin from 3 week old chicks. Procedure used is described in the text.

times, this has not created difficulties, since none of the proteins or antibiotics that we have encountered have contained two unresolvable amino acids.

Elastin has an unusual complement of basic amino acids, some of which are present in very small amounts. The only method of resolution of these amino acids has been a single-column gradient elution system[4]. Fig. 2 shows a chromatogram of the basic amino acids from a hydrolysate of purified chick aortic elastin, obtained with our modified two-column system. As can be seen in this chromatogram, the basic amino acids contained in the elastin hydrolysate have been well resolved. Further, because this system allows one to increase the size of the sample introduced onto the basic column without affecting the analysis of the acidics and neutrals, accurate measurement of the small basic peaks normally obtained becomes possible.

Increasing the length of the basic column to 11 cm increases the time required for an amino acid run using the standard procedure. Retention times in minutes for standard amino acids are: tryptophan, 31; lysine, 46; histidine, 55; ammonia, 68; and arginine, 104.

Difficulty may be encountered if the distilled water used in making the buffers is contaminated with ammonia. Such contamination will cause either a large-skewed ammonia peak or a dramatic increase in the baseline.

Acknowledgement

The author would like to thank Dr. CARL FRANZBLAU for the generous gift of lysinonorleucine.

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Received August 30th, 1968

CHROM. 3710

Chromatographie sur couches minces de quelques composés organiques du phosphore

La littérature signale un grand nombre de méthodes chromatographiques relatives aux composés phosphorés, notamment dans le domaine minéral. En ce qui concerne les dérivés organiques, ces méthodes sont peu nombreuses et s'adaptent principalement à des cas particuliers et non à un ensemble complet d'esters. LEW *et al.*[1], HIGGENS ET BALDWIN[2] et CESARANO ET LEPSCKY[3] ont réalisé la séparation sur colonne des mono- et dialkylorthophosphates. MOULE ET GREENFIELD[4], CASIDA ET PLAPP[5] et CRAMER[6] ont séparé quelques phosphates éthyliques par chromatographie sur papier. Des résultats plus intéressants ont été obtenus par MARKRYLOWA[7], PETSCHICK ET STEGER[8] sur les esters thiophosphoriques et par NEUBERT[9] sur les organophosphites. Cependant, aucune de ces méthodes ne permet la chromatographie simultanée de phosphites, phosphates, phosphonates et esters soufrés correspondants.

Partie expérimentale

La technique de base est celle développée par STAHL[10,11]. Nous n'y avons apporté que quelques modifications dans les détails pratiques.

L'équipement de base est celui de la Firme Desaga. Nous avons utilisé deux types d'adsorbants: le gel de silice "Kieselgel G Merck" et la poudre de cellulose "Powder Pleuger". Ces adsorbants sont déposés sous forme de couches de 0.275 mm d'épaisseur.

Les substances étudiées en solution dans un solvant volatil adéquat, entre 0.5 et 2 % suivant la teneur en phosphore et soufre du composé, sont déposées sous forme de taches de 3 mm de diamètre ou de bandes étroites de 2 mm de largeur. Le dépôt sous forme de bande permet la récupération de quantités plus importantes de produit en vue d'élution et d'analyses quantitatives ultérieures.

Les cinq solvants utilisés sont les suivants: (I) isopropanol–ammoniaque concentrée (75:25); (II) propanol normal–ammoniaque concentrée–eau (60:30:10); (III) acétone–eau–méthanol–acide trichloracétique (75:25:10), (100 mg); (IV) butanol tertiaire–acétonitrile–ammoniaque concentrée (50:40:10); (V) acétone–butanol tertiaire–eau–ammoniaque concentrée (50:40:10:10).

Les proportions données pour ces solvants sont relatives à des volumes. Les différents alcools utilisés, sauf l'alcool butylique tertiaire, ont été fraîchement distillés avant leur utilisation. L'alcool butylique tertiaire, l'acétone, l'acétonitrile et l'ammoniaque concentrée sont des produits "Merck" pour analyses.

Le réactif molybdique utilisé comme révélateur a la composition suivante pour un litre: 250 ml de molybdate d'ammonium en solution à 4 % dans l'eau; 20 ml d'acide chlorhydrique concentré; 50 ml d'acide perchlorique (70 %) et 680 ml d'eau distillée. Après vaporisation, les plaques sont portées 10 min aux températures de 80° pour les plaques de cellulose et 110° pour les plaques de silice. Les esters soufrés se révèlent immédiatement. Pour faire apparaître les esters non soufrés, la plaque doit être ensuite passée sous un courant d'hydrogène sulfuré.

Résultats

Les résultats sont donnés dans les Tableaux I et II. Dans le Tableau I, nous donnons les valeurs R_F d'esters éthyliques dont les groupements phosphorés sont de nature différente. Dans le Tableau II, nous signalons les valeurs R_F de divers composés phosphorés contenant des fonctions supplémentaires (amine, amide ou sulfure) et d'esters non-éthyliques.

TABLEAU I

VALEURS R_F DES DIFFÉRENTS COMPOSÉS ÉTHYLIQUES ANALYSÉS

	Couches de cellulose				*Couches de gel de silice*					
	Solvant (I)		*Solvant (II)*		*Solvant III*		*Solvant IV*		*Solvant V*	
	17°	25°	17°	25°	17°	25°	17°	25°	17°	25°
Esters phosphoriques										
Triéthylphosphate	Impossible à révéler				0.88	0.90			0.89	0.96
Diéthylphosphate	0.61	0.62	0.77	0.79	0.16	0.18			0.43	0.50
Monoéthylphosphate	0.09	0.10	0.32	0.34	0.00	0.00			0.03	0.06
Tétraéthylpyrophosphate	0.92	0.93	0.93	0.95	0.66	0.68			0.82	0.93
Diéthylpyrophosphate	0.36	0.38	0.60	0.61	0.23	0.23			0.54	0.60
Pentaéthyltripolyphosphate (?)									0.55	0.61
Esters thiophosphoriques										
S-éthyl-diéthylphosphate										0.90
Triéthylmonothiophosphate	Impossible à révéler						0.92	0.96	0.94	0.98
Diéthylmonothiophosphate		0.63		0.80			0.55	0.58	0.62	0.65
Monoéthylmonothiophosphate		0.13		0.38			0.05	0.05	0.06	0.08
Diéthyldithiophosphate		0.70		0.83				0.65	0.65	0.70
Tétraéthylmonothiopyrophosphate									0.85	0.94
Esters phosphoreux										
Triéthylphosphite							0.93	0.96	0.93	0.96
Diéthylphosphite							0.83	0.85	0.40	0.55
Monoéthylphosphite							0.08	0.08	0.08	0.10
Acide phosphoreux									0.00	0.00
Acides (ou sels minéraux)										
H_3PO_4 (Na_3PO_4)	0.02	0.03	0.15	0.17					0.00	0.00
$P_2O_7Na_4$	0.00	0.00	0.07	0.08					0.00	0.00
H_3PO_3									0.00	0.00
Dérivés phosphiniques										
Acide diéthylphosphinique										0.55
Acide diéthylthiophosphinique		0.83		0.84				0.82		0.84
		+		+				+		+
		0.63		0.70				0.62		0.72
Acide diéthyldithiophosphinique										0.75
Dérivés phosphoniques										
Éthyl-diéthylphosphonate										0.98
Tétraéthyléthylènediphosphonate										0.95

TABLEAU II

VALEURS R_F DE QUELQUES COMPOSÉS VARIÉS DU PHOSPHORE

Valeurs R_F obtenus avec le solvant (V) à 25°.

	Valeurs R_F
Sulfure de diéthylphosphine $(C_2H_5)_2P(S)H$	0.91
Disulfure de diéthylphosphine $[(C_2H_5)_2PS]_2$	0.95
Diéthylamidophosphate $(C_2H_5O)_2P(O)NH_2$	0.98
O,O-Di(2-aminoéthyl)dithiophosphate $(NH_2\text{-}CH_2\text{-}CH_2\text{-}O)_2P(S)SH$	0.44
Monobenzylphosphate	0.19
Dibenzylphosphate	0.58
Tribenzylphosphate	0.96
Triisoamylphosphate	0.93
Triphénylphosphite	0.98

Ces deux tableaux paraissent incomplets mais ils correspondent en fait à la sélection de solvants adaptés à des séparations particulières.

Les solvants (I) et (II), utilisés par CRAMER[6] pour la chromatographie sur papier permettent, sur couches minces de cellulose, de contrôler rapidement si les séparations obtenues sont suffisantes pour utiliser la radiochromatographie quantitative sur papier avec ces mêmes solvants.

Le solvant (III) n'est valable que pour la séparation des esters phosphoriques.

Le solvant (IV) est principalement intéressant pour les substances qui, comme les phosphites, sont sensibles à l'hydrolyse.

Le solvant (V) est le plus complet car il est valable avec tous les dérivés organiques du phosphore.

La chromatographie sur couches minces de gel de silice nous a permis de révéler les triesters phosphoriques et thiophosphoriques très résistants à l'hydrolyse. C'est le problème de la température, au moment de la révélation, qui a fait échouer toute chromatographie de ces esters sur papier ou sur couche mince de cellulose. La silice qui peut supporter des températures supérieures à 100° pendant des temps prolongés a permis la révélation de ces composés.

Applications

Notre but n'est pas de donner une table de valeurs R_F d'un certain nombre de composés phosphorés mais de montrer que la méthode est applicable à une grande variété de ces composés et de montrer également les possibilités ainsi offertes pour l'analyse de mélanges, la vérification de pureté de composés, la séparation d'isomères et même la récupération de produits.

Les résultats les plus intéressants ont été ceux obtenus sur les esters polyphosphoriques et les esters thiopolyphosphoriques. Nous avons montré[12] que, malgré des analyses élémentaires correctes et des analyses spectrales conformes aux données de la littérature, le pyrophosphate tétraéthylique (et son homologue soufré) n'étaient pas purs mais consistaient en des mélanges de trois composés que nous avons identifiés en tant que monophosphates di- et triéthyliques et pyrophosphate tétraéthylique (ou leurs homologues soufrés). Nos résultats semblent confirmer l'hypothèse, émise par VAN WAZER[13] à la suite d'études par résonance magnétique nucléaire, de l'existence de ces composés sous forme d'équilibres de réorganisation.

Nous avons mis en évidence, par chromatographie, la dégradation rapide du diéthyldithiophosphate en solution dans le tétrachlorure de carbone, l'acétone et le chloroforme. Les produits de dégradation n'ont pu encore être tous identifiés.

Nous avons également mis en évidence, par cette méthode, l'hydrolyse en solution aqueuse du diéthylphosphite avec formation de monoéthylphosphite et d'acide phosphoreux[14].

Quelques séparations d'isomères se sont révélées possibles avec les solvants utilisés. Les formes isomères "thiol" et "thiono" sont correctement séparées avec le solvant (V) (triéthylmonothiophosphate, R_F 0.98 et S-éthyldiéthylphosphate, R_F 0.90), tandis que les formes isomères phosphite–phosphonate sont moins nettement séparées (triéthylphosphite, R_F 0.96 et éthyldiéthylphosphonate, R_F 0.98).

Les modes de révélation des dérivés du phosphore sont nombreux[7–9] mais le réactif molybdique utilisé lors de nos travaux est intéressant pour plusieurs raisons. Ce révélateur est valable pour tous les composés contenant du phosphore. Il permet de distinguer un phosphite d'un phosphate et d'un thiophosphate et de différencier les différents groupements thiophosphorés (Tableau III). Il est encore plus intéressant avec les composés riches en carbone, comme les esters benzyliques, car la séparation est plus nette. Il est également valable pour les phosphates et thiophosphates minéraux. Enfin, il nécessite très peu de manipulations.

TABLEAU III

POSSIBILITÉS OFFERTES PAR L'UTILISATION DU RÉACTIF MOLYBDIQUE COMME RÉVÉLATEUR

Substance	*Vaporisation du réactif molybdique*		*Puis passage à H_2S*
	À froid	*À chaud*	
(I) $>P(=O)-O-$	Pas de révélation	Pas de révélation	Spot bleu (sur fond brun)
(II) $>P(=S)-O-$	Pas de révélation	Spot bleu (sur fond blanc)	Spot bleu (sur fond brun)
(III) $>P(=O)-S-$	Spot bleu (sur fond blanc)	Spot bleu (sur fond blanc)	Spot bleu (sur fond brun)
(IV) $>P(=S)-S-$	Spot rose (sur fond blanc)	Spot bleu (sur fond blanc)	Spot bleu (sur fond brun)

Conclusions

Nous pensons avoir mis au point une méthode valable de détection et de séparation des dérivés organiques du phosphore.

L'utilisation du gel de silice comme adsorbant permet des révélations à des températures supérieures à 100°.

Les cinq solvants mis au point pour le développement ne sont pas limitatifs mais leur variété permet l'adaptation de chacun à une séparation désirée.

L'utilisation du révélateur à base de molybdate d'ammonium permet la révélation de toutes les substances contenant du phosphore.

Nous avons également montré sur des exemples précis, à la suite d'expériences personnelles, les possibilités offertes par la chromatographie dans de nombreux cas: analyse d'un mélange, séparation de composés et d'isomères, détection d'impuretés ou contrôle de pureté, études cinétiques etc... L'intérêt de la méthode réside également dans son adaptation possible à des composés du phosphore plus complexes.

Remerciements

La partie concernant les phosphites a été étudiée avec la collaboration de Monsieur A. FRANCINA. La partie concernant les dérivés phosphoniques et phosphiniques a été étudiée avec la collaboration de Monsieur J. P. MEILLE.

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Reçu le 21 juin 1968; manuscrit modifié reçu le 17 juillet 1968

CHROM. 3767

Separation of acidic amino acids by high voltage paper electrophoresis and paper chromatography

Many methods for the separation of amino acids on paper[1], or using thin layers of cellulose[2–4], starch[5], silica gel[6], or mixed cellulose–silica gel[7], have been recorded, and have been recently reviewed[8]. Although these methods usually separate common amino acids, many specific groups of amino acids require modified procedures, *e.g.*, imino acids[9], iodo-amino acids[10], substituted tryptophans[11] and sulphur- and seleno-amino acids[12,13]. During investigations into the soluble amino acids present in various fern genera in New Zealand, a method was required to separate the numerous acidic amino acids encountered.

High concentrations of sugars, organic acids and salts present in some extracts interfere with amino acid separation during chromatography. To overcome this, high voltage electrophoresis was used in the first dimension, followed by chromatography in the second dimension. A chromatographic step was used in preference to another electrophoretic step because of the structural data that can be gained from the R_F value of the amino acid. This paper reports the most useful separation procedures investigated.

Experimental

Electrophoresis was carried out using Whatman 3MM paper, 43 cm × 53.5 cm in a high voltage apparatus (Miles Hivolt Ltd, Shoreham-by-Sea, Sussex). The paper was dipped in buffer solution, uniformly blotted and the extract in a volume of 10 μl, applied as a narrow 2 cm streak. Amino acid loading of up to 50 μg of each amino acid gave rise to the best separation. Electrophoresis was carried out at pH 3.4 (acetic acid 10 ml, pyridine 0.6 ml in 1 l water) for 30 min at 7.5 kV and 100 mA.

After drying for 1 h in a current of warm air, the paper was folded and descending partition chromatography carried out in a single phase solvent *n*-butanol–acetic acid–water (12:3:5 v/v) for 20 h at 20°.

Amino acids were located by spraying with 1% ninhydrin solution (in 95% ethanol with 1% 2,4,6-collidine added) and the colours allowed to develop at room temperature for several hours before being recorded.

Prior to electrophoresis at pH 3.4, a preliminary separation into neutral, basic and acidic amino acids could be carried out at pH 5.3 (pyridine 10 ml, acetic acid 4 ml in 1 l water) for 10 min at 5.5 kV and 300 mA. A small strip was sprayed with ninhydrin solution as location reagent and the amino acid bands eluted, concentrated and electrophoresed at pH 3.4.

A two-dimensional separation employing electrophoresis at pH 5.3, 30 min, followed by chromatography could also be employed but resolution of many compounds was poor. Electrophoresis at this pH did however yield some structural data.

Results and discussion

All of the compounds listed could be separated by pH 3.4 electrophoresis followed by chromatography in *n*-butanol–acetic acid–water as is shown in Fig. 1. Two of these compounds, α-aminopimelic acid (L) and γ-ethylideneglutamic acid

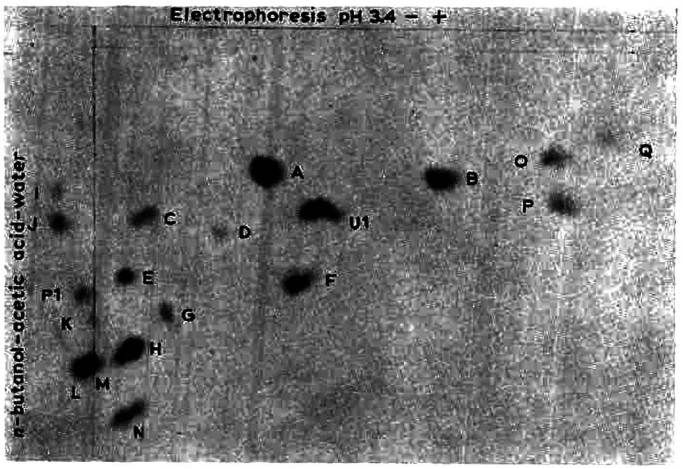

Fig. 1. A two-dimensional separation of various amino acids using pH 3.4 high voltage electrophoresis for the first dimension followed by chromatography in *n*-butanol–acetic acid–water. The key and relative movement data are listed in Table I. β,γ-Dihydroxyglutamic acid is not shown in this figure.

TABLE I

R_{GLU} VALUES, ELECTROPHORETIC MIGRATION DISTANCES (cm)* AND NINHYDRIN COLOURS FOR VARIOUS AMINO ACIDS

Key	*Compound*	*Electrophoresis*		R_{glu}	*Colour*
		pH 3.4	*pH 5.3*		
D	Aspartic acid	9.4	22.7	0.81	grey-blue
B	*threo*-β-Hydroxyaspartic acid	22.3	22.5	0.57	green-grey
A	*erythro*-β-Hydroxyaspartic acid	11.9	21.4	0.56	green-grey
E	Glutamic acid	3.9	19.6	1.0	blue
C	β-Hydroxyglutamic acid**	5.2	18.9	0.72	brown-blue
C	β-Hydroxyglutamic acid**	4.5	18.9	0.76	brown-blue
U1	*threo*-γ-Hydroxyglutamic acid	15.2	20.6	0.72	blue
–	β,γ-Dihydroxyglutamic acid***	9.8	19.5	0.35	blue
H	*erythro*-γ-Methylglutamic acid	4.4	18.7	1.30	blue
H	*threo*-γ-Methylglutamic acid	3.5	18.7	1.34	blue
G	γ-Methyleneglutamic acid	6.1	20.2	1.15	blue
F	*erythro*-γ-Methyl,γ-hydroxy-glutamic acid	13.8	19.7	0.99	grey-blue
M	γ-Ethylideneglutamic acid	1.9	16.9	1.36	grey-blue
N	*erythro*-γ-Ethylglutamic acid	4.4	17.2	1.56	blue
N	*threo*-γ-Ethylglutamic acid	3.2	17.2	1.61	blue
K	α-Aminoadipic acid	1.9	16.7	1.19	blue
L	α-Aminopimelic acid	1.0	14.7	1.42	blue
P1	γ-Hydroxy-α-aminopimelic acid***	1.4	14.9	1.08	blue
Q	Cysteic acid	32.4	25.5	0.39	blue
O	Homocysteic acid	29.2	23.3	0.48	blue
P	Cysteine sulphinic acid	29.4	24.1	0.66	blue
I	Asparagine	0.0	0.0	0.63	brown
J	Glutamine	0.0	0.0	0.78	blue

* As electro-osmotic flow of buffer takes place during electrophoresis, the migration distances recorded have been measured from the position of the common amide asparagine.

** Synthetic, configurations not known.

*** Natural isolate, configuration unknown.

(M) run close together, but the different ninhydrin colour reaction (Table I) allows accurate identification.

During chromatography, lateral diffusion of the spots does take place so the diastereo-isomers which were clearly separate as narrow bands after pH 3.4 electrophoresis have in some cases merged together. Electrophoresis alone at this pH could therefore be a useful method to separate these isomers. At pH 5.3, however, no useful separation of the isomers was found.

Electrophoresis at pH 5.3 showed that although many of the compounds did not separate from one another, they could be considered in groups. The sulphur amino acids were the fastest, although an increase in chain length decreased the migration distance. Likewise as the straight chain length increased from aspartic acid to α-aminopimelic acid, the migration distance decreased. Aspartic acid and the substituted aspartic acids behaved similarly. A hydroxyl-, methyl-, or methylene-substitution of glutamic acid only slightly altered the migration rate. Substitution of an ethylidene-, or ethyl-group however slowed the migration rate appreciably to values approaching α-aminoadipic acid. The substituted α-aminopimelic acid behaved in a similar manner to α-aminopimelic acid. Hence we can use the position of migration of an unknown compound after electrophoresis at this pH as an aid in tentatively arriving at its carbon skeleton. This is certainly the case when considered in conjunction with the chromatographic data.

From the chromatographic data also, structural correlations could be noted. Increase in chain length increased R_F value, while addition of a hydroxyl-group as a side chain decreased the R_F value. Addition of two hydroxyl-groups further reduced the R_F value. Addition of a methyl-group increased the R_F value, and an ethyl-group substitution increased it further still. The respective unsaturated compounds had slightly lower R_F values than the saturated compounds.

Acknowledgements

I should like to thank Prof. L. FOWDEN for the gift of authentic samples of a few of the amino acids used in this investigation.

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Received August 30th, 1968

Book Reviews

CHROM. 3706

Handbuch der Lebensmittelchemie, Vol. 2, Part 2, *Analytik der Lebensmittel*, edited by W. DIEMAIR, Springer-Verlag, Berlin, 1967, 1552 pp., price DM 389, US $ 97.25.

This book is written in that compendious style which is so often found in German texts and which makes them so eminently suitable as works of reference. It contains not only the standard determinations commonly used in food laboratories but also many other procedures, both qualitative and quantitative, which, though not strictly belonging to the realm of food analysis, are rarely described in books on general analysis. The authors deal fully with those classical methods which have been familiar to generations of food chemists, but do not hesitate to add to them many of the modern techniques which have so greatly increased the analyst's armoury. Chromatography in particular features largely in several of the chapters. Many of the methods are described in detail, so that the book is a very good companion in the laboratory. This fact, together with an excellent bibliography, makes it a splendid addition to the library of any food analyst for whom the German language holds no terrors.

The first chapter deals in a most comprehensive fashion with the determination of water. There then follows a chapter concerned with other inorganic constituents of food and describing the various ashing and wet oxidation techniques as well as the analyses for the most commonly encountered mineral substances. The third chapter treats qualitatively and quantitatively those inorganic substances which are normally present in small quantities only. Next are described the analytical procedures for the detection of the elements carbon, hydrogen, nitrogen and sulphur as well as the halogens together with the determinations of the first three and of phosphorus. This chapter is perhaps open to the objection that the methods given are classical in approach, but this is not a serious criticism since in foodstuffs these elements are often present in fair abundance and the analyst, unlike his colleagues in other fields, must frequently take relatively large samples in order to ensure accuracy. Some nitrogen compounds, namely proteins, amino acids and amines, are then dealt with very fully in another chapter, whilst ammonia, nitrites and nitrates form the subject of that following. The next chapter, which contains thirty-four pages, is on serological methods for the examination of proteins, a subject much neglected by food analysts. Then, after ninety pages on enzymes, it is the turn of carbohydrates, to which five chapters are devoted, the first dealing with total carbohydrate content, the second with monosaccharides and oligosaccharides, and the third with some polysaccharides. Pectins apparently merit a chapter of their own and cellulose is similarly segregated. There follow two chapters which give very full accounts of the analytical chemistry of the alcohols, aldehydes and ketones encountered in foodstuffs, a similar chapter on organic acids being found at the end of the book. Vitamins, provitamins and carotenoids

are the next to receive attention and are followed by additives. The latter are contained in five chapters under the headings of preservatives, antioxidants, colours, pesticides and miscellaneous substances, that on colours being relegated to a later part of the book. The total coverage of food additives comprises about three hundred and sixty pages. After a chapter on functional group analysis the book then explains that very important subject, the statistical evaluation of analytical results.

All through the work the policy of the authors seems to have been directed towards a very comprehensive treatment, and this aim has been most admirably achieved.

A few small blemishes may be mentioned. The determination of sulphate, for example, is confined to the use of barium reagents and that of benzidine, the other organic precipitants available not being cited. In a few other places similar faults are found, but in general the book is remarkably up-to-date. A more serious objection stems from the fact that determinations concerned with fat are scattered throughout the book. A chapter devoted to this subject would surely have been appropriate.

The book is highly recommended to all concerned with food analysis, and as an example of its exhaustive treatment one may quote the fact that the first chapter, which is on the determination of water, occupies no fewer than forty-three pages and describes so many techniques in detail that the hazel twig seems to be the only instrument mention of which is omitted.

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Disk-Elektrophorese, Theorie und Praxis der diskontinuierlichen Polyacrylamidgel Elektrophorese, by H. RAINER MAURER, Walter de Gruyter & Co., Berlin, 1968, 221 pp., price DM 36.00.

This small book will be extremely useful to those who are intending to use polyacrylamide gel electrophoresis in their investigations. The reports of a great number of workers are presented enabling the reader to choose a method suitable for his own purpose.

Chapter 2, in particular, is an excellent practical guide to the analytical possibilities of disc electrophoresis. The other chapters are also comprehensive and adequate, with the exception of chapter 4 where a critical treatment of the difficulties with preparative disc electrophoresis is lacking. Despite a number of sophisticated devices, separations on a preparative scale still have numerous drawbacks. In Table 4 an additional column referring to the original literature would be useful.

The list of about 600 references at the end of the book provides a valuable source of information even to those who already have experience in disc electrophoresis.

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Chemical Problems Connected with the Stability of Explosives, Proceedings of Symposium held in Stockholm, May 1967, Laborator Sture Lundin, Sektionen för Detonik ock Förbränning, Försvanets Forskningsanstalt, Stockholm, 1968, 237 pp., paperbound, price Sw. Cr. 30.00.

The papers discuss aspects of sensitivity, stability and compatibility of explosives. Several papers describe use of thin layer chromatography in the separation of explosives and related materials.

Vademecum Chromatograficzne, edited by J. OPIÉNSKA-BLAUTH AND A. SMOCZKIEWICZOWA, Państwowe Wydawnictwo Naukowe, Warsaw, 1968, 206 pp.

This paper-back, in Polish, has a short introductory section (23 pages) on basics of chromatography, followed by 43 pages devoted to equipment and techniques of paper chromatography. About 100 pages are devoted to the chromatography (especially on paper) of sugars, amino acids, organic acids, glycosides and terpenes, alkaloids and steroids. The remainder of the book describes practical exercises.

International Symposium IV, Chromatography, Electrophorese, Proceedings of Brussels 1966 Symposium, Presses Académiques Européennes, Brussels, 1968, 625 pp.

This book contains the papers presented at the Symposium organised by the Belgian Pharmaceutical Society. Discussion is not recorded. In addition to original papers there are plenary lectures on progress in chromatography (E. LEDERER), characterisation and isolation of proteins by electrophoresis (N. HEIMBURGER AND H. G. SCHWICK), ion exchangers in thin-layer chromatography (K. RANDERATH), juridical aspects of chromatography (G. B. MARINI-BETTÒLO) and systematic analysis of steroids by thin-layer chromatography (B. P. LISBOA).

News

New Apparatus

The MT160 from MicroTek, TRACOR (Austin, Texas) is a space-saving instrument available for isothermal or temperature programmed dual column operation to 500° with two detectors. It includes a new total solid state electrometer with three switchable and entirely separate input channels for ultimate convenience in multi-detector operation. The temperature programmer option is also completely transistorized including three electronic timers for functions reproducible to within 0.6 seconds.

An all-glass system is available with the unique MicroTek inlet for on-column injection. This glass system is strongly recommended with the Flame Photometric detector for sensitive work with organo-phosphorus and organo-sulfur compounds.

Other detector options include the dual flame, thermal conductivity, and electron capture detectors.

For further information apply to the publisher under reference No. Chrom. N-142.

Appointment of Waters Associates as the distributor for Corning's new controlled pore diameter glass packings for liquid chromatography was announced by Corning Glass Works at the 1968 Meeting of the Federation of American Societies for Experimental Biology, April 14, in Atlantic City, New Jersey. This newest addition to Waters' extensive line of LC column packings combines the separation properties of certain porous polymers with the rigid and essentially inert characteristics of glass. With Corning's new LC packings, biomedically important materials can now be studied using an all-glass column system thus minimizing the possibility of interaction of biologicals or pharmaceuticals in the liquid chromatographic separation process. These LC glass packings are available in porosities similar to other Waters' column packings for a wide range of liquid chromatographic applications in the biomedical field.

For further information apply to the publisher under reference No. Chrom. N-143.

Errata

J. Chromatog., 35 (1968) 192–200.

Page 192 and running heads of pp. 194–200, name of author, "NGUYEN TRONG AHN" should read "NGUYEN TRONG ANH".

Page 196, 3rd line, "La porte du méthyle" should read "La perte du méthyle".

Page 197, legend to figure, "Fig. 7 et 8" should read "Spectres 1 et 2".

J. Chromatog., 37 (1968) 499–507.

Page 507, references 16 and 17 should read as follows:

Ref. 16 G. PATAKI, *4th Symposium on Chromatography and Electrophoresis, Brussels, 1966*, p. 305.

Ref. 17 G. PATAKI AND E. STRASKY, *4th Symposium on Chromatography and Electrophoresis, Brussels, 1966*, p. 317.

CHROM. 3726

CONTRIBUTION TO THE THEORY OF GAS CHROMATOGRAPHY AND TO THE COMPUTATION OF THE HETP VALUE

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SUMMARY

Results of our investigations on theoretical and practical problems of programmed flow gas chromatography and the data of the paper of fundamental importance by COSTA NETO, KÖFFER AND DE ALENCAR made it possible to solve the problem of the new approximation.

In the new approximation of general validity, the plate and rate theories approximating the gas chromatographic processes, are amalgamated into a harmonic unity. Derivation of the new equation makes the numerical computation of the coefficients possible either traditionally or by means of computers.

Until now, processes occurring in the gas chromatographic columns have generally been approached in two ways: either by the plate theory or by the kinetic, *i.e.* the rate theory.

The plate theory was introduced by MARTIN AND SYNGE[1] to describe chromatographic processes. This theory has been developed further by MAYER AND TOMPKINS[2] to determine the number of theoretical plates necessary for a suitable separation. The stepwise procedure adopted by them was extended by GLUECKAUF[3,4] to cover conditions of real, continuous flow. The kinetic or rate theory describes the kinetics of the process, and the VAN DEEMTER, ZUIDERWEG AND KLINKENBERG[5] equation, which describes the process, is widely used and accepted.

We have no intention of dealing with the plate theory or the VAN DEEMTER equation[6], or with papers and theories suggesting different modifications, *e.g.* the work of KAMBARA[7,8,14], but we wish to point out that many workers including ourselves have attempted to find a better approximation and description of real processes, primarily to clear up the effect of factors having an influence on the HETP value.

Though the coefficients of the VAN DEEMTER equation may be determined graphically or by other approximation methods[9,15], factors influencing the HETP value could not be investigated unambigously, on account of errors peculiar to the approximation methods. Because of this last circumstance, we have developed an approximation-equation which uses simple mathematical means numerically determining the coefficients of the approximation-equation.

Results of our investigation on theoretical and practical problems of programmed flow gas chromatography[10–12], and the data of the paper of fundamental impor-

tance by COSTA NETO, KÖFFER AND DE ALENCAR[13] made it possible to solve the problem of the new approximation.

In the course of the theoretical derivation, it became evident that the two different approximations (plate and kinetic theory, respectively) can be amalgamated harmoniously in the new equation which is of general validity and describes the processes in either packed, open tubular or capillary columns.

THEORETICAL

On the basis of GLUECKAUF's equation[4] n can be expressed by:

$$n = 16 \left(\frac{t_N}{w}\right)^2 \tag{1}$$

where:

n = number of theoretical plates
t_N = net retention time of the component (min)
w = peak width of the component (min).

This expression can be substituted into the well-known fundamental equation for the HETP value:

$$\text{HETP} = \frac{L}{n} = \frac{L}{16} \left(\frac{w}{t_N}\right)^2 \tag{2}$$

where:

HETP = height equivalent to a theoretical plate (cm)
L = length of the column (cm).

Next, using the two fundamental theorems[10–12], obtained by investigating theoretical and practical problems of programmed flow gas chromatography, equation (2) may be rewritten, according to one of the fundamental theorems, as:

$$w = m_1 \Psi + b_1 \tag{3}$$

where:

m_1 = slope of the straight line (ml)
ψ = flow rate factor of the carrier gas (min/ml)
b_1 = axial section Y of the straight line (min).

By definition, the flow rate factor of the carrier gas is[12]:

$$\Psi = \frac{1}{F^2} (F + C^x) \tag{4}$$

where:

F = flow rate of the carrier gas (ml/min)
C^x = constant depending on the quality of substance (ml/min).

According to the other fundamental theorem:

$$t_N = m_2 \cdot \frac{1}{F} \tag{5}$$

where m_2 is the slope of the straight line (ml).

Equations (3) and (5) have first of all to be transformed, in order to have the linear velocity of the carrier gas instead of its flow rate:

$$w' = m_1' \varphi + b'_1 \tag{6}$$

where:

m_1' = slope of the straight line (cm)
φ = linear velocity factor of the carrier gas (sec/cm)
b_1' = axial section Y of the straight line (sec)
w' = peak width of the component (sec)

By definition, the linear velocity factor of the carrier gas is:

$$\varphi = \frac{1}{u^2}(u + C^{xx}) \tag{7}$$

where:

u = linear velocity of the carrier gas (cm/sec)
C^{xx} = constant depending on the quality of the substance (cm/sec).

Equation (5) can be transformed for the linear velocity of the carrier gas as:

$$t'_N = m_2' \cdot \frac{1}{u} \tag{8}$$

where:

t_N' = net retention time of the component (sec)
m_2' = direction tangent of the straight line (cm).

Substituting the respective parts of equations (6) and (8) into equation (2):

$$\mathrm{HETP} = \frac{L}{16}\left(\frac{m_1'\varphi + b_1'}{m_2' \cdot \frac{1}{u}}\right)^2 \tag{9}$$

On substituting the value of φ from equation (7), rearranging the equation and applying all possible reductions, we obtain:

$$\mathrm{HETP} = \frac{L}{16}\left(\frac{b_1'}{m_2'}u + \frac{m_1'}{m_2'} + \frac{m_1' C^{xx}}{m_2'} \cdot u^{-1}\right)^2 \tag{10}$$

After squaring and arranging the equation according to diminishing powers of u:

$$\mathrm{HETP} = \frac{L}{16}\left[\left(\frac{b_1'}{m_2'}\right)^2 u^2 + 2\left(\frac{b_1'}{m_2'}\right)\left(\frac{m_1'}{m_2'}\right)u + \left(\frac{m_1'}{m_2'}\right)^2 + 2\left(\frac{b_1'}{m_2'}\right)\left(\frac{m_1' C^{xx}}{m_2'}\right) + \right.$$
$$\left. + 2\left(\frac{m_1'}{m_2'}\right)\left(\frac{m_1' C^{xx}}{m_2'}\right)u^{-1} + \left(\frac{m_1' C^{xx}}{m_2'}\right)^2 u^{-2}\right] \tag{11}$$

If we now introduce the symbols:

$$A = \frac{L}{16}\left[\left(\frac{m_1'}{m_2'}\right)^2 + 2\left(\frac{b_1'}{m_2'}\right)\left(\frac{m_1' C^{xx}}{m_2'}\right)\right] \tag{12}$$

$$B = \frac{L}{8}\left[\left(\frac{m_1'}{m_2'}\right)\left(\frac{m_1' C^{xx}}{m_2'}\right)\right] \tag{13}$$

$$C = \frac{L}{8}\left[\left(\frac{b_1'}{m_2'}\right)\left(\frac{m_1'}{m_2'}\right)\right] \tag{14}$$

$$D = \frac{L}{16}\left[\left(\frac{m_1' C^{xx}}{m_2'}\right)^2\right] \tag{15}$$

$$E = \frac{L}{16}\left(\frac{b_1'}{m_2'}\right)^2 \tag{16}$$

equation (11) can now be written in a new but conventional form as:

$$\mathrm{HETP} = A + \frac{B}{u} + Cu + \frac{D}{u^2} + Eu^2 \qquad (17)$$

Equation (17) is a new equation of general validity describing gas chromatographic processes, incorporating the HETP value, and is suitable for following the processes occurring in any kind of column. There are fundamental differences between equation (17) and the well-known VAN DEEMTER equation because of the more general character of the approximation, though concordant features can also be observed, especially with respect to the 2nd and 3rd terms of the equation. There is a definite difference both in the interpretation and value of the first term, and in the fact that the new, general equation incorporates two terms with coefficients D and E, which take into account diffusion and represent better the mass transfer conditions respectively, and thus improve the approximation.

Equation (17) due to the mathematical exactness of its coefficients (equations (12) to (16)) creates a new opportunity of investigating the factors which influence the HETP value, and at the same time allows one to follow gas chromatographic processes numerically.

On the basis of the relationships above, there are unambigous answers to many questions, formerly unsettled. For instance whether the term A in the equation can or cannot be neglected; what is the relationship between the diffusion and mass-transfer processes for a given gas rate, etc. Our investigations in this respect are continuing.

The derivation of the equation and the method of computing the coefficients is published now, well before completing our investigations, in order to give other workers the chance to examine the coefficients influencing HETP value, or on the other hand, to evaluate critically both their derivation and equation (17).

EXPERIMENTAL

Numerical evaluation of equation (17), *i.e.* of its constants, is done in the following manner. The net retention time of the tested component is determined for five different linear velocity values of the carrier gas under stationary gas chromatographic conditions. Then, adopting the principle of the least squares[11] to the data obtained, a functional relationship between the net retention time and the reciprocal of linear velocity is obtained, so the linear functional equation (8) will be solved. The slope m_2' of this straight line is the first of the data necessary for the computations.

Next, the relationship between the peak width and the linear gas rate is determined with help of the chromatograms already determined for the net retention time and the linear gas velocity function. Writing equation (6) involves lengthy computations, based on our earlier publications on the determination of these coefficients[12], because of the substance constant figuring in the linear velocity factor, so possibly a computer should be used, so that these computations can be carried out easily. From equation (6), *i.e.* from the substance constant and the linear velocity factor the values of m_1', b_1' and C^{xx}, necessary for the computations, are obtained.

The constants of equation (17) have then to be computed by means of the relationships (12 to 16), derived as discussed in the theoretical part.

The complete series of computations can also be carried out by a computer. The gas chromatographic programme necessary for programming the computer is as follows:

Related data of carrier gas rate–retention time (at a constant temperature and under unchanged gas chromatographic conditions), the relevant peak width, the inlet and outlet pressures of the carrier gas, together with the relevant t_M values are fed into the computer in dimensions corresponding to the equations. t_M stands for the gas hold-up time of the non-sorbed substance, in sec.

The computer is programmed so as to compute first the gas compressibility correction factor j by means of the well-known JAMES–MARTIN equation:

$$j = \frac{3}{2}\left(\frac{P^2 - 1}{P^3 - 1}\right) \tag{18}$$

where:

j = gas compressibility correction factor according to JAMES–MARTIN

$P = (p_i/p_o)$

p_i = inlet pressure of the carrier gas (in kp/cm^2), or another pressure unit

p_o = outlet pressure of the carrier gas (in kp/cm^2, or another pressure unit).

After the evaluation of j, the net retention times have to be computed according to the well-known relationship:

$$t_N' = j(t_R^0 - t_M) \tag{19}$$

where:

t_R^0 = retention time of the component (sec).

Next equation (8) should be computed by applying the principle of least squares and the slope m_2' of the straight line so determined is printed out.

Then the substance constant, the slope and the axial section of equation (6) are determined on the basis of appropriate relationships. These m_1', C^{xx} and b_1' values are also printed out.

Now that all the necessary data for further computations are stored in the memory of the computer, determination and printing out of the constants of equation (17), based on equations (12) to (16), can be carried out. After determining the coefficients, the corresponding linear velocities are co-ordinated to the corresponding constants, and equation (17) for the substance tested is printed out corresponding to the given gas chromatographic system. Finally a computer programme for the HETP value will be presented.

According to our experience, it is expedient to feed the computer a programme, which makes it automatically raise the linear gas rate values first by 0.5 (cm/sec) from the value of 0.5 cm/sec to 50.0 cm/sec in the equation, then by 5.0 cm/sec up to 100.0 cm/sec. In some instances, *e.g.* in high rate investigations, even higher values may be needed, these are advisably programmed separately. Of course, in this stage of the programme all HETP–u values have to be printed out. If the investigations are intended to examine factors influencing the HETP value, it is expedient to have all the computed data printed.

In addition to the well-known advantages (rapidity, exactness, etc.), computer investigations of this character have the great advantage that a general programme has only to be prepared once, and the changing gas chromatographic data merely have to be fed into this established general programme for each case.

To illustrate the above, instead of examples of individual character, the developement of the functional curves for the VAN DEEMTER approximation equation

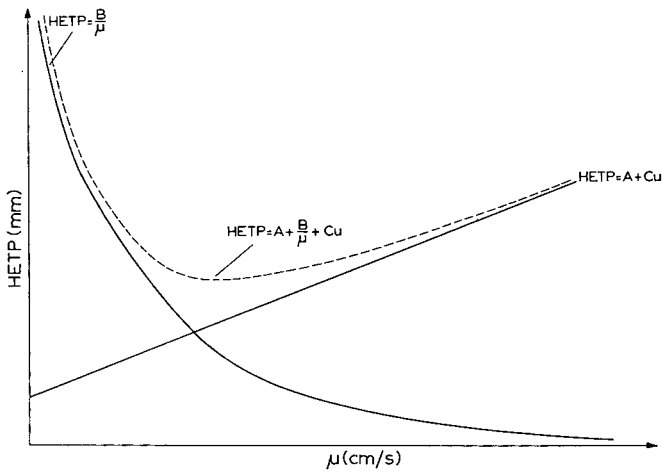

Fig. 1. General functional curve for the approximation by the VAN DEEMTER equation.

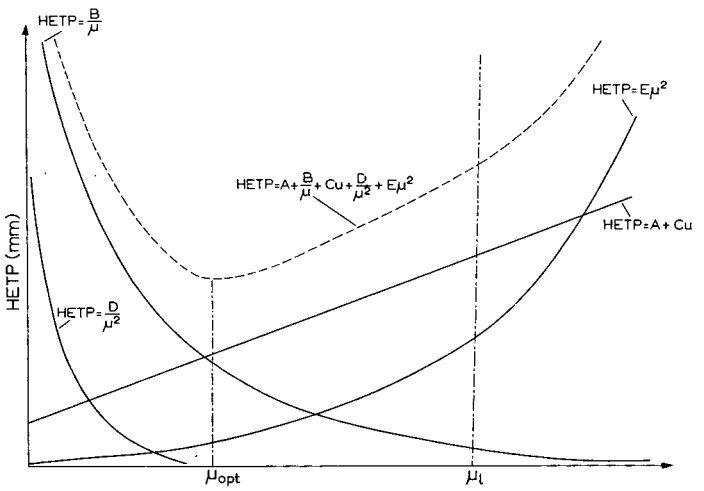

Fig. 2. General aspect of the functional curve for the approximation by equation (17).

(Fig. 1) and for the general approximation by equation (17) are presented, plotting also each component separately (Fig. 2).

DISCUSSION

This initial paper on our work in this field, concentrates on a new approximation of general validity, therefore no attempt has been made to discuss completely all the problems. Nevertheless some problems are outlined here and some of them even discussed.

One of these is the interpretation of the first term "*A*" of the equation, which is formulated in an entirely different manner from the former conventions, though it

must be stressed that the interpretation of the first term of the VAN DEEMTER equation must be incorporated in this new term, because it belongs there intrinsically. Up to now this term has been given much less attention, than justified by its effect on the process. There have even been opinions that its effect on the HETP value could be neglected, its value would be zero, etc. For instance $HETP_{min}$ values depend on this first term in each case in the same way as do diffusion and mass transfer factors.

In our opinion that part of the theory of capillary columns should also be reconsidered according to the new approximation of general validity which is connected with this question, as this term has been neglected in the case of capillary columns, in the earlier interpretations.

Another problem of interest is that the functional curve for HETP–u, occurs in each case above the functional curve of the VAN DEEMTER equation. This is primarily due to the fourth and fifth term of the equation, adding their values to the corresponding function values. This, however, by no means contradicts practical experiences, which in general have higher values than expected according to the VAN DEEMTER equation.

The third problem to which we want to call attention is connected with the upper limit of interval used for gas chromatographic processes; this value is in general $P = 3.00$–3.50. In Fig. 2, the relative position of this upper limit has been indicated and the linear gas velocity (u_1) corresponding to this limit has been specially marked. In former approximations, there was no explicit theoretical basis for this empirical upper limit (Fig. 1), whereas the new approximation gives an unambiguous theoretical explanation for its development and position. Namely the new, fifth term (Eu^3), takes into account more exactly the mass transfer resistance, which has a decisive role here by bending upwards the hitherto straight line and lending a parabolic character to the functional curve by developing an inflexion along it. Practically this inflexion point may be considered as upper limit.

Last but not least, the dimensions of acceleration and of reciprocal acceleration of the 4th and 5th terms, respectively, of the new equation are to be noted as a fact also deserving further investigation. This investigation may yield useful data to complete the existing picture of the gas chromatographic process.

SYMBOLS

n = number of theoretical plates
t_N = net retention time (min)
t_N' = net retention time (sec)
w = chromatographic peak width (min)
w' = chromatographic peak width (sec)
HETP = height equivalent to a theoretical plate (cm)
L = length of column (cm)
m = slope of the straight line (ml)
m' = slope of the straight line (cm)
b'_1 = axial section Y of the straight line (sec)
ψ = flow rate factor of the carrier gas (min/ml)
φ = linear velocity factor of the carrier gas (sec/ml)
F = flow rate of carrier gas (ml/min)

b'_1 = axial section Y of the straight line (min)
u = linear velocity of carrier gas (cm/sec)
C^x = constant depending on the substance quality (ml/min)
C^{xx} = constant depending on the substance quality (cm/sec)
A, B, C, D and E = constants of equation (17)
A = base factor of the gas-chromatographic system (cm)
B = a factor to take into account diffusion processes (cm^2/sec)
C = a factor to take into account mass transfer processes (sec)
D = a further factor to take into account diffusion processes (cm^3/sec^2)
E = a further factor to take into account mass transfer processes (sec^2/cm)

ACKNOWLEDGEMENT

The author wishes to thank Professor L. ERDEY for his continued interest and help.

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CHROM. 3754

ZUR DOPPELT PROGRAMMIERTEN GASCHROMATOGRAPHIE

I. TEIL. TRÄGERGASPROGRAMM NACH TEMPERATURPROGRAMM

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(Eingegangen am 21. August, 1968)

SUMMARY

Double programmed gas chromatography. Part I. Carrier gas programming following temperature programming

The theoretical and practical problems of double programming in gas chromatography are dealt with in the case where the carrier gas programme follows on after the temperature programme. Examination of the efficiency of the double programming showed that, with the double programme described and used here, it was possible to attain, together with appropriate peak resolution, a minimal analysis time.

Durch die Technik der doppelt programmierten Gaschromatographie wird eine raschere und selektivere Analyse ermöglicht, wenn Trägergasprogramm und Temperaturprogramm nicht einzeln, sondern gleichzeitig, u.U. aufeinanderfolgend durchgeführt werden[1–4]. Verträgt z.B. die Verteilungsflüssigkeit keine weitere Erhöhung der Kolonnentemperatur, so lässt sich die Dauer der Analyse lediglich dadurch abkürzen, dass gleichzeitig Druck bzw. Strömungsgeschwindigkeit des Trägergases programmässig erhöht werden. Die Anwendung innerhalb einer einzigen Analyse von Temperatur- und Trägergasprogramm ist jedoch auch in dem Falle gerechtfertigt, wenn unterhalb der oberen Temperaturgrenze der Verteilungsflüssigkeit gearbeitet wird, weil im Fall einer Probe von komplexer Zusammensetzung mit weitem Siedepunktbereich die vorteilhaftesten Analysenverhältnisse so besser gewährleistet werden können, als lediglich durch Temperatur- bzw. Trägergasprogrammierung[5–8].

In vorliegender Arbeit wird die Art der Programmierung[9] behandelt, wo das Trägergasprogramm auf das Temperaturprogramm folgt. Grundlegende Voraussetzungen für die Behandlung des Problems sind, dass:

(1) Während des Temperaturprogramms die Trägergasströmung gleichwertig bleibt;

(2) Die Trägergasprogrammierung stationär, im Grenzfall quasistationär ist.

Zur Behandlung der Frage ist ein Grundchromatogramm erforderlich, das ein Chromatogramm des zu analysierenden Gemisches darstellt, das bei dem optimalen Temperaturprogramm und bei einer der entscheidenden Mehrheit der Komponenten entsprechenden ständigen Trägergasgeschwindigkeit aufgenommen wurde. Bei der

Aufnahme des Grundchromatogramms in Fig. 1 (a) wurde ein Gemisch folgender Zusammensetzung verwendet:

(1) Azeton; (2) Benzol; (3) Toluol; (4) *m*- und *p*-Xylol; (5) *o*-Xylol; (6) *o*-Nitro-Äthylbenzol; (7) *m*-Nitro-Äthylbenzol; (8) *p*-Nitro-Äthylbenzol; (9) Dimethylphthalat.

Das Chromatogramm (a) in Fig. 1 wurde bei den untenstehenden gaschromatographischen Parametern aufgenommen:

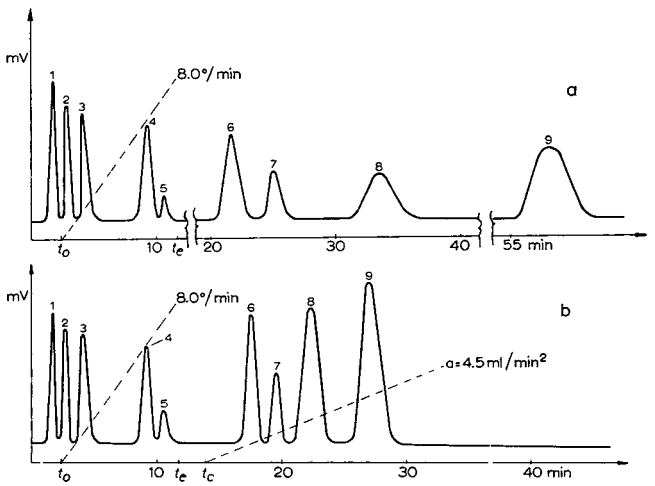

Fig. 1. Grundchromatogramm des analysierten Gemisches bei optimalem Temperaturprogramm und einer für die entscheidende Mehrheit der Komponenten entsprechenden, ständigen Strömungsgeschwindigkeit von $F = 12.0$ ml/min.

Gaschromatograph: Carlo Erba Fractovap Modell D

Detektor: Flammenionisation

Empfindlichkeit: 16 × 100

Einwaage: 1.0 μl mit einer Hamilton-Spritze

Kolonne: Länge 3.0 m, Kupferspirale mit einem Innendurchmesser von 4.0 mm

Kolonnenfüllung: 12.5% Silikonöl DC-550 auf einem Träger Chromosorb W 60/80

Kolonnentemperatur: Bei der Einwaage: 133.0 ± 0.1° nach Abschaltung des Temperaturprogramms: 197.0 ± 0.1°

Temperaturprogramm: Angefahren in der 2.oten Minute nach der Einwaage $\beta =$ 8.0°/min

Abschaltung: bei 200.0° (obere Temperaturgrenze der Anwendbarkeit von Silikonöl DC-550)

Verdampfertemperatur: 250.0 ± 1.0°

Trägergas: Stickstoff

Innendruck des Trägergases: 1.56 kp/cm²

Hilfsgase: Sauerstoff; Eintrittsdruck: 1.74 kp/cm²; Wasserstoff: Eintrittsdruck: 1.25 kp/cm²

Schreiber: Speedomax G; 2.5 mV/Endausschlag; 1.0 sec; Papiergeschwindigkeit: 1.27 cm/min.

Das Grundchromatogramm in Fig. 1 wird durch Anfangs- (t_o) und Endzeitpunkt (t_e) der Temperaturprogrammierung in drei Abschnitte unterteilt:

Von der Einwaage ($t = 0$) bis t_o, isothermer Abschnitt bei ständiger Strömung;

von t_o bis t_e, Abschnitt mit ständiger Strömung bei programmierter Temperatur; und

von t_e bis zum Ende der Analyse, isothermer Abschnitt bei ständiger Strömung.

In Fig. 1 ist auch der vorgesehene Zeitpunkt für das Anfahren des Trägergasprogrammes (t_c) bezeichnet; diese Angabe wird bei den Berechnungen benötigt. Die Frage wird diesen Abschnitten entsprechend behandelt.

Bei einer doppelten Programmierung wird versucht, neben einer entsprechenden Spitzenauflösung eine minimale Analysenzeitdauer zu erreichen, daher kann das Trägergasprogramm lediglich beschleunigend sein[10]. Das Trägergasprogramm wird 1.5–2.0 Min nach Abschaltung des Temperaturprogramms angefahren, auch bei zeitgemässen Gaschromatographen ist nämlich diese Zeit erforderlich, damit nach dem Temperaturprogramm wieder isotherme Verhältnisse eintreten. Durch eine Nichteinhaltung dieser Zeit werden Genauigkeit und Reproduzierbarkeit beeinträchtigt.

Bedient man sich in Anlehnung an das Grundchromatogramm der Beziehung

$$V_{p,z} = j_c F_c \, (t_{Rp,z} - t_c) \tag{1}$$

so werden zuerst die für die einzelnen Komponenten im Abschnitt von Anfahren des Trägergasprogramms (t_c) bis zum Ende der Analyse kennzeichnenden Teilretentionsvolumina berechnet. Es werden die für diesen Abschnitt charakteristischen gaschromatographischen Parameter (j_c,F_c) benutzt. Sind die V_p Werte bekannt, errechnet man die ΔV_p Werte:

$$\Delta V_p = V_{p,z+1} - V_{p,z} \tag{2}$$

sodann wird der niedrigste ΔV_p Wert, $\Delta V_{p,\min}$ ausgewählt. Aus dem Grundchromatogramm bestimmt man die Spitzenbreiten w_{x+1} und w_x der zu $\Delta V_{p,\min}$ gehörigen zwei Komponenten. Hier sei bemerkt, dass theoretisch auch je zwei Komponentenpaare gleiche ΔV_p und $\Delta V_{p,\min}$ Werte haben können. In einem solchen Falle wird bei linearen Trägergasprogramm das sich später eluierende Komponentenpaar gewählt und durch die Indexe x bzw. $x+1$ bezeichnet, während bei einem gestuften Trägergasprogramm die Berechnung des maximalen Trägergasprogrammes durch diesen Fall nicht beeinflusst wird. In der Praxis ist den Verfassern noch kein derartiger Fall vorgekommen. Es wurde bereits darauf hingewiesen[11], dass für jede Komponente der Trägergasprogrammierung gilt:

$$V_{p,z} = \int_{t_c}^{t_{dp,z}} (j_c F_c + at) \, \mathrm{d}t = j_c F_c \, (t_{dp,z} - t_c) + \frac{a}{2} (t_{dp,z}^2 - t_c^2) \tag{3}$$

Wird Gleichung (3) für die x-te und $x+1$-te Komponente aufgestellt und werden die so erhaltenen zwei Gleichungen voneinander abgezogen, erhält man

$$V_{p,x+1} - V_{p,x} = \Delta V_{p,\min} = j_c F_c \, (t_{dp,x+1} - t_{dp,x}) + \frac{a}{2} (t_{dp,x+1}^2 - t_{dp,x}^2) \tag{4}$$

Bei entsprechender Spitzenauflösung wird eine minimale Analysenzeitdauer nur gesichert, wenn

$$t_{dp,x+1} - t_{dp,x} = \frac{w_x + w_{x+1}}{2} \tag{5}$$

ist, wobei Gleichung (4) auch in der Form

$$\Delta V_{p,\min} = j_c F_c \left(\frac{w_x + w_{x+1}}{2}\right) + \frac{a}{2}\left(\frac{w_x + w_{x+1}}{2}\right)\cdot\left(\frac{w_x + w_{x+1}}{2} + 2t_{dp,x}\right) \quad (6)$$

geschrieben werden kann.

Wird "a" aus der für die Komponente $x+1$ aufgestellte Gleichung (3) ausgedrückt und in (6) eingesetzt, erhält man

$$\Delta V_{p,\min} = j_c F_c \left(\frac{w_x + w_{x+1}}{2}\right) + \frac{V_{p,x} - j_c F_c (t_{dp,x} - t_c)}{t_{dp,x} - t_c}\cdot \left(\frac{w_x + w_{x+1}}{2}\right)\cdot\left(\frac{w_x + w_{x+1}}{2} + 2t_{dp,x}\right) \quad (7)$$

Werden die Operationen in (7) durchgeführt und die Gleichung nach abnehmenden Potenzen von $t_{dp,x}$ geordnet, erhält man die Beziehung:

$$t_{dp,x}^2[4\Delta V_{p,\min} + 2j_c F_c(w_x + w_{x+1})] + t_{dp,x}[j_c F_c(w_x + w_{x+1})^2 - 4j_c F_c t_c(w_x + w_{x+1}) - 4V_{p,x}(w_x + w_{x+1})] + 2j_c F_c t_c^2(w_x + w_{x+1}) - 4\Delta V_{p,\min} t_c^2 - V_{p,x}(w_x + w_{x+1})^2 - j_c F_c t_c (w_x + w_{x+1})^2 = 0 \quad (8)$$

Gleichung (8) stellt bereits eine auf $t_{dp,x}$ auflösbare Gleichung zweiten Grades dar, so kann die Retentionsdauer bei doppelter Programmierung für die x-te Komponente ($t_{dp,x}$) ermittelt werden. Ist $t_{dp,x}$ bekannt, und bedient man sich der Gleichung (6), kann "a", die maximale Gasstromvolumenbeschleunigung in einem linearen Trägergasprogramm errechnet werden:

$$a = \frac{8\Delta V_{p,\min} - 4j_c F_c(w_x + w_{x+1})}{4t_{dp,x}(w_x + w_{x+1}) + (w_x + w_{x+1})^2} \quad (9)$$

Nach Auflösung von Gleichung (9) stehen sämtliche zur linearen Trägergasprogrammierung erforderliche Daten[11] zur Verfügung, so kann das entsprechende doppelte Programm ausgearbeitet werden, mit dessen Anwendung bei minimaler Analysenzeitdauer die grösste Trennung erzielt wird. In Fig. 1(b) ist das so erhaltene doppelt programmierte Chromatogramm dargestellt. In der Abbildung ist das doppelte Programm in üblicher Weise[12] durch eine gestrichelte Linie bezeichnet. Die Komponenten sind ebenso wie in Fig. 1(a) numeriert. Auch die gaschromatographischen Parameter waren innerhalb der Reproduzierbarkeitsgrenzen die gleichen, mit Ausnahme des lineare Trägergasprogramms, das bei $t_c = 13.8$ Min. angefahren wurde.

Die Sache ist viel einfacher, wenn nach Abschaltung des Temperaturprogramms eine gestufte Trägergasprogrammierung[13] durchgeführt wird. Da bei einem gestuften Trägergasprogramm, mit Ausnahme einiger Minuten nach Anfahren des Programms die Gasstromvolumenbeschleunigung des Trägergases gleich Null ist, kann Gleichung (9) gleich Null gesetzt werden. Sie ist gleich Null, wenn auf der rechten Seite der Zähler gleich Null ist, also gilt:

$$8\Delta V_{p,\min} - 4j_c F_c(w_x + w_{x+1}) = 0 \quad (10)$$

Wird aus Gleichung (10) $j_c F_c$ ausgedrückt und gekennzeichnet, dass diese nun maximale Programmwerte in einem gestuften Trägergasprogramm darstellen, so schreibt man

$$(j_c F_c)_{max} = \frac{2\Delta V_{p,min}}{w_x + w_{x+1}} \qquad (11)$$

Der einem anhand von Gleichung (11) berechneten, maximalen, gestuften Trägergasprogramm entsprechende Eintrittsdruck wird von einer bei der isothermen Temperatur der Analyse besonders ermittelten Tabelle oder Funktionskurve abgelesen, die für den Eintrittsdruck die Werte des Produkts $j_c F_c$ enthält, damit wird nach dem Temperaturprogramm eine mit gestuftem Trägergasprogramm aufgebaute doppelte Programmierung erzielt.

Eine derartige doppelte Programmierung zeigt Fig. 2. In der Abbildung sind das isotherme (a), das temperaturprogrammierte (b) und das doppelt programmierte Gaschromatogramm (c) des Modell-Gemisches dargestellt.

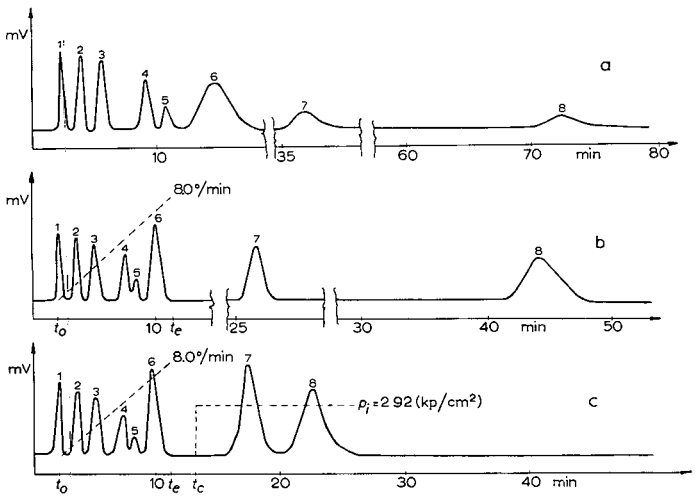

Fig. 2. Das mit Hilfe eines gestuften Trägergasprogramms aufgenommene, doppelt programmierte Chromatogramm des untersuchten Gemisches.

Die Chromatogramme in der Abbildung wurden bei folgenden gaschromatographischen Parametern aufgenommen:

Gaschromatograph: Carlo Erba Fractovap Modell D

Detektor: Flammenionisation

Empfindlichkeit: 16 × 100

Einwaage: 1.0 μl mit einer Hamilton-Spritze

Kolonne: Aluminiumspirale, Länge 2.0 m, Innendurchmesser 4.0 mm

Kolonnenfüllung: 20,0% Silikonöl DC-550, auf einem Träger Chromosorb W 60/80 mesh

Kolonnentemperatur: (a) 128.0 ± 0.1°; (b) bei der Einwaage 128.0 ± 0.1°, in der darauffolgenden zweiten Minute wird mit β = 8.0°/min Aufheizgeschwindigkeit ein Temperaturprogramm angefahren, das bei 200.0° automatisch abschaltet; (c) gleich (b)

Verdampfungstemperatur: 225.0 ± 1.0°

Trägergas: Stickstoff

Eintrittsdruck des Trägergases: (a) 1.76 kp/cm²; (b) 1.71 kp/cm² bwz. 1.79 kp/cm²; (c) 1.71 kp/cm² bzw. 1.79 kp/cm².

Letzterer Wert wurde durch den dem bei $t_c = 13.3$ min angefahrenen Programm $(j_c F_c)_{max}$ (33.9 ml/min) entsprechenden Wert 2.92 kp/cm² abgelöst.

Hilfsgase: Sauerstoff, Eintrittsdruck: 1.75 kp/cm²; Wasserstoff: Eintrittsdruck: 1.25 kp/cm²

Schreiber: wie vor.

Die einzelnen Komponenten in Fig. 2:

(1) Azeton; (2) Benzol; (3) Toluol; (4) *m*- und *p*-Xylol; (5) *o*-Xylol; (6) Nitrobenzol; (7) *p*-Nitro-Äthylbenzol; (8) Di-Methyl-Phthalat.

Die Wirksamkeit der doppelten Programmierung wird zweckmässigerweise auf das Grundchromatogramm, als Bezugsgrundlage, bezogen. Da für eine Probe mit z Komponenten die Analysenzeitdauer (t_A) in einem doppelt programmierten Chromatogramm

$$t_{A,dp} = t_{dp,z} + \frac{w_{z,dp}}{2} \tag{12}$$

und in den Grundchromatogramm

$$t_{A,p} = t_{Rp,z} + \frac{w_{z,p}}{2} \tag{13}$$

beträgt, lässt sich die Wirksamkeit durch folgende Beziehung ausdrücken:

$$E_{dp} = \frac{t_{A,p} - t_{A,dp}}{t_{A,p}} \cdot 100\,\% \tag{14}$$

Als Zahlenbeispiele werden die Wirkungsgrade der doppelten Programmierungen in den Fig. 1 und 2 vorgeführt. Dabei waren

$$E_{dp,\ \text{linear}} = \frac{44{,}2 - 22{,}2}{44.2} \cdot 100 = 49.9\,\%$$

hingegen

$$E_{dp,\text{gestuft}} = \frac{58.0 - 27.0}{58.0} \cdot 100 = 53.5\,\%$$

Die Änderung der Kolonnenwirksamkeit im Laufe der doppelten Programmierung lässt sich anhand der Änderungen der theoretischen Trennstufenzahl bzw. der Höhe einer theoretischen Trennstufe verfolgen. Dazu mussten jedoch theoretische Trennstufenzahl und HETP-Wert für das Grundchromatogramm, als Bezugsgrundlage, sowie die zur doppelten Programmierung gehörige theoretische Trennstufenzahl und der HETP-Wert als zu ermittelnde Parameter, bestimmt werden. Zu diesen Untersuchungen benutzten die Verfasser die Arbeiten von GLUECKAUF[14], PURNELL[15] sowie HARRIS UND HABGOOD[5]. Auf dieser Grundlage wurde die theoretische Trennstufenzahl für das Grundchromatogramm angeschrieben:

$$n_{a,z} = 16 \left(\frac{V_{t_o} + V_{(t_e - t_o)} + V_{(t_c - t_e)} + V_{p,z}}{w_z \cdot j_c F_c} \right)^2 \tag{15}$$

Aus Gleichung (15) lässt sich für eine jede Komponente der Probe der im Grundchromatogramm entsprechende HETP-Wert berechnen

$$\text{HETP}_{a,z} = \frac{L}{n_{a,z}} \tag{16}$$

Für die theoretische Trennstufenzahl der Komponenten eines doppelt programmierten Chromatogramms ist auch das angewandte Trägergasprogramm bestimmend, deshalb müssen die theoretischen Trennstufenzahlen eines doppelten Trägergasprogramms unter Anwendung eines gestuften bzw. eines linearen Trägergasprogramms gesondert behandelt werden. Für die theoretische Trennstufenzahl eines doppelten Trägergasprogramms unter Anwendung eines gestuften Trägergasprogramms gilt:

$$n^{s}_{dp,z} = 16\left(\frac{V_{t_o} + V_{(t_e - t_o)} + V_{(t_c - t_e)} + V_{p,z}}{w^{s}_{dp,z}\cdot(j_c F_c)\max}\right)^2 \tag{17}$$

So errechnet man aus der Gleichung (17) den HETP Wert:

$$\mathrm{HETP}^{s}_{dp,z} = \frac{L}{n^{s}_{dp,z}} \tag{18}$$

Bei doppelter Programmierung mit einer linearen Trägergasprogrammierung erhält man die theoretische Trennstufenzahl aus der Beziehung:

$$n^{1}_{dp,z} = 16\left[\frac{V_{t_o} + V_{(t_e - t_o)} + V_{(t_c - t_e)} + V_{p,z}}{\int_{t_{dp,z} - \frac{w^{1}_{dp,z}}{2}}^{t_{dp,z} + \frac{w^{1}_{dp,z}}{2}} (j_c F_c + at)\mathrm{d}t}\right]^2 \tag{19}$$

Nach Berechnung des Integrals im Nenner der Gleichung (19) wird das Ergebnis in diese Gleichung wieder eingesetzt:

$$\int_{t_{dp,z} - \frac{w^{1}_{dp,z}}{2}}^{t_{dp,z} + \frac{w^{1}_{dp,z}}{2}} (j_c F_c + at)\mathrm{d}t = (j_c F_c + a t_{dp,z})\cdot w^{1}_{dp,z} \tag{20}$$

bzw.

$$n^{1}_{dp,z} = 16\left(\frac{V_{t_o} + V_{(t_e - t_o)} + V_{(t_c - t_e)} + V_{p,z}}{(j_c F_c + a t_{dp,z})\cdot w^{1}_{dp,z}}\right)^2 \tag{21}$$

Ähnlich wie oben ist aufgrund von Gleichung (21) der HETP-Wert

$$\mathrm{HETP}^{1}_{dp,z} = \frac{L}{n^{1}_{dp,z}} \tag{22}$$

KURZBEZEICHNUNGEN

t = die Zeit (min)
t_o = Zeitpunkt des Anfahrens des Temperaturprogramms (min)
t_e = Zeitpunkt der Abschaltung des Temperaturprogramms (min)
t_c = Zeitpunkt des Anfahrens des Trägergasprogramms (min)

j = der bekannte James-Martinsche Korrektionsfaktor des Druckabfalls
F = Stromvolumengeschwindigkeit des Trägergases ohne Korrektion (ml/min)
p_i = Eintrittsdruck des Trägergases (kp/cm²)
t_k und t_{k-1} = ein beliebiger k-ter Zeitpunkt und der diesem unmittelbar vorangehende Zeitpunkt $(k-1)$ (min)
V_p = Teilretentionsvolumen (ml Trägergas)
z = Laufzahl der Komponenten
j_c = Korrektionsfaktor für den zum Zeitpunkt t_c gehörigen Druckabfall
F_c = die zum Zeitpunkt t_c gehörige Trägergasvolumengeschwindigkeit (ml/min)
t_{Rp} = programmierte Retentionszeit für eine beliebige Komponente (min)
ΔV_p = Differenz von zwei Teilretentionsvolumina (ml Trägergas)
$\Delta V_{p,\min}$ = der aus den Teilretentionsvolumina gebildete, niedrigste ΔV_p Wert (ml Trägergas)
w = Spitzenbreite im Grundchromatogramm (min)
t_{dp} = doppelt programmierte Retentionszeit einer beliebigen Komponente (min)
a = Stromvolumenbeschleunigung des Trägergases (ml/min²)
x = Laufnummer der sich früher eluierenden von den beiden Komponenten, die $\Delta V_{p,\min}$ ergeben
$(j_c F_c)_{\max}$ = die zur Bedingung "a" = o gehörige, grösste, durch einen Druckabfall-Korrektionsfaktor verbesserte Trägergasvolumengeschwindigkeit (ml/min) bei gestuftem Trägergasprogramm, die noch keine Aufeinanderprogrammierung verursacht
$t_{A,dp}$ = Analysenzeitdauer in der doppelt programmierten Gaschromatographie (min)
$t_{A,p}$ = Analysenzeitdauer in der programmierten Gaschromatographie (min)
E_{dp} = Wirksamkeit der doppelten Programmierung hinsichtlich der Analysenzeitdauer (%)
V_{t_o} = die von der Einwaage bis zum Anfahren des Temperaturprogramms das System durchströmende Trägergasmenge (ml Trägergas)
$V_{t_e-t_o}$ = die von Anfahren des Trägergasprogramms bis zur Abschaltung das System durchströmende Trägergasmenge (ml Trägergas)
$V_{t_c-t_e}$ = die von Abschaltung des Temperaturprogramms bis Anfahren des Trägergasprogramms das System durchströmende Trägergasmenge (ml Trägergas)
$n_{a,z}$ = die zur z-ten Komponente gehörige theoretische Trennstufenzahl im Grundchromatogramm
$\mathrm{HETP}_{a,z}$ = die einer theoretischen Trennstufe gleichwertige Kolonnenlänge im Falle der z-ten Komponente, berechnet aufgrund des Grundchromatogramms (cm)
L = Kolonnenlänge (cm)
$n^s_{dp,z}$ = die zur z-ten Komponente gehörige theoretische Trennstufenzahl, in einem doppelt programmierten Chromatogramm mit gestuftem Trägergasprogramm
$\mathrm{HETP}^s_{dp,z}$ = die einer theoretischen Trennstufe gleichwertige Kolonnenlänge im Falle der z-ten Komponente, berechnet aufgrund eines doppelt programmierten Chromatogramms mit gestuftem Trägergasprogramm (cm)
$n^1_{dp,z}$ = die zur z-ten Komponente gehörige theoretische Trennstufenzahl, in einem doppelt programmierten Chromatogramm mit linearem Trägergasprogramm
$w^s_{dp,z}$ = Spitzenbreite der z-ten Komponente im doppelt programmierten Chromatogramm, bei gestuftem Trägergasprogramm (min)

$w^1_{dp,z}$ = Spitzenbreite der z-ten Komponente im doppelt programmierten Chromatogramm, bei einem linearen Trägergasprogramm (min)

$HETP^1_{dp,z}$ = die einer theoretischen Trennstufe gleichwertige Kolonnenlänge im Falle der z-ten Komponente, berechnet aufgrund eines doppelt programmierten Chromatogramms mit linearem Trägergasprogramm (cm)

$w_{p,z}$ = Spitzenbreite der z-ten Komponente in einem programmierten Chromatogramm (min).

DANK

Die Verfasser sprechen Herrn Prof. Dr. LÁSZLÓ ERDEY für die weitgehende Ermöglichung der Forschungen ihren Dank aus.

ZUSAMMENFASSUNG

In der Arbeit werden theoretische und praktische Fragen einer doppelten Programmierung behandelt, wo das Trägergasprogramm auf das Temperaturprogramm folgt. Untersuchungen über die Wirksamkeit der doppelten Programmierung zeigen, dass sich mit der beschriebenen und angewandten doppelten Programmierung bei entsprechender Spitzenauflösung eine minimale Analysenzeitdauer erreichen lässt.

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CHROM. 3753

GAS CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF DIMERIZATION PRODUCTS OF α-METHYLSTYRENE*

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(Received July 24th, 1968)

SUMMARY

α-Methylstyrene dimerization products have been separated and identified by gas chromatography.

Although isomeric compounds with a high number of functional groups in the molecule are involved here, the structure of the dimers and the type of the other dimerization products was successfully determined by means of Kovats' retention indices. The results obtained for the structure of the compounds examined are in good agreement with the results of analyses by spectrometric methods (N.M.R., I.R., U.V.).

INTRODUCTION

Dimerization of α-methylstyrene in sulphuric acid results in a compound which acts as a polymerization modifier and can exist in at least four isomeric forms: 1,1,3-trimethyl-3-phenylindane; 2,4-diphenyl-4-methyl-1-pentene; *cis*- and *trans*-2,4-diphenyl-4-methyl-2-pentene[1]. Unsaturated isomers can act as polymerization modifiers but, it has been assumed that their activities, dependent on their structures, are different. The cyclic dimer 1,1,3-trimethyl-3-phenylindane is believed to be substantially inactive as a polymerization modifier. The composition of the mixture obtained through the dimerization of α-methylstyrene in sulphuric acid medium is not completely known. Gas chromatographic analysis of this mixture gave four peaks[1]. The second and the third peak predominate and are incompletely separated, and at present it is not known which dimer gives rise to a particular peak. The author[1] assumes that the dominant peaks originate from unsaturated isomers.

This paper describes the complete analysis of the dimerization products of α-methylstyrene by gas chromatography. The components were identified by Kovats' retention indices, and the results obtained were confirmed by spectrometric methods.

The increment δI used for the determination of the compounds investigated represents the difference in dispersion behaviour of two different compounds, *viz.*: the compound examined and the "parent" compound, the latter closely being related to

* Presented at the 1st Yugoslav Symposium on Gas Chromatography, Zagreb, April 10–12, 1968.

the former. This increment is referred to in the literature as the factor of homomorphy[2], or functional index[3].

The increment ΔI, defined as the difference of the retention indices for one and the same compound on polar and nonpolar stationary liquids, was used for the determination of oxidation products of dimerization.

EXPERIMENTAL

Dimerization of α-methylstyrene is effected by polymerization of α-methylstyrene with H_2SO_4 as catalyst and in the presence of the surface active agent Ambiteric D which forms an emulsion between the sulphuric acid and the monomeric α methylstyrene.

The dimer 1,1,3-trimethyl-3-phenylindane is prepared by dimerization of α-methylstyrene on zeolite[4]. Determination of the retention and functional indices was carried out on the commercially pure components (*n*-paraffins and other hydrocarbons used as "parent" and related compounds) and on the pure components of the mixture examined which were obtained by preparative gas chromatography. The gas chromatograph Varian Aerograph Model 1520 provided with an analytical and a preparative column was used for the work. The measurements of the retention times of the individual compounds, required for the determination of the retention indices of the components of the dimeric mixture, were made on analytical columns. The preparative column was used for the separation and isolation of the dimeric fraction in the α-methylstyrene dimerization product. The mixture of dimeric isomers examined had a high boiling point within a narrow range (299–302°). For such separations silicone columns SE-30 and DC 710 are suitable[5–7]. In our work the best separation of the α-methylstyrene dimers was obtained on an Apiezon L column.

As is shown in Fig. 1 six well separated components in the dimer fraction of the α-methylstyrene dimerization products are obtained.

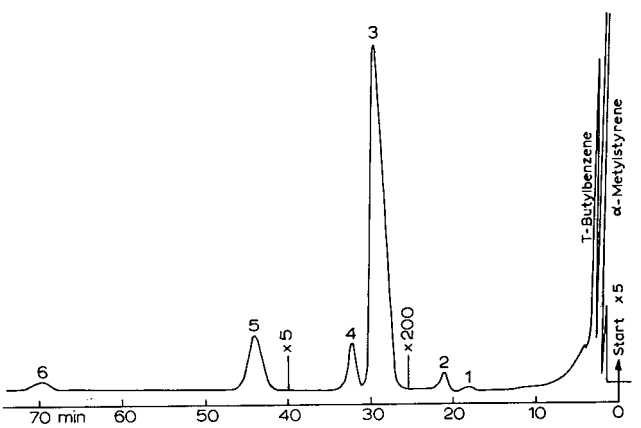


Fig. 1. Gas chromatogram of α-methylstyrene dimerization products (Apiezon L, 10%; Chromosorb W, 80–100 mesh; 1/8 in. × 6 ft.; column temperature 200°; carrier gas flow rate 20 ml/min; carrier gas nitrogen; sample size 0.4 μl).

For the preparative work a silicon rubber SE 30 column was used. The resolution obtained on the analytical SE-30 column was 94.6 %, and had a ten times shorter analysis time than the Apiezon L column, which is a real advantage. The analysis on the preparative column was performed under the following conditions:

Column 50 ft. × 3/8 in. filled with 10 % SE 30 on Chromosorb A (20–30 mesh); carrier gas flow rate 400 ml/min; inlet pressure 60 p.s.i.g.; column temperature 240°; detector temperature 240°; injection block temperature 260°; sample size 1000 μl. The isolated compounds were rechromatographed.

TABLE I

REDUCED RETENTION TIMES AND RETENTION INDICES OF THE DIMERIC COMPONENTS

Component	*SE 30 (180°)*		*Emulphor (200°)*		*Apiezon L (200°)*		$\Delta I = I^E - I^A$
	T'_R (*sec*)	I	T'_R (*sec*)	I^E	T'_R (*sec*)	I^A	
1	105	1555	244	2057.4	883	1723	334.4
2	138	1621	286	2102.0	1013	1756	346.0
3	225	1788	436	2221.2	1478	1843	378.2
4	240	1808	499	2254.6	1638	1866	388.6
5	305	1871	1276	2521.6	2618	1970	551.6
6	395	1938	1256	2516.0	3953	2061	455.0

RESULTS AND DISCUSSION

Table I shows the retention times and indices of the pure components of α-methylstyrene dimerization products, determined on three different columns: SE-30, Emulphor O and Apiezon L. The components of the dimeric mixture are designated with numbers, 1 to 6, according to the sequence of emergence of the peaks on the chromatogram. In the last column of Table I the increments ΔI for each component of the dimeric mixture are given.

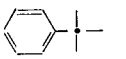
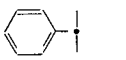
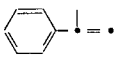
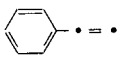
Table II shows the functional indices for methyl and phenyl groups which were obtained through the model compounds on the SE 30 column at 28°. It was found (see also WIDMER[3]) that there is no noticeable temperature dependence of the retention indices on this column. The functional index obtained in this work for the methyl group is in good agreement with the data obtained by WIDMER[3]. It was found that the phenyl group functional index changed depending on the type of carbon atom to which it is linked: *e.g.* quarternary or tertiary carbon atom, or double bond. The latter can again give a different value if a methyl group is found in its neighbourhood. Such cases were examined because in 2,4-diphenyl-4-methyl-1-pentene the phenyl group is linked to a quarternary carbon atom, while in 2,4-diphenyl-4-methyl-2-pentene the phenyl group is attached to a double bond in the neighbourhood of a methyl group.

The retention indices obtained for "parent" compounds of the isomeric dimers of α-methylstyrene were 489 for 1-pentene and 519 for 2-pentene which is in good agreement with the results obtained by WIDMER[3] for the same compounds and column. On the basis of these data and the data for the functional indices of methyl

TABLE II

FUNCTIONAL INDICES

Column SE 30, 5%; column temperature 28°; 5 ft. × 1/8 in; 20 ml N_2/min.

Functional group	*Functional index, δI*
H_3C-	70
	610
	616
	622
	636

and phenyl group (Table II), the retention indices for the dimers which should occur as the main products of α-methylstyrene dimerization[1] were calculated.

$$I_{2,4\text{-diphenyl-4-methyl-1-pentene}} = 1791$$

$$I_{2,4\text{-diphenyl-4-methyl-2-pentene}} = 1821$$

As shown in Table I the retention index of component 3 was $I = 1788$ and component 4 was $I = 1808$; thus it was concluded that component 3 is 2,4-diphenyl-4-methyl-1-pentene and component 4 is 2,4-diphenyl-4-methyl-2-pentene. As 2,4-diphenyl-4-methyl-2-pentene can exist in either *cis*- or *trans*-form, and the large substituents in the *cis*-form contribute less to the total value of the retention index[8], the *cis*-isomer would be expected to have a shorter retention time than the *trans*-isomer. Hence, some of the previous components could belong to the *cis*-isomer. Component 2 was identified as the dimer 1,1,3-trimethyl-3-phenylindane with regard to the retention time of the pure compound. Thus, it can be assumed that component 1 is *cis*-2,4-diphenyl-4-methyl-2-pentene. The N.M.R. analysis confirmed that component 1 is the *cis*-isomer and component 4 is *trans*-2,4-diphenyl-4-methyl-2-pentene.

The difference in the retention indices on polar and nonpolar columns (ΔI) for the first four components is similar. However, the ΔI for components 5 and 6 is much higher implying the polar character of these components. The compounds are obviously of dimeric structure as they emerge from the column in the region of the other dimers. Thus it should be possible to calculate on the basis of the difference of the increments ΔI for hydrocarbon dimers (components 1–4) and the polar components 5 and 6, which group of compounds these components belong to. If the average value of ΔI of the first four components, 361.8 units, (corresponding to ΔI of compounds with two phenyl groups)[9], is subtracted from the ΔI of components 5 and 6, the following values are obtained: $\Delta I'_{(5)} = 1898$ and $\Delta I'_{(6)} = 93.2$ units. The first value corresponds to compounds with a carbonyl group and the second to com-

pounds with an ether group. On the basis of results obtained by gas chromatography, the composition of the α-methylstyrene dimerization product is as follows:

Component 1: *cis*-2,4-diphenyl-4-methyl-2-pentene(?)
Component 2: 1,1,3-trimethyl-3-phenylindane
Component 3: 2,4-diphenyl-4-methyl-1-pentene
Component 4: *trans*(?)-2,4-diphenyl-4-methyl-2-pentene
Component 5: ketone, aldehyde or ester
Component 6: ether

The analysis of each separate component by N.M.R. spectrometry confirmed the above results[10]. Component 5, with help of elementary microanalysis and I.R. and U.V. spectrometric analysis, was identified as 1,3-diphenyl-3-methyl-2-butanone, but further identification of component 6 failed due to the instability of this compound.

TABLE III

QUANTITATIVE COMPOSITION OF VARIOUS SAMPLES OF α-METHYLSTYRENE DIMERIZATION PRODUCT

Sample	*Conditions of dimerization*	*Component* (%)					
		1	*2*	*3*	*4*	*5*	*6*
I	H_2SO_4:αMS = 4:1 50% H_2SO_4 50°, 4 h	0.02	0.09	91.05	8.18	0.54	0.11
III	H_2SO_4:αMS = 4:1 50% H_2SO_4 50°, 3.5 h	0.02	0.08	91.53	7.86	0.41	0.09
VI	H_2SO_4:αMS = 4:1 50% H_2SO_4 70°, 9 h	0.02	0.09	91.70	8.13	0.03	—

A quantitative analysis of the α-methylstyrene dimerization product can be made by the method of normalization from the gas chromatographic data obtained by analysis on Apiezon L at 200° and 20 ml N_2/min (Fig. 1). The composition of the dimeric fraction was quantitatively determined without the light fraction which includes about 10% of α-methylstyrene. The results of the quantitative analysis for several dimer samples, obtained under various operating conditions of dimerization, are given in Table III. It is apparent from Table III that 2,4-diphenyl-4-methyl-1-pentene and *trans*-2,4-diphenyl-4-methyl-2-pentene are the main components under all conditions of dimerization, whereas *cis*-2,4-diphenyl-4-methyl-2-pentene, 1,1,3-trimethyl-3-phenylindane and oxidation products are present only in quantities less than 1%. By increasing the temperature and the duration of dimerization the quantity of oxidation products is considerably reduced.

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CHROM. 3737

ÜBER DIE KLASSIFIZIERUNG GAS-CHROMATOGRAPHISCHER TRENNFLÜSSIGKEITEN UND DAS ABSCHÄTZEN VON RETENTIONEN MIT HILFE INFRAROTSPEKTROSKOPISCHER DATEN

II. MITT. ZUR GEZIELTEN AUSWAHL VON TRENNFLÜSSIGKEITEN FÜR DIE GAS-CHROMATOGRAPHISCHE UNTERSUCHUNG VON HOMOLOGEN UND ISOMEREN METHYLCYCLOPOLYSILOXANEN*

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(Eingegangen am 19. Februar 1968; geänderte Fassung am 30. Juli 1968)

SUMMARY

The classification of gas chromatographic separating liquids and the calculation of retention values with the aid of infrared spectroscopic measurements. Part II. The objective choice of separating liquid for the gas chromatographic study of homologues and isomers of methylcyclopolysiloxanes

A method is described for a simple and rapid selection of a gas chromatographic separating liquid by means of infrared spectroscopic measurements. The method is applied to the analysis of a siloxane mixture.

Methylcyclosiloxanes containing the polar SiH-group are separated selectively from equal or similar boiling methylcyclosiloxanes without SiH-groups using such separating liquids which produce a frequency shift $\Delta \nu_{SiH}^{Ass}$ of the SiH-valence vibration in the I.R. spectrum of the siloxane compounds. $\Delta \nu_{SiH}^{Ass}$ is a measure of the polar intermolecular forces between the SiH-groups and the polar groups of the separating liquids and therefore of the selectivity. It is possible with the aid of $\Delta \nu_{SiH}^{Ass}$ and two empirical constants measured by means of reference substances to calculate the relative retention volumes approximately by following an equation presented earlier.

However, this principle cannot be applied to the separation of position-isomeric methylhydrogencyclotetrasiloxanes. Gas chromatographic and infrared spectroscopic measurements showed that the separation in the latter case is caused by a specific selectivity of special functional groups within the molecules of the separating liquid; in this case, the phenyl groups. By increasing the proportion of phenyl groups in the separating liquid molecule, and with it the number of selective centres, it is possible to improve systematically the separation of the two position-isomeric hexamethylcyclotetrasiloxanes.

* I. Mitteilung: Siehe Ref. 23.

EINLEITUNG

Um die Auswahl von gas-chromatographischen Trennbedingungen sowie die Identifizierung von getrennten Komponenten zu erleichtern, versuchten zahlreiche Autoren, die zu erwartende Elutionsreihenfolge bereits aus den physikalischen und physikalisch-chemischen Eigenschaften der zu trennenden Substanzen und der eingesetzten Trennflüssigkeiten vorauszusagen.

Halbempirisch gefundene Beziehungen zwischen den gas-chromatographischen Daten und den physikalischen Eigenschaften der reinen Probe wie Molgewicht[1–3], Dampfdruck[4–6], Parachor[7], Molrefraktion[8] oder Dipolmoment[9,10] erweisen sich bei der Identifizierung vor allem homologer Reihen sehr nützlich, liefern jedoch keine Hinweise auf die zur Trennung geeigneten Trennflüssigkeiten. Auf Grund des Zusammenhangs zwischen den Dielektrizitätskonstanten verschiedener Trennflüssigkeiten mit den an ihnen gemessenen Retentionsdaten können die Trenneigenschaften stationärer flüssiger Phasen in gewissem Umfang vorausgesagt werden[11]. Der Vergleich gas-chromatographischer Retentionen mit spektroskopisch bestimmten Assoziationskonstanten von charge-transfer-Komplexen zur Ermittelung des Anteils an Donor-Akzeptor-Wechselwirkungen, deren Einfluss auf die Sorption schon früher erkannt wurde[12–16], gestattet vorerst nur überwiegend qualitative Aussagen[17–19].

Versuche von MARTIRE[20], physikalische Konstanten von Probe und Trennflüssigkeit gleichzeitig in einer Beziehung mit den Retentionsdaten zu verknüpfen, erscheinen besonders interessant, da das Retentionsvolumen V_i einer Substanz i bekanntlich nicht nur von ihrem Dampfdruck im reinen Zustand p^0_i, sondern auch von ihrem Aktivitätskoeffizienten γ_{ij} in der Trennflüssigkeit j bestimmt wird. Derartige Berechnungen haben jedoch bisher nur geringe praktische Bedeutung erlangt[21], denn einerseits sind die erforderlichen physikalischen Grössen nur in den seltensten Fällen bekannt bzw. exakt messbar und andererseits führt die Entwicklung geeigneter allgemeingültiger mathematischer Zusammenhänge zwischen Retentionsdaten und Stoffkonstanten zu erheblichen theoretischen Problemen.

Bei der Bestimmung der gas-chromatographisch besonders wichtigen polaren Anteile lassen sich diese Schwierigkeiten, wie in vorhergehenden Arbeiten gezeigt wurde[22,23], umgehen, wenn die im System Probe–Trennflüssigkeit herrschenden und den Aktivitätskoeffizienten beeinflussenden zwischenmolekularen Kräfte I.R.-spektroskopisch gemessen werden können.

In dieser Arbeit werden an siliziumorganischen Verbindungen gemessene I.R.-spektroskopische Daten als Hilfsmittel für die Auswahl der Trennbedingungen sowie zur Voraussage der zu erwartenden Elutionsreihenfolge bei der gas-chromatographischen Untersuchung eines Siloxangemisches benutzt.

PROBLEMSTELLUNG

Bei der Cohydrolyse von Dimethyldichlorsilan ($(CH_3)_2SiCl_2$) und Methyldichlorsilan (CH_3SiHCl_2) entstehen je nach dem eingesetzten Molverhältnis neben linearen auch cyclische Methyl- und Methylhydrogenpolysiloxane mit unterschiedlichem Gehalt an D- bzw. D^H-Gruppen pro Molekül*.

* Die Erklärung der in der siliziumorganischen Chemie üblichen Symbole und die Konstitutionen der in dieser Arbeit hauptsächlich interessierenden siliziumorganischen Verbindungen geht aus Fig. 1 hervor.

Octamethylcyclotetrasiloxan (D_4) als Ausgangsprodukt zur Silikongummisynthese wurde bereits früher gas-chromatographisch untersucht[24,25]. ROTZSCHE UND RÖSLER[24] stellten fest, dass Substanzen mit SiH-Bindungen in Spuren vorliegen, die die Synthese stören, und sie identifizierten die Methyl- und Methylhydrogencyclopolysiloxane an Hand des Zusammenhanges zwischen Retentionsdaten und Molekulargewichten, wobei es jedoch noch nicht gelang, D_4 und D_5^H und etwaige Stellungsisomeren voneinander zu trennen. In einer späteren Arbeit[26] konnte durch Kombination von Gas-Chromatographie und I.R.-Spektroskopie an der empirisch als vorteilhaft erkannten Trennflüssigkeit Methyl-Phenyl-Silikonöl OE 4011 erstmalig nachgewiesen werden, dass die Hexamethylcyclotetrasiloxan-Fraktion ($D_2D_2^H$) keine einheitliche Substanz ist, wie aus einer Arbeit von SOKOLOV[27] hervorgeht, sondern tatsächlich aus den von OKAWARA UND SAKIYAMA[28] sowie ROTZSCHE UND RÖSLER[24] vermuteten Stellungsisomeren besteht.

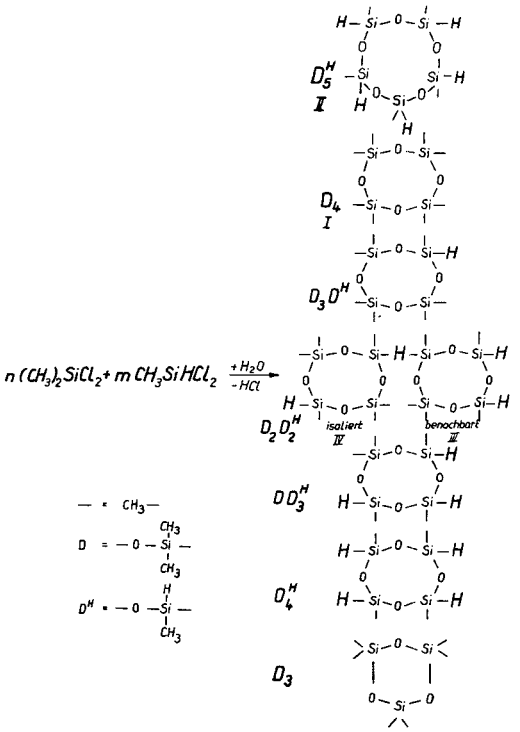

Fig. 1. Strukturen einiger bei der Cohydrolyse von Dimethyldichlorsilan und Methylhydrogendichlorsilan gebildeter Methylcyclosiloxane.

Die erzielte Isomerentrennung genügte jedoch nicht, um durch präparative Gas-Chromatographie reine Substanzen für Raman- und kernresonanzspektroskopische Untersuchungen gewinnen und eine quantitative Bestimmung für die Messung relativer Solvolysegeschwindigkeiten durchführen zu können. Im Rahmen dieser Untersuchungen galt es somit, die gas-chromatographischen Arbeitsbedingungen zur Trennung der D_4-, D_5^H- und der $D_2D_2^H$-Fraktion hinreichend zu verbessern. Ausser-

dem sollten die Cyclotrisiloxane — Hexa-, Penta-, Tetra-und Trimethylcyclotrisiloxan — in die Untersuchungen einbezogen werden, da ihre Spektren rür Konstitutions- und Bindungsbetrachtungen erforderlich waren.

THEORIE

Ein konstant siedendes binäres Gemisch kann mittels Gas-Flüssigkeits-Chromatographie getrennt werden, wenn eine der beiden Komponenten von der Trennflüssigkeit stärker zurückgehalten wird als die andere. Dieser Trenneffekt kann bei konstanter Kolonnenform, -länge und -temperatur nur mittels wirksamerer Trennflüssigkeiten verbessert werden.

Die Verbesserung erreicht man einmal dadurch, dass man Trennflüssigkeiten mit solchen funktionellen Gruppen einsetzt, deren Wechselwirkung mit den polaren Gruppen der einen Komponente zu stärkeren zwischenmolekularen Kräften führt als an der vorher verwendeten Trennflüssigkeit. Die relative Stärke dieser Kohäsionskräfte kann I.R.-spektroskopisch in Form eines "Lösungsmitteleffektes" gemessen werden. Wie an anderer Stelle näher beschrieben[23], wird hierzu die Probe in den zur Auswahl stehenden Trennflüssigkeiten gelöst und die Wellenzahl ν_i (Trennflüssigkeit) der im I.R.-Spektrum erscheinenden Valenzschwingungsbande der massgeblichen funktionellen Gruppe i der Probe gemessen. Bezieht man alle diese Wellenzahlen auf den in einer unpolaren Trennflüssigkeit wie z.B. Squalan erhaltenen Wert ν_i (Squalan), so erhält man sog. Frequenzverschiebungen

$$\Delta\nu_i^{\mathrm{Ass}} = \nu_i \text{ (Squalan)} - \nu_i \text{ (Trennflüssigkeit)},$$

die über die Beziehung

$$lg \frac{V_{\mathrm{polar}}}{V_{\mathrm{unp.}}} = lg\, V_{\mathrm{rel}} = E \cdot \Delta\nu_i^{\mathrm{Ass}} + F \qquad (1)$$
$$T = \text{konst.}$$

unmittelbar mit den Retentionsdaten verknüpft sind. Danach werden polare von gleichsiedenden unpolaren Verbindungen an der Trennflüssigkeit am besten getrennt, die infrarotspektroskopisch den grössten $\Delta\nu_i^{\mathrm{Ass}}$-Wert liefert.

Für die Trennung gleichsiedender Verbindungen unterschiedlicher Polarität kann die wirksamste Trennflüssigkeit somit ohne gas-chromatographische Versuche, allein mit den einfach und schnell zu gewinnenden infrarotspektroskopischen Daten ermittelt werden.

Zum anderen kann nach Untersuchungen von ROTZSCHE[29] angenommen werden, dass der gas-chromatographische Trenneffekt nicht unbedingt durch das gesamte Trennflüssigkeitsmolekül, sondern durch ganz bestimmte in ihm enthaltene Baugruppen, sog. selektive Zentren, hervorgerufen wird. Unter dieser Voraussetzung kann der Trenneffekt auch durch solche Trennflüssigkeiten systematisch vergrössert werden, die pro Molekül eine grössere Anzahl selektiver Zentren enthalten. Eine derartige Beeinflussung des Retentionsverhältnisses ist durchaus verständlich, da der Aktivitätskoeffizient nicht nur durch die Stärke der zwischenmolekularen Kräfte zwischen den polaren Gruppen von Probe und Trennflüssigkeit ΔU_{ij}, sondern auch

durch die Anzahl z der gleichzeitig einwirkenden benachbarten selektiven Zentren bestimmt wird[30]. Im vorliegenden Falle ist z die Anzahl der selektiven Zentren, die mit der polaren Gruppe eines in der Trennflüssigkeit gelösten Probenmoleküls in unmittelbarer Wechselwirkung stehen. Es gilt

$$\ln \gamma_i = z \cdot \frac{\Delta U}{R \cdot T},$$

$$\Delta U = \frac{1}{2}\Delta U_{ii} - \Delta U_{ij} + \frac{1}{2}\Delta U_{jj},$$

wobei ΔU_{ii} die Kräfte zwischen Probenmolekülen, ΔU_{jj} die Kräfte zwischen Trennflüssigkeitsmolekülen und ΔU_{ij} die Kräfte zwischen Probe und Trennflüssigkeit beschreibt. Für ein binäres Gemisch aus Komponenten gleichen Dampfdruckes bei der betreffenden Arbeitstemperatur besteht folgende Beziehung zu den Retentionsdaten:

$$\lg V_{II} - \lg V_I = \lg \frac{V_{II}}{V_I} = \frac{\lg \gamma_{I,j}}{\lg \gamma_{II,j}} = z \cdot A[\Delta U_{IIj} - \Delta U_{Ij}] \qquad (2)$$

da ΔU_{jj} für I und II gleich gross ist und bei der Differenzbildung entfällt und ΔU_{ii} ebenfalls vernachlässigt werden kann, da es wegen der niedrigen Probenkonzentration ohnehin sehr klein ist und sich infolge der Ähnlichkeit der Isomeren, deren Trennung hier beschrieben werden soll, bei der Differenzbildung noch weiter verringert.

Demnach sollte eine Verbesserung der Trennung gleichsiedender Isomerer, die unterschiedliche zwischenmolekulare Kräfte ausüben (d.h. endlicher Wert von ΔU_{IIj}-ΔU_{Ij}), an solchen Trennflüssigkeiten möglich sein, die eine grössere Anzahl selektiver Zentren pro Molekül enthalten, weil damit gleichzeitig mit einer Erhöhung von z zu rechnen ist.

Trennung der homologen Cyclotetrasiloxane

An dem von ROTZSCHE UND RÖSLER[24] verwendeten Methyl-Phenyl-Silikonöl DC 703 wird das Pentamethylpentahydrogencyclopentasiloxan (D_5^H) trotz seines höheren Molekulargewichts (D_5^H: 300; D_4: 296) vor dem D_4 eluiert. Der Trennfaktor ist jedoch sehr klein ($\alpha = 1.05$), so dass es zu starker Überlappung kommt, die eine quantitative Bestimmung erschwert und kleine Anteile nicht mehr erkennen lässt.

Ausgangspunkt für die systematische Verbesserung der Trennung ist eine Betrachtung des am Silikonöl DC 703 erhaltenen Trenneffekts im Hinblick auf die wirksamen zwischenmolekularen Kräfte. Im D_4 wie im D_5^H sind die Methylgruppen zur Dispersionswechselwirkung und die SiOSi-Gruppen zur Assoziation befähigt. D_5^H enthält ausserdem 5 SiH-Gruppen, die auf Grund ihres Gruppenmoments von 1.0 D[31] in polaren Trennflüssigkeiten zusätzliche Beiträge zu den Assoziationskräften liefern sollten.

DC 703 ist ein wenig polares Silikonöl, dessen Trenneigenschaften für Cyclotetrasiloxan denen des Squalans ähnlich sind. Die Retentionsvolumina werden hauptsächlich durch Dispersionswechselwirkung der Methylgruppen bestimmt. Dementsprechend wird das D_4 mit seinen 8 Methylgruppen stärker zurückgehalten als D_5^H mit nur 5 Methylgruppen, wobei der Effekt vermutlich durch das höhere Molekulargewicht des D_5^H etwas kompensiert wird.

Wie aus infrarotspektroskopischen Messungen hervorgeht[32], ist die SiH-Gruppe zur Assoziation mit den polaren Gruppen eines Lösungsmittels befähigt, so dass an

polaren Trennflüssigkeiten zusätzliche Beiträge zu den zwischenmolekularen Kräften zu erwarten sind. Dieser Effekt sollte an den Trennflüssigkeiten am stärksten sein, die für die SiH-Gruppe den grössten $\Delta\nu_{SiH}^{Ass}$-Wert ergeben. Zur Auswahl einer geeigneten Trennflüssigkeit wurde deshalb gemäss Gleichung 1 die Frequenzverschiebung der SiH-Valenzschwingung in verschiedenen Trennflüssigkeiten ermittelt.

Von den geprüften Trennflüssigkeiten schieden die stark polaren nitrilhaltigen wegen ihrer schlechten Lösungseigenschaften für die Cyclotetrasiloxane von vornherein aus. Deutliche Frequenzverschiebungen ergaben sich in Dioctylphthalat und Trikresylphosphat, an denen auch die gas-chromatographischen Daten der Siloxane gemessen wurden. Die Frequenzverschiebungen der SiH-Valenzschwingung, gemessen an der Vergleichssubstanz D_3D^H, die nur eine SiH-Bindung enthält, und die auf D_4 bezogenen relativen Retentionsvolumina sind in Tabelle I zusammengestellt.

TABELLE I

FREQUENZVERSCHIEBUNG DER SiH-VALENZSCHWINGUNG VON D_3D^H UND DESSEN RELATIVE RETENTIONEN IN VERSCHIEDENEN TRENNFLÜSSIGKEITEN

Trennflüssigkeit	νSiH (cm^{-1})	$\Delta\nu_{SiH}^{Ass}$ (cm^{-1})	V_{rel} (D_3D^H/D_4)
Squalan	2166	0	0.81
Dioctylphthalat	2162	4	0.86
Trikresylphosphat	2159	7	0.89

Die Werte wurden in einem $\lg V$–$\Delta\nu^{Ass}$-Diagramm eingetragen und die Konstanten zu Gleichung (1) ermittelt. Man erhält für $E = 0.00623$ und für $F = 0.9085$. Die Verwendung von D_4 als Bezugssubstanz erscheint berechtigt, da es keine SiH-Gruppen enthält und sich die Beiträge der Methyl- und Siloxangruppen annähernd kompensieren (Tabelle II).

Mit den nun bekannten Konstanten E und F kann das interessierende Retentionsverhältnis an den I.R.-spektroskopisch als vorteilhaft erkannten Phasen Dioctylphthalat und Trikresylphosphat berechnet werden, wobei jedoch berücksichtigt werden muss, dass im D_5^H 5 SiH-Gruppen zur Wirkung kommen und daher mit einem 5-fachen Beitrag zu den Kohäsionskräften gerechnet werden muss. $\Delta\nu_{SiH}^{Ass}$ wird deshalb mit dem 5-fachen Betrag in Gleichung (1) eingesetzt. In Tabelle II sind die berechneten den zur Kontrolle gemessenen relativen Retentionsvolumina gegenübergestellt.

TABELLE II

VERGLEICH DER BERECHNETEN MIT DEN GEMESSENEN RETENTIONSVERHÄLTNISSEN VON D_5^H UND D_4

Trennflüssigkeit	$V_{D_5^H}/V_{D_4}$ (*ber. f. 40°*)	$V_{D_5^H}/V_{D_4}$ (*gem. f. 120°*)
DC 703	—	0.952
Dioctylphthalat	1.27	1.115
Trikresylphosphat	1.34	1.21

Tatsächlich wird also an den polaren Trennflüssigkeiten das Retentionsvolumen von D_5^H gegenüber D_4 so erheblich vergrössert, dass die Trennung der Methylhydrogen-Cyclopentasiloxane von den Methyl-Cyclotetrasiloxanen nahezu vollständig ist

(Fig. 2). Erwartungsgemäss liegen die bei 120° gemessenen Retentionsverhältnisse niedriger als die berechneten, denen die bei 40° ermittelten Frequenzverschiebungen zugrunde liegen; bekanntlich nehmen die Orientierungskräfte, die hier für die Verbesserung der Trenneffekte verantwortlich sind, bei Temperaturerhöhung ab.

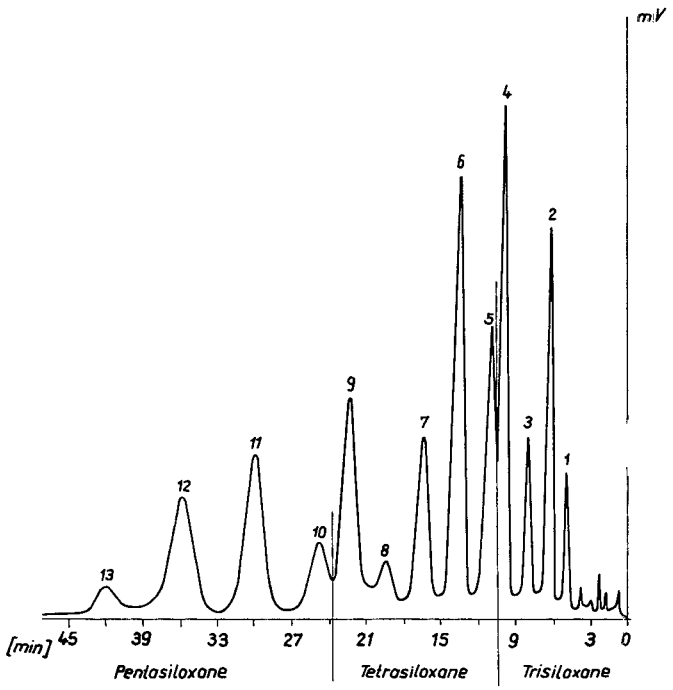

Fig. 2. Trennung der Methylcyclopolysiloxane an Dioctylphthalat bei 120°.

Fig. 2 zeigt das an Dioctylphthalat bei 120° erhaltene Gas-Chromatogramm der Cyclotri-, -tetra- und -pentasiloxane. Dem berechneten Trennfaktor entsprechend werden die Cyclotetrasiloxane und Cyclopentasiloxane gut voneinander getrennt und nunmehr ohne Überschneidungen in der Reihenfolge der Molekulargewichte eluiert (Fig. 3). Vor den Cyclotetrasiloxanen erscheinen die Cyclotrisiloxane ebenfalls in der Reihenfolge steigenden Molekulargewichts. Die in Fig. 2 und 3 verwendete Numerierung entspricht der Reihenfolge der in Tabelle III angegebenen Symbole und relativen Retentionsvolumina. Die an DC 703 (24) gefundenen relativen Retentionsdaten wurden zum Vergleich mit in die Tabelle aufgenommen.

Isomerentrennung

Um zu prüfen, ob dieselben polaren Eigenschaften der Trennflüssigkeiten, die für eine Trennung SiH-haltiger Cyclosiloxane von SiH-freien entscheidend sind, auch die Trennung stellungsisomerer Methylhydrogensiloxane beeinflussen, wurde das aus zwei Isomeren bestehende Hexamethylcyclotetrasiloxan an 12 Trennflüssigkeiten gas-chromatographisch untersucht. Diese sind in Tabelle IV in der früher[23] infrarotspektroskopisch gefundenen Reihenfolge steigender Polarität angeordnet. Der erzielte

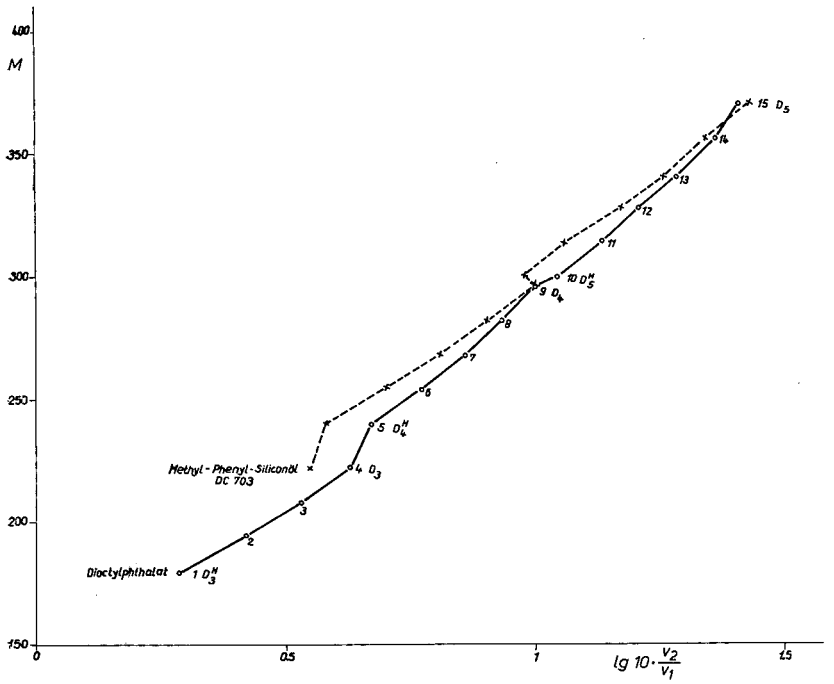

Fig. 3. Zusammenhang zwischen Retentionsvolumina und Molekulargewichten von Methylcyclopolysiloxanen.

TABELLE III

AUF D_4 BEZOGENE RELATIVE RETENTIONSVOLUMINA EINIGER METHYLCYCLOPOLYSILOXANE

Nr. in Fig. 2 und 3	*Symbol*	$V_{rel} = \frac{V_i}{V_{D_4}}$		*Molekulargewicht*
		Dioctylphthalat	*DC 703*[24]	
1	D_3^H	0.195		180
2	DD_2^H	0.265		194
3	D_2D^H	0.338		208
4	D_3	0.425	0.355	222
5	D_4^H	0.468	0.38	240
6	DD_3^H	0.592	0.50	254
7	$D_2D_2^H$	0.725	0.645	268
8	D_3D^H	0.861	0.807	282
9	D_4	1	1	296
10	D_5^H	1.115	0.952	300
11	DD_4^H	1.357	1.178	314
12	$D_2D_3^H$	1.623	1.485	328
13	$D_3D_2^H$	1.917	1.807	342
14	D_4D^H	2.29	2.212	356
15	D_5	2.575	2.693	370

TABELLE IV

Trennflüssigkeit	Strukturelemente	Trennung
Squalan	$-CH_3, -CH_2-, -CH-$	–
OE 4018	$-CH_3, Si-O-Si$	–
OE 4011	$-CH_3$,  , $Si-O-Si$	+
Polyphenyläther		–
Dioctylphthalat		–
Trikresylphosphat		–
Reoplex		–
OE 4178	$CH_3CH_2C{\equiv}N$, $Si-O-Si$	–
ß,ß'-Oxydipropionitril	$N{\equiv}C-CH_2CH_2O-CH_2CH_2C{\equiv}N$	–
Äthylenglykolbispropionitriläther	$-O-CH_2CH_2O-CH_2CH_2C{\equiv}N$	–
Polyäthylenglykol	$HO-CH_2-\left[CH_2O-CH_2\right]_n-CH_2OH$	–
Triäthanolamin	$>N-CH_2CH_2OH$	–

Trenneffekt ist mit + bzw. — angegeben. Das schwach polare Silikonöl OE 4011 ist die einzige zur $D_2D_2^H$-Trennung geeignete Trennflüssigkeit. (Prinzipiell ist daher mit der Trennung dieser Isomeren auch an dem weiter vorn zitierten Methyl-Phenyl-Silikonöl DC 703 zu rechnen[24]; sie wurde jedoch an den seinerzeit verwendeten kurzen Trennsäulen (1.6 m) nicht beobachtet.) Die Ergebnisse zeigen, dass die "Polarität"[23] der Trennflüssigkeiten—wie bei zahlreichen anderen Isomerentrennungen—auch im vorliegenden Falle keine Rolle spielt. Es erschien deshalb interessant, nach den Ursachen der Isomerentrennung zu suchen.

Ein Vergleich der Konstitutionen des Methyl-Phenyl-Silikonöls OE 4011 und des Methyl-Silikonöls OE 4018 führt zu der Schlussfolgerung, dass speziell die sehr leicht polarisierbare Phenylgruppe in OE 4011 für die hohe Selektivität dieser Trennflüssigkeit bei der $D_2D_2^H$-Trennung verantwortlich ist. Im folgenden soll diese Feststellung zunächst durch eine Betrachtung der zwischen den Phenylgruppen und den Isomerenmolekülen möglichen zwischenmolekularen Kräfte und deren Beziehung zum Trenneffekt erhärtet werden, um schliesslich die zur Auswahl wirksamerer Trennflüssigkeiten aus Gleichung (2) resultierenden Schlussfolgerungen ziehen zu können.

Wie schon bei früheren Arbeiten[22,23] wird davon ausgegangen, dass sich das chemische Zusatzpotential μ_i^E, welches den Aktivitätskoeffizienten und demzufolge auch das Retentionsvolumen massgeblich beeinflusst, annährend additiv aus Beiträgen der einzelnen Baugruppen des Moleküls zusammensetzt. μ_i^E kann nach der Gibbs-Helmholtz'schen Gleichung in die Zusatzenthalpie H_i^{Ass} und die Zusatzentropie S_i^{Ass} zerlegt werden, wobei die Energiegrösse H_i^{Ass} in unmittelbarer Beziehung zu den zwischenmolekularen Kräften steht, während über das Entropieglied, das

hauptsächlich sterischen Einflüssen unterliegt, z.Zt. keine Aussagen gemacht werden können. S_i^{Ass} dürfte im vorliegenden Fall allerdings kaum von Einfluss auf den Trenneffekt sein, da beide Isomerenformen sehr ähnliche Struktur und gleiche Molekülgrösse besitzen.

Seitens der $D_2D_2^H$-Moleküle sind es—wie in den oben behandelten Cyclopolysiloxanen—die CH_3-, SiOSi- und SiH-Gruppen, die als Zentren einer lokalisierten Wechselwirkung mit bestimmten Gruppen der Trennflüssigkeit in Frage kommen. Die Beiträge der einzelnen Gruppen addieren sich und eine Isomerentrennung ist nur dann zu erwarten, wenn auf Grund der Struktur hinreichende unterschiedliche Gesamtkohäsionskräfte auftreten.

Die CH_3-Gruppen kommen als Ursache verschiedener Kohäsionskräfte offensichtlich nicht in Betracht, da ihre Anzahl in beiden Molekülen gleich ist und ihre Wechselwirkungseigenschaften (Dispersionskräfte) wegen der im Mittel gleichen Beeinflussung durch Nachbargruppen (SiOSi, SiH) ebenfalls sehr ähnlich sein sollten. Auch für die Siloxangruppen können keine unterschiedlichen Beiträge zum Trenneffekt festgestellt werden. Zwischenmolekulare Kräfte dieser Gruppierung müssten allenfalls durch die Assoziationseigenschaften des Siloxansauerstoffs hervorgerufen werden, was sich durch die Tendenz zur Wasserstoffbrückenbindung infrarotspektroskopisch empfindlich nachweisen liesse[33]. Die Stärke der Wasserstoffbrückenbindung kann durch den Frequenzunterschied zwischen der an einer Siloxangruppe assoziierten und einer nichtassoziierten phenolischen OH-Gruppe ausgedrückt werden. Messungen an den in Fig. 1 dargestellten Cyclotetrasiloxanen ergaben zwar deutliche und systematische Unterschiede in der Reihe D_4, D_3D^H, $D_2D_2^H$, DD_3^H, D_4^H (steigende Anzahl SiH-Gruppen im Ring), die unterschiedliche Stellung der SiH-Gruppe im Ring zeigte jedoch keinen nachweisbaren Einfluss auf die Polarität der SiOSi-Gruppen.

Zum gleichen Resultat kommt man auf Grund des negativen Ergebnisses eines Trennversuches an Triäthanolamin; in diesem Falle sollten Wasserstoffbrücken zwischen OH- und SiOSi-Gruppen den Hauptbeitrag zu den Assoziationskräften liefern.

Demgegenüber ergibt sich aus den I.R.-Spektren, dass die SiH-Gruppen je nach ihrer Stellung im Ring verschieden polar sind[26]. So liegt die SiH-Valenzschwingungsfrequenz im I.R.-Spektrum des Isomeren IV bei 2169 cm^{-1}, beim Isomeren III jedoch bei 2172 cm^{-1}. Der kleine Frequenzunterschied wird durch die Raman-Spektren bestätigt[34]. Da die I.R.-Frequenz bei nichtkoppelnden Schwingungen mit zunehmendem Dipolmoment der schwingenden Gruppe absinkt, folgt, dass die SiH-Gruppen im Isomeren IV ein grösseres Gruppenmoment besitzen als im Isomeren III. Eine zwischenmolekulare Wechselwirkung der SiH-Gruppen mit den leicht polarisierbaren Phenylgruppen des OE 4011 ergibt für IV stärkere Kohäsionskräfte (Induktionskräfte) und somit ein grösseres Retentionsvolumen als für III. Die so erhaltene Elutionsreihenfolge wird durch die spektroskopische Konstitutionsbestimmung der beiden präparativ abgetrennten Fraktionen bestätigt[26].

Dieses Ergebnis stützt die eingangs getroffene Annahme über die spezifische, selektive Wirkung der Phenylgruppen des Silikonöls OE 4011 und liefert die Voraussetzung für die Auswahl wirksamerer Trennflüssigkeiten. Eine bessere Isomerentrennung sollte nämlich an Silikonölen mit einem höheren Phenylgehalt als OE 4011 zu beobachten sein, weil damit gleichzeitig ein grösserer Wert für die Zahl nächster Nachbarn und unter Berücksichtigung von Gleichung (2) ein grösserer Trennfaktor zu erwarten ist.

Es wurden deshalb Silikonöle mit höherem Phenylgehalt sowie Benzyldiphenyl zur Isomerentrennung eingesetzt. Wie die in Tabelle V zusammengestellten Ergebnisse zeigen, steigen die Trennfaktoren mit zunehmendem Phenylgehalt wie erwartet an, wobei aus Fig. 4 hervorgeht, dass der relativ kleine absolute Anstieg der Werte von bedeutendem Einfluss auf das gas-chromatographische Ergebnis ist.

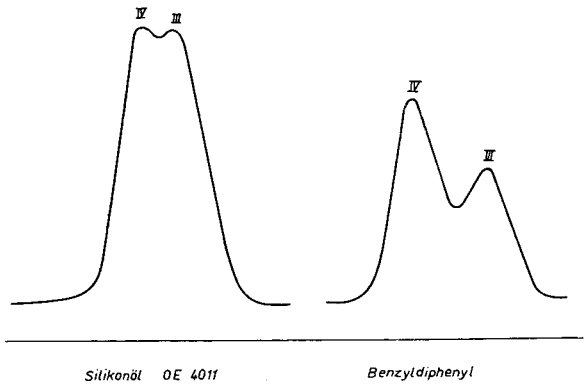

Fig. 4. Trennung der $D_2D_2^H$-Isomeren an Silikonöl OE 4011 und Benzyldiphenyl bei 75°.

TABELLE V

TRENNUNG DER ISOMEREN AN PHENYLHALTIGEN TRENNFLÜSSIGKEITEN

	Phenylgehalt in Mol-%	V_{rel}	*L (m)*
OE 4011	16.67	1.022	6
OE 4008 D	30.75	1.033	6
OE 4007 D	38.5	1.045	6
Benzyldiphenyl		1.042	6
		1.052	8

Während an OE 4011 die Trennsäule bereits bei einer Probenmenge von 10 μl $D_2D_2^H$-Gemisch so stark überladen ist, dass keine Auftrennung mehr erzielt wird, konnte an Benzyldiphenyl die Probenmenge bei präparativer Trennung bis auf 100 μl erhöht werden. Weiterhin konnten in einem Gas-Chromatogramm gleichzeitig alle Methylhydrogencyclotetrasiloxane (Fig. 5) aufgetrennt und quantitativ bestimmt werden, so dass die Messung der relativen Solvolysegeschwindigkeiten der SiH-Bindungen möglich wurde[35].

Ungeklärt bleibt jedoch die Beobachtung, dass an den polaren Trennflüssigkeiten das Isomerengemisch nicht getrennt wird. Ob sich hier ein makroskopisches Dipolmoment des Isomeren III den lokalen Assoziationskräften überlagert und das grössere Gruppenmoment der SiH-Gruppen im Isomeren IV kompensiert, kann erst durch weitere Untersuchungen entschieden werden.

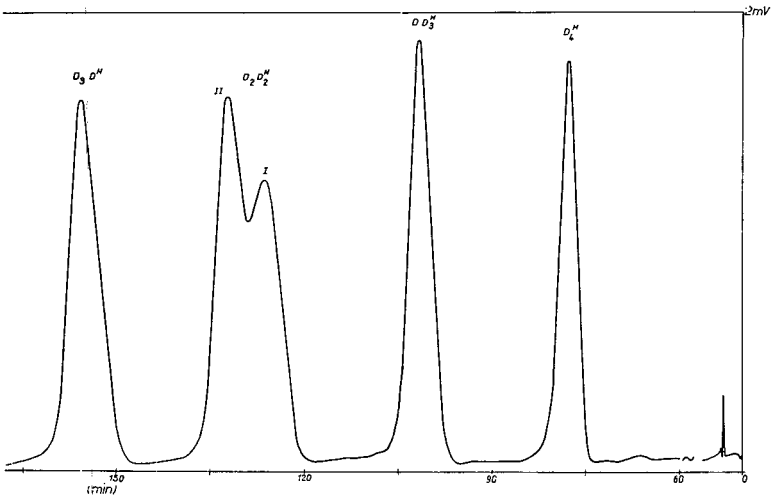

Fig. 5. Trennung des Methylcyclotetrasiloxangemisches an Benzyldiphenyl bei 90°.

EXPERIMENTELLES

Darstellung der Methyl- und Methylhydrogencyclotri-, -tetra- und -pentasiloxane

Dimethyldichlorsilan und Methylhydrogendichlorsilan werden im Molverhältnis 1:1 bei etwa 15–20° hydrolysiert, die organische von der wässrigen Phase getrennt und fraktioniert. Bei der Destillation erhält man bei etwa 130° D_3 und D_4^H, dann folgen die weiteren Methylhydrogencyclotetrasiloxane und schliesslich bei 175° D_4. Oberhalb 175° folgen zunächst die Cyclopentasiloxane. Daneben beobachtet man Nebelbildung durch Depolymerisation der im Destillationskolben noch enthaltenen linearen Polymeren. Im Destillat treten erneut Cyclotrisiloxane auf, diesmal als Depolymerisationsprodukte.

Stationäre Phasen

Die Silikonöle wurden vom Institut für Silikon- und Fluorkarbonchemie zur Verfügung gestellt. Benzyldiphenyl wurde im chemischen Laboratorium des o.g. Forschungsbereiches synthetisiert. Die übrigen Trennflüssigkeiten waren handelsübliche Produkte. Als Trägermaterial diente Sterchamol der Korngrösse 0.2–0.3 mm. Das Gew.-Verhältnis von Trennflüssigkeit zu Träger betrug 20:80.

Gas-Chromatographie und I.R.-Spektroskopie

Die gas-chromatographischen Messungen wurden an einem modifizierten Gas-Chromatographen GCI 1 (Institut für Gerätebau der DAW zu Berlin) und dem Gas-Chromatographen GChF 18/2 (W. Giede KG, Berlin-Oberschöneweide) unter Verwendung U-förmiger Trennsäulen von 6 mm Innendurchmesser, Wasserstoff als Trägergas sowie Wärmeleitfähigkeitsmesszellen durchgeführt. Die Gas-Chromatogramme wurden mit 2 mV-Kompensationsbandschreibern des VEB MAW Magdeburg registriert.

Die Probenaufgabe erfolgte mittels Dosierspritzen der Firma E. Zimmermann, Leipzig, durch einen Serumverschluss.

Die in der Diskussion benutzten I.R.-spektroskopischen Daten wurden mit dem I.R.-Doppelstrahl-Spektralphotometer UR 10 des VEB Carl Zeiss Jena gemessen. Die Ermittlung der Frequenzverschiebungen ist in[23], die Aufnahme der Gas-Spektren von den $D_2D_2^H$-Isomeren in[26] und die Messung der Wasserstoffbrücken in[36] näher beschrieben.

ZUSAMMENFASSUNG

Am Beispiel der Analyse eines Siloxangemisches wird gezeigt, dass die für eine Trennaufgabe vorteilhafteste gas-chromatographische Trennflüssigkeit einfach und schnell durch I.R.-spektroskopische Messungen ausgewählt werden kann.

Methylcyclosiloxane mit SiH-Bindungen wurden gegenüber gleich- oder ähnlichsiedenden SiH-freien Methylcyclosiloxanen an solchen Trennflüssigkeiten selektiv zurückgehalten, die im I.R.-Spektrum eines Methylhydrogencyclosiloxans einen Lösungsmitteleffekt in Form einer Frequenzverschiebung $\Delta \nu_{SiH}^{Ass}$ hervorrufen. Deren Grösse ist ein Mass für die Stärke der polaren Wechselwirkungen zwischen den SiH-Gruppen und den polaren Gruppen der Trennflüssigkeiten und damit der Selektivität. Mit Hilfe von $\Delta \nu_{SiH}^{Ass}$ und zweier an Vergleichssubstanzen ermittelten Konstanten lassen sich nach einer früher mitgeteilten Beziehung relative Retentionsvolumina näherungsweise berechnen.

Dieses Prinzip ist jedoch auf die Trennung stellungsisomerer Methylhydrogencyclotetrasiloxane nicht anwendbar. Gas-chromatographische und I.R.-spektroskopische Messungen ergaben, dass eine spezifische Selektivität bestimmter Baugruppen, des Trennflüssigkeitsmoleküls, in diesem Falle der Phenylgruppen, die Trennung bewirkt. Durch Erhöhung des Phenylgehaltes der Trennflüssigkeit und damit der Anzahl selektiver Zentren konnte die Trennung der beiden isomeren Hexamethylcyclotetrasiloxane systematisch verbessert werden.

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CHROM. 3765

SULFUR-CONTAINING AMINO ACIDS

II. CHROMATOGRAPHY OF DISULFIDES AND TRISULFIDES WITH AN AUTOMATIC ANALYZER*

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SUMMARY

Synthetic mixed disulfides and trisulfides related to cystine were chromatographed on a column of cation-exchange resin. The elution times and colour yields with ninhydrin are reported.

The elution pattern of the disulfides is demonstrated by co-chromatography with several of the common amino acids. Chromatography of trisulfides and unsymmetrical trisulfides is also illustrated.

INTRODUCTION

The biochemistry of sulfur-containing amino acids has been reviewed recently by MAW[1]. Some mixed disulfides occur naturally, *e.g.* cysteine-homocysteine disulfide, and others result from treatment of illnesses, *e.g.* cysteine-penicillamine disulfide. Disulfides involving glutathione also occur naturally and a new mixed disulfide, β-mercaptolactate-cysteine disulfide, has been isolated by AMPOLA *et al.*[2]. Several papers have been published recently on the chromatography of sulfur-containing amino acids. An automatic method for analysis of lanthionine and lysinoalanine has been described by ROBSON *et al.*[3] (It is worth noting that the method also detects cysteine.) Automation of several methods for analysis of sulfur-containing compounds has been described by BARBER[4]. Methods are described for thiols, sulfides, disulfides, etc., and detection of cysteine-homocysteine disulfide is illustrated.

In the course of studying the radiation chemistry of disulfides, we have prepared and examined many sulfur-containing amino acids. In an earlier publication, chromatography of a number of disulfides and related sulfinic and sulfonic acids was reported[5]. Since that time, some new compounds have been synthesised and most of the mixed disulfides have been further purified. This paper reports the chromatographic properties of these compounds using a commercial amino acid analyzer and

* DCBRE Report No. 566.

TABLE I

CHROMATOGRAPHY OF THIOLS, DISULFIDES AND TRISULFIDES ON A COLUMN OF 'CHROMOBEADS'
Flow rate 35 ml/h, temperature 60°.

Compound	*Abbreviation*	*Time (min)*	*Yield (hw,0.25 μE)*	*Ratio (440/570)*
L-Cysteine	CySH	249	3.05	2.25
Glutathione (ox.)	GSSG	275	9.78	0.22
DL + *meso*-Lanthionine	CySCy	296, 319*	2.68 (2.39)	—
D-Penicillamine	PenSH	331	2.20	0.26
L-Cysteine-glutathione disulfide	CySSG	394	10.32	0.33
L-Cystine	CySSCy	473	7.29	0.55
L-Cysteine-D-penicillamine disulfide	CySSPen	504	7.84	0.33
D-Penicillamine disulfide	PenSSPen	545	7.42	0.19
DL + *allo*-Cystathionine	CySHo	548, 554*	impure	—
L-Isoleucine	Ileu	566	13.62	0.19
L-Cysteine trisulfide	CySSSCy	595	5.47	0.66
L-Cysteine-D-penicillamine trisulfide	CySSSPen	616	impure	0.48
L-Cysteine-L-homocysteine disulfide	CySSHo	618	10.23	0.42
L-Homocysteine-D-penicillamine disulfide	HoSSPen	640	9.31	0.27
D-Penicillamine trisulfide	PenSSSPen	655	6.62	0.31
L-Cysteine-L-homocysteine trisulfide	CySSSHo	675	impure	0.52
L-Homocystine	HoSSHo	723	12.77	0.31
D-Penicillamine tetrasulfide	PenSSSSPen	735	impure	0.25
Cysteamine-glutathione disulfide	CyaSSG	737	impure	0.24
L-Homocysteine trisulfide	HoSSSHo	796	11.36	0.35
L-Cysteine-cysteamine disulfide	CySSCya	976	4.65	0.56

* Twin peaks.

the relationship of the disulfides to some of the common amino acids is illustrated. The abbreviations used for the sulfur compounds are explained in Table I.

MATERIALS AND METHODS

Commercially available thiols, sulfides and disulfides were purchased from Calbiochem or Mann Research Labs.

Unsymmetrical disulfides were synthesised using the method of SCHÖBERL AND GRÄFJE[6], *i.e.*

$$2\ R'SH + R''\overset{\overset{O}{\|}}{S}SR'' \rightarrow 2\ R'SSR'' + H_2O$$

This reaction is rapid, without byproducts and gives good yields. A similar method, starting from cystine-S,S-dioxide, was used by ERIKSSON AND ERIKSSON to prepare cysteine-glutathione disulfide[7]. The monoxides of cystine, homocystine and cystamine, which were required for the various mixed disulfides, were prepared by oxidation of the disulfide with peracetic or performic acid as described by SAVIGE *et al.*[8]. In the case of cystamine-S-monoxide, the method described by KLAYMAN AND MILNE[9] was also used and is probably superior. The preparation of cysteine-penicillamine disulfide is described in detail elsewhere[10]. After recrystallization, the mixed disulfides were obtained analytically pure with the exception of cysteamine-glutathione disulfide.

The trisulfides of cysteine, homocysteine and cysteamine were obtained by bubbling H_2S through an aqueous solution of the S-monoxide. After crystallization from aqueous ethanol, pure compounds were obtained. Penicillamine trisulfide (PenSSSPen) was prepared by the method of FLETCHER AND ROBSON[11]; addition of sulfur to PenSH. The product was purified by chromatography on Dowex 50W cation-exchange resin with pyridine–acetic acid buffers[12] and recrystallized to give pure PenSSSPen[10]. In the course of separating the trisulfide, some tetrasulfide (PenSSSSPen) was isolated and its composition confirmed by sulfur analysis. The same method, addition of sulfur to thiols, was utilized to produce mixed trisulfides. Thus a mixture of CySSSCy, CySSSPen, and PenSSSPen was obtained from a mixture of cysteine and penicillamine. Similarly, a mixture containing CySSSHo was prepared. The latter compound was also obtained by bubbling H_2S through an aqueous solution of cystine-S-monoxide and homocystine-S-monoxide.

The amino acid analyzer was a Technicon, Model NC-1, with a column of "chromobeads" Type A cation-exchange resin (0.6 × 150 cm). It was operated as recommended by the manufacturer except for the minor modifications described previously[5]. The column temperature was maintained at 60° throughout the runs as this was satisfactory for the compounds being studied. The "Autograd" gradient elution device was used with 75 ml of buffer in each chamber as follows: 1 to 4, pH 2.875; 5, pH 3.80; 6, pH 3.80 + pH 5.0 (1:2); 7 to 9, pH 5.0. This differs only slightly from the recommended autograd composition and does not affect the order of elution of the compounds described. A buffer flow rate of 35 ml per hour was used.

RESULTS AND DISCUSSION

The results obtained with a number of thiols, sulfides, disulfides and trisulfides are contained in Table I. The yields given are typical yields obtained with the purified amino acids but some variation was observed between different runs, different columns, and different batches of reagent. The yields given are height × width at half height for 0.25 μE where E is the equivalent weight with respect to ninhydrin positive groups. For exact calculations, standards were chromatographed with the same reagents etc., as the compounds being studied. Table I also gives the ratio of the peak heights at 440 and 570 mμ. This ratio was useful in identifying derivatives of cysteine, most of which had higher than average values. It was found that the mixed disulfides were reasonably stable in water (pH 4 to pH 6) and no disulfide exchange

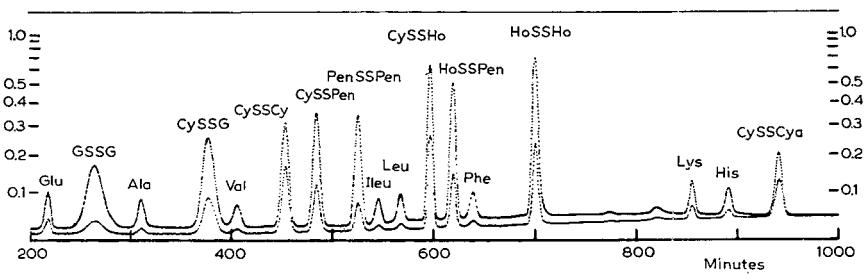

Fig. 1. Chromatography of disulfides and mixed disulfides on a column of cation-exchange resin at 60°. Buffer flow rate, 35 ml/h.

was observed with solutions after two weeks at 5°. Above pH 7 conversion to a mixture occurs and at very low pH acid catalyzed disulfide exchange may take place.

The mixed disulfides were co-chromatographed with the corresponding symmetrical disulfides and several of the natural amino acids. This provided a useful illustration of the elution pattern and the chart obtained is shown in Fig. 1. 0.25 μE of each disulfide was used while the quantity of the other amino acids was kept much smaller (0.025 μE). Chromatography of the trisulfides is shown in Fig. 2 which shows the trisulfides together with the corresponding disulfides and thiols. As with the mixed disulfides, the order of elution of these compounds was reproducible and almost independent of flow rate. The trisulfide of cysteamine was also tested but it was not eluted from the column.

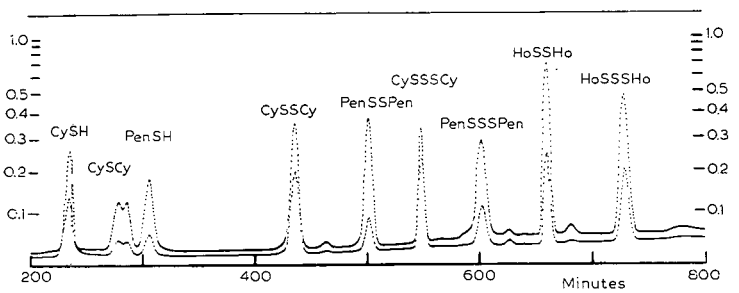

Fig. 2. Chromatography of thiols, sulfides, disulfides and trisulfides on a column of cation-exchange resin at 60°. Buffer flow rate, 35 ml/h.

The result obtained with a mixture containing a mixed trisulfide, CySSSPen, prepared as described above, is illustrated in Fig. 3. The mixed trisulfide was not isolated for characterization but it is likely that the peak occurring between CySSSCy and PenSSSPen is CySSSPen. The mixed trisulfide of cysteine and homocysteine was obtained by two different methods and it behaved in the same manner. In Fig. 3, a compound tentatively identified as the tetrasulfide, PenSSSSPen, co-chromatographed with ammonia. At a pump rate of 30 ml/h it separates from ammonia. The

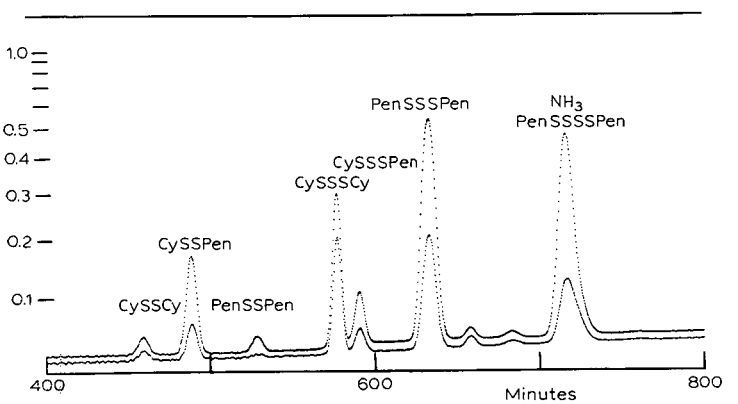

Fig. 3. Chromatography of a mixture containing disulfides, trisulfides and tetrasulfides of penicillamine and cysteine at 60° and 35 ml/h.

two minor peaks between PenSSSPen and PenSSSSPen may be due to CySSSSCy and CySSSSPen. FLETCHER AND ROBSON[11] also obtained a tetrasulfide when they prepared CySSSCy by the sulfur method.

Thus it can be seen that mixed disulfides chromatograph between the corresponding symmetrical disulfides and mixed trisulfides run between the symmetrical trisulfides as would be expected. However the unsymmetrical compounds do not always lie exactly midway between the symmetrical compounds so a synthetic mixture or a specific sulfur reaction may be necessary for satisfactory identification when they are expected.

In general terms, the elution times increase in proportion to the size of the molecule in a series; sulfide, disulfide, trisulfide and tetrasulfide. The amino acid functional groups probably change very little between members in the series. In homocystine for example there are two methylene groups between the sulfur atoms and the functional groups and still the increase in elution time between the disulfide and the trisulfide is similar to the increase in elution time between cystine and cysteine trisulfide. Hence the increase in elution times must be due to adsorption chromatography or to an increasing ability to reach two ionic sites on the resin at the same time. In the absence of more information about these compounds, a more detailed analysis is not possible.

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CHROM. 3775

SELECTIVE SEPARATION OF "NONBASIC" NITROGEN COMPOUNDS FROM PETROLEUM BY ANION EXCHANGE OF FERRIC CHLORIDE COMPLEXES

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SUMMARY

"Nonbasic" nitrogen compounds in petroleum products are quantitatively removed from hydrocarbons and other nonhydrocarbons by complex formation with ferric chloride supported on kaolin. The colored complexes remaining adsorbed on the kaolin are easily desorbed with 1,2-dichloroethane and other polar organic solvents. The free nitrogen compounds are quantitatively recovered by contacting a dichloroethane solution of the complexes with a strong anion exchange resin of the quaternary ammonium hydroxide type.

INTRODUCTION

The petroleum industry has been concerned for many years with the problem of isolating various types of nonhydrocarbon impurities from petroleum for purposes of identification. Although nitrogen compounds generally represent only a small percentage of these nonhydrocarbon constituents, they are among the most deleterious to refining catalysts and confer adverse stability properties. Numerous investigations have been made on the basic nitrogen compounds since they are readily accessible. The majority of nitrogen compounds, though, do not contain basic functional groups and consequently present greater problems in characterization studies. This paper briefly discusses techniques that are unique and useful in the separation of trace levels of "nonbasic" nitrogen compounds in a variety of petroleum products. The techniques include the independent use of coordination and ion exchange reaction chemistry. The study shows that nonbasic, as well as basic, nitrogen compounds present in petroleum are capable of forming isolable complexes with ferric chloride supported on clay mineral kaolin. The resulting complexes can be quantitatively

* The work upon which this report is based was done under a cooperative agreement between the Bureau of Mines, U.S. Department of the Interior, and the University of Wyoming.

cleaved by means of anion exchange resin chromatography to yield the original nitrogen compounds.

EXPERIMENTAL

Ferric chloride is supported on the clay mineral kaolin, 60–100 mesh, by stirring the latter with an equal volume of hot saturated methanolic ferric chloride hexahydrate for several minutes, followed by a rapid filtration. The cold filter cake is broken and washed with two portions of cold methanol (room temperature) to remove entrained nonadsorbed metal salt, and two additional washings are made with benzene to remove the methanol. The filter cake is then dried at 30° in a stirred air bath producing a light yellow powder containing approx. 2.2 % iron. The powder is easily used in conventional chromatographic column techniques.

TABLE I

EFFECT OF $FeCl_3 \cdot 6H_2O$ ON NITROGEN CONTENT OF PETROLEUM

Sample	*Pretreatment*	*Nitrogen, p.p.m.*	
		Original	*After reaction* *
1 Kuwait heavy gas oil	None	810	280
2 Kuwait heavy gas oil	Bases removed	466	Trace
3 Wilmington heavy gas oil	Acids and bases removed	2000	Trace
4 Kuwait furnace oil	Bases removed	12	Trace
5 Texas transformer oil	Bases removed	19	Trace
6 Kuwait cracked naphtha	Bases removed	150	10

* No basic nitrogen remains after each reaction as determined by titration with 0.1 *N* perchloric acid–glacial acetic acid.

Nitrogen compounds are removed by slowly percolating light petroleum distillates or hexane solutions of high-boiling petroleum fractions through beds of the synthetic adsorbent or by stirring the adsorbent with the distillate in successive portions and filtering (batch treatment); the former method is perferred. As the distillate reacts with the adsorbent, a rapid color change to green or blue occurs, indicating the formation or iron complexes on the surface of the kaolin. Entrained oil is removed from the adsorbent by exhaustive hexane elution. The effluent oils may be freed from traces of entrained colored complexes by contacting with a strong anion exchange resin.

The colored complexes of nitrogen compounds are desorbed from the kaolin by exhaustive elution with 1,2-dichloroethane. The solution of complexes is blue-green, exhibiting a broad adsorption band at 630 mμ. This solution is percolated through a bed of strong anion exchange resin (Amberlyst A-29)* in the hydroxide form. The resin breaks the complexes as indicated by a loss of blue color and the disappearance of the 630 mμ band. The metal salt is retained on the resin while the free nitrogen compounds are recovered in resulting effluents.

* Mention of specific products or brand names is made for identification only and does not imply endorsement by the Bureau of Mines.

RESULTS AND DISCUSSION

Table I shows some typical data obtained on different types of petroleum products that have been successfully denitrogenated by ferric chloride supported by kaolin. Except for sample No. 1, the nitrogen compounds in the original oils are only the neutral or nonbasic types, since basic compounds had been removed with mineral acids[1] or cation exchange resins[2] prior to treatment with ferric chloride. Removal of 92–100 % of "nonbasic" nitrogen is normally observed. Samples 1 and 2 are actually the same oil except the latter had been previously extracted with aqueous hydrochloric acid. The data show that 65.4 % of the total nitrogen could be removed in one experiment (sample 1) and essentially 100 % of the nitrogen was removed in a second experiment (sample 2). No basic nitrogen could be detected in the treated samples. These two experiments demonstrate that when basic nitrogen compounds are present in the oil they will be the type initially removed by ferric chloride; if total denitrogenation is desired, the oil must be contacted successively with fresh portions of the ferric chloride–kaolin adsorbent. Since basic nitrogen compounds are easily separated by mineral acids, most oils are pretreated in this manner to improve the overall efficiency of the ferric chloride–kaolin reagent; the neutral nitrogen compounds, as a class, are therefore more easily isolated by using the pretreated oils. Efficiency of nitrogen removal is also improved if acidic compounds are removed prior to treatment with ferric chloride–kaolin. Sample No. 3 in Table I is an example of large amounts of neutral nitrogen being removed when both acidic and basic constituents are previously removed. Complete removal of nitrogen compounds also could have been achieved without the prior removal of acids and bases, if selectivity for nonbasic types were not desired. Sample 6 seems to show a slight loss of efficiency of the reagent for severely cracked petroleum nitrogen compounds; a thorough study of these nitrogen compounds may provide an explanation for this observation. However, sample 6 could be completely denitrogenated with excess reagent.

Because ferric chloride will react in differing stoichiometries with different nitrogen functional groups (and phenols), one cannot precalculate the exact amount of reagent for any given type of oil. The fact that some reactive sites are not actually in positions suitable for a surface reaction with nitrogen donor groups is a further point for consideration. Numerous experiments in these laboratories indicate that when the ratio of pretreated oil to adsorbent is 1:2 (by weight), all "neutral" nitrogen compounds could be removed even if the nitrogen content is as high as 0.2 %, as shown in sample No. 3, Table I. The data in Table I were obtained by using a 1:2 ratio of oil to reagent.

The ferric chloride–kaolin technique is an improvement over the original approach to forming metal complexes of petroleum nitrogen compounds[3]. The present technique provides a means of isolating the nitrogen compounds from the hydrocarbons and heterocyclic oxygen and sulfur compounds; all of the latter types that are known to be present in petroleum are unreactive to the reagent. The kaolin can be coated with numerous transition metal salts in a manner similar to that described for ferric chloride if other types of complexes are desired; this may be useful where selectivity in removing certain types of nitrogen compounds is desired. Experiments in these laboratories have shown, though, that ferric chloride is the reagent of choice where complete denitrogenation of an oil is desired. After removing the complexes

from the kaolin with appropriate polar solvents, the kaolin may be reactivated and coated with additional metal salt.

Quantitative recovery of the original nitrogen compounds from their ferric chloride complexes is best achieved by using a strong anion exchange resin of the quaternary ammonium hydroxide type; these complexes could also be decomposed by heating[3] or by purging with ammonia or hydrogen sulfide[4]. MAYER's complexes of alkaloids have previously been decomposed on anion exchange resins to yield the free alkaloid[5]. This technique of recovering the free nitrogen compounds is very rapid and quite desirable, since these compounds are most stable in the basic environment. Also, small quantities of the resin are sufficient for large quantities of the complexes since the resin possesses a high capacity for the ferric ion. If desired, the resin can also be regenerated for re-use by standard techniques.

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CHROM. 3774

AN IMPROVED METHOD OF DENSITOMETRIC THIN LAYER CHROMATOGRAPHY AS APPLIED TO THE DETERMINATION OF SAPOGENIN IN *DIOSCOREA* TUBERS

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SUMMARY

A cheap, easily made and generally applicable template and syringe assembly is described which gives a more rapid and accurate densitometric thin layer chromatographic procedure. This is illustrated by the estimation of sapogenin in *Dioscorea* tubers. Factors limiting the precision of the assay method have been investigated and a standard deviation of 1.1 % has been obtained.

INTRODUCTION

The commercial production of biologically active steroids commonly begins from steroidal sapogenins. The latter may be accurately estimated in plant material by infra-red spectrometry and the improved procedure of BRAIN *et al.*[1] is rapid and affords the biologically interesting data of the amounts of the 25α- and 25β-epimers contributing to the total sapogenin. The method has the limitations that the sample must yield about 50 mg of sapogenin and that, in certain cases, interference in the infra-red spectrum, due to absorption by non-sapogenin compounds in the solution of crude sapogenin, has been observed.

These limitations were partially overcome by BLUNDEN, HARDMAN AND MORRISON[2] when they applied densitometric TLC to the determination of diosgenin in *Dioscorea* tubers, with an error of approximately 7 %. Recently they have extended the method to include the estimation of the mono- and di-hydroxy sapogenins of species of *Yucca* and *Asparagus*[3].

Our work, on the factors controlling the sapogenin available from harvested plant material, required a more accurate means of estimating the sapogenin and we have used densitometric TLC to give a method accurate to $\pm$ 2 %.

It is well known that one of the major sources of error in quantitative TLC is the spotting procedure and we have devised a relatively simple and inexpensive application apparatus which optimises the requirements of speed and accuracy.

BLUNDEN *et al.*[2,3] found that a linear relationship held between the logarithm of the weight of sapogenin applied to the plate and the square root of the absorbance

TABLE I

ERRORS IN THE APPLICATION OF SAMPLES TO THIN LAYER CHROMATOGRAPHIC PLATES

No.	*Method*	*Number of plates*	*Number of spots on each plate*	*Total number of spots scanned*	*Mean % S.D.*	*Variation of % S.D.*
i	Hand-held disposable micropipette[a]	6	9	54	9.3	± 5.4
ii	Stand-held 10 μl microsyringe[b]. Hand set	4	8	32	10.6	± 0.4
iii	Stand-held 10 μl microsyringe[b]. Automatic setting by Chaney adaptor	5	7	35	7.9	± 3.4
iv	Stand-held Agla micrometer syringe[c]. Hand set	6	7	42	7.7	± 2.7
v	Hand-held 250 μl microsyringe with repeating dispenser[b] and special template[d]	9	7	63	2.8	± 0.3
vi	As (v) but with polythene sleeve on tip of the needle	6	7	42	6.4	±1.9

[a] Drummond Scientific Co., Broomall, Pa., U.S.A.
[b] Hamilton Co., P.O. Box 307, Whittier, Calif., U.S.A.
[c] Burroughs, Wellcome and Co. Ltd., Dartford, England.
[d] Described in this paper.

value of the spot produced. However, under our conditions, a direct relationship was found between the weight of sapogenin applied and the integrated absorbance reading. This may well be due to the different chromatographic procedures used and hence to the different shape, both in area and profile, of the spots of sapogenin, as well as the use of the Chromoscan* rather than the Vitatron** densitometer.

EXPERIMENTAL

Isolation of crude sapogenin

The sapogenin was isolated in the manner described by BRAIN *et al.*[1] but the final residue was dissolved in sufficient chloroform to give a total sapogenin concentration of approximately 0.1 %.

Thin layer chromatography

A slurry was prepared by shaking 30 g of Silica Gel G (Merck) with 60 ml of distilled water and was applied as a 250 μ thick layer to 20 × 20 cm glass plates using a Shandon adjustable spreader and Unoplan leveller. The plates were air-dried for 15 min before activation at 110° for 30 min.

Application of the samples. When some commonly used procedures[4] for the application of samples to TLC plates were tried it became obvious that the application process was a major source of error. Therefore an evaluation of six different methods was undertaken. By each procedure a series of 5 μl spots of standard sapogenin solution, 1 μg/μl, was applied, the chromatogram developed, sprayed and scanned by

* Joyce Loebl & Co. Ltd., Gateshead, England.
** Fison's Scientific Apparatus Ltd., Loughborough, England.

transmittance. The % S.D. for each plate and the mean % S.D. for each series of plates were calculated (Table I).

Procedure v, using the template and syringe assembly described below (Fig. 1) was the best both for speed and accuracy. The template was constructed of perspex as follows: the base-plate, 21.5 × 21.5 cm, had two opposite side pieces, 17 × 0.5 × 2 cm high, cemented to it at the edges, and a third side, the end-piece, 21.5 × 0.5 × 2

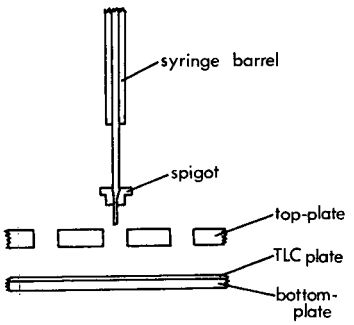

Fig. 1. Template and syringe assembly.

cm, was added. A top-plate, 21.5 × 0.5 × 2 cm, was fixed at the lower edge, across the side and end pieces, and a series of twelve holes, 0.5 cm in diameter, with 1.5 cm centres, was drilled through it, 1.5 cm from the end-piece. A 250 μl Hamilton microsyringe was fitted into a Hamilton PB 600 repeating dispenser, which, on depression of the spring-loaded button, moved the syringe piston through one-fiftieth of its total travel length, thus expelling a fixed volume of liquid, 5 μl. A perspex spigot was constructed of shaft diameter 0.49 cm with a collar of 1 cm diameter. A hole of 0.3 mm diameter was drilled through the centre of the spigot. This allowed the final centimetre of the syringe to pass through but the spigot was a tight fit at the position where the needle diameter expanded to 0.5 mm.

The syringe was filled with the test solution in the usual manner, then held vertically, in one hand, over the template. It was lowered until the spigot engaged with the first template hole and a single 'shot' of liquid ejected. This was repeated with the same solution for alternate holes and the intermediate positions spotted in the same manner with standard solution. The procedure was extremely rapid and required only about 15 sec to apply five spots of one solution. In the sapogenin assay five spots, each of 5 μl, of a standard sapogenin solution of 1 μg/μl, and another five spots, each of 5 μl, of an unknown solution of approximately equal concentration, were applied alternately to each of two plates.

Solvent system. Each plate was developed in a Desaga S-chamber for a distance of 15 cm (25–30 min), with *n*-hexane–acetone (8:1). The chromatographic separation is shown in Fig. 5.

Detection of sapogenin. Antimony trichloride, 300 g, in concentrated hydrochloric acid, 100 ml, was used to char the sapogenin spots. The plate was air-dried for 2 min before residual solvent was removed at 110° for 30 min in a mechanical convection oven. The plate was allowed to cool for 15 min before scanning.

Densitometry

A Chromoscan double-beam recording and integrating densitometer with thin layer scanner (for optical system see SHELLARD AND ALAM[5]) was used for quantitation in the transmission mode under the following conditions: wedge 2, cam C, gain 5, aperture 1005, tungsten lamp with no filter.

TABLE II

MEAN VARIATION OF THE DENSITOMETER READINGS ACROSS THE THIN LAYER CHROMATOGRAPHIC PLATE

Standard spots	*Unknown spots*	*Ratio of adjacent spots*
2.8%	3.2%	3.6%

The plate was positioned with the slit 1.5 cm to higher R_F value of the first sapogenin spot, the baseline count set to one unit per 5 sec, and the spot scanned at a sample:record expansion ratio of 1:4. The area under the densitometer curve was automatically calculated and expressed as a readout on the built-in digital integrator (see Discussion). Subsequent spots were scanned similarly, working from right to left, and then each spot was scanned a second time, working from left to right.

Calibration curves

Mixtures of the C_{25} epimers, for example diosgenin with yamogenin, normally occur in nature and the relative proportions of the two components can readily be determined by infrared spectrometry[1]. A 0.1% w/v solution in chloroform of a natural mixture, having approximately the same 25α:25β ratio as the test sample, was used as the reference material to minimise any difference in response of the C_{25} isomers to the assay procedure.

Three spots, each of 5 μl, of four standard diosgenin/yamogenin solutions of concentrations ranging from 2 to 14 μg/μl were applied to a TLC plate as described above. Fig. 2 shows the relationship between functions of concentration and integrator readings.

In another experiment three spots, each of 5 μl, of five standard sapogenin solutions of concentrations ranging from 0.88 to 1.28 μg/μl were applied to each of four TLC plates and treated similarly. The linear relationship between the integrator reading and the sapogenin concentration is shown in Fig. 3.

TABLE III

DETERMINATION OF THE ACCURACY OF THE PROCEDURE

	Mean % S.D. of duplicate determinations
(a) Thirteen TLC plates each scanned twice	0.80
(b) Six sapogenin solutions each determined twice	0.95
(c) Four plant samples each assayed twice	1.10

RESULTS AND DISCUSSION

The precision of the densitometer was determined by scanning a single 5 μg spot of sapogenin 26 times without resetting the scan path. The integrator reading was noted in each case and the % S.D. of the readings was 1.1 %. The built-in digital integrator was used successfully in this work as there was negligible fluctuation observed in the baseline.

There must always be some degree of spatial separation between the standard and unknown on a TLC plate and it is impossible to spray the whole surface evenly. To minimise the effects of this each plate was spotted with five standard and five unknown spots and the mean values calculated. The variation in the densitometer readings of 40 spots, five each of standard and unknown on each of four plates is given in Table II. The variation in the values for individual spots was slightly lower than the variation in the ratio of adjacent spots.

The precision of the assay procedure was estimated in three ways (Table III). The mean % S.D. was calculated from the results of thirteen TLC plates each scanned twice. This gave a figure of 0.80 % for the error in the densitometric determination. The errors involved in the chromatography and detection stages were estimated from the means of six sapogenin solutions each determined twice. The value of 0.95 % was only slightly greater than the figure above indicating that there was little error introduced at these stages. The accuracy of the extraction procedure was determined by assaying each of four plant samples twice. Again the figure of 1.10 % was only slightly greater than the previous figure indicating complete and repeatable extraction.

The basis of the high precision of the whole assay procedure is repeated determination. The final result is the mean of ten separate spot values each determined twice. This duplication of the estimation is readily achieved with the aid of the template and syringe assembly.

The figures for the errors in the application of samples to TLC plates by various methods (Table I) show that not only does the precision of application vary from method to method but also wide variation can occur in the accuracy of a given procedure. For example method (i): a hand-held disposable micropipette, had an error range of approximately 5–15 %. This variation in the accuracy is due in part to the artifice of the particular operator.

Our finally adopted procedure (method v) has the advantage that variation caused by the operator's manipulations are minimised and controlled: location of the spigot and depression of the plunger are the only actions required. Connection of the tip of the needle to the TLC plate by means of a piece of flexible polythene tubing (method vi) failed: the accuracy was lower and damage to the surface of the plate was frequent. Method (v) was as rapid as method (i) and these procedures were considerably faster than any of the others; the time taken to spot 5 × 5 μl of two different solutions to each of two plates was approximately 3 min.

Recently FAIRBAIRN AND RELPH[6] have published the results of an investigation of the errors in spotting paper and thin layers. They quote a coefficient of variation of 9.74 % for delivery of 25 μl of a methanolic solution of morphine from a Hamilton repeating dispenser. BRIDGER AND RELPH[7] have constructed a machine for automatic application with a coefficient of variation of 2.5 % and our value of 2.8 % compares

very favourably with this, especially when the simplicity of our apparatus is noted.

Considerable variation has occurred in the results of different workers as to the relationship between the absorbance and the concentration of the compound in both densitometric PC and TLC. Several authors[8–16] have found that a direct relationship occurs under suitable conditions between the integrated area under a densitometric peak, the absorbance value of the centre of the spot, or the optical density of the whole spot, and the quantity of the compound applied. A variety of compounds have been studied including Solanaceous alkaloids[8], *Digitalis* glycosides[9], anthraquinones[10,11], cholesterol[12], cholesterol and monopalmitin[13], methyl and cholesteryl palmitates[14], sugars[15], and amphetamines and barbiturates[16].

PURDY AND TRUTER[17] found that there was a linear relationship between the logarithm of the weight of substance applied and the square root of the area of the spot produced. BLUNDEN *et al.*[2] found that this relationship also held for the logarithm of the weight of sapogenin against the square root of the integrated absorbance reading produced.

JOHNSTONE AND BRINER[18] found a linear relationship between the logarithm of the absorbance area and the logarithm of the concentration of certain purines and SHELLARD AND ALAM[5] have found a linear relationship between the curve area and the square root of the amount of compound for the *Mitragyna* oxindole alkaloids.

If the BEER–LAMBERT law were obeyed then a linear relationship would be expected between the logarithm of the integrated area and the concentration of the compound in the layer. However, this law is not obeyed as the thin layer plate is translucent and a high proportion of the light is scattered. The KUBELKA–MUNK[19] equation for the absorption of light in highly scattering media has recently been applied by GOLDMAN AND GOODALL[20] to the problem of quantitative analysis of thin layer chromatograms. The KUBELKA–MUNK equation overlaps the BEER–LAMBERT law at low concentrations and a direct relationship between the logarithm of the curve area and the compound concentration can occur over short distances[21].

In the case of the mixture of diosgenin with yamogenin under our experimental conditions, various functions of our results were plotted and Fig. 2 shows these over the range 10–70 μg of total sapogenin. All deviated to some extent from a linear relationship and no method was better than the direct one of sapogenin concentration plotted against integrator reading. Over a narrow range of 4.5 to 6.5 μg of total sapogenin (Fig. 3) a linear relationship was obtained. This relationship between integrator reading and sapogenin concentration is not direct and a correction curve (Fig. 4) was drawn up for use with a 5 μg standard. The apparent sapogenin concentration was calculated from the standard and unknown readings assuming a direct relationship and this was then correlated with the absolute concentration by means of the correction graph and expressed as a percentage of the original plant material on a moisture-free basis.

Fig. 5 shows the TLC separation and corresponding densitometer trace for the same extract from a sample of *Dioscorea deltoidea* tuber powder under (A) the conditions of BLUNDEN *et al.*[2], *n*-hexane–ethyl acetate (4:1) in an ordinary saturated TLC tank, (B) using *n*-hexane–ethyl acetate (4:1) in an S-chamber, and (C) our present conditions of *n*-hexane–acetone (8:1) in an S-chamber. Under condition (A) a diffuse sapogenin spot was produced of diameter 15 mm. Using the same solvent system but in the S-chamber the sapogenin was deposited as a circular spot of diameter 10 mm.

In the S-chamber with the *n*-hexane–acetone solvent system a narrow 2 × 8 mm band was produced.

The distribution of spots in depth in thin and thick layers has been the subject of speculation. Under conditions of perfect saturation there should be an even distribution of the compound throughout the thickness of the layer but under normal chromatographic conditions there is a concentration of the substance towards the

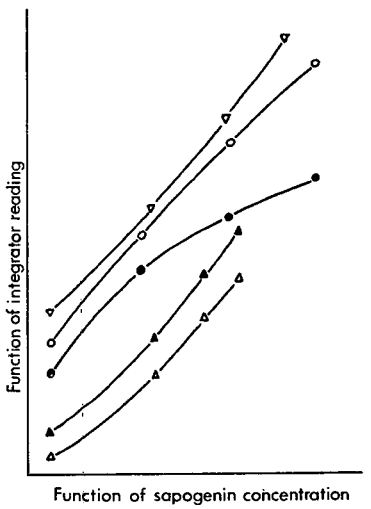

Fig. 2. Relationship between various functions of integrator reading and of sapogenin concentration over the range 10–70 μg of diosgenin and in the transmission mode. (○—○) Integrator reading *vs.* sapogenin concentration; (●—●) log integrator reading *vs.* sapogenin concentration (BEER–LAMBERT law); (▲—▲) log integrator reading *vs.* log sapogenin concentration; (△—△) √(integrator reading) *vs.* log sapogenin concentration; (▽—▽) integrator reading *vs.* √(sapogenin concentration).

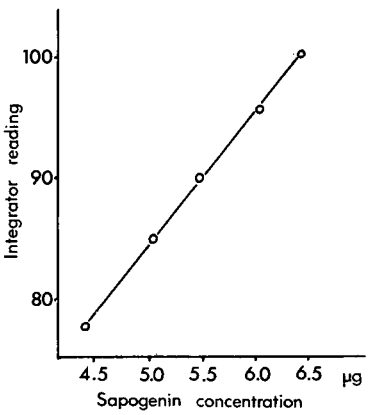

Fig. 3. Relationship between integrator reading and sapogenin concentration in the transmission mode over the range 4.5–6.5 μg of diosgenin.

upper surface of the layer. The S-chamber gives a close approximation to perfect saturation and suppresses the movement of solvent towards the surface of the layer giving a more even distribution in depth and hence a sharper spot edge and narrower band width.

Using transmission scanning (true densitometry) the same amount of light will be absorbed by the compound no matter what the thickness of the layer although the background absorbance will vary with layer thickness. A problem encountered using transmission methods has been the preparation of TLC plates of even thickness but we have found that, provided the plates were run in the direction of spreading, this gave no difficulty especially as the high contrast of the spot and background allowed the use of a low cam setting.

With reflectance scanning the distribution of the compound in the layer is of greater importance as reflectance takes place to the greatest extent at the surface of the layer. We found that the response rapidly dropped off with increasing concentration (Fig. 6) and we opted for the transmission mode.

In transmission scanning the light passes through a certain amount of the layer before it reaches the spot and according to the length of this path so the scatter will vary. As the spot becomes more compact scatter becomes relatively less important. SHELLARD AND ALAM[5] found in the case of the *Mitragyna* alkaloids that if the plate was sprayed with petroleum–liquid paraffin (1:1) to make it transparent before

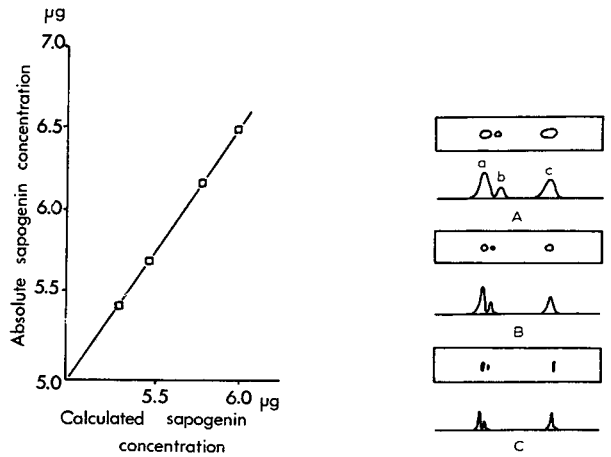

Fig. 4. Correction curve using 5 μg standard and transmission scanning.

Fig. 5. Chromatographic separations and densitometer traces from an extract of *Dioscorea deltoidea* tuber run under different conditions, *viz.* (A) *n*-hexane–ethyl acetate (4:1) in an ordinary tank; (B) *n*-hexane–ethyl acetate (4:1) in an S-chamber; (C) *n*-hexane–acetone (8:1) in an S-chamber. a = diosgenin/yamogenin; b = sterol; c = spirostan-3,5-diene.

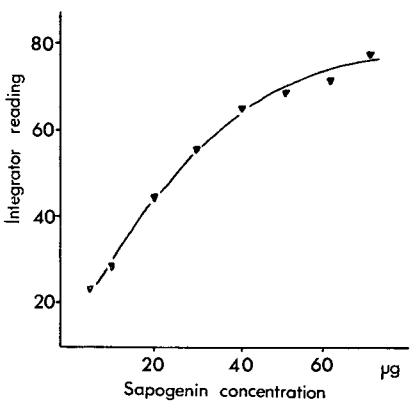

Fig. 6. Relationship between integrator reading and sapogenin concentration in the reflectance mode over the range 5–70 μg of diosgenin.

transmission scanning a linear relationship existed between peak area and the amount of alkaloid present whereas if the untreated plate was scanned a curvo-linear relationship was obtained. It is suggested that due to the even distribution in depth of the compound throughout the layer under our conditions the situation approximates to that found by SHELLARD AND ALAM[5] and the layer acts as if it were transparent.

ACKNOWLEDGEMENTS

We wish to thank the National Research Development Corporation for financial assistance and one of us (K.R.B.) thanks the Tropical Products Institute, Ministry of Overseas Development, for the award of a research studentship. Mr. A. G. KENYON and Mr. H. L. PEERMAN, of the Institute and Corporation respectively, are thanked for their assistance with supplies of plant material, and Mr. R. M. SADLER for his technical assistance.

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ABTRENNUNG VON TERPENALKOHOLEN AUS TERPENGEMISCHEN ALS 3,5-DINITROBENZOATE AUF KIESELGEL-DÜNNSCHICHTEN

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(Eingegangen am 25. Juli 1968)

SUMMARY

Separation of terpene alcohols from terpene mixtures as their 3,5-dinitrobenzoates on Kieselgel thin layers

The separation of terpene alcohols from volatile oils and mixtures of turpentine oils in the form of their 3,5-dinitrobenzoyl esters is studied in this work. The following results have been obtained by use of thin-layer chromatography.

(1) The dinitrobenzoyl esters of the following terpene alcohols have been isolated in crystalline form from turpentine oils: (25 % excess reagent used) citronellol, geraniol, nerol, borneol, menthol, *d*-isomenthol, *d*-neomenthol, *d*-neoisomenthol, α-terpineol and linalool.

(2) The primary and secondary alcohols can be separated by their different reaction time from the tertiary alcohols. In this way α-terpineol and linalool could be separated on thin layers from the other terpene alcohols by using 3,5-dinitrobenzoyl chloride; as well as from turpentine oil and from coriander oil, lavender oil, pelargonium oil, sweet orange peel oil, orange flower oil and rose oil. For complete reaction the tertiary alcohols need a reaction time of at least one hour, whereas the reaction for the primary and secondary terpene alcohols is complete after five minutes.

(3) The secondary alcohols, borneol and menthols do not yield coloured spots with antimony(V) chloride before the plates have been dried at 120° and can accordingly be differentiated from the tertiary alcohols.

(4) Neryl 3,5-dinitrobenzoate has mustard yellow crystals, m.p. 31–32°.

(5) Menthyl 3,5-dinitrobenzoate can be separated from isomenthyl 3,5-dinitrobenzoate as well as neomenthyl 3,5-dinitrobenzoate from neo-isomenthyl 3,5-dinitrobenzoate.

Schon im Jahre 1926 hat REICHSTEIN[1] gezeigt, dass 3,5-Dinitrobenzoylchlorid zur Identifizierung von Alkoholen geeignet ist. Seitdem ist diese Verbindung häufig dazu benutzt worden[2,3]. Mit den meisten Alkoholen werden durch scharfe Schmelzpunkte charakterisierte Ester gebildet. Die verschiedenen 3,5-Dinitrobenzoate (DNB) weisen oft auch grosse Differenzen im Schmelzpunkt auf. Öfters ist es auch möglich Alkohole aus Gemischen durch Herstellung der 3,5-DNB und darauffolgende

fraktionierte Kristallisation zu trennen. Besonders zur Trennung von stereoisomeren Mentholen ist die Methode brauchbar[4–9].

Von den quantitativen Bestimmungsmethoden für Alkohole ist die von JOHNSON UND CRITCHFIELD[10] zu nennen. Durch kolorimetrische Methoden konnten die Alkohole quantitativ bestimmt werden. Weiter haben ROBINSON und Mitarb.[11] eine potentiometrische Titriermethode ausgearbeitet, mit deren Hilfe es möglich ist, durch Verwendung von 3,5-Dinitrobenzoylchlorid den Alkohol quantitativ zu bestimmen.

Die 3,5-DNB lassen sich auch chromatographisch trennen. Durch Anwendung von Säulenchromatographie haben WHITE UND DRYDEN[12] die 3,5-DNB aliphatischer Alkohole und MONTES UND CLAVET[13] sowie HOLLEY UND HOLLEY[14] die 3,5-DNB einiger Terpenalkohole isoliert.

Auch einige papierchromatographische Arbeiten, die die 3,5-DNB von Alkoholen behandeln sind bekannt[15–17]. Es handelt sich meistens um 3,5-DNB von aliphatischen Alkoholen, die mit Naphtylamin oder Rhodamin sichtbar gemacht werden können. Dagegen gibt es von Terpenalkoholen weniger Auskunft.

Schon KIRCHNER UND MILLER[18] haben 3,5-DNB zur Identifizierung von Terpenalkoholen auf Dünnschichten benutzt. Die Methode ist von DHONT UND ROOY[19] weiter entwickelt und auch zur Reindarstellung von Alkoholen aus Terpengemischen benutzt worden. MEHLITZ, GIERSCHNER UND MINAS[20] haben die Methode zur Trennung von Alkoholen in Fruchtaromen benutzt und GRAF UND HOPPE[21] zum Nachweis von Isomenthol in Menthol. BRAUN[22] hat die 3,5-DNB der Alkohole in Weichmachern durch direkte Umesterung dargestellt und dann die Verbindungen dünnschichtchromatographisch identifiziert. Schliesslich hat OKINAGA[23] versucht auf Mikro-Platten die 3,5-DNB einiger Terpenalkohole zu separieren, die sich jedoch mit dieser Methode nicht befriedigend trennen liessen.

In vorliegender Arbeit wurden die Möglichkeiten zur Isolierung der Terpenalkohole aus Terpengemischen als 3,5-DNB untersucht. Die isolierten Ester wurden weiter dünnschichtchromatographisch getrennt um festzustellen, ob die Alkohole so leichter zu trennen sind als in freier Form.

DIE 3,5-DINITROBENZOATE DER TERPENALKOHOLE AUS LÖSUNGEN IN TERPENTINÖL

Durch Verwendung von 3,5-Dinitrobenzoylchlorid in 25 %igem Überschuss war es möglich die 3,5-DNB in ziemlich guter Ausbeute auch aus Terpentinöllösungen darzustellen. Tabelle I gibt Auskunft über die dargestellten Ester. Aus der Tabelle I ist auch zu ersehen, dass die Ausbeute der Ester aus den tertiären Alkoholen viel kleiner ist als aus den primären und sekundären. Es ist schon seit langem bekannt, dass sich Acylchloride schwierig mit tertiären Alkoholen umsetzen. Nach REICHSTEIN[1] setzt sich 3,5-Dinitrobenzoylchlorid mit primären und sekundären Alkoholen recht glatt um. Auch mit tertiären Alkoholen werden Ester gebildet aber viel langsamer.

TRENNUNG DER 3,5-DINITROBENZOATE AUF KIESELGEL-DÜNNSCHICHTEN

Mit Benzol–Äthylacetat (95 + 5) als Laufmittel konnten einige Alkohole von einander getrennt werden (Tabelle II). Zur Trennung der 3,5-DNB war dieses Laufmittel zu polar.

TABELLE I

DARGESTELLTE 3,5-DINITROBENZOATE

Alkoholderivat (3,5-DNB)	*Ausbeute* %	*Mal umkrist.*	*Schmp.*	*Schmp. in Litt.*	*Litt. Hinweis*
Citronellyl-	67.1	2	22–24	30	13
Geranyl-	58.8	2	53–55	63	1
Neryl-	84.4	7	31–32	—	—
Bornyl-	63.8	10	147–149	154	24
Menthyl-	71.0	1	150–152	153	9
d-Isomenthyl-	80.0	3	141–143	145	6
d-Neomenthyl-	79.8	3	149–151	155	7
d-Neoisomenthyl-	58.2	6	94–96	101	8
α-Terpinyl-	44.5	18	78–79	79	1
Linalyl-	32.8	2	85–87	89	13

TABELLE II

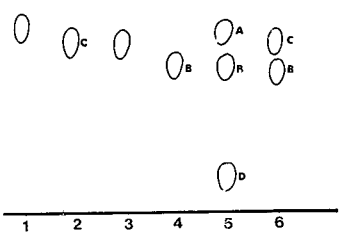
R_F-Werte der untersuchten Terpenalkohole und ihrer 3,5-Dinitrobenzoate

Laufmittel	*Nach Besprühen mit Antimon(V)chlorid*	*Nach Trocknung*	R_F-*Wert* × 100
Benzol–Äthylacetat (95 + 5)			
Citronellol	gelbbraun	braun	14
Nerol	gelbbraun	braun	16
Geraniol	gelbbraun	braun	15
Linalool	gelbbraun	braun	25
α-Terpineol	gelbbraun	braun	16
Menthol	—	braun	21
d-Neomenthol	gelbbraun	braun	29
d-Neoisomenthol	gelbbraun	braun	29
Borneol	—	braun	17
Citronellyl-3,5-DNB	gelb	braun	70
Neryl-3,5-DNB	gelb	braun	70
Geranyl-3,5-DNB	gelb	braun	67
Linalyl-3,5-DNB	gelb	braun	67
α-Terpinyl-3,5-DNB	gelb	braun	68
Menthyl-3,5-DNB	—	braun	64
d-Neoisomenthyl-3,5-DNB	—	braun	64
d-Neomenthyl-3,5-DNB	—	braun	64
d-Isomenthyl-3,5-DNB	—	braun	65
Bornyl-3,5-DNB	—	braun	64
*Benzol–Petroläther (1 + 1)**			
Citronellyl-3,5-DNB	gelb	braun	33
Neryl-3,5-DNB	gelb	braun	34
Geranyl-3,5-DNB	gelb	braun	32
Linalyl-3,5-DNB	gelb	braun	35
α-Terpinyl-3,5-DNB	gelb	braun	35
Menthyl-3,5-DNB	—	braun	49
d-Neoisomenthyl-3,5-DNB	—	braun	43
d-Neomenthyl-3,5-DNB	—	braun	48
d-Isomenthyl-3,5-DNB	—	braun	45
Bornyl-3,5-DNB	—	braun	38

* Zweimal eluiert.

Mit Benzol–Petroläther (1 + 1) wurde eine bessere Trennung erreicht.

Von den freien Alkoholen gingen Citronellol, Nerol, Geraniol und α-Terpineol in demselben Fleck, während Linalool sich ziemlich gut von diesen trennt. Borneol trennt sich schlecht von Linalool, und Menthol geht mit Linalool fast in demselben Fleck. Die Mentholisomeren trennten sich befriedigend von einander als 3,5-DNB und auch gut von Citronellyl-, Geranyl-, Neryl-, Linalyl- und α-Terpinyl-3,5-DNB. Der Fleck des Bornyl-3,5-DNB lag zwischen den Flecken dieser Gruppen (Fig. 1 und 2).



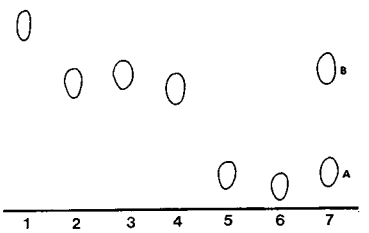


Fig. 1. Dünnschichtchromatogramm (DC) von einigen isolierten 3,5-Dinitrobenzoaten. 1 = Menthyl-3,5-DNB; 2 = Neoisomenthyl-3,5-DNB (C); 3 = *d*-Isomenthyl-3,5-DNB; 4 = Bornyl-3,5-DNB (B); 5 = Neomenthyl-3,5-DNB (A) + Bornyl-3,5-DNB (B) + Linalool (D); 6 = Neoisomenthyl-3,5-DNB (C) + Bornyl-3,5-DNB (B). Laufmittel: Benzol–Petroläther (1 + 1); Sprühreagenz: Antimon(V)chlorid. Laufstrecke 15 cm, zweimal eluiert.

Fig. 2. DC von einigen isolierten 3,5-Dinitrobenzoaten. 1 = Menthyl-3,5-DNB; 2 = Citronellyl-3,5-DNB; 3 = Neryl-3,5-DNB; 4 = Geranyl-3,5-DNB; 5. = Linalool; 6. = α-Terpineol; 7 = Linalool (A) und Citronellyl-3,5-DNB (B). Laufmittel: Benzol–Petroläther (1 + 1); Sprühreagenz: Antimon(V)chlorid. Laufstrecke 15 cm, zweimal eluiert.

Bei Benutzung von Antimon(V)chlorid als Sprühmittel traten die Flecke der 3,5-DNB der primären und tertiären Alkoholen schon beim Sprühen hervor, während die der sekundären Alkohole erst nach Trocknung der Platten hervortraten. Weder Phenyläthylalkohol noch Amylalkohol noch deren 3,5-DNB lassen sich mit Antimon(V)chlorid kenntlich machen.

Primäre und sekundäre Alkohole lassen sich durch ihre unterschiedliche Reaktionsgeschwindigkeit mit 3,5-Dinitrobenzoylchlorid von den tertiären Alkoholen trennen. Aus Gemischen können die primären und sekundären Alkohole durch kurzzeitige Einwirkung von 3,5-Dinitrobenzoylchlorid entfernt werden und nachher, nach längerer Einwirkung, die tertiären Alkohole.

ÄTHERISCHE ÖLE

Folgende ätherische Öle, deren Gehalt an Alkoholen schon früher durch gaschromatographische Analyse ermittelt war wurden untersucht.

1. Corianderöl:	
Linalool / Linalylacetat	61.5 %
Borneol	1.1 %
Geranylacetat	2.3 %
Nerol	0.9 %
Geraniol	2.1 %
2. Lavendelöl:	
Amylalkohol, Cineol und Limonen	12.8 %
Linalool	19.2 %
Linalylacetat	29.7 %
Bornylacetat	<2.7 %
Citronellylacetat	<3.7 %
Borneol / Terpinylacetat	2.2 %
Geranylacetat	1.6 %
Geraniol	2.2 %
3. Pelargoniumöl:	
Linalool / Isopulegol	10.3 %
Citronellylformiat	9.0 %
Geranylformiat	7.3 %
Citral	2.3 %
Citronellol	8.8 %
Geraniol	16.1 %
Citronellylacetat	8.1 %
4. Süsses Orangenschalenöl	
Linalool / Linalylacetat	5.3 %
Terpinen-4-ol	<0.4 %
α-Terpineol	1.6 %
Nerylacetat / Geranylacetat	2.2 %
Nerol / Geraniol	3.2 %
Citronellol	2.2 %
5. Orangenblütenöl:	
n-Heptanol	0.44 %
Linalool	37.4 %
Linalylacetat	11.92 %
Terpinen-4-ol	0.17 %
α-Terpineol	1.72 %
Nerylacetat	0.79 %
Geranylacetat	1.52 %
Nerol	0.60 %
Geraniol	1.52 %
Phenyläthylalkohol	6.77 %
Eugenol	0.05 %

6. Rosenöl:

Linalool	2.9 %
Linalylacetat	0.1 %
Citronellylacetat	2.5 %
Citral	0.5 %
Citronellol / Geranylacetat	29.5 %
Nerol	6.7 %
Geraniol	26.3 %
Phenyläthylalkohol	0.4 %
Eugenol	0.8 %

TABELLE III

R_F-WERTE DER ALKOHOLE UND DEREN 3,5-DINITROBENZOATE AUS ÄTHERISCHEN ÖLEN

	Freier Alkohol $R_F \times 100$		*Nach 5 Min*	*Nach 30 Min*	*Nach 60 Min*	*Nach 75 Min*	*3,5-DNB* $R_F \times 100$	
	*Laufmittel**						*Laufmittel*	
	A	*B*					*A*	*B*
1. Corianderöl								
Geraniol und Nerol	4	20	—	—	—	—	43	70
Linalool	9	33	+	+	(+)	—	45	70
Borneol	11	40	—	—	—	—	45	70
Linalyl- und Geranylacetat	29	60	+	+	+	+		
2. Lavendelöl								
Geraniol	6	22	—	—	—	—	45	
Linalool	10	33	+	+	(+)	—	45	
Borneol	12	38	—	—	—	—	45	
Linalyl-, Citronellyl-, Geranyl-, α-Terpinyl- und Bornylacetat	30	60	+	+	+	+		
3. Pelargoniumöl								
Geraniol und Citronellol	5		—	—	—	—	45	
Linalool	10		+	+	(+)	—	45	
Citral	17		+	+	+	+		
Geranylacetat	29		+	+	+	+		
Citronellyl und Geranylformiat	32		+	+	+	+		
4. Süsses Orangenschalenöl								
Geraniol, Nerol, Citronellol und α-Terpineol	6		—	—	—	—	42	
Linalool	9		+	+	(+)	—	42	
Linalyl-, Geranyl- und Nerylacetat	30		+	+	+	+		
5. Orangenblütenöl								
Geraniol, Nerol und α-Terpineol	5		—	—	—	—	40	
Linalool	9		+	+	(+)	—	40	
Linalyl-, Geranyl- und Nerylacetat	30		+	+	+	+		
6. Rosenöl								
Geraniol, Nerol und Citronellol	6	22	—	—	—	—	40	69
Linalool	9	32	+	+	—	—	40	69
Citral	16	44	+	+	+	+		
Eugenol	16	48	+	—	—	—	—	
Geranyl-, Citronellyl und Linalylacetat	28	55	+	+	+	+		

* Laufmittel: A = Benzol–Petroläther (1 + 1); B = Benzol–Äthylacetat (90 + 10).

Die Bildung der 3,5-Dinitrobenzoate der tertiären Alkohole in den ätherischen Ölen liess sich dadurch verfolgen, dass die Reaktionszeit allmählich verlängert wurde.

Tabelle III zeigt die R_F-Werte der identifizierten Verbindungen und die Reaktionszeiten, die erforderlich waren, um auch die tertiären Alkohole der Öle quantitativ in ihre 3,5-DNB umzusetzen (vgl. Fig. 3). Es ist ersichtlich, dass Linalool mindestens eine Reaktionszeit von einer Stunde braucht, um vollständig reagiert zu haben. Die Betrachtung der Flecke in den Figs. 3 und 4 zeigt, dass die Linaloolflecke sich vermindern, während die 3,5-DNB sich vergrössern. Linalyl-3,5-DNB lässt sich chromatographisch nicht von den Geranyl-, Citronellyl- und Nerylderivaten trennen. Linalool kann aber wegen der Trägheit der Reaktion mit 3,5-Dinitrobenzoylchlorid in dieser Weise gut neben den primären und sekundären Terpenalkoholen identifiziert werden.

Sowohl in Corianderöl, Lavendelöl und Orangenblütenöl, die erhebliche Mengen von Linalool enthalten, konnte freies Linalool in dieser Weise neben den anderen Alkoholen und neben Linalylacetat deutlich gezeigt werden.

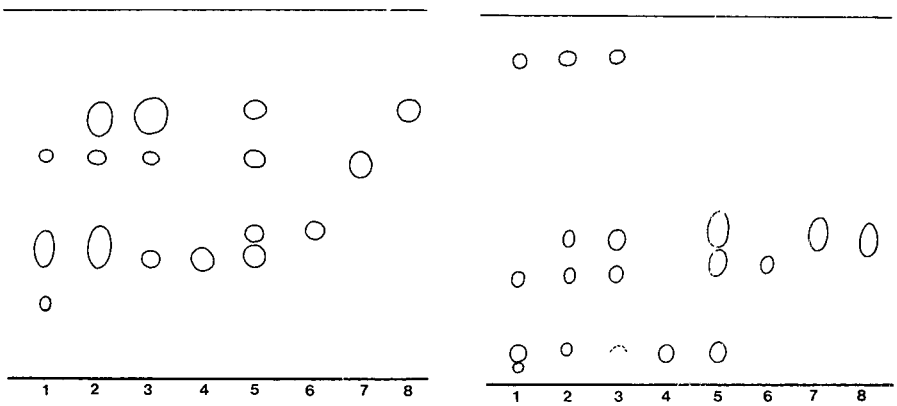


Fig. 3. DC von 1 = Corianderöl; 2 = mit 3,5-Dinitrobenzoylchlorid behandeltes Corianderöl (5 Min); 3 = mit 3,5-Dinitrobenzoylchlorid behandeltes Corianderöl (60 Min); 4 = Linalool; 5 = Linalool, Geraniol, Nerol, Borneol und Linalylacetat; 6 = Borneol; 7 = Linalylacetat; 8 = Linalyl-3,5-DNB. Laufmittel: Benzol–Äthylacetat (90 + 10); Sprühreagenz: Vanillin–Schwefelsäure 3%.

Fig. 4. DC von 1 = Orangenblütenöl; 2 = mit 3,5-Dinitrobenzoylchlorid behandeltes Orangenblütenöl (5 Min); 3 = mit 3,5-Dinitrobenzoylchlorid behandeltes Orangenblütenöl (60 Min); 4 = Linalool; 5 = Linalool, Linalylacetat, Linalyl-3,5-DNB und Geranyl-3,5-DNB; 6 = Linalylacetat; 7 = Linalyl-3,5-DNB; 8 = Geranyl-3,5-DNB. Laufmittel: Benzol–Petroläther (1 + 1); Sprühreagenz: Antimon(V)chlorid.

EXPERIMENTELLES

Herstellung der 3,5-Dinitrobenzoate

Von den Terpenalkoholen wurde eine 10%ige Lösung in Terpentinöl bereitet. 10.0 g davon wurden mit 6 ml Pyridin versetzt und mit einer heissen Lösung von 2 g 3,5-Dinitrobenzoylchlorid in 10 ml frisch destilliertem Benzol vermischt. Nach Kochen unter Rückfluss (die Reaktionszeit wurde verschieden gewählt, von 5 Min für primäre und sekundäre bis sogar 75 Min für tertiäre Alkohole) wurden die Reak-

tionsprodukte in Äther aufgenommen, erst mit 25 ml 10 %iger Salzsäure und dann mit 10 ml 10 %iger Natriumhydroxydlösung geschüttelt und mit Wasser bis neutrale Reaktion gewaschen. Von dem nach Trocknung und Destillation der ätherischen Phase erhaltenen Rückstand wurde das Terpentinöl unter verminderten Druck abdestilliert und der Rest wurde durch mehrfache Umkristallisation aus Petroläther gereinigt (vgl. Tabelle I).

Aus den ätherischen Ölen wurden die 3,5-DNB-Verbindungen in ähnlicher Weise abgetrennt. Im allgemeinen wurde 1 g 3,5-Dinitrobenzoylchlorid pro 2 g Öl verwendet und die Ätherextrakte wurden auf 3 ml eingeengt.

Dünnschichtchromatographie

Die Dünnschichtplatten wurden in üblicher Weise mit Kieselgel G "Merck" bedeckt und im Trockenschrank 30 Min bei 110° aktiviert. Die 3,5-DNB und die freien Alkohole wurden in Äther gelöst (10 %ige Lösung) und davon 1 μl auf die Platte appliziert. Laufzeit 35–40 Min. Laufstrecke 15 cm. Sprühmittel: 10 % Antimon(V)-chlorid in Chloroform.

ZUSAMMENFASSUNG

In vorliegender Arbeit werden die Möglichkeiten zur Abtrennung von Terpenalkoholen aus ätherischen Ölen und Terpentinölgemischen mit Hilfe der 3,5-Dinitrobenzoylester studiert. Unter Zuhilfenahme der Dünnschichtchromatographie wurden folgende Ergebnisse erzielt.

(1) Die Dinitrobenzoylester folgender Terpenalkohole liessen sich aus Terpentinöllösungen in kristallinischer Form isolieren: (Reagenz in 25 %igem Überschuss) Citronellol, Geraniol, Nerol, Borneol, Menthol, *d*-Isomenthol, *d*-Neomenthol, *d*-Neoisomenthol, α-Terpineol und Linalool.

(2) Primäre und sekundäre Alkohole lassen sich durch ihre Reaktionsgeschwindigkeit von den tertiären Alkoholen trennen. In dieser Weise konnten α-Terpineol und Linalool auf Dünnschichten nach Entfernung der anderen Terpenalkohole mittels 3,5-Dinitrobenzoylchlorid getrennt werden, sowohl aus Terpentinöllösungen als aus Corianderöl, Lavendelöl, Pelargoniumöl, Süsses Orangenschalenöl, Orangenblütenöl und Rosenöl. Die tertiären Alkohole benötigen wenigstens einstündige Reaktionszeit zur vollständigen Reaktion, während die Reaktion bei primären und sekundären Terpenalkoholen schon nach fünf Minuten beendet ist.

(3) Die sekundären Alkohole, das Borneol und die Menthole treten beim Sprühen mit Antimon(V)chlorid erst nach Trocknung der Platten bei 120° hervor und sind dadurch von den primären und tertiären Alkoholen zu unterscheiden.

(4) Neryl-3,5-dinitrobenzoat, senfgelbe Kristalle, Schmelzpunkt 31–32°.

(5) Menthol-3,5-dinitrobenzoat lässt sich von Isomenthyl-3,5-dinitrobenzoat trennen, Neomenthyl-3,5-dinitrobenzoat entsprechend von Neoisomenthyl-3,5-dinitrobenzoat.

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CHROM. 3769

PRECISE LOCATION OF STEROIDS IN THIN-LAYER CHROMATOPLATES WITH MARKER DYES*

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(Received August 15th, 1968)

SUMMARY

A method is described whereby the location of steroids in developed chromatoplates can be determined to ± 1 mm without spraying, by the use of marker dyes which do not interfere in subsequent quantification of steroid extracted from the adsorbant. Applications to urinary steroids are discussed.

INTRODUCTION

A systematic method for the qualitative and quantitative analysis of steroids based on the combined use of thin-layer and gas–liquid chromatography (TLC–GLC) was established in this laboratory[1–8].

By submitting total steroids to TLC in a chloroform–methanol system[1,2], a first separation based mainly on differences in polarity yielded a series of distinct TLC zones characterized by molecules which contained specific numbers of hydroxyl and carbonyl groups. Quantitatively removed and eluted groups of steroids[8] could be successfully analyzed by GLC.

As shown in a series of recent articles[9–11] the development in this laboratory of highly efficient nonpolar columns permitted excellent complementary separations of TLC-zone components on the basis of molecular weight and stereoconfiguration. Segregation into TLC subzones of group components that otherwise would form mixtures difficultly separable by GLC was observed[9,10].

The systematic simplicity of the method is undoubtedly dependent on zone and subzone boundaries being consistently and precisely located on chromatoplates.

The use of so-called "nondestructive" sprays is considerably limited for this purpose if quantification of very small amounts of steroids is contemplated; indeed none of such sprays effectively fulfils both the following requisites: (1) ability to reveal the position of very small amounts of steroids, (2) complete absence of interference in subsequent quantification of small amounts of steroids by GLC, fluorometry, UV, or IR spectrophotometry.

* Contribution No. 324, Animal Research Institute, Research Branch.

In addition, sprays will not permit unequivocal location of individual steroids when relatively large amounts of other steroids are present in the same TLC zone.

Undoubtedly the use of UV light-induced fluorescence is helpful in the case of urinary steroids[5]. However, required trace amounts of fluorescing impurities accompanying urinary steroids are generally lacking in steroid samples of different origin. Hence UV light visualization of thin-layer chromatograms is not generally possible.

The present article proposes the use of marker dyes which consistently permit the location of steroid band centers within $\pm$ 1 mm, and do not interfere in subsequent quantification. Relationships between dye- and steroid-band positions were established by examination of a large number of sprayed chromatoplates in the manner described below.

EXPERIMENTAL

Materials and solutions

Adsorbosil 4 (10 % $CaSO_4$); Applied Science, Inc., State College, Pa.

Sudan Blue: No. 3788, Harleco.

p-Aminoazobenzene (No. 3477); 2,4-diaminoazobenzene (HCl) No. 12087; 4,4'-dihydroxyazobenzene (No. 4736): K & K, Plainview, N.Y. 11803.

Steroids: Steraloids Inc., Flushing, N.Y.

Phosphomolybdic acid: Analar, British Drug House.

Methanol: redistilled, Analar grade, British Drug House.

Chloroform: redistilled, Fisher C-754 reagent.

Developing solvent: $CHCl_3$–MeOH–H_2O (200:9:0.75, v/v/v).

Dye solution: Sudan Blue, 100 mg; *p*-aminoazobenzene, 10 mg; 2,4-diaminoazobenzene, 20 mg; 4,4'-dihydroxyazobenzene, 20 mg per 100 ml of $CHCl_3$–MeOH (2:1, v/v).

Steroid stock solutions: 1 mg steroid per ml of $CHCl_3$–MeOH (1:2,v/v).

EQUIPMENT

Desaga adjustable spreader, coating template, and 200/200/3.8 mm selected[6] glass plates.

Shandon developing tanks and storage cabinets.

Semi-automatic TLC sample applicator[6], Supelco, Inc., P.O. Box 581, Bellefonte, Pa. 16823.

Hamilton microsyringes.

Desaga spotting template.

METHODS

Steroids test solutions

250 μl of each of the required steroid stock solutions were successively evaporated at 56° under a stream of nitrogen in a 1-ml tube; 500 μl of the dye solution were added to the dry residue. The upper section of the ground-glass stoppers were lightly smeared with silicone grease and the stoppered tubes kept at —5° between uses.

Test solutions containing from eight to twelve different steroids were prepared. Component steroids were selected on the basis of R_b values previously observed in a similar system[1,2] to obtain distinctly separated bands in developed plates. A total of about 100 steroids were included in 26 test solutions, most steroids in more than one solution. A sprayed chromatogram obtained with one of the mixtures is shown in Fig. 1. Fig. 2 shows chromatograms obtained with urinary steroids.

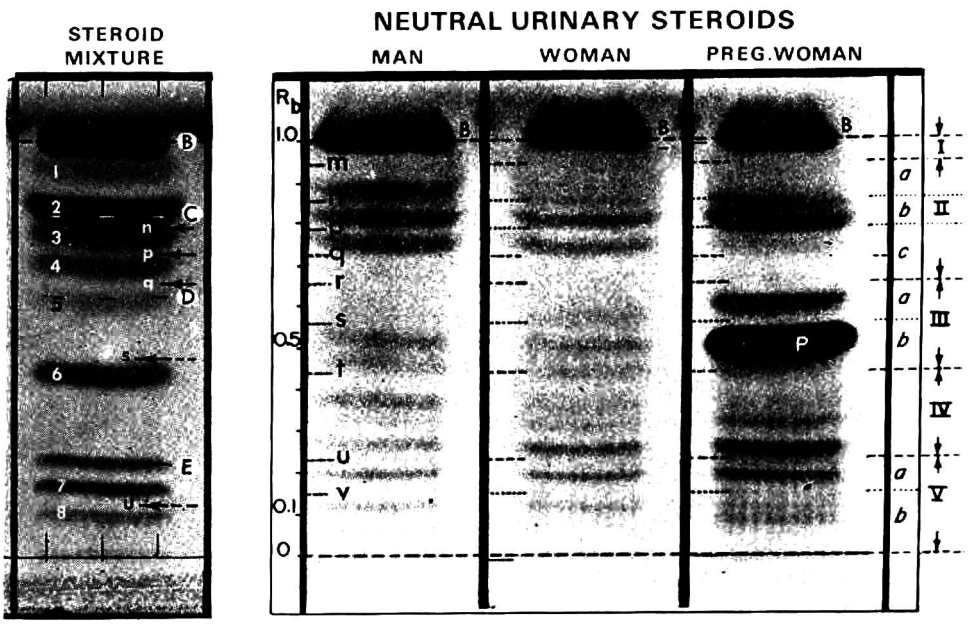

Fig. 1. Sprayed chromatogram of a mixture with the following composition; capital letters refer to marker dyes, numbers to steroids: (B) Sudan Blue; (1) progesterone (pregn-4-ene-3,20-dione); (2) cholesterol (3β-hydroxycholest-5-ene); (C) *p*-aminoazobenzene; (3) androsterone (3α-hydroxy-5α-androstan-17-one); (4) etiocholanolone (3α-hydroxy-5β-androstan-17-one); (D) 2,4-diaminoazobenzene (HCl); (5) 11-ketoandrosterone (3α-hydroxy-5α-androstane-11,17-dione); (6) pregnanediol (3α,20α-dihydroxy-5β-pregnane); (E) 4,4'-dihydroxyazobenzene; (7) pregnanetriol (3α,17α,-20α-trihydroxy-5β-pregnane); (8) estriol (3,16α,17β-trihydroxyestra-1,3,5(10)-triene). For conditions: *cf.* text and caption of Fig. 4. Dye-band centers are indicated by a dashed line at both ends and middle of dye bands. Arrows indicate positions m, n, p... in intervals between steroid bands which correspond to zone and subzone boundaries indicated by the corresponding letter in Fig. 2 (*cf.* discussion). Dyes have the following colours in unsprayed chromatograms: (B) blue; (C, D, and E) pale yellow. In chromatograms sprayed with phosphomolybdic acid, then heated, B is very dark blue, C and E are bright red, D stays pale yellow; most steroid bands turn dark blue; only a few, progesterone, for example, are faint.

Fig. 2. Chromatograms of neutral urinary steroids obtained under conditions similar to those used for the chromatogram in Fig. 1; dyes were not included. Note correspondance of zone and subzone boundaries m, n, p... with points (arrows) within steroid-band intervals designated by the same letters in Fig. 1. The procedures used to obtain neutral urinary steroids from 5 ml of urine are described in ref. 5.

Measurement of migration distance

The dry developed plate was placed on a light box. A plastic Desaga spotting template (MNPQ, Fig. 3a) was placed over the plate, both as a protective cover and to provide a straight edge MN perpendicular to dye bands. By sliding the template

in a direction parallel to base line OO', MN was made to coincide successively with one end, the middle, and the other end of dye bands. In each of these positions the center of each dye band was carefully marked with a fine needle.

Distances OB, OC, and OE from base line OO' to the marked dye-band centers were measured to the nearest 0.25 of a millimeter or better by using the simple device shown in Fig. 3 b.

The plate was sprayed with 10% phosphomolybdic acid solution and heated to reveal steroid-band positions[1,2]. The positions of steroid-band centers, of zone and subzone boundaries (cut-lines) were marked and measured in the manner described for dye-band centers.

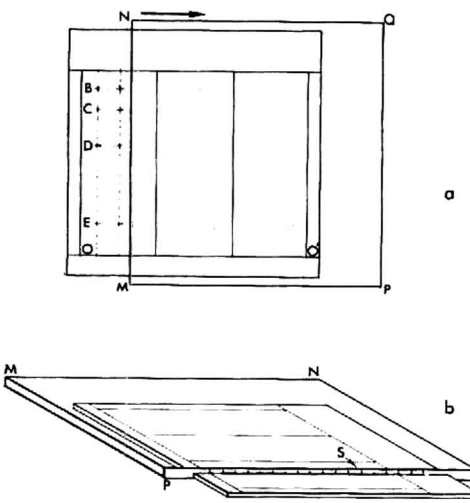

Fig. 3. Illustrating marking and measuring techniques. (a) *cf.* text. (b) Scale S is cemented to side PQ of Desaga spotting template MNPQ. Zero of scale graduated in 0.5 of a millimeter is made to coincide with plate origin.

Plotting data

R_b values. R_b values of dye-band centers C, D and E were calculated as the ratio of dye-band center distances OC, OD, and OE to the corresponding OB distance. R_b values for steroid-band centers, X, were similarly determined. The diagram in Fig. 4 shows a grouping of steroids according to average R_b values. Grouping was obviously dependent on specific numbers of hydroxyl and carbonyl groups as was previously observed in a similar solvent system[1-5].

Migration data for dyes C, D, and E, and steroids included in a typical test solution are given in Table I.

Steroid-dye positional relationship. From dye-band center and steroid-band center data, distances XY_n from steroid-band center, zone or subzone boundary, X, to nearest dye-band center Y_n, and distances BC, CD, DE, between dye-band centers were determined.

Plots relating XY_n to ratios of distances OB, OC, OD, OE, BC, CD, and DE were made with the purpose of finding that which expressed a consistent positional

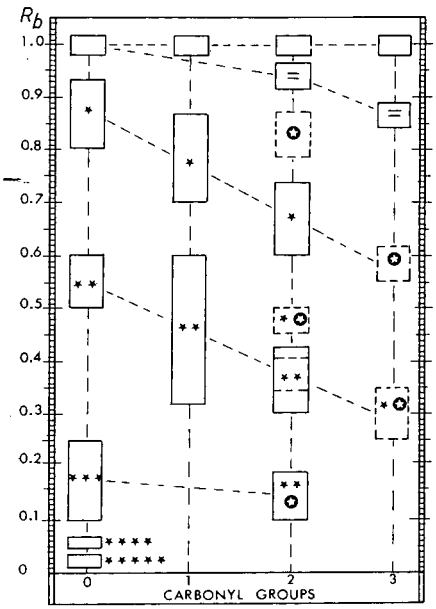

Fig. 4. Diagram showing relative migration distance R_b of steroids on TLC plates in relation to hydroxyl group (0 to 5) and carbonyl group (0, 1, 2 or 3) content. The 20 × 20 cm TLC plates were coated with a 0.25 mm layer of Adsorbosil 4 (Applied Science) and developed in $CHCl_3$–MeOH–H_2O (200:9:0.75, v/v/v). For details on coating, conditioning, loading, and developing, *cf.* refs. 3, 6, and 7. $R_b = 1$ is migration distance of Sudan Blue included in the samples. Rectangles indicate spread of band centers for components of specific groups of steroids; number of hydroxyl groups is indicated by number of stars, number of carbonyl groups by figure at bottom of relevant column. Dashed contours are used for corticosteroids; white star in black circle indicates 21-hydroxyl group. Two horizontal dashes indicate double bonds. Tetra- and pentahydroxysteroids (4 and 5 black stars at bottom of first column) also include 21-corticosteroids.

relationship of X to dye-band centers. Such a plot, characterized by an approximatively linear, tight grouping of points is exemplified by plots a and b in Fig. 5 for steroid-band centers.

In the combined TLC–GLC method of analysis, the positions of zone and subzone boundaries are required. These correspond to centers of characteristic intervals between zones and subzones observed, for instance, in sprayed chromatoplates obtained with urinary steroids exemplified in Fig. 2.

Plots relating the positions of zone and subzone boundaries to dye-band center positions were obtained. Examples of such plots are given in Fig. 6.

DISCUSSION

Difficulties in locating steroid bands and zone boundaries in unsprayed chromatoplates stem from the narrowness of such features; as seen in Figs. 1 and 2, they measure as little as 3 mm. Hence a method leading to points located within $\pm$ 1.5 mm of their actual center 99 % of the time, *i.e.*, a method corresponding to a standard deviation $\sigma < 0.58$, is required.

TABLE I

MIGRATION DATA[a] FOR A NUMBER OF STEROIDS[b] AND FOR DYES B, C, D, AND E

Dye or steroid[t]	*Mean XO* (*mm*)	$\Delta_{XO}max$[c] (± *mm*)	σ_{XO} (*mm*)	R_b (*av.*)	$\Delta_p max$[c] (± *mm*)	$\sigma\Delta_p$ (*mm*)	*Score*[d]
B	136.0	6.0	2.4	1.000	3.0	1.2	80
Progesterone	130.0	6.0	2.4	0.958	3.4	1.3	76
Cholesterol	112.5	6.0	2.4	0.827	3.6	1.4	72
C	111.5	6.0	2.4	0.819	4.0	1.6	64
Androsterone	106.0	7.5	3.0	0.778	4.8	1.9	57
Etiocholanolone	94.8	9.5	3.8	0.697	5.9	2.3	48
D	89.4	10.5	4.2	0.657	6.0	2.4	46
11-Ketoandrosterone	84.0	12.2	4.9	0.617	5.4	2.2	50
Pregnanediol	61.8	9.8	3.9	0.454	4.9	1.9	57
E	25.5	7.2	2.9	0.187	4.0	1.6	65
Pregnanetriol	19.6	6.4	2.6	0.144	2.7	1.1	82
Estriol	9.9	3.7	1.5	0.073	1.3	0.5	100

[a] Solvent front migrated 150 mm under conditions described in caption of Fig. 4.

[b] Chromatogram for this mixture is shown in Fig. 1; UPAC designations of compounds are given in caption of Fig. 1.

[c] $\Delta_{XO}max$ is maximum deviation from mean XO observed in a set of twenty chromatograms measuring from centers at both ends and middle of bands (60 values); $\Delta_p max$ is maximum difference observed for XO measured from the center at middle of bands for chromatograms in the same plate (usually three chromatograms per plate).

[d] Number of successful locations predicted from $\sigma\Delta_p$ for 100 independent experiments; *cf.* discussion.

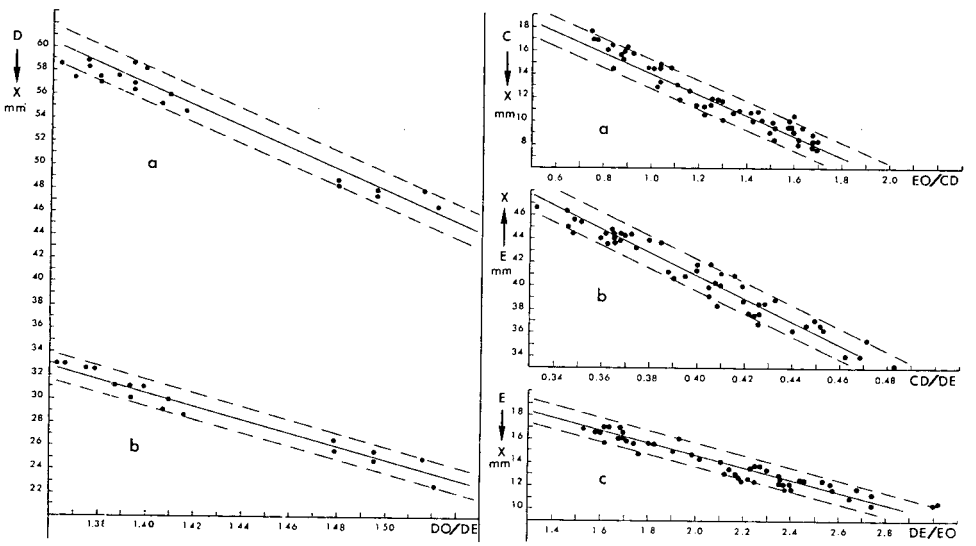

Fig. 5. DX *vs.* DO/DE plots obtained for single steroids. (a) Cortexolone (17α,21-dihydroxypregn-4-ene-3,20-dione); R_b value (average) = 0.471; σ_{DX} = 0.7. (b) Cortisone (17α,21-dihydroxypregn-4-ene-3,11,20-trione); R_b value (average) = 0.289; σ_{DX} = 0.55.

Fig. 6. (a) CX *vs.* EO/CD plot for interval *p* (Fig. 1) or subzone boundary *p* (Fig. 2); *cf.* discussion; σ = 0.6. (b) EX *vs.* CD/DE plot for interval *s* (Fig. 1) or subzone boundary *s* (Fig. 2); *cf.* discussion; σ = 0.67. (c) EX *vs.* DE/EO plot for interval *u* (Fig. 1) or subzone boundary *u* (Fig. 2); *cf.* discussion; σ = 0.5.

The XO data listed in columns 1, 2 and 3, Table I indicate remarkably little variation for migration data obtained with a multicomponent solvent system. Yet in all cases σ_{XO} was too large for a satisfactory prediction of X positions from average R_b values listed in column 4.

When use was made of the classical method consisting in spraying a chromatogram on one side of a plate, determining X positions for chromatograms on the unsprayed side from R_b data obtained from the sprayed one, chances of falling within $\pm$ 1.5 mm of actual centers were not considerably greater. This is evident from $\Delta_P$max (largest difference observed within a plate) and related $\sigma\Delta_P$ data given in columns 5 and 6, respectively. Except for estriol ($\sigma\Delta_P$ = 0.5 mm) scores shown in column 7 indicate as few as one successful positioning operation out of two trials by this method; yet $\Delta_P$max data concerned centers in the middle of bands, *i.e.*, centers for-which Δ_P variation was least.

In plots shown in Fig. 5 and 6, solid lines represent regression lines of experimental points; broken lines indicate fiducial limits containing 95 % of points obtained from about 50 chromatograms; points actually shown resulted from plotting data from a series of chromatograms obtained in the following three months.

In plot a, Fig. 6, for example, the 95 % fiducial limits corresponds to a $\pm$ 1.25 mm variation of CX. Hence CX values determined from the regression line by using measured EO/CD ratios were within $\pm$ 1.5 mm of actual values 98 % of the time. In the case of plot c, Fig. 6, the score was even better: EX was within $\pm$ 1 mm of the actual center 95 % of the time.

The predictability of X positions by this method was lowest for positions which corresponded to $R_b \approx 0.65$; the variability of XO values (Table I) was also greatest in this case. However, X positions could still be predicted within $\pm$ 1.5 mm of the actual one 95 % of the time (Fig. 5a).

XY_n distances were represented by the general expression

$$XY_n = kR + h \qquad (1)$$

where k is the slope of the regression line, h a constant intercept, and R the ratio of two distances determined by three points; these were either three dye-band centers or two dye-band centers and O, the origin. Satisfactory vectors in ratio R were determined by trial and error; no rationale was found to guide this choice. However, reliable plots for X positions not too distant from each other were obtained with the same R (Fig. 5a and b). When the distance was less than 5 mm, parallel regression lines were obtained. Hence X positions in appreciably large sections of chromatoplates corresponded to the same R ratio.

In view of the overall variability of the TLC system indicated, for example, by the range of EO/CD values (a, Fig. 6) and DE/EO values (c, Fig. 6), a consistent relationship of X positions to such ratios is remarkable. Evidently, steroid migration is governed by laws whose complexity induces the apparent lack of coordination observed in the raw data (Table I, XO values.)

Properties indicated by plots exemplified in Fig. 5 and Fig. 6 lead to obvious applications. Since specific steroids can be located precisely, the adsorbant area which needs to be removed and eluted for subsequent analysis is smaller; hence, the mixture of accompanying steroids will be less complex and subsequent analysis

less difficult. Precise location of zone or zone boundaries likewise results in more easily analyzed steroid mixtures. Zone and subzone boundaries found in urinary steroid chromatograms, in Fig. 2, for example, result from a grouping of steroids according to rules already discussed[1,2,9,10]. In zone II, a subdivision in subzones a, b, and c is particularly apparent for male urinary steroids. Together with other minor components, the following steroids were found in these subzones[12]. Note GLC retention times* in square brackets.

Subzone II, a: 3β-hydroxy-5β-androstan-17-one [2.75]; 3α-hydroxy-5β-pregnan-20-one (epipregnanolone) [4.54]; 20β-hydroxy-5α-pregnan-3-one (6.44]; 5β-hydroxy-cholest-5-ene (cholesterol) [15.36].

Subzone II, b: 3α-hydroxy-5α-androstan-17-one (androsterone) [2.78]; 17β-hydroxy-5α-androstan-3-one (allodihydrotestosterone) [3.78]; 20β-hydroxypregn-4-en-3-one [7.99].

Subzone II, c: 3α-hydroxy-5β-androstan-17-one (etiocholanolone) [2.88]; 3β-hydroxyandrost-5-en-17-one (DHA) [3.37]; 17α-hydroxyandrost-4-en-3-one (epitestosterone) [3.87]; 17β-hydroxyandrost-4-en-3-one (testosterone) [4.59]; 3β-hydroxypregna-5,16-dien-20-one [4.96]; 3β-hydroxypregn-5-en-20-one (Δ^5-pregnenolone) [5.38].

As seen from their retention times, many of the above compounds would form difficultly separable pairs without a preliminary separation into subzones. On the other hand, retention times of compounds within each subzone differ by at least 6 %, *i.e.*, enough to be sufficiently separated and precisely quantified with our efficient 6.000-theoretical plate JXR columns[9].

Note that in the chromatogram shown in Fig. 1, compound 2, cholesterol, is characteristic of subzone II, a; compound 3, androsterone, is characteristic of subzone II, b; compound 4, etiocholanolone, is characteristic of subzone II, c. Hence band separations for these compounds correspond to subzone separations in Fig. 2. Indeed plots a, b, c, Fig. 6, and others obtained with simple mixtures of steroids, are applicable to the precise location of zone and subzone boundaries in chromatograms of urinary and other steroids.

Plot b, Fig. 6, for example, corresponds to boundary *s* between subzones III, a and b. Subzone III, b contains pregnanediol (P in Fig. 2) whereas subzone III, a contains a number of isomeric pregnanediols; among these is the 5α-epimer of pregnanediol which constitutes as much as 30 % of total pregnanediols in the urine of pregnant women. A preliminary TLC separation followed by analyses of subzones III, a and b allows separate estimations of pregnanediol and its isomers to be made[12].

Dyes, B, C, D or E do not interfere in the quantification of TMS derivatives of steroids by GLC. Addition of any of these dyes before TMS derivatization of many steroids did not alter the GLC response of the derivatives; relatively small peaks induced by dyes appeared well ahead of steroid peaks.

Analyses by fluorometry, UV or IR spectrophotometry of steroids eluted from TLC zones located between dye bands suffer no interference; if analyses by this method of steroids which migrate with or near dyes B, C, D or E are contemplated, other dyes of the same type with different R_b values should be used: *p*-hydroxyazobenzene (K. & K.), for example, migrates somewhat above dye D (R_b = 0.700).

* At 230°; *cf.* ref. 9.

While the present method could be viewed as a means of compensating for variations in migration distance of steroids, it should not be regarded as a corrective for poor TLC techniques. The described useful relationships undoubtedly depend on the consistent use of TLC procedures conducive to reproducibility[1–5], including the use of a sample applicator[6].

Presumably, somewhat different TLC procedures, if consistently applied, might lead to results equally useful in the present problem. In any event, the method is empirical and requires careful calibration.

Errors in the marking of band and zone-boundary centers, and errors in measuring distances between marks account for at least half the variability in XY distances computed by the present method. While measurements made on photostatic reproductions of marked plates are more accurate, the simple and expeditive techniques illustrated in Fig. 3 appear satisfactory for most purposes.

ACKNOWLEDGEMENT

The technical assistance of Mr. R. COCHRANE was very much appreciated.

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CELLULOSE THIN LAYERS FOR ANTHOCYANIN ANALYSIS, WITH SPECIAL REFERENCE TO THE ANTHOCYANINS OF BLACK RASPBERRIES

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(Received August 27th, 1968)

SUMMARY

The anthocyanins of the American black raspberry (*Rubus occidentalis*) were reinvestigated, since quite different glycosidic characteristics had been attributed to them by different authors. New evidence was presented by means of two-dimensional thin-layer chromatography to show that the four black raspberry anthocyanins are: cyanidin 3-glucoside, cyanidin 3-rutinoside, cyanidin 3-sambubioside, and cyanidin 3-xylosylrutinoside.

Boysenberries were also reinvestigated and found to contain the following four anthocyanins: cyanidin 3-glucoside, cyanidin 3-rutinoside, cyanidin 3-sophoroside and cyanidin 3-glucosylrutinoside.

INTRODUCTION

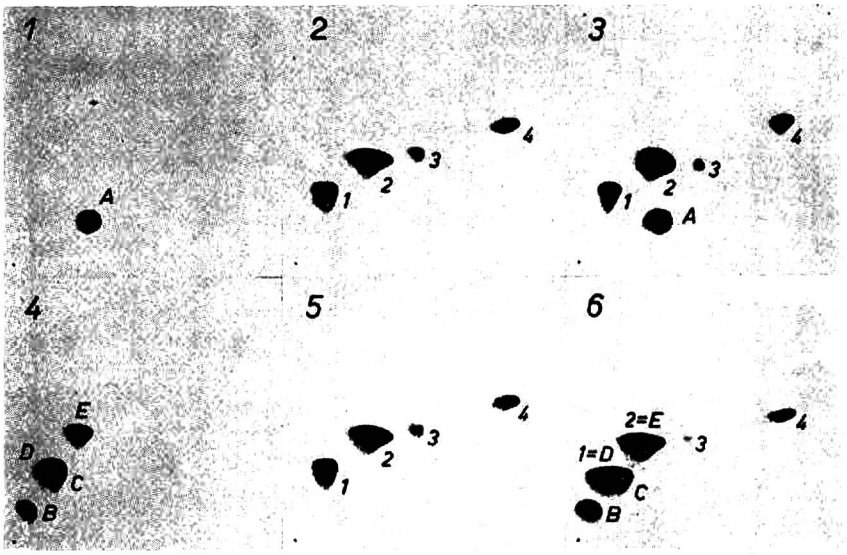
The American black raspberry, belonging to the species *Rubus occidentalis*, was shown by HARBORNE AND HALL[1] to contain four cyanidin glycosides, all with the sugars attached to the 3-position, namely glucoside, rutinoside, sambubioside and xylosylrutinoside. The same number and nature of the sugar elements had been found also by the present writer[2,3].

In contrast to these findings, extensive studies led DARAVINGAS AND CAIN[4] to describe the four pigments as 3-rhamnoglucoside, 3,5-diglucoside, 3-diglucoside and 3-rhamnoglucoside-5-glucoside. In a new publication[5] these authors retain their interpretation, which was felt to be a reason for reinvestigating the matter.

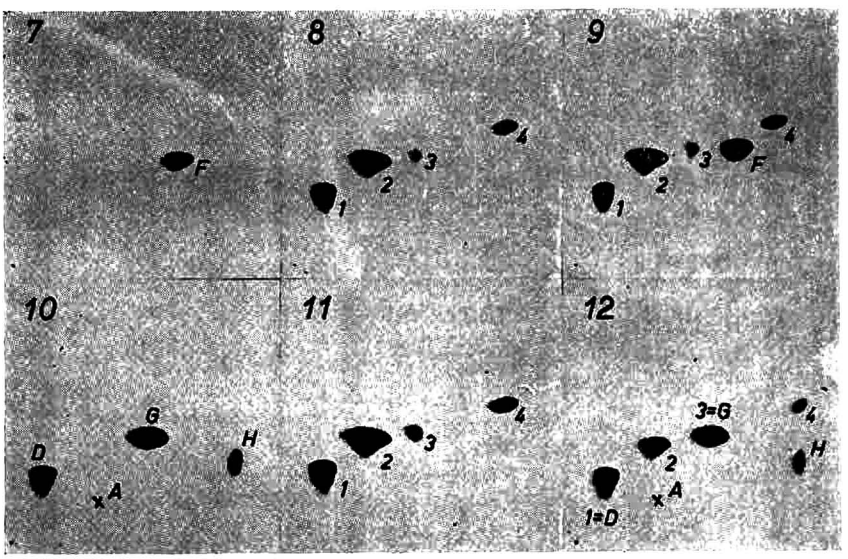
The anthocyanins of boysenberries were also checked, as the cyanidin 3-rhamnoglucoside 5-glucoside was reported from this type of berry by LUH *et al.*[6]; this being also at variance with the results of HARBORNE AND HALL[1] and with unpublished results of the present author.

EXPERIMENTAL

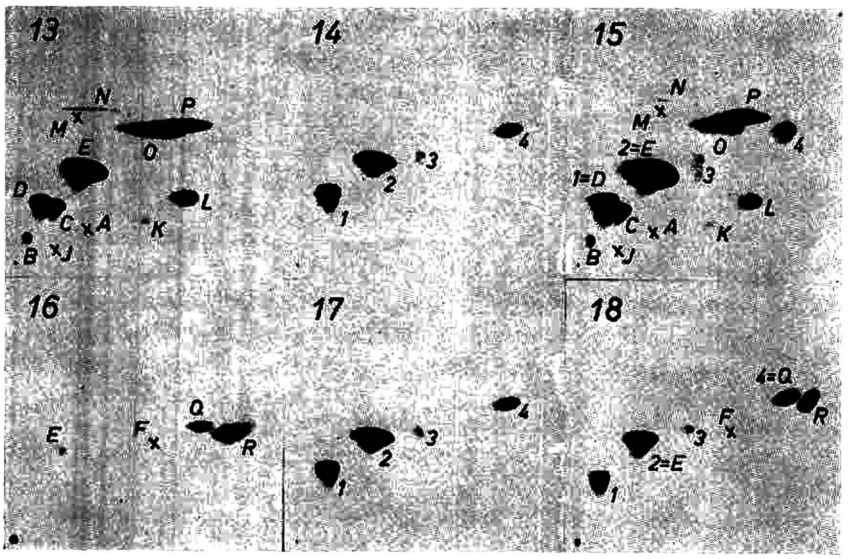
A special system of thin-layer chromatography was used[3,7]. Glass plates 6 in. × 6 in. were coated with a suspension of Merck microcrystalline cellulose, slit width 0.35



Figs. 1–6. Photo-copies of anthocyanins on two-dimensional thin-layer chromatograms. Letters refer to Table I. Fig. 1. Extract of cornflower petals. Fig. 2. Extract of black raspberries, cv. Bristol. Fig. 3. Cornflower and black raspberries co-chromatographed. Anthocyanin A does not occur in black raspberries. Fig. 4. Extract of black-currants. Fig. 5. Extract of black raspberries, cv. Bristol. Fig. 6. Black-currants and black raspberries co-chromatographed. Anthocyanins D and E match with black raspberry pigments 1 and 2, respectively.



Figs. 7–12. Photo-copies of anthocyanins on two-dimensional thin-layer chromatograms. Letters refer to Table I. Fig. 7. Extract of hibiscus petals. Fig. 8. Extract of black raspberries, cv. Bristol. Fig. 9. Hibiscus and black raspberries co-chromatographed. Anthocyanin F does not occur in black raspberries. Fig. 10. Extract of elderberries. Fig. 11. Extract of black raspberries, cv. Bristol. Fig. 12. Elderberries and black raspberries co-chromatographed. Anthocyanin G matches with black raspberry pigment 3. The x signs show the position of spots visible in the original chromatograms but too weak to show up in the prints.



Figs. 13–18. Photo-copies of anthocyanins on two-dimensional thin-layer chromatograms. Letters refer to Table I. Fig. 13. Extract of petals of Swiss pansy. Fig. 14. Extract of black raspberry, cv. Bristol. Fig. 15. Swiss pansy and black raspberries co-chromatographed. Anthocyanin L does not occur in black raspberries. Fig. 16. Extract of red-currants belonging to the species *Ribes petraeum*. Fig. 17. Extract of black raspberries, cv. Bristol. Fig. 18. *Ribes petraeum* and black raspberries co-chromatographed. Anthocyanin matches with black raspberry pigment 4. The x signs show the position of spots visible in the original chromatograms but too weak to show up in the prints.

mm. After application of the extracts, the plates were stacked on top of each other[7], so that up to 15 plates could be processed at the same time.

Two different solvent systems, were used:

(A) 1st direction, 5 parts *n*-BuOH + 2 parts conc. HCl + 1 part water; 2nd direction, 8 parts water + 4 parts conc. HCl + 1 part conc. formic acid.

(B) 1st direction, 6 parts *n*-BuOH + 1 part conc. acetic acid + 2 parts water; 2nd direction, 10 parts water + 2 parts conc. HCl + 3 parts conc. propionic acid.

Corresponding results, though with somewhat different R_F values, were obtained with the two solvent systems (*cf.* Figs. 19 and 20). The chromatograms in Figs. 1–18 were all made with solvent system A.

Before application on the plates, all pigments were purified with the aid of Dowex cation exchanger, 50 W-X 4 (ref. 3). In several cases, acid or alkaline hydrolysis was made in order to check the aglyconic or glycosidic pattern of the anthocyanins used for comparison.

RESULTS

The results are summarized in Figs. 1–18, which are reflex photocopies of actual chromatograms, reduced *ca.* 3.5 times in printing. Anthocyanins of known constitution were extracted from various plants and compared, by means of co-chromatography, with the pigments of the black raspberry cultivar Bristol.

In this way, Figs. 1–3 show that cyanidin 3-glucoside-5-glucoside (A in Figs.

TABLE I

CHEMICAL COMPOSITION OF ANTHOCYANINS REFERRED TO IN THE FIGURES

Reference letter	*Anthocyanin*
A	Cyanidin 3-glucoside-5-glucoside
B	Delphinidin 3-glucoside
C	Delphinidin 3-rutinoside
D	Cyanidin 3-glucoside
E	Cyanidin 3-rutinoside
F	Cyanidin 3-sophoroside
G	Cyanidin 3-sambubioside
H	Cyanidin 3-sambubioside-5-glucoside
J	Delphinidin 3-glucoside-5-glucoside
K	Delphinidin 3-rutinoside-5-glucoside
L	Cyanidin 3-rutinoside-5-glucoside
M	Delphinidin 3-coumaroylglucoside (?)
N	Cyanidin 3-coumaroylglucoside (?)
O	Delphinidin 3-coumaroylrutinoside-5-glucoside
P	Cyanidin 3-coumaroylrutinoside-5-glucoside
Q	Cyanidin 3-xylosylrutinoside
R	Cyanidin 3-glucosylrutinoside

1 and 3), as isolated from cornflower (*Centaurea cyanos*), does not occur in Bristol. On the other hand, the comparison between Bristol and black-currants (*Ribes nigrum*) in Figs. 4–6 indicates that the Bristol pigments 1 and 2 are the same as the two black-currant anthocyanins D and E, cyanidin 3-glucoside and cyanidin 3-rutinoside (*i.e.* rhamnoglucoside).

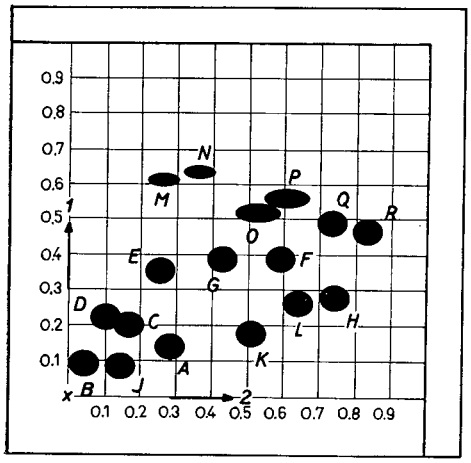
The chemical composition and R_F values of the anthocyanins dealt with in this paper and/or occurring in the figures are summarized in Table I and in Figs. 19–20.

Figs. 7–12 deal with the third Bristol pigment. The comparison with cyanidin 3-sophoroside (*i.e.* diglucoside, F in Figs. 16 and 18) from hibiscus (*Hibiscus rosa-sinensis*) shows that this anthocyanin does not occur in Bristol. The cyanidin 3-sambubioside (*i.e.* xyloglucoside, G in Fig. 10) from elderberry (*Sambucus nigra*) corresponds, however, completely with pigment 3 of the black raspberry.

Bristol pigment number 4 finally is dealt with in Figs. 13–18. The idea that pigment 4 would be cyanidin 3-rutinoside-5-glucoside (L in Fig. 13) is not supported by co-chromatography with this anthocyanin from petals of the Swiss pansy (*Viola x wittrockiana*, dark red flowers of unknown cultivar). The red-currants derived from the species *Ribes petraeum* differ from other red-currants by the presence of a branched triglucoside, containing one molecule each of glucose, rhamnose and xylose[1,3]. The comparison in Fig. 18 indicates that this cyanidin 3-xylosylrutinoside (Q in Figs. 16 and 18) corresponds to pigment 4 of Bristol.

In addition to Bristol, the same anthocyanin constitution was found in the black raspberry cultivars John Robertson, Dundee and Black Hawk, as well as in a selected clone without name.

Further evidence that the anthocyanin composition described for these cultivars is typical for *Rubus occidentalis* is given by the study of various purple raspberry cultivars, *i.e.* hybrids between *Ribes idaeus/strigosus* and *R. occidentalis*, in the first place the cultivars Clyde, Marion and Sodus. All these types have six cyanidin 3-



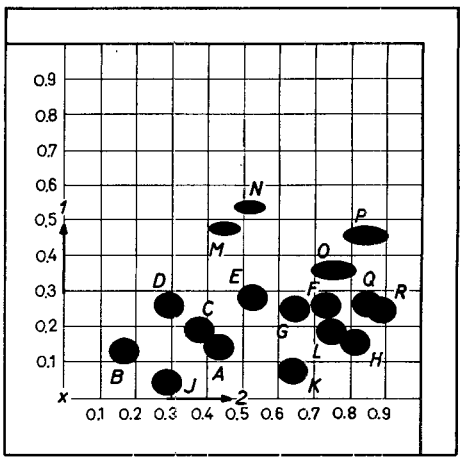


Fig. 19. The composite two-dimensional separation pattern of various anthocyanins on cellulose thin-layer. Solvent system A. Letters refer to Table I.

Fig. 20. The composite two-dimensional separation pattern of various anthocyanins on cellulose thin-layer. Solvent system B. Letters refer to Table I.

glycosides, namely glucoside, rutinoside, sambubioside, sophoroside, xylosylrutinoside and glucosylrutinoside. Some of these compounds have been inherited from the red raspberries (sophoroside and glucosylrutinoside), whereas others are typical of the black raspberries (sambubioside and xylosylrutinoside); glucoside and rutinoside being common to both types of parents. Some raspberries, red, purple and black may contain rather much of the related pelargonidin glycosides.

An analysis of the pigments of the raspberry–blackberry hybrids Boysen and Logan clearly showed that these consist of the four red raspberry anthocyanins: cyanidin 3-glucoside, cyanidin 3-rutinoside, cyanidin 3-sophoroside and cyanidin 3-glucosylrutinoside. This is in agreement with the results of HARBORNE AND HALL[1], but does not support the findings of LUH *et al.*[6], who reported cyanidin 3-rutinoside 5-glucoside in boysenberries.

It should be mentioned that the 3,5-anthocyanins of cyanidin, as well as the 5-glucoside and the cyanidin itself, are all red-fluorescent in U.V. light. None of the anthocyanins of black raspberries or boysenberries are fluorescent in U.V. light.

DISCUSSION

This reinvestigation of the pigments of black raspberries and boysenberries thus would show that the earlier results by HARBORNE AND HALL[1] and by the present author[2,3] are still valid. This is not of academic interest only. According to the present writer's experience different types of glycosides behave in rather different ways, at least towards acid hydrolysis. Whether or not this is also true with regard to the anthocyanin destruction in fruit products certainly invites further study. For such technological investigations[5] it seems to be desirable to know, however, what substances one is working with.

Considering the chromatographic procedure, it is obvious that two-dimensional separation is far more elucidative than one-dimensional. Also, thin-layer chromatography does yield considerably more clear-cut results than conventional paper chromatography. Finally, co-chromatography must be considered more reliable than the comparison of R_F-values, as these may vary considerably.

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Notes

CHROM. 3781

Gaschromatographische Trennung von stellungs- und konfigurationsisomeren, verzweigten Octenen

Über die gaschromatographische Trennung von ungesättigten Kohlenwasserstoffen durch mit Silbersalzen imprägnierte Säulen berichteten wir bereits in früheren Arbeiten[1,2]. Dabei war uns die Trennung aller stellungs- und konfigurationsisomeren *n*-Octene, *n*-Hexene und Hexadiene mit konjugierten und kumulierten Doppelbindungen gelungen.

Auf Grund dieser guten Ergebnisse versuchten wir, auch verzweigte Octene auf die gleiche Weise durch mit Silberborfluorid imprägnierte Säulen in die *cis*- und *trans*-Isomeren aufzutrennen. Die Versuche verliefen nicht zufriedenstellend. Wahrscheinlich verhindert die Verzweigung an der Doppelbindung eine für die Trennung ausreichende unterschiedliche Komplexbildung der *cis*- und *trans*-Isomeren. Diese Annahme wird durch eine Arbeit von MUHS UND WEISS[3] bestätigt, die für die Gleichgewichtskonstanten der Komplexe Silber-*cis*-3-methylpenten-(2) und Silber-*trans*-3-methylpenten-(2) in Silbernitrat–Äthylenglykol-Säulenfüllmaterial gleiche Werte fanden. Wir versuchten deshalb, die Trennung der isomeren verzweigten Octene an unpolaren Säulen durchzuführen. Ähnliche Versuche sind bekannt.

SMITH, OHLSON UND LARSON[4] gelang 1963 an unpolaren Säulenfüllungen zwar die Trennung von *cis*- und *trans*-3-Methylpenten-(2) und *cis*- und *trans*-3-Methylhexen-(3), nicht aber die Trennung von *cis*- und *trans*-3-Methylhexen-(2). Diese Analyse gelang HIVELY[5] u.a. mit einer Di-*n*-decylphthalat-Säule, und zwar trennte er nebeneinander die geometrischen Isomeren des 3-Methyl-hexen-(3) und des 3-Methylhexen-(2). In der vorliegenden Arbeit wird gezeigt, dass auch wir mit dieser Säulenphase eine Trennung verzweigter Octene erreichen konnten.

Im Rahmen unserer Arbeiten auf dem Gebiet höhermolekularer Olefine standen wir vor der Aufgabe, alle einfach verzweigten Octene, vor allem solche, die die Doppelbindung und Verzweigung am gleichen C-Atom tragen, zu trennen.

Apparatives

Alle aufgeführten Trennungen wurden unter folgenden Bedingungen durchgeführt:

Gaschromatograph: WCLID 1680 (Warner-Chilcott).

Trennsäule: Material Edelstahl (SH 2); Länge 200 m, Innendurchmesser 0.5 mm; stationäre Phase Di-*n*-decylphthalat.

Trägergas: Helium, Durchflussmenge 3.7 ml/min.

Detektor: Flammenionisation.

Temperatur: Säule 25°; Verdampfer 110°; Detektor: 250°.

Einspritzmenge: 0.05 μl, Probenteilung 1:40.
Schreiber: Honeywell-Brown 1 mV Kompensations-Schreiber, Dämpfung 30.

Trennung der 3-methylverzweigten Octene

Bei der Isomerisierung von 2-Äthylhexen-(1) mit Eisenpentacarbonyl erhielten wir, wie zu erwarten[6–8], ein Gemisch aller doppelbindungs- und konfigurationsisomeren 3-methylverzweigten Octene. Unter den genannten Bedingungen konnten wir diese Octene vollständig in die elf möglichen Isomeren auftrennen. Fig. 1 zeigt

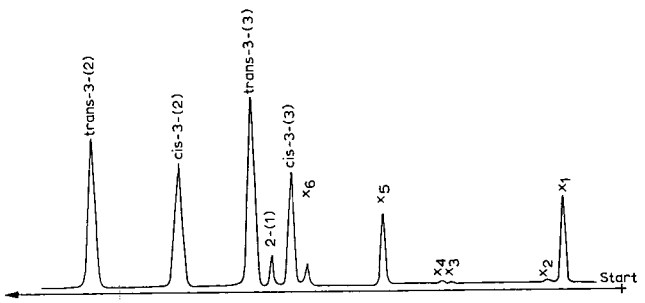

Fig. 1. Gaschromatogramm der elf doppelbindungs- und konfigurationsisomeren 3-methylverzweigten Octene. *cis*-3-(3) = *cis*-3-Methylhepten-(3); 2-(1) = 2-Äthylhexen-(1); *trans*-3-(3) = *trans*-3-Methylhepten-(3); *cis*-3-(2) = *cis*-3-Methylhepten-(2); *trans*-3-(2) = *trans*-3-Methyl-hepten (2); X_1–X_6 = 3-methylverzweigte Octene, die die Doppelbindung nicht am gleichem C-Atom wie die Verzweigung haben.

TABELLE I

RETENTIONSINDICES VERZWEIGTER OCTENE

Substanz	*Retentionsindex*
n-Heptan	700
cis-3-Methylhepten-(3)	795
2-Äthylhexen-(1)	798
trans-3-Methylhepten-(3)	800
cis-3-Methylhepten-(2)	807
trans-3-Methylhepten-(2)	817
n-Nonan	900

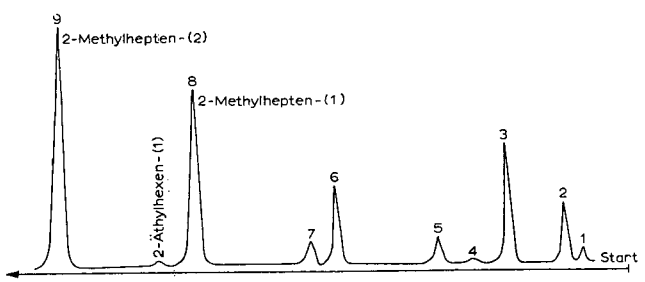

Fig. 2. Gaschromatogramm der neun doppelbindungs- und konfigurationsisomeren 2-methylverzweigten Octene.

das Gaschromatogramm. Fünf der gefundenen elf Peaks konnten wir mit Hilfe von Testsubstanzen als jene Octene identifizieren, bei denen Doppelbindung und Verzweigung am dritten Kohlenstoffatom sitzen. Bei den sechs nicht identifizierten Isomeren—im Gaschromatogramm mit X_1 bis X_6 bezeichnet—befindet sich die Doppelbindung nicht am tertiären C-Atom, sondern im geraden Teil der Olefinkette. Den Beweis für die Richtigkeit dieser Annahme werden wir zu einem späteren Zeitpunkt mit Hilfe von Testsubstanzen, die noch herzustellen sind, erbringen.

Für die bekannten 3-methylverzweigten Olefine (s. Fig. 1) berechneten wir die in Tabelle I aufgeführten Retentionsindices nach KOVATS[9,10] unter Zuhilfenahme der Retentionszeiten des *n*-Heptans und des *n*-Nonans.

Trennung der 2-methylverzweigten Octene

Analog zu der Darstellung der 3-methylverzweigten Octene erhielten wir die 2-methylverzweigten Octene durch Isomerisierung von 2-Methylhepten-(1) mit Eisenpentacarbonyl[11]. Das Gaschromatogramm (Fig. 2) dieses Isomerengemisches zeigt

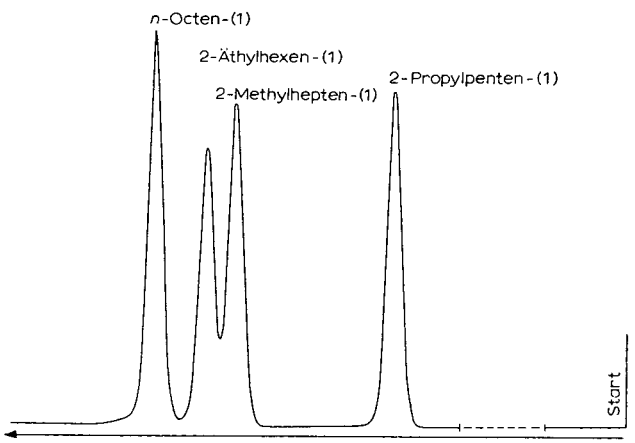

Fig. 3. Gaschromatogramm der vier α-Octene aus C_8-Oxo-Alkoholen.

TABELLE II

RETENTIONSINDICES DER VIER α-OCTENE AUS C_6-OXO-ALKOHOLEN

Substanz	*Retentionsindex*
2-Propylpenten-(1)	785
2-Methylhepten-(1)	795
2-Äthylhexen-(1)	797
n-Octen-(1)	800

alle Peaks der möglichen neun doppelbindungs- und konfigurationsisomeren 2-methylverzweigten Octene. Da bisher nur 2-Methylhepten-(1) und 2-Methylhepten-(2) synthetisiert worden sind, konnten nur diese beiden Isomeren zugeordnet werden. Die restlichen sieben sollen zu einem späteren Zeitpunkt mit Hilfe von Testsubstanzen identifiziert werden.

Trennung von zwei 4-methylverzweigten Octenen

Die Isomerisierung von 2-Propylpenten-(1) mit einem silicathaltigen Katalysator* führte zu einem Produkt, das, wie die gaschromatographische Analyse ergab, aus *cis*- und *trans*-4-Methylhepten-(3) bestand. Die Identifizierung erfolgte durch Testsubstanzen. Andere 4-methylverzweigte Octene haben wir bisher gaschromatographisch nicht untersucht.

Trennung einiger α-Octene

Fig. 3 zeigt die Trennung der vier α-Olefine, die aus den vier möglichen C_8-Oxo-Alkoholen durch Dampfphasepyrolyse ihrer Essigester hergestellt werden können. Um eine gute Trennung zu erreichen, mussten die Bedingungen für die gaschromatographische Analyse teilweise abgeändert werden:

Gaschromatograph: RSCO 60-10 (Warner-Chilcott).

Trennsäule: Material Edelstahl (SH 2); Länge 200 m, Innendurchmesser 0.5 mm; stationäre Phase Di-*n*-decylphthalat.

Trägergas: Helium, Durchflussmenge 5 ml/min.

Detektor: Flammenionisation.

Temperatur: Säule: 30°; Verdampfer: 110°; Detektor: 110°.

Einspritzmenge: 0.1 μl, Probenteilung 1:35.

Schreiber: Honeywell-Brown 1 mV Kompensationsschreiber, Dämpfung 100.

In Tabelle II sind die α-Octene mit den Retentionsindices aufgeführt, die wir nach KOVATS[9,10] anhand der Retentionszeiten des *n*-Heptans und des *n*-Octans berechneten.

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Eingegangen am 11. September 1968

* Veröffentlichung in Vorbereitung.

CHROM. 3778

Deposits on flame ionization detectors with silicone gum rubber columns

During the preparative gas chromatography of some organophosphorus esters using a silicone gum rubber column, a copious white deposit was formed on the collector electrode of the flame ionization detector over a period of 2 weeks. No similar deposits have been noticed on the detector when used with "Reoplex" (polypropylene glycol adipate) and poly-*m*-phenyl ether columns under similar conditions.

In addition a saucer-shaped depression was formed on the inner surface of each silicone rubber injection port septum used. White material in the depression crumbled very readily although the remainder of the septum retained its initial appearance and flexibility.

The gas chromatograph used was an F & M 776 Prepmaster Jr. with a flame ionization detector. The column was 80 in. of ¾ in. O.D. stainless steel packed with 20% W 98 on 60–80 Chromosorb P. Column temperatures used ranged from 100° to 140°; the injection port and detector block were maintained at 220°.

Although the deposits from both detector and injection port appeared similar to the naked eye, analysis by the Debye-Scherrer X-ray diffraction method and by optical emission spectroscopic techniques showed that the detector deposits were mainly silicon phosphate ($2\ SiO_2.P_2O_5$), and the injection port samples were alpha cristobalite (SiO_2).

Silicon phosphate formation could occur at the very high temperature of the hydrogen-air flame as phosphorus compounds elute in the presence of silicone vapour due to column bleed. Bleeding off of the stationary phase was confirmed by a marked decrease in retention times of the injected samples.

SPEAKMAN[1] in a paper on limitations set by column bleed using the flame ionization detector, mentioned the formation of white deposits on the collector electrode when he was using SE 30 columns but did not identify them. Since he was studying column bleed only and no samples were injected, the deposits were probably SiO_2.

The formation of cristobalite is probably due to the action of the hot (up to 220°) vapours, from volatilization of the phosphorus esters, on the silicone rubber septa. Two alternatives seem possible for the origin of the SiO_2; either liberation of the SiO_2 used as a filler in the preparation of the silicone rubber or oxidation of the silicone rubber itself. Temperature appears to be critical since a septum placed in refluxing triethyl phosphite (b.p. 160°) for six hours was unaffected.

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Received September 2nd, 1968

CHROM. 3792

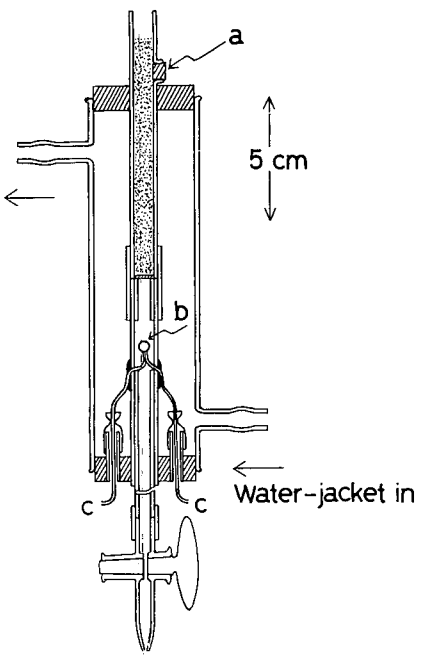
Thermal conductivity detection in liquid chromatography

Use of a micro adsorption detector in liquid chromatography has been reported by NAONO AND PRCHAL[1] and HUPE AND BAYER[2]. They detected the heats of adsorption and desorption in the chromatographic process by means of a micro thermistor placed in the center of the cell which was filled with a suitable adsorbent material. The chromatogram obtained did not show the ordinary Gaussian shape but rather its differential peak shape because the detector measures the gain and loss of heat. The liquid chromatograph constructed on the basis of measurement of adsorption-desorption heat was supplied by the Japan Electron Optics Laboratory (Nihon Denshi) and Varian Aerograph.

In this report we tried to introduce into liquid chromatography thermal conductivity detection by means of thermistors and to measure the temperature change due to the difference in thermal conductivities between eluent and eluted materials.

Experimental

Fig. 1 shows the column assembly. A thermistor, Ten KD-27, 4.2 mm in diameter and 2.2 mm thick, disk type, having an electrical resistance of 270 Ω at 25°, was used. It was coated with Araldite (Ciba Co.) to avoid electric contact with eluent,



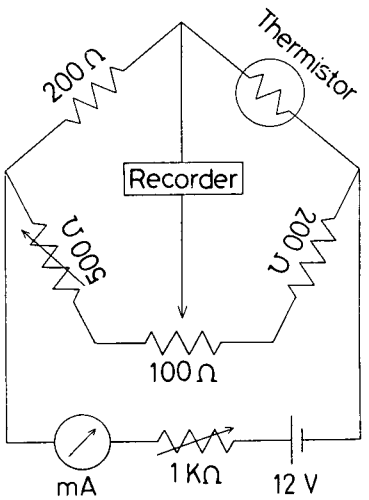


Fig. 1. Column assembly. (a) = Silicone-rubber stopper for sample injection; (b) = thermistor; (c) = lead wire.

Fig. 2. Bridge circuit.

and was placed in the center of a glass tube, 7.5 mm in diameter, connected directly to the column outlet. The sample was injected by means of a syringe through the silicone-rubber stopper inserted into the hole on the side of the column inlet. The column, made of glass tube, 7.5 mm in diameter, was packed with Sephadex G-25 Coarse (Pharmacia Co., Uppsala) and had an effective height of 8.3 cm. The temperature of column and detector was maintained at 10.7 $\pm$ 0.1° by a well-water circulation. The sample, Blue Dextran 2000 (Pharmacia Co.) and sodium chloride aqueous solution, was eluted with deionized water. The flow rate was kept constant by controlling the level of eluent in the reservoir with a stopcock.

In the bridge circuit (Fig. 2) a single active bridge arm was used in order to simplify the experimental work.

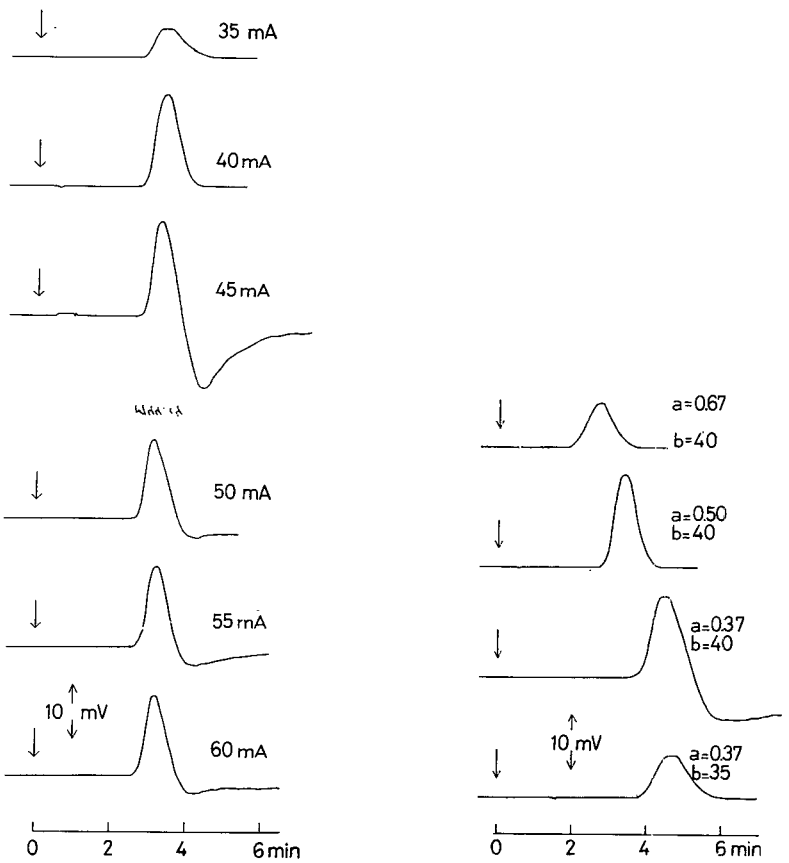


Fig. 3. Chromatograms of Blue Dextran 2000 eluted from the Sephadex column with water. The figures show the bridge current. When the bridge current exceeds 45 mA, a negative deflection appears in the chromatogram. The arrows indicate the injection point. The flow rate was 0.5 ml/min.

Fig. 4. Effect of flow rate on the chromatogram. (a) = Flow rate (ml·min^{-1}); (b) = bridge current (mA).

Results and discussion

The chromatograms recorded are given in Figs. 3–6. It may be safe to say that in principle the measurement of the electric resistance of the current-fed thermistor, cooled by eluate of various compositions, can be applied to liquid chromatography.

With increasing bridge current and decreasing flow rate, a slight negative deflection appears in the chromatogram, as shown in Figs. 3 and 4. The reason for this is not clear, however.

The chromatograms of Blue Dextran 2000 and sodium chloride are shown in Fig. 5 together with that of their mixture. The reproducibility of retention volume is good.

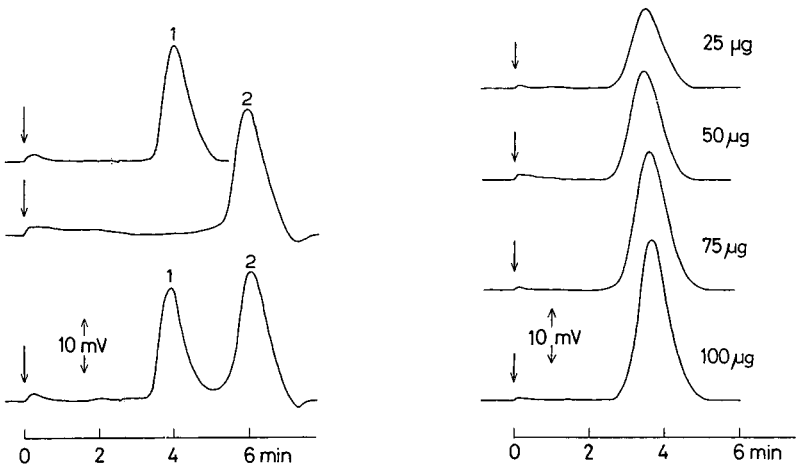


Fig. 5. Chromatograms of Blue Dextran 2000 (1) and NaCl (2), each 50 µg, and their mixture.

Fig. 6. Variation of the chromatogram with amount of Blue Dextran 2000 injected.

Variation of chromatogram with the amount of Blue Dextran 2000 injected is shown in Fig. 6. The peak area shows a linear relationship with the sample size, but the proportionality is not good.

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Received August 23rd, 1968

CHROM. 3776

The purification of synthetic oxytocin and analogues by gel filtration on Sephadex G-15

The procedures most commonly employed for the purification of laboratory synthesized oxytocin and analogues have been counter current distribution[1] and partition chromatography on Sephadex G-25[2]—methods which entail the careful selection of a suitable solvent system for the purification of each individual analogue. The present communication describes a purification procedure by gel filtration[3] on Sephadex G-15 which requires the use of just two standard solvents. It is an adaptation of the gel filtration procedure on Sephadex G-25, described by PORATH AND SCHALLY in the purification of oxytocin isolated from natural sources[4]. The procedure is both rapid and effective, having already been used in this laboratory for the purification of several synthetic analogues of the neurohypophysial hormones.

Experimental

The synthetic oxytocin used in the purification was prepared by the Merrifield method of solid phase peptide synthesis[5] as described previously[6]. The crude lyophilized material obtained after reductive deprotection and oxidation of the protected nonapeptide intermediate served as the starting material for the purification. At this stage the sample contained, in addition to the active peptide, inorganic salts and some dimeric peptide. The first step in the purification entailed desalting of the peptide with 50% acetic acid. In a typical run the procedure used was as follows: The crude lyophilized powder (300 mg, obtained by reduction of 75 mg of protected intermediate) was dissolved in 2.0 ml of 50% acetic acid and applied to the top of a column of Sephadex G-15 (Pharmacia Fine Chemicals, Uppsala, particle size 40–120 μ, column size 110 × 1.2 cm) which had been pre-equilibrated with 500 ml of 50% acetic acid. The sample was then washed into the column with an additional one ml of 50% acetic acid and eluted with 50% acetic acid at a rate of 8–10 ml per hour. Two ml aliquots were collected using an automatic fraction collector. The eluate was assayed for peptide by the method of LOWRY *et al.*[7] and the salts were located by flame photometry for potassium and sodium, and by precipitation with silver nitrate for chloride. The peptide material was eluted in two partially resolved peaks (Fig. 1), clearly separated from the salt (tubes 60–80). The second peak (tubes 31–37), containing most of the active peptide as detected by oxytocic assay[8], was pooled, diluted with two volumes of distilled water and lyophilized. Meanwhile the column was being re-equilibrated with 500 ml of 0.2 *N* acetic acid over a period of 20 h. The lyophilized powder (35 mg) from peak 2 was dissolved in 2.0 ml of 0.2 *N* acetic acid, applied to the column, washed with a further one ml of 0.2 *N* acetic acid and then eluted and collected as before, this time at a rate of 12–15 ml per hour. The active peptide emerged as a single nearly symmetrical peak preceded by a small amount of dimer (Fig. 2). The contents of this main peak (tubes 44–51) were pooled, diluted with two volumes of distilled water and lyophilized to give the desired product as a white fluffy powder (30 mg). This material was found to have an oxytocic activity of approx. 480 U/mg on the isolated rat uterus[8], and was adjudged pure by the criteria of electrophoresis, thin

layer and paper chromatography, optical rotation and microchemical and amino acid analyses[6].

In addition to oxytocin, the method has also been used to purify glumitocin (4-Ser 8-Gln oxytocin)[9], 8-Phe and 8-Gln oxytocins[10] as well as several other analogues of oxytocin. The total time required to carry out one such purification, starting with a column which had been pre-equilibrated with 50% acetic acid, was approx. 64 h, *i.e.* 12 h for each of the two elution steps and two 20 h re-equilibration periods.

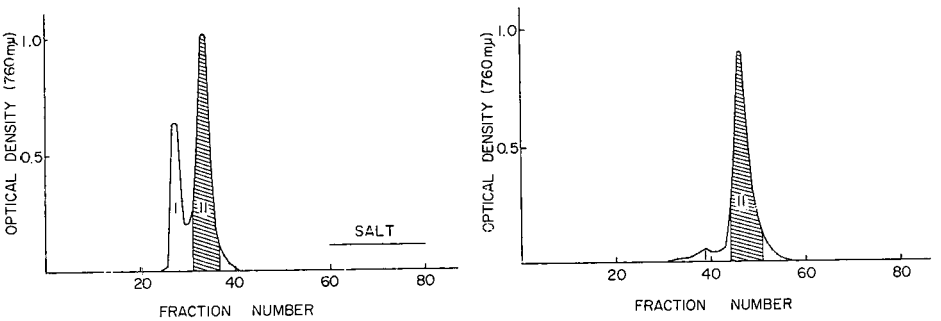

Fig. 1. Elution profile for first step in purification of synthetic oxytocin. 300 mg crude lyophilized material (dimeric peptide, active peptide and salt) dissolved in 2 ml of 50% acetic acid and applied to a column of Sephadex G-15. Eluting solvent: 50% acetic acid, flow rate 8–10 ml per hour; fraction size 2 ml. Peptide measured by LOWRY method; (I, dimer; II, oxytocin). Salt located by standard determinations as mentioned in text. Material from shaded area under peak II lyophilized.

Fig. 2. Elution profile for second step in purification of synthetic oxytocin. 35 mg of active peptide plus small amount of dimer dissolved in 2 ml of 0.2 *N* acetic acid and applied to a column of Sephadex G-15. Eluting solvent: 0.2 *N* acetic acid; flow rate 12–15 ml per hour; fraction size 2 ml. Peptide measured as in Fig. 1; (I, dimer; II, oxytocin). Material from shaded area under peak II lyophilized.

A number of further points are worth noting with regard to the development of this procedure. (1) In preliminary experiments, when only 0.2 *N* acetic acid was used for elution, the salt present in the crude lyophilized sample retarded the peptide material on the column, resulting in the active peptide being eluted with the salt. This led to the incorporation of a second step in which 50% acetic acid was used to desalt the peptide. (2) Elution with 50% acetic acid resulted in contamination of the final product with trace amounts of Sephadex dissolved out by this solvent. By reversing the two steps *i.e.* by eluting first with the 50% acetic acid solution and following with the 0.2 *N* acetic acid elution, these impurities were removed and thus the problem was eliminated. (3) In our experience, as much as 300 mg of crude lyophilized material can be applied to a column of the above size to give a satisfactory separation by this procedure.

Conclusion

Gel filtration on Sephadex G-15 can be used for the rapid and effective purification of those neurohypophysial hormones and their analogues which, because of their individual solubility characteristics, cannot readily be purified by either counter current distribution or partition chromatography until a suitable solvent system is first found. Furthermore, since the solvents which are used to effect purification of the

desired peptide by this procedure, *i.e.* acetic acid and water, are both readily removed by lyophilization, the final product is obtained in the highest possible state of purity free from contamination by eluting solvents. Thus the problem of contamination of the final product by less volatile solvents such as pyridine and benzene, which are commonly used in partition systems, is obviated. We feel that these distinct advantages, coupled with the inherent speed and simplicity of gel filtration, make this procedure a useful complement to the aforementioned methods for the purification of those synthetic analogues of the neurohypophysial hormones in which the only contaminants present are inorganic salts and dimer.

Acknowledgement

Supported in part by a studentship (to J.W.M.B.) and a grant from the Medical Research Council of Canada and an award from the Banting Research Foundation (to T.C.W.). The authors wish to express their appreciation to Dr. MURRAY SAFFRAN for generous use of laboratory facilities.

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Received September 10th, 1968

CHROM. 3773

Reduction and detection of aromatic nitro-compounds on thin layers of silica gel

Aromatic nitro-compounds are important derivatives for the chromatography of alcohols and carbonyl compounds as their 3,5-dinitrobenzoates (DNB) and 2,4-dinitrophenylhydrazones (DNPH) respectively. Unfortunately detection of these derivatives on thin layer chromatograms in very small amounts has proved difficult, despite the intense colour of DNPH derivatives. Therefore attempts were made to devise more sensitive methods of detection.

Attempted saponification of a model compound (1,2-dinitrobenzene) on thin layers of silica gel with alcoholic NaOH (4 %) at 105° gave no colouration on subsequent diazotisation and coupling according to FEIGL's method[1] for polynitro-aromatic compounds. Similarly an attempt to reduce the nitro group to an amino group by spraying an ethereal solution of lithium aluminium hydride on to the spotted silica gel layer was unsuccessful. Visualisation of amine (by diazotisation with acidified 5 % $NaNO_2$ solution followed by coupling with 0.1 % N-1-naphthyl-ethylenediamine dihydrochloride (NEDDI) in water to develop the azo dye[2]) showed no colour. Presumably reduction had not taken place.

However a second attempt using tin and HCl to reduce the nitro group succeeded. As indicated below, the technique used did not interfere with the separation of a mixture of DNB derivatives. TLC plates were prepared by shaking silica gel (Whatman SG41) containing finely powdered tin (5 % w/w) with water for 1 min, spreading, drying and activation at 105° before spotting the layer with model compounds. The plate was placed in a developing tank containing HCl vapour, left overnight, by which time the specks of tin had disappeared, and sprayed with acidified $NaNO_2$ solution (5 % $NaNO_2$). After standing for about 1 min the plate was sprayed with NEDDI solution. An intense pink-purple colour developed on the model compound spots with very little discolouration of the background.

Comparison of the reducing power of different finely powdered metals incorporated into silica gel thin layers showed that tin and zinc gave rise to vivid spots on reduction and visualisation without disruption of the surface of the layer. Magnesium and iron gave less strongly coloured spots. The Fe/silica gel layer became yellow in the HCl tank, presumably due to the formation of ferric compounds. Incorporation of tin or zinc in cellulose thin layers (5 % w/w with Whatman CC41) failed to give consistent colour production on subsequent reduction and visualisation. Probably BARTON's procedure[3] of spraying a suspension of, or dusting with, zinc powder followed by reaction with HCl spray is more effective for thin layers of cellulose.

The sensitivity of the method for silica gel thin layers was determined by the application of aliquots of standard solutions of *iso*-butyl methyl ketone DNPH and of a mixture of the DNBs of ethanol, *n*-propanol, *n*-butanol and 3-methyl-butan-1-ol and development of the colours. Spots containing 0.1 μg of derivative on 0.25 mm thick layers of silica gel were too faint to be seen but spots containing 0.5 μg or more DNB or DNPH were readily visible. Chromatography of the mixture of DNBs in diethyl ether: 60/80° petroleum spirit (5:100) indicated that 0.3 μg was detectable

after development and visualisation while 1.0–5 μg quantities of the mixture were readily visualised as four distinct pink-purple spots.

This technique is currently being used to locate DNB and DNPH derivatives of compounds present in honey aroma after separation by TLC. It could have wider application as a general technique for reductions on thin layer chromatograms.

Acknowledgements

This work was supported by the Hopkins and Cotterell Bequests for Honey Research.

I thank Dr. M. E. U. TAYLOR and Dr. J. R. L. WALKER for suggestions and encouragement in this study.

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Received September 9th, 1968

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CHROM. 3779

Separation of cello-oligosaccharides by thin-layer chromatography*

While studying the initial products of hydrolysis of cellulose by rumen micro-organisms of the sheep it was found that separation of the cello-oligosaccharides by paper chromatography was time consuming and resulted in poor resolution. To achieve better separation of the oligosaccharides it was decided to make use of thin-layer chromatography (TLC).

The application of TLC for the separation and identification of malto-oligosaccharides has been reported by many workers[1–3]. On the other hand, little information is available on the resolution of cello-oligosaccharides by this method. BECKER *et al.*[4] reported on the separation of cellulose degradation products eluted from a charcoal–celite column by TLC on Kieselguhr G using *n*-butanol–ethanol–water (50:30:20, v/v). A suitable technique for the separation of cello-oligosaccharides by TLC is described in this communication.

Experimental

The chromatoplates (20 cm × 60 cm) were coated with Kieselgel G or Kieselguhr G (Merck & Co) to a thickness of 250 μ, according to the procedure described by STAHL[5]. Solutions of cello-oligosaccharides** (cellobiose, cellotriose, cellotetraose,

* Supported by the National Research Council of Canada, Projects A-132 and A-4913.
** Gift from Mr. R. JEFFRIES, Shirley Institute, Didsbury, Manchester, 20.

cellopentaose, cellohexaose) were spotted on the plates in concentrations of 10 to 15 μg. Ascending chromatography was accomplished in closed glass tanks containing sheets of filter paper saturated with the developing solvent. Two different solvent systems were used for developing the chromatograms, *viz.* (a) isopropanol–water–ethyl acetate (1:2:1, v/v), and (b) *n*-propanol–ethyl acetate–water (6:1:3, v/v). When the solvent front reached 50 cm from the origin, the chromatoplates were removed and air dried. The presence of the cello-oligosaccharides was detected by spraying the plates with aniline phosphate reagent[6] and heating at 115° for 20 min. Alternatively, plates were sprayed with a 0.5% solution of potassium permanganate in 1 *N* sodium hydroxide[7] and heated at 100° for 1–2 min.

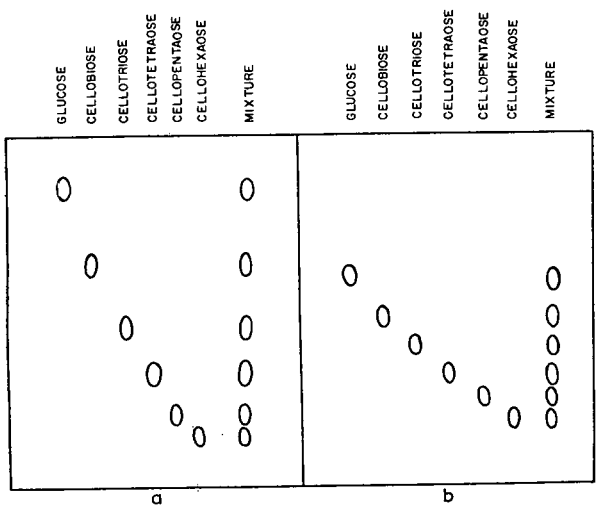

Fig. 1. Separation of cello-oligosaccharides by TLC using the following solvent systems: (a) isopropanol–water–ethyl acetate (1:2:1, v/v), and (b) *n*-propanol–ethyl acetate–water (6:1:3, v/v).

Results and discussion

Cello-oligosaccharides appeared as well-defined brown spots against a white background after treatment with aniline phosphate and as pale yellow spots on a purple background after treatment with alkaline permanganate. Fig. 1a and b shows the separation of individual and a mixture of oligosaccharides achieved using the two solvent systems. The R_F values of the oligosaccharides in the solvent systems studied are given in Table I. Under the conditions of the experiment solvent system (a) gave a better resolution of oligosaccharides than solvent system (b). To obtain repeatable results it was found necessary to have the atmosphere of the developing chamber saturated with the solvent system for a minimum of 30 min prior to development. This is easily achieved by placing a folded filter paper sheet soaked in the solvent so that it forms an inner lining to the wall of the chamber. It was also observed that the use of thick layers (300–350 μ) of Kieselgel G or Kieselguhr G rendered the detection of small quantities of oligosaccharides difficult. Comparatively Kieselgel G gave a better resolution of oligosaccharides than Kieselguhr G; the activation of the

TABLE I

R_F VALUES (× 100) OF GLUCOSE AND CELLO-OLIGOSACCHARIDES

Solvent systems: (a) isopropanol–water–ethyl acetate (1:2:1, v/v); (b) *n*-propanol–ethyl acetate–water (6:1:3, v/v).

	Solvent system	
	a	*b*
Glucose	45	46
Cellobiose	33	36
Cellotriose	23	30
Cellotetraose	15	23
Cellopentaose	8	18
Cellohexaose	5	13

prepared plates was not necessarily required. Spraying with the aniline phosphate reagent gave distinct spots whereas spraying with the alkaline permanganate reagent yielded spots which faded rapidly.

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Received September 2nd, 1968

CHROM. 3719

Improved thin-layer chromatographic separation of amanita toxins on Silica Gel G chromatoplates*

Amanita toxins are polypeptides that contain unusual amino acids[1–3]. These toxins are present in certain species of mushrooms and thus are of interest to physiologists, pharmacologists, and biochemists. Because they are large complex molecules, study of their structures can provide knowledge ultimately useful in the stereochemistry of biological molecules. The chemistry and toxicology of amanitins have been studied by WIELAND[2,3]. A thin-layer chromatographic method for the separation and detection of α- and β-amanitins in mushroom extracts is described by SULLIVAN, BRADY AND TYLER[4]. A methanolic extract of the mushroom is chromatographed on a Silica Gel G chromatoplate, which is then developed with methanol–methyl ethyl ketone. The positions of the amanitins are detected by spraying the plate with a solution of *trans*-cinnamaldehyde in methanol, drying the sprayed plate, and exposing it to the vapors from hydrochloric acid. Discrete lavender-purple areas indicate the positions of the amanitins. The application of the method to the isolation and detection of the toxins from mushrooms is described in further works[5,6].

A project was undertaken by C. K. JOHNSON (Chemistry Division, ORNL) and E. F. PHARES (Biology Division, ORNL) to prepare significant quantities of the amanitin toxins by batch fermentation growth of the mycelium of the mushroom *Galerina marginata* according to the method of BENEDICT, TYLER, BRADY AND WEBER[5]. The thin-layer chromatographic method of SULLIVAN, BRADY AND TYLER[4] was used to follow the progress of the production. During this work, some improvement in the chromatographic separation was realized by means of shaped chromatoplates and, for some samples, also overrun of the development.

Experimental

Apparatus. The Desaga/Brinkmann Minimum Recommended Assembly for TLC (Brinkmann Instruments Inc., Westbury, N.Y.) was used to prepare Silica Gel G chromatoplates of 200-μ nominal layer thickness. The chromatoplates were activated at 105° for 90 min, as recommended[4], and were stored at 50° (ref. 7); Boekel model No. 1078 ovens were used. The activated chromatoplates were shaped by means of a polyethylene nozzle of ~ 1-mm-width tip, which was attached to a vacuum line, and a No. 6 cork borer. Lang-Levy micropipets were used to deposit the extracts on the chromatoplates. The developing tank was the Desaga type. Developed chromatoplates were exposed to vapors of concentrated hydrochloric acid in a 7 1/8 in. wide by 9 3/8 in. long by 12 3/4 in. deep chromatographic jar provided with a plate-glass cover; the chromatoplates were set edgewise on a crystallizing dish, in the jar, that contained concentrated hydrochloric acid.

Reagents. The reference source of amanitins was a portion of a standard sample of *Amanita phalloides* powder provided by Professor R.G. BENEDICT, College of Pharmacy, University of Washington. Concentrated methanolic extracts of the

* Research sponsored by the U.S. Atomic Energy Commission under contract with the Union Carbide Corporation.

Amanita phalloides were obtained by Soxhlet extraction and flash evaporation of the Soxhlet extract.

Silica Gel G, "according to Stahl" (E. Merck & Co.), was purchased from Brinkmann Instruments Inc.

trans-Cinnamaldehyde was Reagent 77 from Distillation Products Industries, Rochester, N.Y.

All other reagents were analytical-reagent grade.

Procedures. The general procedures used were those described by SULLIVAN, BRADY AND TYLER[4]. The exact details of the procedures are recorded as a method[8].

Overrun of the development was achieved by removing a narrow band (~ 1 mm wide) of the Silica Gel G from along a line located at the desired maximum distance of solvent travel and then allowing the chromatoplate to remain in the developing solvent for some time (usually 10 min) after the solvent front had reached that line.

Results and discussion

The chromatograms compared in the color prints (Fig. 1) indicate the better resolution of the amanitins and the greater band sharpness that are achieved by use of shaped layers of adsorbent; the distance of travel of the developer was 10 cm from the origin.

The sensitivity of the method is difficult to define. It is reported that as little as 0.3 μg of pure α- or β-amanitin can be detected with certainty by this procedure[6]. However, limitations with respect to the total amount of an amanitin and the total volume of the test solution spotted on the chromatoplate may vary. Test-portion volumes of 2 μl, spotted in replicate one on top of the other with drying of each spot before deposition of the next, have been satisfactory with 200-μ-thick layers of Silica Gel G. The success of the separation and the effectiveness of the detection of the amanitins depend somewhat on the condition of the Silica Gel G layer and the freshness of the developer solvents. It has been recommended that chromatoplates older than three days should not be used[7]. Some chromatoplates older than this were used satisfactorily, but they seemed to be less sensitive than freshly prepared chromatoplates and to show a faint-violet background color when sprayed with *trans*-cinnamaldehyde reagent. Commercially available Silica Gel G plates did not give satisfactory results. Methyl ethyl ketone used from the one-quarter that remained in a 2-l bottle opened sometime before gave a pronounced violet background color. For maximum sensitivity, the time between the spotting and the development of a plate should be kept as short as possible; chamber saturation during development is recommended.

In the formation on the chromatoplate of the lavender-purple complexes of the amanitins with *trans*-cinnamaldehyde, it is important that the chromatoplate be surrounded by strong vapors from concentrated hydrochloric acid. The lavender-purple color begins to appear about 5 min after a plate has been exposed to the vapors; maximum color intensity may not be reached until 20 to 30 min later. Because the color fades as soon as a plate is removed from the vapors, the chromatograms must be documented almost immediately. Photocopying the developed chromatoplates by the Xerox method is a quick way to document them.

The R_F values for the amanitins vary with the shape, degree of activation, thickness, and age of the Silica Gel G chromatoplates. Also, the amount of extraneous material in the test portion affects the R_F values. The magnitude of the R_F values of

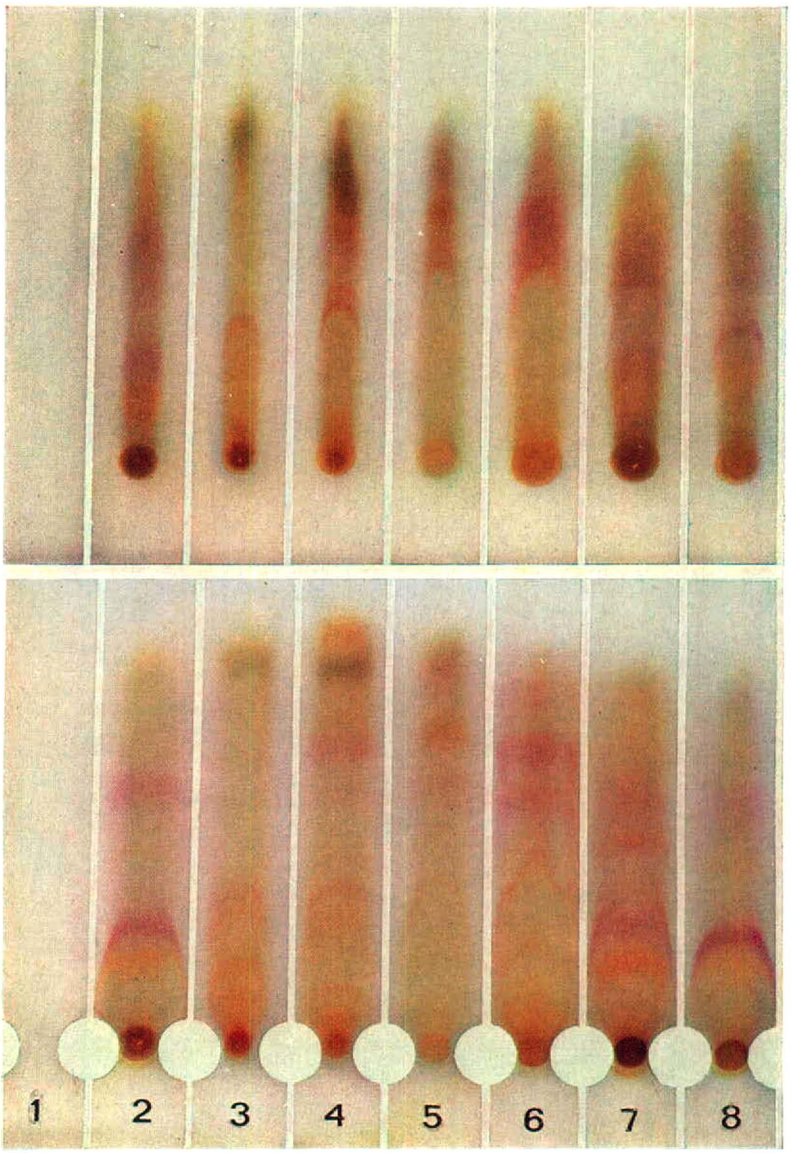

Fig. 1. Photographs of chromatograms of amanitins that show the improved separation achieved with shaped chromatoplates. 1 = Reagent blank; 2 = reference *Amanita phalloides* (lipid-free old extract); 3 = *Galerina marginata* (after 7-day fermentation); 4 = *Galerina marginata* (after 18-day fermentation); 5 = *Galerina marginata* (composite from low-level samples); 6 = *Galerina marginata* (composite from higher-level samples); 7 = reference *Amanita phalloides* (lipid-containing fresh extract); 8 = reference *Amanita phalloides* (lipid-free fresh extract).

the three types of amanitins is in the order: $\beta < \alpha < \gamma$; R_F values for the amanitins on Silica Gel G are given in three publications[4–6].

By this method, some 130 methanolic extracts from *Galerina marginata* and *Amanita phalloides* were analyzed satisfactorily. Both lipid-containing and lipid-free extracts were among them. The samples were derived from pellets harvested during evaluations of fermentation equipment and of anti-foam agents and during studies of the effects of antibiotics on bacteria-contaminated fermentation batches. The results were used to establish suitable fermentation conditions and optimum pellet-harvest times, to measure the effectiveness of the fermentation runs, and thus to guide the course of the preparation of the amanita toxins.

Acknowledgements

Information conveyed by personal communications to C. K. JOHNSON from Professors R. G. BENEDICT and L. R. BRADY of the College of Pharmacy, University of Washington, Seattle, Washington, made this work possible. H. L. HOLSOPPLE of the ORNL Analytical Chemistry Division prepared the methanolic extracts. The invaluable assistance of these persons is gratefully acknowledged.

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Received July 31st, 1968

CHROM. 3788

High resolution techniques of peptide mapping. Separation of bovine carotid actin peptides on cellulose thin layers and of the corresponding dansyl-peptides on polyamide thin layers

Comparative studies of globular actin, extracted from smooth or skeletal muscles from different animals, have failed in general to reveal significant differences in amino acid composition[1]. Bovine carotid actin is not an exception in this respect, its amino acid composition being similar to that of actin from rabbit skeletal muscle[2]. The same uniformity also occurs in the case of their peptide maps. Previous investigations on tryptic digests of carboxymethylated actin, performed by peptide mapping[3,4] after paper chromatography and electrophoresis, revealed about thirty ninhydrin positive spots. Using elaborate combined techniques of column chromatography and high voltage paper electrophoresis, MARTONOSI[5] detected forty-one peptides. On the basis of arginine and lysine content and for an assumed molecular weight of 60000[6,7], more than fifty peptides are theoretically liberated during trypsin digestion, whereas about forty would be expected for a molecular weight of 47000, the latter having been proposed more recently[4,8]. The number of peptides obtained on paper maps is insufficient evidence for determining minor variations appearing in the primary structure of actin extracted from different sources. The technique of MARTONOSI, even if it does allow an approach to the theoretical figures, is too complicated to be useful in comparative studies. For this reason, we undertook an extensive study in order to develop simple techniques, whereby improved resolution of the tryptic peptides of G-actin could be achieved. One hundred and fifty tests were carried out by means of two-dimensional techniques on thin layers of cellulose, silica gel and polyamide, using more than fifty chromatographic mixtures. Two techniques were finally selected: the first makes use of a cellulose thin layer, and allows detection of 46 ninhydrin positive spots, whereas the second, using dansyl-peptides, reveals 44 U.V. fluorescent spots on a polyamide layer.

Materials

Bovine carotid actin has been prepared by GASPAR-GODFROID *et al.*[9], according to the method of CARSTEN AND MOMMAERTS[10]. The carboxymethylation of the protein was performed with iodoacetate in 8 *M* urea in the presence of β-mercaptoethanol[11] whereas the tryptic digestion of the carboxymethylated actin was carried out as described by GOSSELIN-REY *et al.*[2].

Cellulose MN 300 (Macherey, Nagel, Düren, Germany) was used to prepare the thin layers.

Polyamide layers were purchased in 15 × 15 cm sheets from Cheng Chin Trading Co. Ltd., Taipei, Taiwan.

Dansyl chloride (98 %) was obtained from Fluka A.G.

Other chemical reagents were "Analytical grade". Note that the pyridine (Merck No. 9728) was redistilled on ninhydrin (500 ml pyridine–1 g ninhydrin) to avoid a coloured background.

Experimental

The peptide map on a cellulose thin layer. Standard 20 × 20 cm glass plates are spread (Desaga Equipment) with a 500 μ layer of cellulose (32 g cellulose, 192 ml water, 8 ml ethanol, stirred 2 min in a Waring blender). The plates are dried overnight in a cupboard at ordinary temperature and then washed with the chromatographic solvent; after washing, the upper yellow band is scraped off and 15 μl of peptide solution (5 mg/ml) is applied 10 cm from one edge and at 2 cm from the bottom. The plate is equilibrated as described by BONDIVENNE AND BUSCH[12], for 30 min in the chromatography tank, and then developed with amyl alcohol–isobutanol–propanol–pyridine*–water (10:10:10:30:30) for 6 h.

After drying the plate at ordinary temperature, electrophoresis is started at right angles to the first direction using pyridine–acetic acid–water (300:10:2700) at pH 6.5. The temperature of the cooling plate of the Desaga apparatus is kept at 5° with the aid of a circulating bath. After 50 min at 1000 V (20 mA) the plate is dried and then dipped in a ninhydrin reagent (1 g ninhydrin, 700 ml ethanol, 20 ml 2,4,6-collidine, 210 ml glacial acetic acid). The plate is heated at 110° for 15 min in an oven; 46 violet or gray coloured spots appear on a white background (Fig. 1).

The dansyl-peptide map on a polyamide layer. The dansylation of the peptides is performed as described by GRAY[13]. Peptides (0.5 mg) are dissolved in 50 μl 0.2 *M*

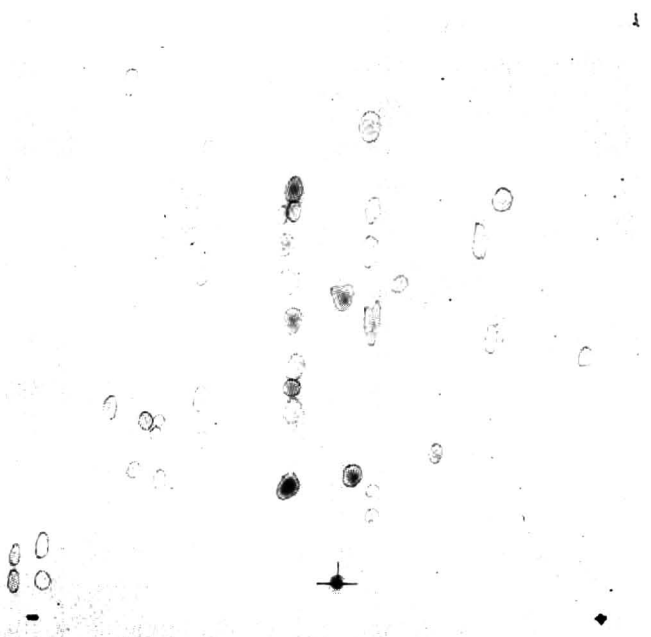

Fig. 1. Map of the peptides of a tryptic digest of bovine carotid actin on a 500 μ thin layer of cellulose. First direction (bottom to top): Chromatography in amyl alcohol–isobutanol–propanol–pyridine–water (10:10:10:30:30); second direction: Electrophoresis at pH 6.5 in pyridine–acetic acid–water (300:10:2700) for 50 min at 1000 V (20 mA).

* See section Materials.

$NaHCO_3$, 60 μl of an acetone–dansyl chloride solution (5 mg/600 μl) are added and the mixture, at pH 9, is incubated at 37.5° for 1 h.

After evaporation *in vacuo*, the residue is redissolved in 50 μl of deionized water and the dansylation process repeated twice more; care being taken to readjust the pH to 9 by addition of 0.1 *N* NaOH and to avoid any precipitation by adding acetone.

The mixture is then evaporated to dryness and 1 ml of 0.01 *N* acetic acid is added. This solution is passed through a column of Dowex 50 X 2 H^+ (7 × 0.9 cm), previously equilibrated with 0.01 *N* acetic acid[14]. The bulk of the by-products is eliminated by washing with 150 ml of 0.01 *N* acetic acid and the dansyl-peptides are eluted with a 25 % acetone solution in 1 *M* NH_4OH. U.V.-fluorescent fractions are pooled and evaporated to dryness *in vacuo*. The residue is dissolved in 500 μl of an acetone–acetic acid mixture (50:50) and the solution is kept at 2°.

To resolve the dansyl-peptides, 5 μl of the solution is applied to the right corner of a 15 × 15 cm plate of polyamide thin layer. The plate is developed first with a chromatographic solvent proposed by WOODS AND WANG[15] (see legend of Fig. 2) for 1 h; after drying, a second development is carried out at right angles using the following mixture, xylene–pyridine–acetic acid (10:1:1), for 2 h.

After drying, the chromatogram is exposed to a U.V.-lamp; 44 fluorescent spots were detected (Fig. 2).

This method, using dansyl-peptides and a polyamide layer, can be compared favourably with the other one in view of its simplicity, its low cost and its twenty times higher sensitivity. However, great care must be taken in the peptide-labelling process to avoid incomplete reaction which can provide additional spots.

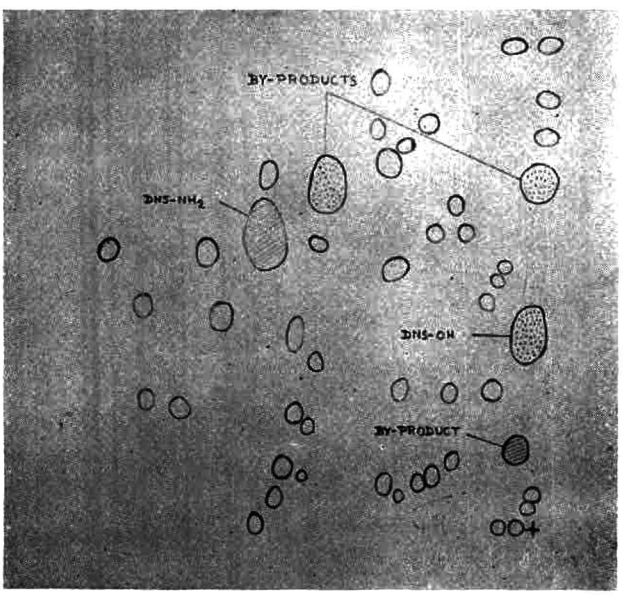

Fig. 2. Map of the peptides of a tryptic digest of bovine carotid actin on a polyamide layer using dansyl-peptides. First direction (bottom to top): Chromatography in water–formic acid (200:2.7); second direction (right to left): Chromatography in xylene–pyridine–acetic acid (10:1:1). Dotted area: blue fluorescent spots. Striated area: strongly yellow fluorescent spots.

Conclusions

An increase of about 30% over the number of peptides detected in G-actin with previously reported techniques of peptide mapping on paper is obtained by means of two simple thin layer techniques described in this paper. The number of spots detected is close to the maximal value theoretically expected and thus indicates that G-actin must consist of either one unique[8] or two different polypeptide chains and not of two very similar subunits as proposed by JOHNSON *et al.*[4].

The present results suggest that both techniques might be advantageously used, independently or together, to reassess the so-far observed similarity of G-actin extracted from different sources, as well as to undertake comparative studies on any homologous proteins.

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Received September 5th, 1968

CHROM. 3772

Identification and quantitation of α-hydroxy and α-keto acids with a ceric ammonium nitrate reagent

The use of ceric ammonium nitrate for the identification of α-hydroxy acids, α-keto acids and mercaptans in paper chromatograms was reported[1]. In continuation of the above, the reagent has now been stabilized and modified for thin-layer chromatograms. A quantitative procedure has also been developed.

Experimental

Identification. The paper or thin-layer chromatogram is dried and sprayed with a fresh solution of 10 % ceric ammonium nitrate in methanol or ethanol. The chromatogram is allowed to dry at room temperature for approximately 10 min. α-Hydroxy acids, α-keto acids and mercaptans show up as light spots on an orange background. Tryptophan reacts to form a brown color, and tyrosine forms a yellow color. The chromatogram is now sprayed with 0.25 % indole in methanol or ethanol. The spots become clearer due to the greater contrast since the background turns dark brown with the spots retaining their original color. These spots are stable both on paper and on thin-layer plates for over 3 weeks.

Quantitative determination. The spots are cut from the paper or scraped from the thin-layer plate and are placed in a centrifuge tube. One ml nitric acid (1:3) is introduced. After mixing for 5 min, the material is centrifuged (low speed, clinical centrifuge). Any paper shreds or silica precipitate immediately.

One-half ml of the clear supernatant is transferred to the center compartment of a Warburg vessel. One-half ml of ceric ammonium nitrate (30 %, w/v) in the nitric acid (1:3) is placed in a side arm. The thermobarometer contains 0.5 ml of the above nitric acid and 0.5 ml of the ceric ammonium nitrate solution. The vessel is equilibrated at 30° for 10 min, and then the two solutions in the vessel are mixed. The volume of gas produced in 20 min is measured.

Results

Identification. The following acids reacted in the range of 0.1 to 1.0 μmoles.

α-Hydroxy acids: α-Hydroxyglutaric acid, lactic acid, ascorbic acid, erythorbic acid, glycolic acid, malic acid, β-methylmalic acid, citramalic acid, glucuronic acid, gluconic acid, glyceric acid and tartaric acid.

α-Keto acids: Glyoxylic acid, α-ketoglutaric acid, pyruvic acid, oxalacetic acid.

Mercaptans: Cysteine, cysteamine and thioglycolic acid.

The other acids mentioned previously[1] reacted as recorded in that paper.

Quantitative determination. The relative volumes of CO_2 released from the α-keto and α-hydroxy acids are listed in Table I.

TABLE I

VOLUME OF CARBON DIOXIDE RELEASED WHEN α-HYDROXY ACIDS OR α-KETO ACIDS REACT WITH CERIC AMMONIUM NITRATE IN A STRONGLY ACID MEDIUM

Acid	*Gas volume μl per μmole*	*Linear range μmole*
Pyruvic acid	21	0.3 –3.5
Lactic acid	22	0.3 –3.5
Glyoxylic acid	24	0.3 –3.5
Malic acid	25	0.2 –2.0
α-Ketoglutaric acid	25	0.2 –2.0
Oxalic acid	42	0.2 –1.5
Tartaric acid	45	0.15–2.0
Citramalic acid	48	0.15–2.0
Glucuronic acid	66	0.1 –1.4
Citric acid	68	0.1 –1.2

The results are linear in the range listed in the table. Other carboxylic acids (neither α-keto nor α-hydroxy) such as butyric acid, palmitic acid, linoleic acid, succinic acid and glutaric acid, did not give off any gas under identical conditions.

Discussion

The ceric ammonium nitrate reagent described in this paper was developed from reagent A of TROP *et al.*[1]. When the above reagent was used, however, the orange background color faded quite rapidly due to reduction by the cellulose of the paper. In addition, the method of drawing the paper through a solution of reagent resulted in a slight diffusion of the colored spots during the time the paper was soaked with the reagent. An aqueous solution was also not suitable for spraying in thin-layer chromatography. The present reagent can be sprayed on paper or on thin layers. It dries rapidly and there is little or no diffusion.

The orange background is quite striking at first. This is due to the complex of the hydroxyl groups of the cellulose with the ceric ammonium nitrate[2]. The orange color is stable as long as the alcohol does not evaporate on thin-layer plates which do not contain cellulose. The contrast is greatly increased by the indole spray, the indole reacting with the unreduced ceric ammonium nitrate to form a dark brown background. The indole does not react with the reduced ceric ammonium nitrate, the spots remaining light.

The oxidation of α-hydroxy and α-keto acids in a strongly acid solution causes the release of CO_2 which can be measured with a Warburg manometer. When sodium hydroxide is placed in the center well, no manometer change is noted. Simple α-hydroxy and α-carbonyl acids (lactic acid, malic acid, glyoxylic acid, pyruvic acid and α-ketoglutaric acid) release almost a stoichiometric volume of gas. Polyhydroxy acids, or acids with the carbonyl groups in a position to allow them to interact with the ceric ammonium nitrate, will release greater quantities of CO_2, the volume of gas not being stoichiometric.

The above is not a great disadvantage because the volume of CO_2 released is linear in the ranges listed, and the quantitative determination can be made with an accuracy of $\pm 6\,\%$.

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Received September 9th, 1968

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Book Reviews

Experiments in Modern Methods of Chemical Analysis, edited by R. L. PECSOK, Wiley, New York, 1968, v + 135 pp., price £ 1.18.0.

This spiral-bound paper-backed edition is the laboratory manual for use by elementary classes in organic and biochemistry in the University of California, Los Angeles. It is used in conjunction with a text for the lecture part of the course *Modern Methods of Chemical Analysis* by R. L. PECSOK AND L. D. SHIELDS.

The experiments are divided into three sections. The first section contains basic experiments in separation techniques including extraction, distillation, crystallisation, GLC, TLC and column chromatography. The second section gives the student experience in the interpretation of infrared, nuclear magnetic resonance and mass spectra. The third group of experiments presents a fundamental approach to equilibrium studies in aqueous and non-aqueous systems. The instructions given for each experiment are very full and explicit. The very few printing errors may not be obvious to a student.

The student who completes all the experiments will then have had experience of a wide selection of the modern techniques that are so helpful in separation and structure determination. For example, in chromatography he will have used GLC, TLC, PC, ion exchange and gel filtration. Also, these methods have been presented, not in isolation, but integrated with a variety of other techniques.

The reviewer cannot agree with the author's statement given in the preface that "elementary analytical chemistry is no longer an exercise in patience, perseverance and strict attention to detail". This is poor advice to a student!

The author has presented a novel approach to the teaching of analytical chemistry. Although, on glancing through the contents, the experiments may seem to be oriented more towards biochemistry, all organic and physical chemistry lecturers who organise laboratory courses will find that this manual will repay careful study.

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Gelchromatographie, by H. DETERMANN, Springer-Verlag, Berlin, Heidelberg, New York, German edition, 1967, 204 pp., price DM 32.00; English edition, 1968, 195 pp., price $ 8.00.

Gel chromatography, also known as gel filtration or gel permeation chromatography, is a relatively new, powerful and rapid method of separating molecular particle distributions. Reviews on various aspects of the technique have appeared in several books and journals, but this is the first work to deal exclusively with it. The method is applicable to a wide variety of materials and molecular sizes and should be of interest to workers in many fields of science. This versatility is reflected in the contents of the book. The first chapter presents a general introduction into the mechanism, terminology and history of gel chromatography. This is followed by a detailed description of materials and methods. The preparation of the various hydrophilic and organophilic gels and their properties are outlined. An extensive list of auxiliary equipment is given, together with a very useful compilation of manufacturers and distributors. The second chapter closes with a discussion of several experimental variations of gel chromatography, *i.e.* column techniques. thin-layer chromatography and centrifugation. Chapter 3 is concerned with the theoretical basis of the method. In keeping with the stated intention of the author to write a laboratory handbook, the presentation does not go into all details. This chapter, perhaps more than the others, is also somewhat handicapped by the fact that the literature throughout the book is covered only up to the middle of 1966. Since then, some more insight has been gained into the mechanism of gel chromatography. The fourth chapter outlines various principles of application, such as desalting, molecular weight measurement, determination of molecular weight distributions and separations on the basis of affinity between solutes and gel. The final chapter presents a very useful and detailed compilation and discussion of published results in a wide range of areas of work.

The book is written in a very readable style and produced well. It should be valuable to anyone concerned with separation science.

Airco, Inc., Murray Hill, N.J. (U.S.A.) M. J. R. CANTOW

News

Meetings

SIXTH INTERNATIONAL GEL PERMEATION CHROMATOGRAPHY SEMINAR

The Sixth International GPC Seminar was held in Miami Beach, Florida at the Eden Roc Hotel, October 7th–9th, 1968. The program format and activity schedule was similar to that of the Fourth International Seminar.

The 1968 Program included 25 technical papers and two panel discussions—one on *Application of GPC to Small Molecules* and the other on *Universal Calibration*. Dr. FRED BILLMEYER, Rensselar Polytechnic Institute and Dr. DON BLY, DuPont, were the panel moderators. A special feature of the program was a discussion of computer correction techniques by Dr. ARCHIE HAMIELEC, McMasters University, Hamilton, Ontario. The 25 technical papers covered a broad spectrum of subjects ranging from sophisticated theoretical concepts to practical GPC applications.

For abstracts of the Seminar papers, contact the Chairman, Sixth International GPC Seminar, c/o Waters Associates, Inc., 61 Fountain Street, Framingham, Massachusetts 01701, U.S.A.

Manufacturers' literature

A folder entitled *Merck Preparations for Chromatography* recently came to hand in this office. The leaflets within the folder were both in English and German. They include descriptions of

(i) thin film procedures and materials;
(ii) TLC plates ready for instant use, both for analytical and preparative usage;
(iii) the Neatan "new" Merck reagent for preservation of thin film chromatograms;
(iv) adsorbents for preparative thin film chromatography;
(v) specialised silica gels for work demanding extra high purity layers, or silanised material for use as a hydrophobic gel especially suited for partition chromatography.

For further information apply to the publisher under reference No. Chrom. N-170.

New apparatus

The new Phoenix SI-4A *Automatic Sample Injector* from Phoenix Precision Instrument Co, Philadelphia, extends automation of amino acid analysis to the near-ultimate level. With the capability of charging up to four cartridges with samples for automatic sequential application to a chromatographic column, a user of a Phoenix Amino Acid Analyzer now can maintain his instrument in round-the-clock operation with the barest minimum of attendance time.

The Automatic Sample Injector is provided in modular form to supplement the Phoenix Varipump for single-column gradient elution chromatography by the Piez-Morris method. On the front panel of the instrument, four miniature ion exchange resin-filled glass columns are mounted to which the operator applies his samples. The sample mixture is adsorbed by the resin and tightly held by ionic bonds. The top fitting of each column is the termination of tubing extending from a motor-driven four-way rotary valve. This valve receives eluting buffer from the Varipump through a fitting at the rear of the chassis. The flow is then directed, in sequence, to each of the four sample-holding columns in response to an electrical pulse received at the initiation of each Varipump elution program cycle. Indicator lights, positioned over each column, identify the sample being eluted at any time. Teflon capillary tubing, connected to the bottom connector of each column, leads to a junction disc of zero dead volume and thence to a take-off port for connection to a chromatographic column. In this way, each of four samples may be eluted automatically from the sample cartridge into a chromatographic column for analysis.

For further information apply to the publisher under reference No. Chrom. N-165.

The MT160 from MicroTek, Tracor Inc., Austin, Texas, is a space-saving instrument available for isothermal or temperature programmed dual column operation to 500° with two detectors. It includes a new total solid state electrometer with three switchable and entirely separate input channels for ultimate convenience in multi-detector operation. The temperature programmer option is also completely transistorized including three electronic timers for functions reproducible to within 0.6 seconds.

An all-glass system is available with the unique MicroTek inlet for on-column injection. This glass system is strongly recommended with the Flame Photometric detector for sensitive work with organo-phosphorus and organo-sulfur compounds.

Other detector options include the dual flame, thermal conductivity, and electron capture detectors.

For further information apply to the publisher under reference No. Chrom. N-163.

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REFERENCE HIGH-EFFICIENCY NONPOLAR PACKED COLUMNS FOR THE GAS–LIQUID CHROMATOGRAPHY OF NANOGRAM AMOUNTS OF STEROIDS

PART I. RETENTION TIME DATA*

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(Received August 15th, 1968)

SUMMARY

The preparation and conditioning of reproducible, long-lasting, 6000 theoretical plate nonpolar columns are described. Retention data of TMS derivatives at 215, 230 and 240° are given for 140 standard steroids of the androstane, pregnane and cholestane series. Additive incremental factors permitting a precise prediction of retention times are given for many specific structural features and functional groups. Operational conditions that permit highly reproducible results with nanogram amounts of steroids are described and discussed.

INTRODUCTION

The analysis of total steroids extracted from most biological materials offer difficult problems which arise from the number, structural variety, similarity in physical properties and very low concentrations of many components. Such problems can be solved by a combined thin-layer and gas–liquid chromatography (TLC–GLC) method[1–8]; by this method, total steroids are first separated in several fractions which are characterized by molecules containing specific numbers of hydroxyl and carbonyl groups. Each of these fractions is then resolved by GLC.

Routine examination of TLC fractions by GLC, whether for the purpose of component identification of quantification, is particularly efficient and reliable with columns of high resolving power when these columns show unaltered characteristics over a long period of time.

The present paper describes the preparation and detailed properties of readily reproduced nonpolar columns which in three years of almost continuous use have

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consistently displayed identical characteristics. Their resolving power, obtained from the Keulemans expression[9] corresponds, for example, to 5500 and 6150 theoretical plates for the trimethylsilyl ether (TMS) derivatives of cholesterol and stigmasterol, respectively.

With such columns, retention times are reproducible enough to permit unequivocal discrimination between compounds which differ very little in this respect; a precise prediction of retention times from structure-dependent increments[1,2] can be made for the entire life-span of the columns.

Furthermore, reliable quantitative work can be made on the basis of calibration curves which remain valid over long periods of time.

These important advantages are obtained under simple operational conditions provided that the conditions are used consistently.

EXPERIMENTAL

Equipment

Perkin Elmer 800 gas chromatograph equipped with dual flame ionization detector fitted with ceramic jets.

Perkin Elmer 900 gas chromatograph.

Philips PR 2500 recorder, 1 mV full scale.

Perkin Elmer No. 194 Printing Integrator.

Maximum signal-to-noise ratio was obtained as follows: Chromatograph and recorder were connected to separate electrical power lines, that used for the recorder being free of noise-generating equipment. Recorder gain was adjusted for incipient "hunting" by pen-drive motor, and the chromatograph chassis was earthed by connecting to a water main. Under full operational conditions at attenuation $\times 1$, the base line was consistently straight with occasional transient deflections not exceeding 0.5 % of the chart span.

The oven of the P.E. 800 instrument was fitted with a 200–260° Anschütz thermometer (Fisher 15-165E) calibrated *in situ* and under operating conditions against a precision resistance thermometer.

Materials

JXR, 3 % dimethylpolysiloxane on 100/120 Gas Chrom Q, prepared by, and received from Dr. W. SUPINA, now with Supelco, Inc., Bellefonte, Pa. 16823, U.S.A.

Hexamethyldisilazane and trimethylchlorosilane, redistilled.

CS_2, Fisher No. C 184 reagent.

Standard steroids from Steraloids Inc., P.O. Box 127, Pawling, N.Y. 12564.

Standard steroids from the Steroid Reference Collection (*cf.* Acknowledgements) indicated by S.R.C. in Tables VIII and IX.

Steroids obtained by reduction of standard steroids (*cf.* Discussion and Tables VIII and IX).

Methods

Preparing columns. Two 9 ft. straight lengths of 1/8 in. O.D. stainless steel tubing cut from the same stock were cleaned internally by connecting one end with polyethylene tubing to a partially evacuated flask and slowly aspirating chloroform–

methanol (2:1) through a polyethylene tubing connected to the other end. The tubes were dried with a stream of N_2 and clamped vertically side by side at about 2 in. from each other. Bottom ends of the tubes were plugged; each of the upper ends were connected by a short section of polyethylene tubing to one of twin funnels described in Fig. 1A.

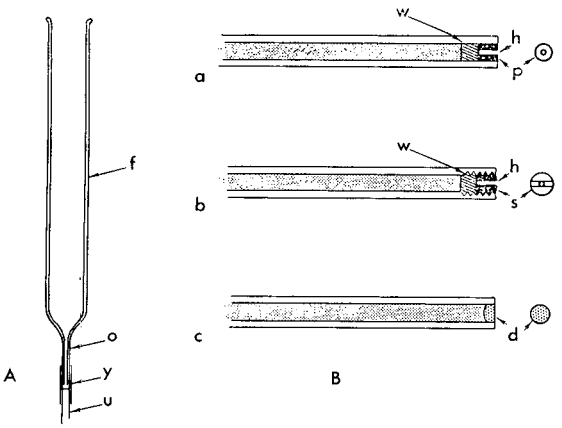

Fig. 1. (A) Glass funnel for the filling of columns. f = Graduated body, 14.3 mm I.D.; o = outlet, 1.5 mm I.D., 1/8 in. O.D., 20 mm long; y = polyethylene connective tubing; u = column. Both funnel and column are clamped on the same support. (B) Three methods for column plugging: (a) Solid plug method (w = fine Pyrex glass wool, Corning No. 3950, silanized; h = 1-mm diam. hole; p = stainless steel plug). (b) Screw method (w, as above; s = 3-48 NC screw; h = 1-mm diam. hole; a size 3-48 NC tap is used to thread the column). (c) Porous disk method (d = stainless steel porous disk, Perkin Elmer Cat. No. 008-1216).

Four grams of 3 % JXR on 100–120 mesh Gas Chrom Q were placed in each of the funnels and the columns were tapped with a pencil about their middle section. Rapid, alternated tapping between the two columns caused just enough vibration to make the powder flow evenly and at the same speed, from both funnels. Packing was completed when levels of excess powder in the funnels remained constant after sustained tapping progressing from the bottom to the top of the columns. The funnels were disconnected carefully and the weights of excess powder were compared to detect a possible discrepancy. On the average, each column contained 3.60 g of packing material of density 0.3 g/ml.

Both ends of the columns were then plugged. Three ways of effecting this operation are described in Fig. 1B and related caption. In plugging columns by methods a and b (Fig. 1B) the packing material in completely filled columns was first pushed 1/4 in. inside with a metal rod. The resulting space was then filled completely with fine silanized glass wool. Insertion of either plug, p, or screw, s, then resulted in further compression of the wool. When method b was used, the column ends were threaded over 1/2 in. before cleaning and filling the tubes. With method c, damage to the pores of disks, d, was avoided by pressing (rather than tapping) the disks in position. In all cases, damage to the terminal 1/2 in. of outer surface was avoided to permit a tight fitting of Swagelock connectors. The columns were coiled on a 2 in. O.D. mandrel.

The packed columns were connected to the gas chromatograph by their inlet end; connection to the detector was absolutely avoided. Helium flow through each column was adjusted to 60 ml/min. with a bubble meter. The gas flow was then completely cut off and the conditioning schedule shown in Table I was observed.

TABLE I

CONDITIONING[a] SCHEDULE FOR TWIN 9-FOOT, 1/8 INCH OUTER DIAMETER HIGH-EFFICIENCY JXR COLUMNS

Time (*h*)	*Temperature*[b] (°C)	*Helium flow rate* (*ml/min*)
0	Room temperature	0
8	250	0
24	250	0
32	300	0
48	300	0
49	250	0
52	300	30
72	300	30
73	300	60
76	Room temperature	60

[a] Helium flow rates through the columns must first be adjusted to the same value, *i.e.*, 60 ml/min (bubble meter). Thus both columns have been flushed with, and are full of helium when cut-off valve is turned off to begin conditioning.

[b] Changes in temperature must be gradual over the period indicated.

Setting-up conditions

Adjustment of conditions permitting duplication with any JXR column of retention times listed for 230° in Tables II–IX was made as follows. A standard CS_2 solution containing 30 ng of TMS derivative of 3β-hydroxy-5α-androstane (3β-androstanol)[4,5] and 50 ng of 5α-cholestane per μl was prepared. With the oven temperature set at 230°, standard conditions described in Table II were used. Two microliters of the standard solution were injected. Corrected retention times of both compounds were recorded with attenuation setting $\times$10 for the P.E. 800 instrument ($\times$1, $\times$40 with P.E. 900). If the ratio of the retention time of cholestane to that of the derivative of 3β-androstanol was *higher* than 3.575 (3.575 = 680/190) the temperature was *increased* by 0.05° for every 0.001 units difference in this ratio; conversely, the temperature was *lowered* by this amount when the observed ratio was *smaller* than 3.575. The helium flow was then adjusted to bring the retention time of cholestane to 6.80 min. If the retention time ratio still differed from the correct one the operation was repeated. It should be noted that the retention time of cholestane varied by 3.7 % per °C, whereas the corresponding variation for 3β-androstane TMS was 3.2 % only.

Preparing TMS derivatives. Up to 1 mg of hydroxylated steroid were reacted in a glass-stoppered flask with 50 μl of hexamethyldisilazane and 50 μl of 10 % trimethylchlorosilane in chloroform (v/v), the reagents being added in that order. With larger amounts of steroids correspondingly larger volumes of reagents were used. Brief

mixing by stirring or vibration was applied after each addition; the top of the stopper was greased with silicone lubricant. If the steroid had been obtained by evaporating a solution, moisture and last traces of solvent were removed before adding the reagents by leaving the flask for 2 h in an evacuated dessicator over P_2O_5.

The reaction mixture was left at room temperature for at least 3 h[1,2]. Excess solvent and reagents were removed as described in ref. 2. CS_2, or a solution in CS_2 of the selected standard was then added to the flask contents. These, including residual ammonium chloride formed in the reaction, dissolved completely.

Standard solutions. It will be shown in Part II[17] of the present series of papers that the retention time of a given steroid under a given set of conditions is minimum when the quantity injected lies within a range which is specific for the structural group to which the steroid belongs.

Concentrations in the standard solutions used to produce the present data were such that 2 μl of injected solution contained amounts of individual steroids falling within the specific range for constant, minimum retention time. These concentrations were also adjusted to produce peaks of roughly comparable size at the same attenuation setting.

To begin with, solutions containing the TMS derivative of single steroids along with at least one standard steroid were prepared and the retention times were determined at 230° under adjusted operational conditions (*cf.* above). Next, several mixtures containing from 20 to 30 steroids were prepared and treated with TMS reagents. The components were selected according to the known retention times so as to produce an uninterrupted series of peaks with minimum overlap when injected together. An example of chromatograms thus obtained is given in Fig. 2. Most steroids were included in at least two of the twelve different solutions that were prepared. Most of

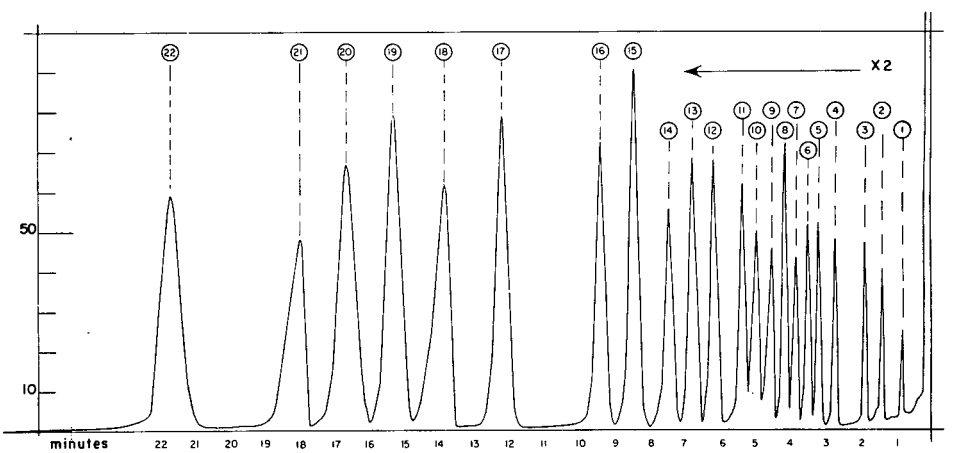

Fig. 2. Chromatogram of standard steroid mixture at 230°. Conditions: *cf.* Tables II–IX. The components, designated by peak number, group and number in group, and Table number, were as follows: 1 = A2,II; 2 = A3,II; 3 = C2,IV; 4 = D1,V; 5 = C4,IV; 6 = D5,V; 7 = D8,V; 8 = E6,VI; 9 = D11,V; 10 = D13,V; 11 = D15,V; 12 = A4,II; 13 = A7,II; 14 = A8,II; 15 = G1,VIII; 16 = G5,VIII; 17 = C5,IV; 18 = B14,III; 19 = C7,IV; 20 = C9,IV; 21 = B16,III; 22 = C16,IV. Some of the amounts injected were, in nanograms: A2, 1.0; C2, 6.0; D15, 20.0; G1, 30.5; C7, 50.1. Note separation of peak 19 (cholesterol, C7) and peak 20 (desmosterol, C9) on JXR column of 5500 theoretical plates (for cholesterol).

six steroids considered as potential internal standards in future work were included in all solutions. These were the TMS derivatives of 3α-hydroxy-5α-androstane, 3α,20α-hydroxy-5β-pregnane (pregnanediol), 3β-hydroxycholest-5-ene (cholesterol), and the hydrocarbons 5α-pregnane, 5α-cholestane and stigmastane.

Using these solutions it was possible to obtain readily the retention times of a large number of steroids and to correlate these retention times to those of the above six key steroids.

It was observed that standard solutions of TMS derivatives prepared in the way described were stable under the following simple conditions[1,2]: cold storage (−5°) between uses of at least 5 ml of solution in narrow-necked flasks fitted with ground-glass stoppers lubricated with silicone grease; *bringing to room temperature* before opening a flask and minimizing exposure to moisture during use.

Retention time data

A Precision-Scientific "Time It" instrument giving a digital reading to the nearest 1/100th of a minute was started (zero time) when a rapid deflection of the recorder pen following injection marked the solvent vapour surge into the detector. This occurred 23 sec. after injections at 230°, for example. For each of the successive peaks, time was read as the recorder pen just left the peak apex on its downward course. Thus "corrected" retention times were recorded within ± 1/100th of a minute.

The retention time data listed in Tables II to IX were obtained with the P.E. 800 chromatographer at 215.0, 230.0, and 240.0° with the 3-year-old columns. In these experiments the temperature, as indicated by the Anschütz thermometer, never varied more than ± 0.1° in the course of each experiment (≈ 30 min). Very slow cyclic variations within ± 0.25° were observed in the course of many hours: thus the temperature from experiment to experiment varied to some extent and the observed retention times varied accordingly.

However, when observed retention times were normalized to the nominal, or set temperature of the experiment, normalized retention times agreed within ± 0.2 %. Normalization was obtained by multiplying an observed value by the ratio $t'_{T_n^s}/t'_{T_e^s}$

TABLE II

CORRECTED RETENTION TIMES[a] t'_R AND LOG t'_R OF STEROIDS ON JXR COLUMNS AT 215, 230 AND 240[c]

GROUP A—HYDROCARBONS

No.	*Compound*	*Trivial name*	$10^2 \times t'_R$ (min)			$10^3 \times \log t'_R$		
			215°	230°	240°	215°	230°	240°
1	5β-Androstane	Etiocholane	113	77	61	2053	1887	1784
2	5α-Androstane	Androstane	124	84	66	2093	1924	1819
3	5α-Pregnane	Allopregnane	218	141	108	2338	2150	2033
4	5β-Cholestane	Coprostane	1089	620	435	3037	2792	2638
5	5α-Cholest-2-ene		1175	667	469	3070	2824	2671
6	Cholest-5-ene	Cholestene	1200	678	475	3079	2831	2676
7	5α-Cholestane	Cholestane	1200	680	477	3079	2833	2678
8	Cholesta-3,5-diene		1332	748	522	3124	2874	2717
9	5α,24β-Ethyl-cholestane	Stigmastane	2038	1118	763	3309	3048	2882

[a] Operational conditions: Inlet temperature, 270°; detector = oven temperature. Inlet pressure to chromatographer, 60 lb.; helium flow rate, 60 ml/min. Hydrogen inlet pressure, 18 lb.; air, 50 lb.

TABLE III

CORRECTED RETENTION TIMES[a] t'_R AND LOG t'_R OF STEROIDS ON JXR COLUMNS AT 215, 230 AND 240°

GROUP B—MONO- AND POLYKETONES

No.	*Compound*	*Trivial name*	$10^2 \times t'_R$ (*min*)			$10^3 \times \log t'_R$		
			215°	*230°*	*240°*	*215°*	*230°*	*240°*
1	5α-Androstan-17-one	Androstanone	241	155	119	2382	2190	2076
2	5α-Androstan-3-one		264	169	127	2421	2228	2104
3	5β-Pregnan-3-one		429	262	194	2632	2418	2288
4	5β-Androstan-17-one		468	288	213	2670	2459	2328
5	Androst-4-ene-3,17-dione		635	384	287	2803	2584	2458
6	Androsta-1,4-diene-3,17-dione		690	416	304	2839	2619	2483
7	5β-Pregnane-3,20-dione	Prenanedione	760	449	325	2880	2652	2512
8	Androst-4-ene-3,11,17-trione		798	475	341	2902	2676	2533
9	5α-Pregnane-3,20-dione		840	497	360	2924	2696	2556
10	5β-Pregnane-3,11,20-trione		960	572	411	2982	2757	2614
11	Pregn-4-ene-3,20-dione	Progesterone	1030	613	440	3013	2788	2643
12	5α-Pregnane-3,11,20-trione		1090	649	467	3038	2812	2669
13	5β-Cholestan-3-one	Coprostanone	2340	1255	860	3369	3098	2934
14	5α-Cholestan-3-one	Cholestanone	2605	1390	951	3415	3143	2977
15	Cholesta-3,5-dien-7-one		2875	1525	1039	3458	3183	3017
16	Cholesta-4,6-dien-3-one		3497	1905	1224	3544	3256	3087

[a] Operational conditions: *cf.* Table II.

where $t'_{T_c^s}$ is the retention time observed in the same experiment for an internal standard included in the mixture, and $t'_{T_n^s}$ its retention time at the nominal temperature of the experiment. In most experiments normalization of the data could be effected by using several internal standards. In no case were the necessary corrections larger than 1%.

From retention time logarithms listed in Tables II to IX incremental factors of these logarithms shown in Tables X and XII were computed. The values obtained for specific functional groups or combination of groups were often averages derived from data on several steroids which included the relevant structural features. Variations observed for individual values of such increments were usually less than 1%; thus retention times could be accurately predicted from the structural formulae simply by adding up appropriate increments[1,2]. Examples applying to androstane- and pregnanediols are given in Table XI. Table XII gives incremental values for many combinations of structural features concerning sterols.

Incremental values of this type were first described by KNIGHTS AND THOMAS[9]. Their use in the detection and structural identification of steroids will be discussed in detail in Part II[17] of the present series of papers. Quantification problems at the nanogram level will be discussed in Part III.

DISCUSSION

The importance of minimizing vibration during the packing of columns must be stressed. By tapping the columns as described the flow of packing material was slow and even; air pockets did not form, the powder packed almost maximally, and about 20 min were required to fill the columns. Further tapping usually resulted in some increase in packing density. Excess vibration, on the other hand, did not increase

TABLE IV

CORRECTED RETENTION TIMES[a] t'_R AND LOG t'_R OF TRIMETHYLSILYL DERIVATIVES OF HYDROXYLATED STEROIDS ON JXR COLUMNS AT 215, 230 AND 240°

GROUP C—MONOHYDROXY COMPOUNDS

No.	*Compound*	*Trivial name*	$10^2 \times t'_R$ (*min*)			$10^3 \times \log t'_R$		
			215°	*230°*	*240°*	*215°*	*230°*	*240°*
1	3α-Hydroxy-5α-androstane	3α-Androstanol	239	150	113	2378	2175	2053
2	3β-Hydroxy-5α-androstane	3β-Androstanol	305	190	141	2484	2278	2149
3	3α-Hydroxy-5β-pregnane	3α-Pregnanol	444	266	192	2647	2424	2283
4	3β-Hydroxy-5α-pregnane	3β-Allopregnanol	540	321	232	2732	2506	2365
5	3β-Hydroxy-5β-cholestane	3β-Coprostanol	2305	1227	825	3362	3088	2916
6	3α-Hydroxy-5β-cholestane	3α-Coprostanol	2439	1268	850	3387	3103	2929
7	3β-Hydroxycholest-5-ene	Cholesterol	2936	1536	1022	3467	3186	3009
8	3β-Hydroxy-5α-cholestane	3β-Cholestanol	3005	1574	1047	3477	3197	3020
9	3β-Hydroxycholesta-5,24-diene	Desmosterol	3217	1673	1108	3507	3224	3044
10	3β-Hydroxycholesta-5,7-diene	7-Dehydrocholesterol	3250	1677	1117	3511	3225	3048
11	3β-Hydroxycholest-7-ene	Lathosterol[b]	3360	1750	1150	3526	3243	3060
12	3β-Hydroxycholesta-8,24-diene	Zymosterol[b]	3365	1754	1155	3527	3244	3063
13	3β-Hydroxycholesta-5,7,24-triene	[b]	3540	1841	1200	3549	3265	3089
14	3β-Hydroxy-24β-methylcholesta-5,7,22-triene	Ergosterol	3640	1885	1268	3561	3275	3103
15	3β-Hydroxy-24α-methylcholest-5-ene	Campesterol	3905	1998	1316	3591	3301	3119
16	3β-Hydroxy-24β-ethylcholesta-5,22-diene	Stigmasterol	4263	2170	1425	3623	3336	3153
17	3β-Hydroxy-4α-methylcholest-7-ene	Methostenol[b]	4310	2195	1435	3634	3341	3157
18	3β-Hydroxy-4,4',14α-trimethyl-5α-cholesta-8,24-diene	Lanosterol	4725	2410	1573	3674	3382	3197
19	3β-Hydroxy-24α-ethylcholest-5-ene	β-Sitosterol	5002	2511	1634	3699	3399	3213
20	3β-Hydroxy-4,4'-dimethylcholest-7-ene	[b]	6070	2568	1670	3783	3410	3223

[a] Operational conditions: *cf.* Table II.

[b] Samples obtained from FUMAGALLI, confirmed by mass spectrum by GALLI AND MARONI[10].

TABLE V

CORRECTED RETENTION TIMES[a] t'_R AND LOG t'_R OF TRIMETHYLSILYL DERIVATIVES OF HYDROXYLATED STEROIDS ON JXR COLUMNS AT 215, 230 AND 240°

GROUP D—MONOHYDROXY, MONOKETO COMPOUNDS

No.	*Compound*	*Trivial name*	$10^2 \times t'_R$ (*min*)			$10^3 \times \log t'_R$		
			215°	*230°*	*240°*	*215°*	*230°*	*240°*
1	3β-Hydroxy-5β-androstan-17-one		458	275	201	2661	2439	2303
2	3α-Hydroxy-5α-androstan-17-one	Androsterone	462	278	202	2664	2444	2304
3	3α-Hydroxy-5β-androstan-17-one	Etiocholanolone	485	288	207	2686	2459	2316
4	3β-Hydroxyandrost-5-en-17-one	DHA	573	337	244	2758	2527	2387
5	3β-Hydroxy-5α-androstan-17-one	Epiandrosterone	593	350	253	2773	2544	2403
6	3-Hydroxyestra-1,3,5(10)-trien-3-one	Estrone	637	372	267	2804	2570	2421
7	17β-Hydroxy-5α-androstan-3-one	Allodihydrotestosterone	642	378	272	2807	2577	2434
8	17α-Hydroxyandrost-4-en-3-one	Epitestosterone	660	387	278	2819	2587	2444
9	17β-Hydroxy-19-norandrost-4-en-3-one	19-Nor-testosterone	695	399	284	2842	2600	2453
10	3β-Hydroxy-5β-pregnan-20-one	Pregnanolone	732	434	308	2864	2638	2489
11	17β-Hydroxyandrost-4-en-3-one	Testosterone	785	459	329	2895	2662	2517
12	3α-Hydroxy-5β-pregnan-20-one	Epipregnanolone	792	454	321	2898	2657	2506
13	17β-Hydroxypregna-1,4-dien-3-one		865	503	358	2937	2701	2553
14	3β-Hydroxypregna-5,16-dien-20-one		866	496	348	2937	2695	2541
15	3β-Hydroxypregn-5-en-20-one	Pregnenolone	944	538	380	2975	2730	2580
16	3β-Hydroxy-5α-pregnan-20-one	Allopregnanolone	973	553	389	2988	2742	2590
17	20β-Hydroxy-5α-pregnan-3-one		1140	644	454	3057	2809	2657
18	20β-Hydroxypregn-4-en-3-one		1441	799	552	3158	2899	2742

[a] Operational conditions: *cf.* Table II.

TABLE VI

CORRECTED RETENTION TIMES[a] t'_R and LOG t'_R OF TRIMETHYLSILYL DERIVATIVES OF HYDROXYLATED STEROIDS ON JXR COLUMNS AT 215, 230 AND 240°

GROUP E—DIHYDROXY COMPOUNDS

No.	*Compound*	*Trivial name*	$10^2 \times t'_R$ (*min*)			$10^3 \times \log t'_R$		
			215°	*230°*	*240°*	*215°*	*230°*	*240°*
1	3α,17β-Dihydroxy-5α-androstane	Dihydroandrosterone	586	339	241	2768	2530	2382
2	3β-17α-Dihydroxyandrost-5-ene		586	336	240	2767	2526	2380
3	3α,6α-Dihydroxy-5β-pregnane		645	367	258	2809	2564	2410
4	3β,17β-Dihydroxyandrost-4-ene	Δ^4-Androstenediol	695	401	282	2842	2602	2450
5	3β,16α-Dihydroxy-5α-androstane	16α-Androstanediol	698	401	282	2843	2602	2450
6	3β,17β-Dihydroxyandrost-5-ene	Δ^5-Androstenediol	724	415	294	2859	2618	2468
7	3,17α-Dihydroxyestra-1,3,5(10)-triene	17α-Estradiol	728	414	289	2862	2617	2461
8	3β,17β-Dihydroxy-5α-androstane		736	426	300	2867	2628	2477
9	3,17β-Dihydroxyestra-1,3,5(10)-triene	Estradiol	809	460	322	2908	2663	2507
10	3β,20β-Dihydroxy-5β-pregnane		1006	564	393	3002	2751	2594
11	3α,20β-Dihydroxy-5β-pregnane		1058	585	402	3024	2767	2604
12	3α,20α-Dihydroxy-5β-pregnane	Pregnanediol	1154	634	437	3062	2802	2640
13	3β,20α-Dihydroxypregna-5,16-diene		1080	599	414	3033	2777	2617
14	3β,20β-Dihydroxypregn-4-ene	Pregnenediol	1238	687	470	3092	2837	2762
15	3β,20β-Dihydroxy-5α-pregnane		1315	728	503	3118	2862	2701
16	3β,20α-Dihydroxy-5α-pregnane		1403	770	529	3147	2886	2723

[a] Operational conditions: *cf.* Table II.

TABLE VII

CORRECTED RETENTION TIMES[a] t'_R AND LOG t'_R OF TRIMETHYLSILYL DERIVATIVES OF HYDROXYLATED STEROIDS ON JXR COLUMNS AT 215, 230 AND 240°

GROUP F—MONOHYDROXYDIKETONES AND DIHYDROXYMONO- AND DIKETONES[b]

No.	*Compound*	*Trivial name*	$10^2 \times t'_R$ (*min*)			$10^3 \times \log t'_R$		
			215°	*230°*	*240°*	*215°*	*230°*	*240°*
1	3α-Hydroxy-5α-androstane-11,17-dione	11-Ketoandrosterone	583	346	250	2765	2539	2398
2	3α-Hydroxy-5β-androstane-11,17-dione	11-Ketoetiocholanolone	591	350	252	2771	2544	2402
3	12α-Hydroxy-5β-pregnane-3,20-dione		934	534	381	2970	2727	2581
4	3α-Hydroxy-5β-pregnane-11,20-dione		1070	599	419	3029	2777	2622
5	3β-Hydroxy-5α-pregnane-11,20-dione		1325	746	510	3122	2873	2708
6	11α-Hydroxypregn-4-ene-3,20-dione	11α-Hydroxyprogesterone	1720	932	634	3235	2970	2802
7	11β-Hydroxypregn-4-ene-3,20-dione		1995	1041	705	3299	3017	2848
8	3α,6α-Dihydroxy-5α-androstan-17-one		692	390	274	2840	2591	2436
9	3α,11β-Dihydroxy-5α-androstan-17-one	11β-Hydroxyandrosterone	753	437	311	2877	2640	2492
10	3α,11β-Dihydroxy-5β-androstan-17-one	11β-Hydroxyetiocholanone	784	445	317	2894	2648	2502
11	3β,17α-Dihydroxy-5β-pregnan-20-one		1090	609	425	3037	2785	2628
12	3α,17α-Dihydroxy-5β-pregnan-20-one		1097	614	430	3040	2788	2633
13	3α,6α-Dihydroxy-5β-pregnan-20-one		1135	617	430	3055	2790	2633
14	3β,17β-Dihydroxypregn-5-en-16-one		1128	628	435	3052	2798	2638
15	3β,16α-Dihydroxypregn-4-en-20-one		1376	769	533	3138	2886	2727
16	3β,17α-Dihydroxy-5α-pregnan-20-one				[c]		[c]	
17	17α,20β-Dihydroxypregn-4-en-3-one			1337			3126	
18	17α,20α-Dihydroxypregn-4-en-3-one			1391			3143	
19	3α,5α-Dihydroxy-5α-cholestan-6-one		7175	3560	2294	3855	3551	3360
20	3β,17α-Dihydroxy-5α-pregnane-11,20-dione				[c]		[c]	
21	3α,17α-Dihydroxy-5β-pregnane-11,20-dione				[c]		[c]	
22	11α,17α-Dihydroxypregn-4-ene-3,20-dione			1416			3151	

[a] Operational conditions: *cf.* Table II.

[b] More of these compounds are listed wiht 21-hydroxy corticosteroids, Table IX.

[c] Some decomposition; the reduced compounds chromatograph very well: *cf.* Table VIII, G16, G18.

TABLE VIII

CORRECTED RETENTION TIMES[a] t'_R AND LOG t'_R OF TRIMETHYLSILYL DERIVATIVES OF HYDROXYLATED STEROIDS ON JXR COLUMNS AT 230°

GROUP G—TRI- AND TETRAHYDROXY COMPOUNDS[b]

No.	*Compound*	*Trivial name and source*[c]	$10^2 \times t'_R$ (*min*)	$10^3 \times \log t'_R$
			230°	*230°*
1	3,16α,17β-Trihydroxyestra-1,3,5(10)-triene	Estriol	846	2926
2	3β,17α,20β-Trihydroxy-5β-pregnane	Red. F11, Table VII	845	2926
3	3α,17α,20β-Trihydroxy-5β-pregnane	S.R.C.: also Red. F12	857	2933
4	3β,11β,20β-Trihydroxy-5β-pregnane	Red. B10, Table III	865	2936
5	3α,11β,20β-Trihydroxy-5β-pregnane	Red. F4	869	2939
6	3α,17α,20α-Trihydroxy-5β-pregnane	Pregnanetriol	942	2974
7	3β,11α,20β-Trihydroxypregn-4-ene	Red. F6	973	2988
8	3α,11β,20α-Trihydroxy-5β-pregnane	Red. B10, G4 = main product	984	2992
9	3β,17α,20β-Trihydroxypregn-4-ene	Red. F17	1038	3016
10	3β,11β,20β-Trihydroxypregn-4-ene	Red. F7	1066	3028
11	3α,11α,20β-Trihydroxypregn-4-ene	Red. F6, G7 = main product	1076	3032
12	3β,17α,20α-Trihydroxypregn-4-ene	Red. F19	1088	3037
13	3β,17α,20β-Trihydroxy-5α-pregnane	Red. F16	1117	3048
14	3β,11β,20β-Trihydroxy-5α-pregnane	Red. F5; also Red. B12	1165	3066
15	3α,11β,20β-Trihydroxypregn-4-ene	Red. F7, G10 = main product	1175	3070
16	3β,11β,20α-Trihydroxy-5α-pregnane	Red. B12, G14 = main product	1287	3110
17	3α,11β,17α,20β-Tetrahydroxy-5β-pregnane	also Red. F21	1248	3096
18	3α,11β,17α,20α-Tetrahydroxy-5β-pregnane	S.R.C.	1416	3151
19	3α,11α,17α,20β-Tetrahydroxypregn-4-ene	Red. F22	1638	3214

[a] Operational conditions: *cf.* Table II.

[b] More of these compounds and also pentahydroxysteroids are listed among 21-hydroxycorticosteroids in Table IX.

[c] Estriol and pregnanetriol obtained from Steraloids; S.R.C. = Steroid Reference Collection. Compounds the source of which is indicated as Red. followed by a letter and number, have been obtained by reduction of the steroid thus designated. Example: F2 is compound No. 2 in Group F (Table VII).

TABLE IX

CORRECTED RETENTION TIMES[a] t'_R AND LOG t'_R OF TRIMETHYLSILYL DERIVATIVES OF HYDROXYLATED STEROIDS ON JXR COLUMNS AT 230°

GROUP H—21-HYDROXYCORTICOSTEROIDS

No.	*Compound*	*Trivial name and source*[b]	$10^2 \times t'_R$ (*min*)	$10^3 \times \log t'_R$
1	21-Hydroxy-5β-pregnane-3,20-dione		957	2981
2	21-Hydroxypregn-4-ene-3,20-dione	Cortexone	1340[c]	3127
3	11β,21-Dihydroxypregn-4-ene-3,20-dione	Corticosterone	2250[c]	3352
4	17α,21-Dihydroxypregn-4-ene-3,20-dione	Cortexolone	[c]	[c]
5	17α,21-Dihydroxy-5β-pregnane-3,20-dione		[c]	[c]
6	21-Hydroxypregn-4-ene-3,11-20-trione	11-Dehydrocortisone	1647[c]	3214
7	11β,17α,21-Trihydroxypregn-4-ene-3,20-dione	Cortisol	[c]	[c]
8	17α,21-Dihydroxypregn-4-ene-3,11,20-trione	Cortisone	[c]	[c]
9	3β,17α,21-Trihydroxy-5α-pregnane-11,20-dione		[c]	[c]
10	3α,17α,21-Trihydroxy-5β-pregnane-11,20-dione		1074[c]	3031
11	17α,21-Dihydroxy-5β-pregnane-3,11-20-trione		[c]	[c]
12	3α,17α,20α,21-Tetrahydroxy-5β-pregnan-11-one	S.R.C.	2090	3320
13	3β,20β,21-Trihydroxy-5β-pregnane	Red. H1	1204	3080
14	3β,20β,21-Trihydroxypregn-4-ene	Red. H2	1462	3165
15	3β,17α,20β,21-Tetrahydroxy-5β-pregnane		1728	3238
16	3β,17α,20β,21-Tetrahydroxypregn-4-ene	S.R.C. also Red. H4	2100	3320
17	3β,11β,20β,21-Tetrahydroxypregn-4-ene	Red. H3, also Red. H6	2254	3354
18	3α,11β,17α,20α,21-Pentahydroxy-5β-pregnane	S.R.C. also Red. H11	2436	3387
19	3α,11β,17α,20β,21-Pentahydroxy-5β-pregnane	also Red. H10	2528	3403
20	3β,11β,17α,20β,21-Pentahydroxy-5β-pregnane	also Red. H11	2544	3404
21	3β,11β,17α,20β,21-Pentahydroxypregn-4-ene	Red. H7, also Red. H8	3180	3502
22	3β,11β,17α,20β,21-Pentahydroxy-5α-pregnane	S.R.C. also Red. H9	3520	3547

[a] Operational conditions: *cf.* Table II.

[b] Compounds 7 to 11, 16, 19 and 20 were obtained from Steraloids, Inc.; S.R.C. = Steroid Reference Collection. Compounds the source of which is indicated as Red. followed by letter and number, have been obtained by reduction of the steroid thus designated. Example: H2 = steroid number 2 in Group H of this table.

[c] Decomposition—All compounds from 12 to 22 are polyhydroxysteroids whose TMS derivatives are very stable.

TABLE X

VALUES OF $10^3 \times \Delta \log t'_R$ AT 230° ON JXR COLUMNS UNDER STANDARD CONDITIONS (TABLES II–IX) FOR FUNCTIONAL GROUPS IN THE ANDROSTANE (A) AND PREGNANE (P) SERIES

Group	*With*	*A-Ring features*											
		5α[a]	*5β*[a]	*5β,3β*	*5α,3α*	*5β,3K*	*5β,3α*	*5α,3K*	*5α,3β*	*Δ⁴,3β*	*Δ⁴,3K*	*Δ⁵,3β*	*Δ¹,⁴,3K*
A		1924	1887	2175	2178	2190	2194	2227	2280	2255	2322	2264	2359
P		2150	2113	2401	2404	2416	2420	2453	2506	2481	2548	2490	2585
11α	P									153	178		
11β	P			170			170		203	193	226		
16α	A[b]								322				
17α	A[b] or P[b]	260	260	260	260	260	260	260	260	260	260	260	260
17α	A[b] or P[b]	350	350	350	350	350	350	350	350	350	350	350	350
20α	P[b]	380	380	380	380	380	380	380	380	380	380	380	380
20β	P[b]	354	354	354	354	354	354	354	354	354	354	354	354
17α,20α	P						554			556	595		
17α,20β	P			526			512		539	535	578		
17α,20K	P			384			368		391		425		
11K	A				90		90						
11K	P						118		118				
17K	A or P[b]	266	266	266	266	266	266	266	266	266	266	266	266
20K	P[b]	243	243	243	243	243	243	243	243	243	243	243	243
21-OH	20K[b]	335	335	335	335	335	335	335	335	335	335	335	
21-OH	20β[b]	315	315	315	315	315	315	315	315	315	315	315	
21-OH	17α,20β	305	305	305	305	305	305	305	305	305	305	305	
21-OH	17α,20α	215	215	215	215	215	215	215	215	215	215	215	

[a] No other feature in A-ring.
[b] No substitution in D-ring.

TABLE XI

CALCULATED AND EXPERIMENTAL CORRECTED RETENTION TIMES[a] t'_R AT 230° FOR DIOLS[b] OF THE ANDROSTANE (A) AND PREGNANE (P) SERIES

Diol (TMS)	t'_R (10^{-2} *min*)		*Diol (TMS)*	t'_R (10^{-2} *min*)	
	Calculated	*Experimental*		*Calculated*	*Experimental*
5β-A-3β,17α-	272		5β-P-3β,20β-	567	564
5α-A-3α,17α-	274		5α-P-3α,20β-	573	
5β-A-3α,17α-	284		5β-P-3α,20β-	593	585
Δ⁴-A-3β,17α-	327		5β-P-3β,20α	603	
5β-A-3β,17β-	335		5α-P-3α,20α-	608	
Δ⁵-A-3β,17α-	335	336	5β-P-3α,20α-	631	630
5α-A-3α,17β-	338	339	Δ⁴-P-3β,20β-	684	686
5α-A-3β,17α-	349		Δ⁵-P-3β,20β-	692	
5β-A-3α,17β-	350		5α-P-3β,20β-	730	731
Δ⁴-A-3β,17β-	403	401	Δ⁴-P-3β,20α-	726	
Δ⁵-A-3β-17β-	412	415	Δ⁵-P-3β,20α-	742	
5α-A-3β,17β-	430	426	5α-P-3β,20α-	774	770

[a] Operational conditions: *cf.* Table II.
[b] *Cf.* Table VI.

packing density, yet caused breakdown of particles and exposure of unsilanized material detrimental to the performance of the columns.

The absence of gas flow during the first stages of conditioning (Table I) presumably allowed condensation of vaporized stationary phase on the column wall and prevented movement of vaporized phase toward the column exit. Heating under these conditions would at first promote a more even distribution of phase within particles, then further polymerization of JXR on both support and column wall. Eventually, fully polymerized stationary phase could no longer be displaced even under high gas flow at high temperature.

In routinely obtained chromatograms examplified in Fig. 2, peaks were generally symmetrical. Response to small amounts of steroid injected was excellent; decrease in response was appreciable at the low nanogram level only. This effect was reproducible and amenable to precise calibration.

Several duplicates of the columns prepared and conditioned in the manner described have been prepared in this and other laboratories. All showed properties identical to those of the 3-year old column pair in effecting separations demonstrated in Tables II to IX: relative retention times were the same and incremental factors listed in Tables X and XII were applicable in all cases. The only variations observed were small; they concerned the number of theoretical plates (from 5400 to 5700 for cholesterol) and the temperatures at which the listed values of relative retention times were obtained. These temperatures varied from column to column within a range of about 2° and must be regarded as life-time characteristics of each column.

Curtailing the conditioning schedule shown in Table I did not seem to modify retention time characteristics appreciably; it did, however, reduce the separating power in two ways: the number of theoretical plates was decreased and some tailing of peaks became apparent particularly when initial heating in the absence of gas flow was shortened.

TABLE XII

CALCULATED VALUES OF $10^3 \times \log t'_R$ ON JXR COLUMNS UNDER STANDARD CONDITIONS (TABLE IV) FOR STRUCTURAL FEATURES OF STEROLS

$10^3 \times \log t'_R = 3197^a + 10^3 \times \Delta \log t'_R{}^b$.

	C 27	*28*	*29*	*29*	*28*	*28*	*30*
	—	*14αMe*	*14αMe* *4αMe*	*14αMe* *24Me*[c]	*4αMe*	*24Me*[c]	*4,4'Me* *14αMe*
	1	*2*	*3*	*4*	*5*	*6*	*7*
(a) 5:22	−74.3	−103	− 7	12	22	40	64
(b) 5:8:22	−64	− 92	4	22	32	51	74
(c) 22	−64	− 92	4	22	32	51	74
(d) 8:22	−53.6	− 82	14	32	42	61	85
(e) 5:7:22	−33.3	− 61	35	53	63	80	105
(f) 7:22	−17.7	− 46	50	68	78	96	121
(g) 5	−10.4	− 39	57	76	86	104	128
(h) —	0	− 28.3	+ 67.7	86.4	96.0	114.7	138.3
(i) 5:8	0	− 28	68	86	96	115	138
(j) 8	+10.3	− 18	78	96	106	125	149
(k) 5:24	26.7	− 2	94	112	123	141	165
(l) 5:7	30.8	+ 2	98	116	127	146	169
(m) 24	37.0	9	105	123	133	152	175
(n) 5:8:24	37.0	9	105	123	133	152	175
(o) 7	46.3	18	114	133	142	161	185
(p) 8:24	47.3	19	115	134	143	162	186
(q) 5:7:24	67.8	40	136	154	164	183	206
(r) 7:24	83.4	55	151	170	179	198	222

[a] $3197 = 10^3 \times \log t'_R$ of 3β-hydroxy-5α-cholestane (cholestanol) at 230° under the same conditions.

[b] Example: Compound a11 is 3β-hydroxy-24β-ethylcholesta-5,22-diene (stigmasterol): $10^3 \times \log t'_R = 3197 + 140 = 3337$. The experimental value for stigmasterol (Table IV, C16) is 3336.

[c] α or β.

The longevity of columns undoubtedly stems also from factors other than preparation and conditioning. Among these the proportion of JXR to support (3 % by weight) is high by comparison with columns generally used for the GLC of steroids. In addition, consistent operation at a very low level of injected material would tend to minimize interactions with the liquid phase of steroids and their thermal decomposition products which tend to affect the stability and separation characteristics of the packing material. Observations at low attenuation following injections clearly showed a considerable increase in the persistence of residual background with increased quantity injected: both residence time and amounts of decomposition products were thereby very much increased. Hence the rate and extent of physical modification of the stationary phase by thermal breakdown products and by polymeric material arising from these products were greatly minimized at the level of injected material used in the present work.

As shown in Fig. 2, low background and stability were obtained by injecting nanogram amounts at very low attenuation. As shown in Part III of this series, precise quantifications were routinely achieved at these levels. The method is therefore entirely suitable for the analysis of very low concentrations of steroids found in most biological materials.

Samples of biological origin invariably contain amounts of extraneous material

29	*30*	*29*	*29*	*31*	*30*	*30*	*32*	*31*
4,4'Me	*14αMe 24Et*[c]	*4αMe 24Me*[c]	*24Et*[c]	*4,4'Me 14αMe 24Me*[c]	*4,4'Me 24Me*[c]	*4αMe 24Et*[c]	*4,4'Me 14αMe 24Et*[c]	*4,4'Me 24Et*[c]
8	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*
92	111	136	140	179	207	236	278	306
103	122	147	150	189	217	246	288	317
103	122	147	150	189	217	246	288	317
113	132	157	160	199	227	256	298	327
133	153	178	181	220	248	277	319	348
149	168	193	196	235	263	292	334	363
156	175	201	204	243	271	300	342	370
166.6	185.7	210.7	214	253	281.3	310.0	352.3	380.6
167	186	211	214	253	281	310	352	381
177	196	221	224	263	291	320	362	391
193	212	237	241	280	308	337	378	407
197	217	242	245	284	312	340	383	411
204	223	248	251	290	318	347	389	417
204	223	248	251	290	318	347	389	417
213	232	257	260	299	328	356	399	426
214	233	258	261	300	329	357	400	427
234	254	280	282	321	349	382	420	448
250	269	294	297	336	365	393	436	463

often far in excess of the complex steroid mixtures to be analyzed. Quantification thus generally requires preliminary clean-up processes as well as preliminary fractionation procedures. The systematic use of TLC[1–8] allows both these requirements to be met. Injection of "clean" samples obtained from TLC plates undoubtedly contributed to the longevity of the present columns by minimizing possible interactions of the stationary phase with extraneous materials, their thermal breakdown and polymeric products. In four years of uninterrupted use neither the injection port, nor the detector of the P.E. 800 chromatograph has required cleaning.

The use of CS_2 as a solvent for TMS derivatives may have contributed to the maintenance of column characteristics. Among advantages derived from the use of CS_2 which have been discussed by one of us[11,12], the abolition of solvent trailing (Fig. 2) and the ability of CS_2 to dissolve TMS derivatives and reaction products (*cf.* above) are noteworthy. In addition, this solvent shows little affinity for most stationary phases and therefore, will not cause much physical alteration of the packing material.

TMS derivatives[13] in contrast to others[1,2] are of general application in the GLC of steroids. With the present method, complete conversion of most steroids was obtained in 3 h at room temperature[1,2]. Very few steroids required longer reaction times; among these, estriol required 10 h. It should be noted that some steroids are

sensitive to direct contact with dimethylsilylchloride. With these, reversing the order of addition of reagents (*cf.* above) will lead to abnormal products.

Trace amounts of unreacted trimethyldisilazane in the final product could have beneficial effects in eliminating "active sites" within the column[1,2]. The injection of large amounts of this compound does, however, undesirably modify column characteristics. The high separating power of the present columns partly resulted from the use of 100–120 mesh Gas Chrom Q[14]. With another silanized support the number of theoretical plates per foot was 500 at best[1,2] although mesh size, percentage JXR and conditioning were identical.

JXR, a dimethylsilane polymer is more stable thermally than the analogous phase SE 30. Our experience with OV1 is too limited to warrant an estimation in this respect. Separation characteristics of the three phases were similar yet appreciably different. Differences observed with OV1 did not confer advantages to this phase over JXR in the present type of work.

Although longer, more efficient columns can undoubtedly be prepared by the present procedure, their use is restricted by present instrumental limitations in permitting required high carrier-gas fore-pressures. Internal flow control systems would have to be changed or by-passed. Longer columns have a significant damping effect on pressure changes which could obviate the need for an internal control system. With 20-ft. columns, the number of theoretical plates could be at least 10,000. However, residence time of steroids in such columns would be twice that in 9-ft. columns for the same flow rate: thermal destruction at low level of steroids may then become significant.

The stability of TMS derivatives in CS_2 solutions has been discussed[1,2]. Under the conditions described, retention times of TMS derivatives obtained with standard solutions prepared three years ago and used repeatedly over this period did not vary; hence the columns characteristics remained constant in spite of extensive use involving many thousands of injections during this period. The thermal stability of these derivatives under present GLC conditions was generally excellent. Among the few that were unstable (Tables VII and IX), some produced well defined peaks; however, the retention times clearly corresponded to compounds of smaller molecular size produced by decomposition. In other cases, several overlapping peaks emerged in close succession. The instability of unsaturated 21-corticosteroids including the steroid hormones is well known[15]. Treatment of all these steroids with sodium borohydride, under conditions to be described in a forthcoming publication, resulted in their quantitative conversion to polyhydroxysteroids whose TMS derivatives could be readily chromatographed (Tables VIII and IX). The retention times of the reduced steroids (TMS) were distinct and specific. Polyhydroxylated steroids thus obtained could also be converted to halogenated derivatives suitable for quantification by electron capture.

The choice of standard temperatures used in the present study was governed by the following consideration. Mixed steroids generally obtained from biological material could be adequately separated at 230°; at this temperature a complete chromatogram was usually obtained in 30 min. With urinary steroid hormones and metabolites a somewhat better separation resulted at 215° in 30 min. On the other hand, the GLC of high molecular weight sterols from molds, bacteria or algae, and also reduced 21-corticosteroids could be achieved more readily at 240°. Operation at 230° was adequate in all cases.

The sensitivity of retention times to temperature changes explains the necessity for precisely controlled oven temperature. Under the described set of conditions, very little change could be observed in retention times. That of cholestane, for example, never varied by more than $\pm$ 0.02 min ($\pm$ 0.3 %) in several hours.

From the data in Tables II to VII the accuracy of the following relation can be demonstrated for all steroids:

$$10^3 \times \log t'_{Ri} = A_i + 10^3 \times B_i T^{-1} \quad (1)$$

where A_i and B_i are constants independent of T, the absolute temperature.

A similar relation can be found for standard steroids included in a mixture, hence:

$$10^3 \times \log t'_{Rs} = A_s + 10^3 \times B_s T^{-1} \quad (2)$$

From eqns. (1) and (2) the following expression

$$10^3 \times \log t'_{Ri}/t'_{Rs} = A_i - A_s + (B_i - B_s)T^{-1} \quad (3)$$

is obtained for the relative retention time t'_{Ri}/t'_{Rs} of a given steroid. Since this is a constant for a given temperature within a relatively wide range of carrier gas flow rates,

$$\bar{A}(i,s) = A_i - A_s \text{ and } \bar{B}(i,s) = B_i - B_s \quad (4)$$

are constants independent of both temperature and carrier gas flow rate. Hence accurate determinations of relative retention times at two temperatures give access to specific constants $\bar{A}(i,s)$ and $\bar{B}(i,s)$ by which unknown steroids can be identified with considerable certainty. Use of these factors, for which we suggest the name of Retention Constants (R.C.), will be described in detail in Part II of the present series.

Incremental factors listed in Tables X and XII are simply additive. When corresponding functional groups or features are sufficiently separated in the molecule to prevent a crowding effect, the values apply in all cases; thus values for 17α-hydroxy, 17β-hydroxy, and all other values which are repeated in every column of Table X indicate independence of the corresponding features on the presence of other groups. Values for 11α- and 11β-hydroxy do not show this independence since they vary with different A-ring features; the value for 21-hydroxy depends on the nature of neighboring C20 or D-ring substitution. A crowding effect is indicated by aggregate values for 17α,20α; 17α,20β; 17α,10K, etc. being smaller than the sum of values for component functional groups taken singly.

With the values listed in Tables X and XII, most retention times can be predicted within $\pm$ 1 %. Discrepancies higher than 2 % are exceptional. Table XI lists calculated and observed values for the TMS derivatives of androstane- and pregnanediols. Discrepancies observed with sterols (Table XII) are even lower.

No values corresponding to Δ^4,3α are given in Table X since a determination for this group in the absence of crowding effect awaits the availability of suitable compounds. However, at least three compounds which include this A-ring feature are listed as G11, G15 and G19 in Table VIII. If incremental values listed under

Δ^4,3β in Table X for other groups included in these compounds are used, a value of 2525 for Δ^4,3α (pregnane) is found in all cases. Hence it is probable that values listed in Table X for Δ^4,3β apply to Δ^4,3α also, and that 2525 is the value for Δ^4,3α (pregnane).

It should be noted that in the two first lines of Table X, aggregate values have been entered for convenience. Each of the values listed in these lines corresponds to a sum, including either 1924 (5α-androstane, *cf.* column 3) or 2150 (5α-pregnane, *cf.* column 3) with the specific value for each A-ring feature; for example, in column 5, 2175 = 1924 + 251 and 2401 = 2150 + 251; in column 6, 2178 = 1924 + 254 and 2404 = 2150 + 254; in column 7, 2190 = 1924 + 266 and 2416 = 2150 + 266; etc. Hence the specific increment corresponding to each A-ring feature is the same for compounds of both androstane and pregnane series.

These and other increments also apply to compounds of the cholestane, or C series. A comparison of incremental values found for the C series with corresponding values in the androstane (A) and pregnane (P) series shows the following correspondences: 5αC,3K = 310 (5αA,3K = 5αP,3K = 307); 5αC,3β = 363 (5αA,3β = 5αP,3β = 355); 5βC,3K = 265 (5βA,3K = 5βP,3K = 269); 5βC,3α = 270 (5βA,3α = 270); 5βC,3β = 255 (5βA,3β = 5βP,3β = 248). Note that 114.7 being the value for methyl in the chain (Table XII, 24Me), 6 × 114.7 = 688. Hence the increment for 5αC should be 2150 (pregnane) + 688 = 2838. The value found for 5αC is 2833.

Independent incremental factors of log t'_R were observed with nonpolar stationary phases only. Polar phases induce phase–steroid interactions which vary in extent with each different configuration. Since values of incremental factors vary with each structural situation, accurate prediction of retention times from structural features is impossible with polar phases.

It is generally believed that separations on nonpolar phases are induced mainly by differences in molecular weight. Furthermore, the name selective is applied to polar columns to indicate a greater sensitivity to differences in structural features. This appellation is misleading. The data presented in Tables II to XII undoubtedly show considerable discrimination by nonpolar JXR columns of subtle structural differences (*cf.* Table XI, for example). The common belief that polar phases have generally useful selective properties is likewise unfounded since such polar phases do not allow many separations which are readily achieved with nonpolar phases.

Use of TLC as a preliminary step in the separation of steroids affords a means of effecting a complementary separation on the basis of polarity. Zones containing steroids differing as to the number of carbonyl and hydroxy groups, and subzones containing steroids of different stereoconfiguration are sharply separated on TLC plates[1–5]. The problem of locating the center of any given zone or subzone within ± 1 mm has been solved in this laboratory by including several pilot dyes in the mixture analyzed and relating the position of interest to that of the nearest dye pair. A forthcoming paper[18] on this procedure will demonstrate that steroids which are poorly separated on JXR columns migrate in separate TLC subzones which can be independently removed from the plates and quantified by GLC. Such is the case, for example with several pairs of compounds listed in Table V: etiocholanolone (D2) and androsterone (D3); testosterone (D11) and epipregnanolone (D12); pregnenolone (D15) and allopregnanolone (D16).

In addition, silver nitrate TLC[16] permits preliminary separations on the basis of differences in unsaturation. Hence separation problems generally believed to

require the use of polar columns can be solved by the combination of TLC with GLC on high-efficiency JXR columns.

ACKNOWLEDGEMENTS

The technical assistance of Mr. R. COCHRANE was very much appreciated. We are grateful to Dr. R. FUMAGALLI and Dr. G. GALLI of the Institute of Pharmacology, Milan, Italy, and to Professor W. KLYNE and Dr. N. KIRK of Westfield College, London, Administrators of the Steroid Reference Collection, for the supply of several steroid samples.

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CHROM. 3784

A VERSATILE LITHIUM BUFFER ELUTION SYSTEM FOR SINGLE COLUMN AUTOMATIC AMINO ACID CHROMATOGRAPHY

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SUMMARY

A new chromatographic system employing lithium citrate buffers is described for use on a single 120 cm resin column of the Technicon amino acid analyzer. With this system, adequate resolution of a large number of amino acids up to and including arginine can be achieved in a chromatographic run of 21 hours. The amides asparagine and glutamine are well separated from other amino acids. This chromatographic procedure should prove useful when it is necessary to quantitate each of a large number of amino acids and related compounds in physiological fluids or tissue homogenates.

INTRODUCTION

Most standard techniques for the column chromatographic determination of amino acids fail to separate glutamine, asparagine, threonine, and serine from one another, when all four amino acids are present in a mixture. In addition, some buffer elution systems commonly used on automatic amino acid analyzers fail to separate homocystine, β-alanine, γ-aminobutyric acid and ethanolamine from ammonia. These can be serious handicaps if one is examining physiological fluids or homogenates of tissues, and wishes to determine quantitatively all of the amino acids and related compounds that are present.

BENSON *et al.*[1] and PETERS *et al.*[2] have recently described the separation of asparagine and glutamine from other amino acids by substitution of lithium citrate buffers for the usual sodium citrate buffers. Their techniques were employed for separation of the acidic and neutral amino acids only, using the Beckman/Spinco model 120C amino acid analyzer.

We wish to describe the successful application of lithium buffers to a one-column separation of amino acids on the Technicon amino acid analyzer. With this system it is possible to achieve on a single chromatogram adequate separation of the great majority of acidic, neutral, and basic amino acids likely to be encountered in tissues or physiological fluids, including the amides asparagine and glutamine, as well as several small peptides.

EXPERIMENTAL

Apparatus

The version of the Technicon automatic amino acid analyzer available in 1965 was used in this study. This analyzer utilizes the PIEZ AND MORRIS system[3] of a single chromatographic column, and a continuous buffer gradient. The only modification made to the apparatus was the addition of a second thermoregulator to the recirculating heating bath for the column jackets. This was connected with a reset interval

TABLE I

COMPOSITION AND PREPARATION OF LITHIUM BUFFERS

pH	*Molarity of lithium*	*Preparation*
2.80	0.2	14.09 g $Li_3C_6H_5O_7 \cdot 4H_2O$ + 25 ml 2 *M* LiOH + 10 ml Brij 35 solution[a] + 5 ml thiodiglycol + 900 ml water[b]. Titrate with 6 *N* HCl to pH 2.80 on an accurate pH meter, make up to 1000 ml with water, and make final adjustment of pH.
3.80	0.2	Same as pH 2.80 buffer, except solution adjusted to pH 3.80.
6.10	1.2	14.09 g $Li_3C_6H_5O_7 \cdot 4H_2O$ + 25 ml 2 *M* LiOH + 42.09 g LiCl + 10 ml Brij 35 solution + 900 ml water. Titrate with 6 *N* HCl to pH 6.10, make up to 1000 ml with water, and make final adjustment of pH.

[a] 100 g Brij 35 dissolved in 200 ml water.
[b] Glass-distilled water demineralized by passage through column of Dowex 50 x 12.

TABLE II

BUFFER ELUTION GRADIENT FOR 21-HOURS CHROMATOGRAM

Autograd chamber	*Methanol*	*pH 2.80, 0.2 M Li^+ buffer*	*pH 3.80, 0.2 M Li^+ buffer*	*pH 6.10, 1.2 M Li^+ buffer*
1	5 ml	70 ml		
2	3 ml	72 ml		
3		75 ml		
4			75 ml	
5			75 ml	
6				75 ml
7				75 ml
8				75 ml
9				75 ml

timer and a double-pole, double-throw relay, so that the columns could be programmed to operate at two different temperatures. A nine-chambered Autograd was used to supply the gradient elution buffer. Except for the use of the lithium buffers described below, and for the modifications to the recirculating heating bath, the Technicon analyzer was operated in the recommended manner[4].

Buffers

Lithium citrate buffers were prepared as shown in Table I. The amounts of each buffer placed in the 9 chambers of the Autograd are shown in Table II.

Resin columns

The 140 × 0.6 cm glass columns were filled at room temperature with Technicon Chromobeads Type B resin (17μ spherical particles). The resin column when poured and packed and in operation was about 120 cm in length. Before columns were poured, the resin was carefully washed successively with acetone, water, 6 *N* nitric acid, water, 2 *M* lithium hydroxide, and water. After the resin columns were poured, and after each chromatographic run, the resin in the column was regenerated by pumping 0.2 *M* lithium hydroxide through it for 30 min, followed by pH 2.80, 0.2 *M* lithium buffer for a further 90 min. Columns were regenerated at 70°.

TABLE III

ELUTION TIMES OF AMINO ACIDS AND RELATED COMPOUNDS

Compound	*Elution time of peak (min)*[a]	*Compound*	*Elution time of peak (min)*[a]
Cysteic acid	45	Cystathionine[c]	548
Cysteine sulfinic acid	46	3,4-Dihydroxyphenylalanine	552
Homocysteic acid	46	Isoleucine	561
Phosphothreonine	48	Selenomethionine	570
Taurine	72	Glucosamine	571
Homotaurine	72	Leucine	575
Phosphoethanolamine	81	Cysteine-homocysteine mixed disulfide	590
2-Aminoethylphosphonic acid	96	Norleucine	593
Urea	98	Galactosamine	615
Hypotaurine	100	Tyrosine	615
Aspartic acid	190	Phenylalanine	645
Glutathione (reduced)	198	Homocystine	686
Hydroxyproline	201	β-Alanine	696
Methionine sulfoxide[b]	226, 236	β-Aminoisobutyric acid	714
Threonine	234	5-Hydroxytryptophan	753
Serine	249	Δ-Aminolevulinic acid	757
Asparagine	275	Kynurenine	759
Glutamic acid	288	γ-Aminobutyric acid	795
Homoserine	300	Tryptophan	863
Glutamine	305	S-Adenosylhomocysteine	879
Sarcosine	328	Ethanolamine	912
Proline	367	Ammonia	950
α-Aminoadipic acid	377	Hydroxylysine	957
Glutathione (oxidized)	394	Ornithine	1007
Glycine	403	Lysine	1023
Alanine	419	Histidine	1064
Citrulline	433	1-Methylhistidine	1085
α-Amino-*n*-butyric acid	443	Anserine	1098
Valine	466	3-Methylhistidine	1102
Cystine	493	Carnosine	1111
Homocitrulline	513	Homocarnosine	1114
Methionine	523	Arginine	1247
Allo-isoleucine	540		

[a] Elution times listed refer to the number of minutes after the chromatogram was started before each peak appeared on the recorder chart. The mean effluent volume in ml at which each compound emerged from the bottom of the ion exchange column can be calculated by subtracting 16 from each figure listed, and dividing the resulting number by 2.

[b] Two peaks are produced by DL-methionine-*dl*-sulfoxide.

[c] Time refers to peak of authentic L-cystathionine. DL- and allo-cystathionine gives a double peak.

Operation of columns

The eluting buffer was pumped through the resin column at a rate of 30 ml/h, for 21 h. The column was operated at 35° for the first 6½ h, and at 70° for the remainder of the chromatogram, the temperature of the circulating heating bath being automatically changed when the reset timer activated the 70° thermoregulator. Maximum operating presssures were 325–375 p.s.i. when the column was operated at 35°, and 175–200 p.s.i. at 70°. The running time of 21 h for each chromatogram, and regeneration time of 2 h, allowed a new sample to be applied to the column each day.

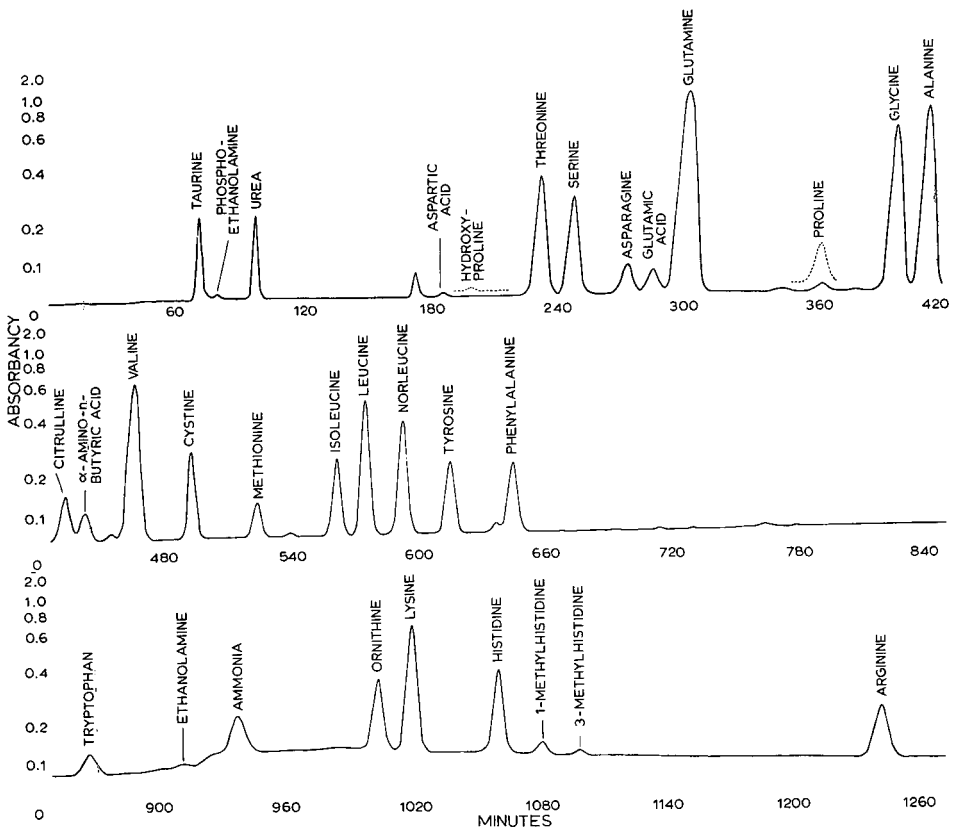

Fig. 1. Tracing of a chromatogram of 1.0 ml of deproteinized fasting human blood plasma. Solid line indicates optical density at 570 mμ. Broken line indicates optical density at 440 mμ. Internal standard = 0.1 μmole of norleucine. Figures below tracings indicate elution time in minutes.

RESULTS AND DISCUSSION

Table III lists the mean elution times of a number of authentic amino acids and related compounds when chromatographed on a 120 cm resin column by the technique described. Table III includes all the amino acids normally found in physiological fluids and tissues, as well as a number of less common amino acids of biological interest. Times listed are those at which the peak optical density for each compound appeared

on the chromatographic chart. With the amino acid analyzer that we used, each compound actually emerged from the bottom of the resin column approximately 16 min earlier than recorded on the chromatogram, and shown in Table III. Slight variations in lengths of resin columns, and in buffer pump rates, resulted in minor variations in the absolute elution times for the amino acids listed, but their relative elution times were unaltered.

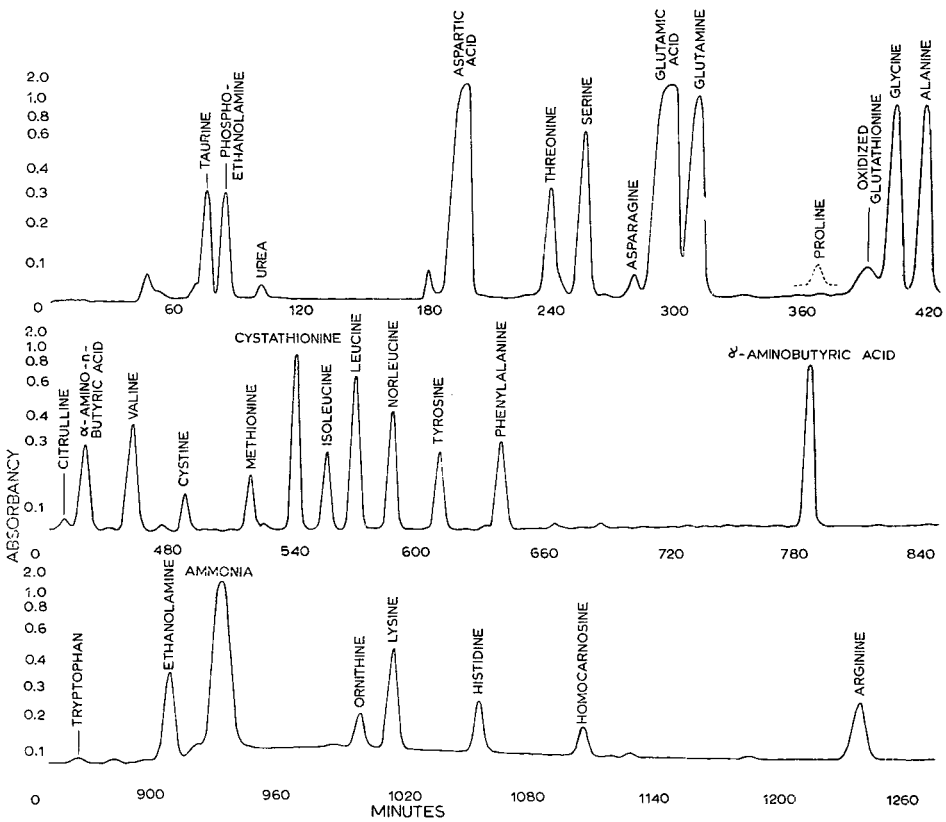

Fig. 2. Tracing of a chromatogram of a deproteinized homogenate of 0.2 g of human frontal cortex. Solid line indicates optical density at 570 mμ. Broken line indicates optical density at 440 mμ. Internal standard = 0.1 μmole of norleucine. Figures below tracings indicate elution time in minutes.

Fig. 1 is a tracing of a chromatogram obtained by this technique from 1.0 ml of fasting human blood plasma deproteinized with sulfosalicylic acid. Thirty mg of solid sulfosalicylic acid was added for each ml of fresh plasma in a stoppered centrifuge tube, the tube was shaken to mix, and the clear supernatant was separated from the denatured protein by centrifugation. Fig. 2 is a tracing of a chromatogram prepared from an aqueous homogenate of 0.2 g of human frontal cortex, similarly deproteinized with sulfosalicylic acid. It can be seen in both figures that the separation of the identified amino acids is sufficiently good to make possible accurate quantitation of almost all of them. The chromatogram of deproteinized brain homogenate was deliberately

over-loaded with respect to certain amino acids (aspartic acid, glutamic acid, glutamine), in order to illustrate some of the lesser components present. Separation would have been improved had only ¼–½ as much brain homogenate been applied to the resin column.

The amides, asparagine and glutamine, can be readily separated with this system from threonine, serine, and glutamic acid. At the same time, a good separation is achieved for almost all the other major ninhydrin-positive components likely to be encountered in physiological fluids and tissue homogenates. An advantage of our procedure as compared to the lithium buffer systems previously reported[1,2] is that acidic, neutral, and basic amino acids are all separated on a single column in one chromatographic run.

Column temperature is maintained at 35° for the first 6½ h, primarily to ensure accurate determination of glutamine, which is easily cyclized to the ninhydrin-negative compound ammonium pyrrolidone carboxylate at higher temperatures[5]. Additional reasons for maintaining a low operating temperature this long are to avoid possible cyclization of the γ-glutamyl peptide glutathione[5] and to improve the separation between alanine, citrulline, and α-amino-*n*-butyric acid. In determining the ratio of the ninhydrin colour yield of the amino acid used as an internal standard to that of glutamine, it is important to select the same column temperature that is to be used in analyzing unknown samples. We found that appreciably less colour is produced when the same amount of authentic glutamine is chromatographed at 45° instead of at 35°. The latter part of each chromatogram is run at 70°, partly to speed up the elution of the basic amino acids, but chiefly in order to elute tryptophan well before ammonia. As pointed out by HAMILTON[6], the speed of elution of tryptophan is unusually sensitive to the temperature at which the ion exchange column is operated.

A minor disadvantage to the use of lithium in place of sodium buffers is that columns of Technicon Chromobeads B resin seem to pack more tightly with lithium buffers. Excessive packing of the column decreases the excellence of resolution of amino acids. We have found several practical steps which help avoid undue column packing. Columns should not be operated longer than necessary at 35°, since operating pressure is higher at the low temperature, and they should be regenerated at 70°. The molarity of the final buffer in respect to lithium should not be increased beyond 1.2. When we used a pH 6.10 buffer 2.4 *M* in lithium, the basic amino acids were eluted from the column sooner, but resolution was poor after a few runs. It is particularly important not to apply the sample to the column dissolved in sucrose solution, as is recommended by Technicon for use with sodium citrate buffer systems[4]. When the sample is blown into the resin column with nitrogen pressure, rather than layering it above the top of the column in sucrose solution, many more good chromatograms can be obtained before it is necessary to repour the column. Regular removal of the top few millimeters of the resin in the column when it becomes discoloured with pump packing, or installation of a resin filter in the high pressure line, also increases the useful life of the column.

Inspection of Table III shows several limitations of our technique. The first 4 amino acids listed are eluted with the buffer front, and are not separable. One of the peaks of the methionine sulfoxides is eluted at virtually the same point as threonine, so that threonine cannot be quantitated accurately if a physiological fluid such as plasma or urine also contains methionine sulfoxide. In measuring amounts of the

unusual sulfur-containing amino acids in the physiological fluids of patients with homocystinuria, one must use an internal standard other than norleucine, since the mixed disulfide of cysteine and homocysteine present in the plasma and urine of such patients is eluted from the column simultaneously with norleucine. Finally, the slight separation of homocarnosine from carnosine is inadequate to obtain accurate quantitation of these dipeptides if both are present in the same specimen. In actual practice, most of the unusual amino acids listed in Table III are either absent from physiological fluids, or are present in such small amounts that they do not interfere with quantitation of the more common amino acids emerging from the ion exchange column at similar effluent volumes.

ACKNOWLEDGEMENTS

The authors thank Miss DONNA L. LOVE for competent technical assistance. This research was supported by grants from the Medical Research Council of Canada.

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CHROM. 3786

A SENSITIVE GAS CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF PENTACHLOROPHENOL IN HUMAN BLOOD*

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SUMMARY

A method for the determination of pentachlorophenol residues in human blood has been designed which involves the simultaneous application of acid pH, mild heat, and agitation of the sample with benzene extractant for the isolation of the pesticide from blood. It is applicable to quantities of one milliliter or less of blood and the detectability limits of the pesticide are in the low parts per billion range.

INTRODUCTION

Pentachlorophenol and/or its sodium salt is extensively used as a wood preservative in areas where insect infestation is a problem, as a contact herbicide in agricultural areas, as a household treatment for termites, and as a disinfectant and mildew retardant. Pentachlorophenol (PCP) is a significant problem in occupational and industrial usage and, to a degree, a hazard to the public; the magnitude of the problem is manifested by the frequency of acute intoxications and occurrence of fatalities from this material. Examples recently reported are industrial intoxications in Canada[1] where one case was fatal; and in Texas[2] where two cases were fatal. Incidents of public intoxication of children from the chemical have been reported in England[3] and, most recently, in the United States[4] where two cases were fatal.

Despite the frequency of acute intoxication for some years, data have been sparse on the subacute or chronic effects of PCP. In Hawaii, where the use of the chemical is heavy, a program has been in progress to search for possible effects of chronic exposure. One facet of the study has included a periodic sampling of the State's general population, including a detailed study of a group occupationally exposed to the chemical[5].

To evaluate chronic effects and to understand more fully the kinetics of human

* Published with the approval of the Director of the Hawaii Agricultural Experiment Station, University of Hawaii, as Journal Series No. 996.
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excretion of PCP, a sensitive method was needed for the analysis of this compound in blood. The analytical procedure developed for PCP in urine[6], although sensitive and yielding excellent recoveries, was not applicable to blood analysis, and the available methods for the analysis of this chemical in blood lacked specificity, sensitivity, reproducibility, or convenience[7]. The proposed method described below is relatively rapid, sensitive, and reliable.

APPARATUS AND MATERIALS

Gas chromatographs

Aerograph Model 204-B electron capture detector; 1/8 in. × 5 ft. spiral borosilicate glass column packed with 3 % SE-30 silicone on Chromosorb W, acid-washed (AW) and treated with dimethyldichlorosilane (DMCS), 80–100 mesh; column temperature 160°; injection temperature 200°; detector temperature 200°; nitrogen carrier gas, flow rate 30 ml/min.

F & M Model 810, electron capture detector; 1/4 in. × 4 ft. spiral borosilicate glass column packed with a mixture of 5 % QF-1 silicone (10,000 cS) and 3.3 % DC-200 silicone (12,500 cS) on Chromosorb W (AW, DMCS), High Performance grade, 80–100 mesh; column temperature 170°, injection temperature 200°, detector temperature 200°; argon–methane 90:10) carrier gas, flow rate 75 ml/min.

Both instruments were equipped with Leeds and Northrup Speedomax H recorders, 1 mV full scale, chart speed 0.5 in. per min.

Thin-layer chromatography apparatus

Desaga-Brinkmann apparatus (Brinkmann Instruments Inc., Westbury, N.Y.), including 20 × 20 cm glass plates and the Model S-11 applicator.

Infrared spectrophotometer

Perkin-Elmer Model No. 337, 4X-Beam condenser.

Reagents

Sulfuric acid, reagent grade, 0.1 *N* solution.
Benzene, Mallinckrodt Nanograde.
Heptane, Matheson Coleman and Bell, reagent grade, redistilled.
Hexane, Mallinckrodt Nanograde.
Aluminum Oxide G (E. Merck AG, Darmstadt, Germany).
Rhodamine B, 1 % solution in ethanol.
Diazomethane solution; prepared as previously described[6].

Pentachlorophenol (Eastman Kodak No. 3462) solutions

Stock solution preparation was 100 mg PCP in 100 ml 0.1 *N* sodium hydroxide. Standard solutions of methylated PCP were prepared at concentrations which would allow injections of 10–200 pg of PCP in 5 μl into the gas chromatograph. An aliquot of the stock solution was added to 1 ml of 0.1 *N* sulfuric acid and 5 ml of distilled water in a 30-ml separatory funnel; the mixture was extracted three times with 5-ml portions of benzene. The benzene fractions were combined in a centrifuge tube and evaporated to about 1 ml on a steam bath (40°) with the aid of a stream of filtered air. One milliliter of diazomethane solution was added to the concentrate and the mixture

was allowed to stand for 15 min; then it was aerated with a gentle stream of filtered air, at room temperature, to remove any excess diazomethane, and made to a suitable volume for analysis of the derived methylated pentachlorophenol by gas chromatography.

PREPARATION OF BLOOD SAMPLES

Exploratory studies with citrated whole blood, to which PCP was added, indicated no inherent problems that would inhibit the recovery or measurement of the PCP component with the proposed analytical procedure. Freshly drawn blood, stored in heparinized tubes, was analyzed within 24 h of the time of receipt. Additional blood samples were centrifuged immediately after receipt, the plasma fractions were removed and the red cell fractions were washed twice with physiological saline solution; the saline washes were added to the plasma fractions, and the plasma and red cell fractions were analyzed separately for PCP.

The sample (1–5 ml) was placed in a 125-ml glass-stoppered Erlenmeyer flask to which was added 20 ml of 0.1 *N* sulfuric acid, 12 ml of benzene and a 1-in. Teflon magnetic stirring bar. The flask was stoppered and the contents heated at 50° with constant stirring on a combination hot plate magnetic stirrer for 20 min. At the end of the heating period, the flask was removed from the heater and cooled immediately in an ice bath. The contents of the flask were transferred to a 40-ml centrifuge tube and centrifuged for 10 min; the upper benzene layer was removed by pipette and placed in a graduated test tube. The centrifugate was washed three times with 5-ml portions of benzene, with centrifugation between each wash, and the benzene layers were combined with the first benzene fraction. The benzene fraction was concentrated to about 1 ml on a steam bath (40°) with the aid of a stream of filtered air. One ml of diazomethane solution was added to the concentrate and the procedure continued as described above for the preparation of the standard PCP solutions.

ANALYTICAL PROCEDURES

Standard curves of the pentachlorophenol methyl ether were prepared from data obtained from the gas chromatograph. The curves were linear in the range of 10–200 pg. The volumes of the prepared blood samples were adjusted so that 2–10 μl of the solution would produce chromatographic responses within the linear range.

Thin-layer chromatography was used as a qualitative confirmatory procedure and also as a means for acquiring a sufficient amount of the suspect compound for conclusive identification by I.R. absorption analysis. The diazomethane-treated blood concentrates and PCP methyl ether standards were chromatographed on aluminum oxide G plates (250 μ thickness) by ascending chromatography, using heptane as the developing solvent. The developed plates were air-dried and sprayed with Rhodamine B solution; the blood samples and the PCP ether standards were compared with 3600 Å U.V. light.

The aluminum oxide in the area containing the fraction of the composite sample of the blood of occupationally exposed workers comparable to the R_F value of the PCP ether was scraped from the plate, extracted three times with 1-ml portions of hexane, and the hexane extract was concentrated to about 100 μl with a stream of

filtered air at room temperature. The concentrate was mixed with potassium bromide, made into a 1.5 mm pellet and compared with a similar preparation of a PCP methyl ether standard by I.R. spectrometry.

RESULTS

The PCP standards, the fortified whole blood, and the freshly drawn blood from occupationally exposed individuals agreed closely in the several analytical characteristics examined. The retention times on the columns of 3 % SE-30 silicone and the QF1-DC200 silicone mixture were similar at 3.5 min and 6.3 min, respectively. All samples on thin-layer chromatograms, visualized with Rhodamine B and U.V. light, demonstrated R_F values of about 0.65. The comparative I.R. spectra were practically identical. The spectra for the PCP methyl ether standard and the material isolated from the composite blood sample of the occupationally exposed workers are shown in Fig. 1. All of the data provided convincing evidence that the material isolated from the human blood samples was pentachlorophenol. Ten replicate experiments gave a mean value of 0.81 p.p.m., with a standard deviation of 0.08. The analytical results of fortified bloods are shown in Table I; recoveries of PCP varied from 87 to 100 %, with a mean of 92 %. Pentachlorophenol residue data on the blood of individuals, most of whom were regularly and directly exposed to the chemical at work, are given in Table II. Apart from the expected variations of PCP found in the bloods, the lowest values were those obtained from samples of the bloods of office workers in the wood-treatment plants. Except for subject number 8, the plasma contained virtually all of the PCP in the blood; the cell fraction contained about 1 % of the compound after saline washing. It is noted that as little as 20 parts per billion (p.p.b.) were detected. Although no attempt was made to define the lower limit of

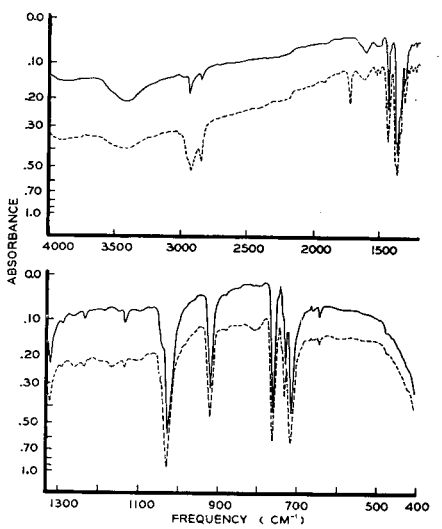

Fig. 1. I.R. spectra of pentachlorophenol methyl ether standard (———) compared with fraction isolated from blood of occupationally exposed workers (– – – –).

TABLE I

RECOVERY OF PENTACHLOROPHENOL FROM BLOOD

Sample[a]	*Amount PCP added* (*μg*)	*Amount PCP recovered* (*μg*)	% *recovered*
1	0.25	0.23	92
2	0.25	0.22	88
3	0.50	0.48	96
4	0.50	0.45	90
5	0.50	0.50	100
6	5.00	4.65	93
7	5.00	4.75	95
8	25.0	21.8	87
9	50.0	44.5	89

[a] 5.0 g blood sample.

TABLE II

PENTACHLOROPHENOL IN HUMAN BLOOD[a]

Subject	*Plasma*	*RBC*	*Whole blood*
1			0.34
2			0.84
3			0.87
4			2.45
5			2.57
6			3.95
7			6.01
8	0.99	0.08	
9	4.31	0.02	
10	4.91	0.03	
11	7.59	0.09	
12	8.26	0.04	
13	9.06	0.12	

[a] In p.p.m.

detection in this study, the value of 20 p.p.b. compares favorably with the limits of 3–10 p.p.b. found for the analytical method applied to urine[6].

DISCUSSION

The identification of pentachlorophenol has been sufficiently well established in these studies to warrant the use of the method for the analysis of this compound in blood. Initial studies involving extraction of the blood samples with both polar and nonpolar solvents under variable pH (2 to 12) conditions, or refluxing the samples for variable periods of time at temperatures ranging from 50° to 100° at variable pH (2 – 12) followed by extraction with various solvents were unsuccessful; in all instances, little or no pentachlorophenol was recovered. The precise mechanism by which the described method was successful when others failed is not known. However, it is noted that the conditions of acid pH, heat, agitation, and the presence of the

benzene extractant were simultaneously involved in the proposed extraction procedure. Pentachlorophenol is a weak acid which readily combines with strong bases to give the corresponding water-soluble salt. The highly reactive hydroxyl group of the compound suggests a sufficiently strong bonding to blood components to make simple extraction procedures impossible.

Similar difficulties with other chlorinated pesticides have been reported. For example, DALE *et al.*[8] reported poor recoveries of several chlorinated insecticides from blood extracted with hexane; improved recoveries were experienced by pretreatment of the samples with heat[9]. DALE and coworkers suggested that some of the pesticide was bound to the lipoprotein components of the blood. Similar analytical difficulties have been noted for phenolic products in sweet corn and milk[10] and in pineapple[11]. An obverse situation is among the postulates suggested to explain the relatively low recoveries of dieldrin by hexane extraction in human and dog blood, *viz.*, that the dieldrin may bind to a hydroxy or amino group of the blood components[12]. From human urinary excretion data on PCP[5], it has been suggested that a binding of this type occurs in the blood. This postulate is partially supported by *in vitro* studies with bovine serum albumin[13].

Any detailed correlative analysis or interpretation of the PCP data obtained from the blood and urine of the subject cases is outside the scope of this paper and will be reported separately. The proposed method of analysis for PCP in blood, which involves a benzene–aqueous interface coupled with mild heat and low pH, is sufficiently sensitive, rapid, and reliable to provide data that will permit a better understanding of the biological behavior of concentrations of pentachlorophenol in humans.

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CHROM. 3785

GAS–LIQUID CHROMATOGRAPHIC ANALYSIS OF NUCLEIC ACID COMPONENTS*

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SUMMARY

The aim of this investigation was to develop a quantitative gas–liquid chromatographic method of analysis for the components of nucleic acids (*i.e.*, purine and pyrimidine bases, nucleosides, or nucleotides), and to apply this method to the analysis of biological material. The N-methyl derivatives of the purine and pyrimidine bases, prepared by thermal dissociation of their tetramethylammonium salts were found to be unsuited for quantitative analysis due to the formation of multiple chromatographic peaks for cytosine, adenine, and guanine. The trimethylsilyl (TMS) derivatives of the bases were found to be far superior to the N-methyl compounds. Bis(trimethylsilyl)acetamide (BSA) was evaluated and found to be a good reagent for silylation of the bases. The optimum derivatization conditions were heating the bases in a closed tube at 150° for 45 min, with a 100 molar excess of BSA to total bases, and a 3:1 v/v acetonitrile/BSA ratio. Calibration curves for the five main bases (U, T, C, A, and G) were prepared and found to be linear over a sample weight range of 25–2000 μg of base. The relative standard deviations ranged from 1.1 % for uracil to 3.1 % for cytosine. The minimum detectable amount (MDA) using the hydrogen flame ionization detector, was determined to be 3 to 5 $\times$ 10^{-9} g or *ca.* 3 $\times$ 10^{-11} moles of each base injected. Comparative studies were made of BSA as a silylation reagent with bis(trimethylsilyl)trifluoroacetamide (BSTFA), and reagent solutions of 0.1 v/v % TMCS in BSA, and 1.0 v/v % TMCS in BSA. There were no significant differences in regard to analytical derivatization yield with any of these reagents. Important advantages of BSTFA include "cleaner" chromatograms with fewer extraneous peaks and complete miscibility of BSTFA in the solvent. A column of 8 w/w % SE-30 on 100–120 mesh Supelcoport provided good resolution and stability for the TMS bases and was superior to all chromatographic columns investigated.

Application of the developed method to the analysis of biological materials was accomplished using perchloric acid hydrolysis and anion-exchange removal of the bases from biological background prior to derivatization and GLC analysis. The combined hydrolysis and purification procedures were shown to give a recovery of the

* Contribution from the Missouri Agricultural Experiment Station, Journal Series No. 5496. Approved by the Director. Supported in part by a grant from the National Aeronautics and Space Administration (NGR 26004-011).

** Experimental data taken in part from masters thesis, University of Missouri, June, 1968.

bases of *ca.* 82 % with the recovery of the four bases being equivalent (± 1.5 %). Base ratio analysis of purified yeast RNA showed that the method was precise giving an average relative standard deviation of 3.0 %. Experiments are in progress on the analysis of microgram amounts of RNA and DNA.

INTRODUCTION

Many areas of biochemistry, molecular biology and other related fields of research are involved either directly or indirectly with the study of the nucleic acids.

Because of the importance of the genetic code it is mandatory to know as much as possible about the chemical compositions of these compounds, in order to study their functions. Determination of the base composition is made possible by hydrolysis of these polymeric compounds into the various monomers. Of particular interest in many areas is the determination of the purine and pyrimidine base ratios.

Methods of determining the bases and other nucleic acid components, including nucleosides and nucleotides, have already been developed using various analytical, chromatographic, and instrumental techniques. These include ion-exchange chromatography, COHN[1]; paper chromatography, VISCHER AND CHARGAFF[2]; paper electrophoresis, GORDON AND REICHARD[3]; and thin-layer chromatography, RANDERATH[4].

The development of gas–liquid chromatography (GLC) and its successful application to many similar analytical problems in the field of biochemistry as steroids, fatty acids and amino acids, suggested that a similar approach for the analysis of nucleic acid components might be developed.

The speed, accuracy, and sensitivity afforded by gas–liquid chromatography offers definite advantages over these other analytical techniques. However, before gas chromatographic analysis of the purine or pyrimidine bases, nucleosides, or nucleotides can be accomplished, these compounds must first be converted to volatile derivatives with suitable chromatographic properties.

In 1962, the first report of the analysis of nucleic acid components by GLC was published by MILES AND FALES[5]. They investigated only the nucleosides, and the derivatives they chose to study were acetyl, methyl, and/or isopropylidene. MACGEE[6] investigated the N-methyl derivatives of the purine and pyrimidine bases, and applied the method to the analysis of hydrolysates of nucleic acids. The procedure yielded useful data on as little as 2.5 nanomoles of each base, but only if the bases were present in an equimolar distribution. Further, only a few of the naturally occurring nucleic acids have an equimolar base ratio. The primary disadvantage of their method involved multiple derivatives, with as many as four chromatographic peaks derived from one particular purine, adenine. These multiple peaks required the use of correction factors in the calculations.

GLC of the trimethylsilyl (TMS) derivatives of nucleosides was also reported by HANCOCK AND COLEMAN[8]. The derivatives were synthesized using hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS) in pyridine. HANCOCK[9] reported later the successful gas chromatography of various adenosine derivatives which included 5-adenosyl-methionine and the nucleotides adenosine monophosphate and adenosine diphosphate.

In 1966, HASHIZUME AND SASAKI[10] reported the gas chromatographic separation

of the ribonucleotides utilizing the trimethylsilyl derivatives. Tris (TMS) phosphate was synthesized initially by reacting trisodium or tripotassium phosphate with a mixture of HMDS and TMCS. This led to the successful preparation of the TMS nucleotides by refluxing 10 mg of the alkali salts of the nucleotides for one hour in a solution of 0.2 ml anhydrous pyridine, 0.1 ml HMDS and 0.05 ml TMCS. Later the same year, SASAKI AND HASHIZUME[11], demonstrated the applicability of their procedure for preparing the TMS derivatives of the purine and pyrimidine bases and nucleosides. Pure TMS derivatives of selected bases and nucleosides were prepared in macro amounts and purified by vacuum distillation. These derivatives were characterized by elemental analysis, N.M.R., and I.R. spectra. Although quantitation was not demonstrated, the relative error of the individual molar responses was within $\pm 4\%$.

In July of 1966, KLEBE *et al.*[12] reported silylation studies with compounds other than nucleic acid components with a new reagent, bis(trimethylsilyl)acetamide (BSA). This reagent was demonstrated to be a more potent trimethylsilyl donor than previously known silylating reagents.

In 1968, GEHRKE and coworkers[13] reported a new silylating reagent for amino acids, bis(trimethylsilyl)trifluoroacetamide (BSTFA), which is an analog of BSA. BSTFA reacts similarly to BSA, is more volatile, and with some compounds reaction is faster and more complete.

The purpose of this investigation was to develop a method for the quantitative analysis of nucleic acid components using gas–liquid chromatography with particular emphasis on chemistry of derivatization, chromatography, quantitative analysis, and application to biological samples. GEHRKE *et al.*[14] in late 1967 reported a communication on some of the initial phases of this investigation.

EXPERIMENTAL

(1) *Apparatus*

An F and M Model 402 Biomedical Gas Chromatograph (F and M Scientific, Division of Hewlett Packard, Avondale, Pa.) equipped with dual hydrogen flame ionization detectors was used.

Area determinations of the chromatographic peaks were made with a Disc Integrator Model 228-A (Disc Instruments Inc., Santa Ana, Calif.).

For elevated temperature reactions, a magnetically stirred, high temperature oil bath (100–200°), with a variable temperature control system ($\pm 2°$), was constructed in this laboratory. The oil bath was placed behind a safety shield.

A freeze-dry apparatus, or lyophilizer, made by the Scientific Instrument Shop, University of Missouri, was used to dry the nucleic acid samples following hydrolysis and ion-exchange clean-up.

The ion-exchange columns used in this study were 9×150 mm pyrex glass with teflon stopcocks, and were obtained from Fischer and Porter Co., Warminster, Pa.

(2) *Reagents*

All of the purine and pyrimidine bases, nucleosides and nucleotides were obtained from Mann Research Laboratories, and were chromatographically pure. Bis-(trimethylsilyl)acetamide (BSA) was purchased from Aldrich Chemical Co., and bis-(trimethylsilyl)trifluoroacetamide (BSTFA) from Regis Chemical Co. The other

silylating reagents, hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS) were obtained from Pierce Chemical Co. Acetonitrile was of "nanograde" purity and was obtained from Mallinckrodt Chemical Works. The ion-exchange resin used was Dowex 1-x2 (100–200 mesh) and was purchased from J. T. Baker Chemical Co. The yeast RNA was obtained from Dr. James Ross, Department of Plant Pathology, University of Missouri.

(3) Instrumental and chromatographic conditions

The chromatographic columns used in the initial phases of this investigation were 4 w/w % SE-30 (straight chained polymethylsiloxane) on 80–100 mesh High Performance Chromosorb G (Johns Manville Inc., New York, N.Y.). The columns used in the latter part of the study were 8 w/w % SE-30 on 100–120 mesh Supelcoport (Chromosorb W type). These column materials were packed in glass columns (borosilicate) which were 1 m long, with an inside diameter of 4 mm.

The instrumental conditions were varied using both 7.5 and 10°/min temperature programs. These programs usually ran from 100° to 250°, however, nucleosides or nucleotides demanded higher final temperatures, approximately 300°. The detector was operated in the 280–320° region, and the injection heater was at approximately 180°. The gas flow rates were as follows with line pressures of 40 p.s.i.g. for all three gases: N_2, 80 ml/min; H_2, 40 ml/min; air, 300 ml/min.

The oven heaters were not capable of following the control setting for the temperature rate of increase. It was determined that the average rate of increase at a setting of 7.5°/min was 6.7°/min.

(4) Determination of optimum reaction conditions for silylation of the purine and pyrimidine bases using BSA

The determination of the reaction conditions necessary to achieve complete silylation involved a study of time, temperature, and reagent concentration. Initial investigations on the five main bases, uracil, thymine, cytosine, adenine, and guanine, demonstrated that guanine was the most difficult base to silylate. Therefore, guanine was chosen to further define the critical reaction conditions.

Molar excess of silylating reagent. The molar excess of BSA required for maximum yield of silylated product was determined as follows:

Samples containing guanine (5.0 mg) and internal standard (phenanthrene, 5.0 mg) were weighed into 16 × 75 mm screw-top culture tubes containing 8 × 15 mm teflon covered magnetic stirring bars. After the addition of acetonitrile and 0.10, 0.50, 1.00, or 2.00 ml of BSA, the tubes were tightly capped with a teflon lined cap. Acetonitrile was added to give a 3:1 v/v acetonitrile to BSA ratio. The different amounts of BSA added corresponded to 5, 25, 50, and 100 molar excess, respectively. The samples were heated in the closed tubes for 30 min at 150° in an oil bath with magnetic stirring, then cooled to room temperature and aliquots of 3 to 5 μl were injected into the gas chromatograph. The molar response of the TMS base relative to phenanthrene, $RMR_{b./phen.}$, was calculated from the measured areas of the two experimental chromatographic peaks as follows:

$$RMR_{B./Phen.} = \frac{\frac{A_{B.}}{W_{B.}/GFW_{B.}}}{\frac{A_{Phen.}}{W_{Phen.}/GFW_{Phen.}}}$$

$$RMR_{B./Phen.} = \frac{A_{B.}/moles_{B.}}{A_{Phen.}/moles_{Phen.}}$$

$RMR_{B./Phen.}$ = Molar response of base relative to phenanthrene
$A_{B.}$ = Area of chromatographic peak for base
$A_{Phen.}$ = Area of chromatographic peak for phenanthrene (I.S.)
GFW = Gram formula weight

The data presented in Fig. 1 show that a minimum of a 50 molar excess of BSA was necessary to obtain a maximum yield of the TMS derivative of guanine.

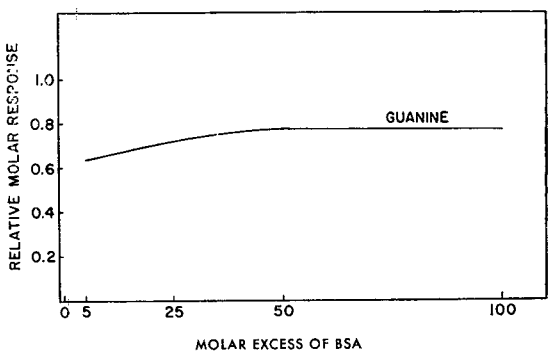
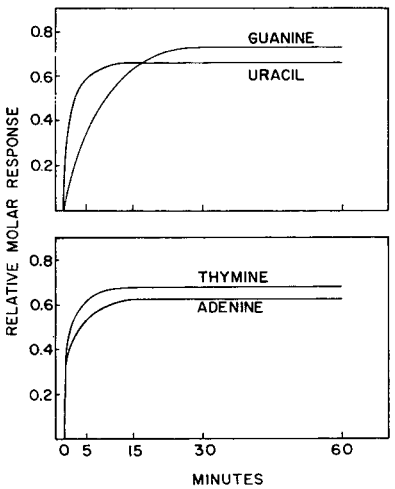


Fig. 1. Effect of reagent concentration on silylation in a closed tube at 150° for 30 min.

Time and temperature required for maximum derivatization. Samples containing *ca.* 1.0 mg of each of two bases (A and T, and, G and U) and phenanthrene were accurately weighed into sample vials (described in previous section) and 0.80 ml of BSA (*ca.* 100 molar excess) and 2.4 ml of acetonitrile were added. Duplicate samples were then stirred for 5, 15, 30, and 60 min at temperatures of 125, 150, and 170°. The samples were then immediately cooled to room temperature and 3–5 μl aliquots were analyzed by GLC. The relative molar response, $RMR_{B./Phen.}$, for each base was calculated according to the previously described formula and plotted as a function of reaction time (Fig. 2). Identical curves were obtained at temperatures of 125° and 170° for uracil, thymine, and adenine. Guanine gave a significantly lower molar response at temperatures of 100° and 125° at all reaction times studied. Also, silylation at 170° severely increased the number of tube cap failures and sample leakages, and did not significantly reduce the time required to obtain maximum conversion of guanine to its TMS derivative.

From these experiments, the optimum silylation conditions for the purine and pyrimidine bases were found to be heating at 150° (in a closed tube) for 45 min with a 50–100 molar excess of BSA.

As a result of using the large molar excess of BSA, a problem was encountered with the base, cytosine. This increase in amount of BSA resulted in the formation of two chromatographic peaks for cytosine, and the peak area of either of the two cytosine peaks was a function of silylation time and temperature. It was also observed that a single peak (the first) with a relatively high response was obtained for cytosine on silylation at room temperature for 30 min with a 100 molar excess of BSA. However, as previously demonstrated, silylation of the other bases at room temperature was not complete, but the area for the first eluted cytosine peak was constant and re-



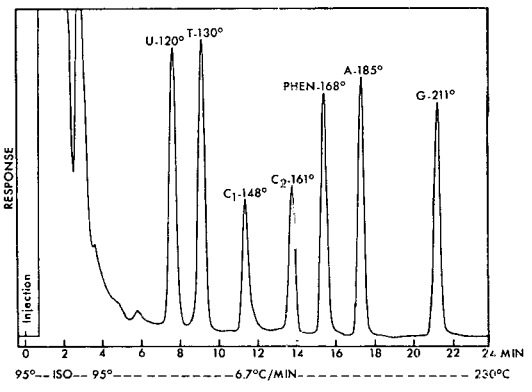


Fig. 2. Effect of silylation time at 150° on relative molar response.

Fig. 3. Chromatographic separation of purine and pyrimidine TMS derivatives. Column: 4 w/w % SE-30 on 80–100 mesh, High Performance Chromosorb G, 1 m × 4 mm I.D. Each peak represents *ca.* 1 μg of each base.

producible at 150° for 45 min. Therefore, these reaction conditions can be used for the quantitative analysis of the five main bases.

Relative molar response of the purine and pyrimidine bases (hydrogen flame ionization detector). Optimum conditions for silylation of the five main bases (U, T, C, A, and G) using BSA were chosen from the results of the previous experiments. These conditions were then used for the determination of $RMR_{B./Phen.}$ for each of the following purine and pyrimidine bases: uracil, thymine, purine, cytosine, 5-methyl cytosine, hypoxanthine, adenine, xanthine, and guanine. The results of these determinations are given in Table I including standard deviations for the $RMR_{B./Phen.}$ of four of the bases, U, T, A, and G. Two peaks were observed for 5-methyl cytosine similar to those obtained for cytosine. The $RMR_{C.}$ shown for cytosine was determined by stirring the samples at room temperature for 30 min (until the second peak just began to appear). All of the chromatographic peaks exhibited desirable peak shapes with very little tailing. A typical chromatogram of the five main bases is shown in Fig. 3. This chromatogram represents 1 μg of each base injected.

(5) Quantitative analysis of the five main purine and pyrimidine bases

Calibration curves were prepared to demonstrate the quantitative aspects and reproducibility of the linear range of response for the formation of the TMS-base derivatives with BSA. A sample weight range of 25–2000 μg of each base was used. Phenanthrene was used as the internal standard.

Preparation of stock solution of bases. A stock solution containing the five main bases was prepared to facilitate the handling of samples containing amounts of material less than could be accurately weighed. No single organic solvent was suitable to dissolve all five bases, therefore, ammonium hydroxide, which could be removed by

TABLE I

RELATIVE MOLAR RESPONSE OF THE TMS DERIVATIVE OF PURINE AND PYRIMIDINE BASES USING A F.I.D.[a]

Compound	*Retention temperature*[b] (°C)	*RMR*[c]	*S.D.*[d]
Uracil	120	0.67	0.010
Thymine	130	0.68	0.012
Purine	140	0.38	
Cytosine	148; 161	0.54[e]	
5-Methyl cytosine	152; 165	0.45[f]	
Hypoxanthine	176	0.58	
Adenine	185	0.60	0.004
Xanthine	202	0.71	
Guanine	211	0.72	0.006
Phenanthrene (I.S.)	172	1.00	

[a] F.I.D. = flame ionization detector.
[b] Chromatographic conditions are described in experimental, section 3.
[c] RMR determined from at least two independent determinations. RMR = relative molar response to phenanthrene as I.S.

$$\text{RMR} = \frac{\text{molar response of bases}}{\text{molar response of phenanthrene}}$$

[d] Standard deviation calculated from more than 4 independent determinations.
[e] Silylation for 30 min at room temperature.
[f] Response based on first eluted peak.

evaporation, was used. The stock solution was prepared by weighing 25.0 mg of each of the five bases (U, T, C, A, and G) into a 100 ml volumetric flask and dissolving the bases with 7.5 *N* ammonium hydroxide, then diluting to volume. Complete solution of the bases was achieved.

Preparation of known samples. Exact aliquots of the stock solution (8.00, 4.00, 1.00, 0.50, and 0.10 ml) containing 2.000, 1.000, 0.250, 0.125, and 0.025 mg, respectively of each base were placed in screw necked culture tubes. The samples were dried by directing a stream of dry filtered air into the tubes while heating on a steam bath, then vacuum dried over P_2O_5 at 50° for 24 h.

Silylation and analysis of samples. An internal standard solution of phenanthrene in acetonitrile (50 ml of 0.177 mg/ml) was prepared. Exactly 10.00, 6.00, 1.50, 0.75, and 0.45 ml of this solution were added to the known samples prepared from aliquots of the stock solution of the bases, respectively. BSA (volumes of 4.0, 2.0, 0.50, 0.25, and 0.15 ml, respectively) was added to each sample. A magnetic stirring bar was placed in each tube; the tube was then tightly capped and heated for 45 min at 150° with magnetic stirring.

After cooling to room temperature, the samples were analyzed by GLC and the experimental peak area of each base relative to the phenanthrene peak area was calculated. Each of the samples contained approximately a one hundred molar excess of BSA and had a 3:1 v/v ratio of acetonitrile to BSA. The actual concentrations of the bases in each sample were in the range of 0.04–0.143 mg/ml.

The calibration curves shown in Fig. 4 demonstrate that the derivatization procedure gave linear calibration curves over an 80 fold increase in sample size. Excellent linearity and reproducibility was obtained over this sample weight range with

a relative standard deviation ranging from 1.1 % for uracil to 3.1 % for cytosine. The calibration curve for cytosine was prepared using only the first eluted peak area. These calibration curves demonstrate that precise and accurate quantitative analysis of the purine and pyrimidine bases can be achieved from an aqueous sample.

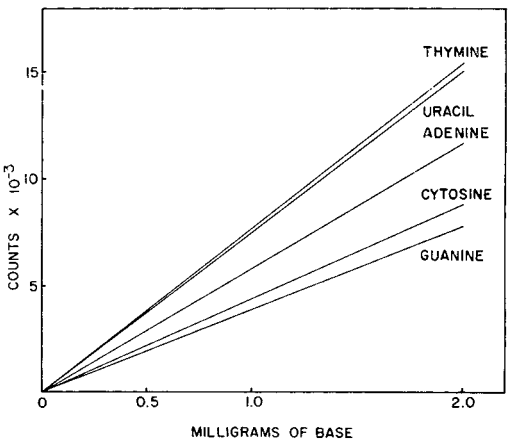


Fig. 4. Calibration curves for the purine and pyrimidine bases.

Minimum detectable amount (MDA). The second largest calibration sample was used for the determination of the minimum detectable amount (MDA). This sample contained 1.0 mg of each of the five main bases in a total volume of 8.0 ml (6.0 ml of acetonitrile and 2.0 ml of BSA). An aliquot of this solution was diluted 1:5 with acetonitrile thus containing 1 mg/40 ml (solution A). An aliquot, 1.00 ml, of solution A was then diluted 1:5 with acetonitrile (solution B) containing 1 mg of each base/200 ml. These solutions were then analyzed by GLC using various size injections and adjusting the instrument attenuation (*i.e.*, sensitivity) until a signal to noise ratio of 3:1 was obtained. This was considered the MDA. The MDA for the bases injected was 3–5 ng, or *ca.* 3×10^{-11} moles of each base.

(6) Determination of the relative molar response of selected nucleosides and nucleotides

Selected nucleosides and nucleotides were subjected to silylation under the conditions previously chosen for the purine and pyrimidine bases. The samples were subsequently analyzed by GLC to determine their retention temperatures and molar response values relative to phenanthrene. Even though these conditions were not necessarily optimal for the nucleosides and/or nucleotides, the response values could be useful in the analysis of biological samples containing mixtures of all of the nucleic acid components.

Individual samples containing *ca.* 1.0 mg of the following nucleosides or nucleotides with *ca.* 1.0 mg of phenanthrene (I.S.) were prepared: uridine, thymidine, adenosine, guanosine, uridylic acid, and adenylic acid. BSA (0.5 ml, *ca.* 100 molar excess) and 1.5 ml of acetonitrile were added to each nucleoside, and 0.8 ml of BSA (*ca.* 100 molar excess) and 2.4 ml of acetonitrile were added to each of the nucleotides. The samples were reacted at 150° for 45 min, then cooled to room temperature and analyzed by GLC.

TABLE II

RELATIVE MOLAR RESPONSE OF SELECTED NUCLEOSIDES AND NUCLEOTIDES AS TMS DERIVATIVES

Compound	*Retention temperature* (°C)	*RMR*[a]	*RMR range*
Thymidine[b]	230		
Uridine	234	1.19	± 0.10
Adenosine	260	1.60	± 0.10
Guanosine	265	1.37	± 0.09
Uridylic acid	270	0.61	± 0.05
Adenylic acid	276	0.98	± 0.03

[a] Calculated from at least two independent observations; samples heated for 45 min at 150° with a 100 molar excess of BSA.

[b] RMR not calculated due to two unresolved peaks for thymidine. RMR = relative molar response to phenanthrene as I.S.

Single chromatographic peaks were obtained for the compounds investigated which contained ribose; however, when the same procedure was used for thymidine, a deoxyribose containing compound, two unresolved peaks resulted. A chromatographic peak for cytidylic acid was obtained on room temperature silylation for extended periods of time (*e.g.* 24 h). HASHIZUME AND SASAKI[10] using HMDS and TMCS reported unsuccessful silylation of cytidylic acid. The relative molar responses of some of the nucleosides and nucleotides have been determined and are given in Table II. A mixture of ribonucleosides was chromatographed under the conditions used for the purine and pyrimidine bases, but complete separation was not achieved at these conditions as shown in Fig. 5. In particular, cytidine and guanosine were not separated at all. Further investigations are necessary to delineate the optimum silylating, chromatographic, and instrumental conditions for the successful analysis of the nucleosides and nucleotides.

(7) *Silylation using hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS), and BSA*

The results of SASAKI AND HASHIZUME[11] showed that a single peak for cytosine was obtained using the HMDS–TMCS reagents. This was not the case, however, using the BSA silylation procedure, since cytosine under these conditions yielded two peaks. These differences necessitated a comparison of these two derivatization procedures under similar experimental and chromatographic conditions. The HMDS–TMCS and BSA procedures are described as follows:

HMDS–TMCS method. A mixture of *ca.* 2.0 mg of each of the five main bases (uracil, thymine, cytosine, adenine, and guanine) and phenanthrene (I.S.) was weighed into a 25 ml Claisen flask. HMDS (0.2 ml), TMCS (0.1 ml), and 0.7 ml of pyridine as solvent were added and the mixture was refluxed for 1 h under anhydrous conditions, then cooled to room temperature.

BSA method. A mixture of *ca.* 1.0 mg of adenine and *ca.* 1.0 mg of phenanthrene was silylated with 0.4 ml of BSA and 1.2 ml of acetonitrile at 150° for 45 min in a closed tube.

The derivatized samples for both methods were chromatographed under the same instrumental conditions on the 4 w/w % SE-30 column.

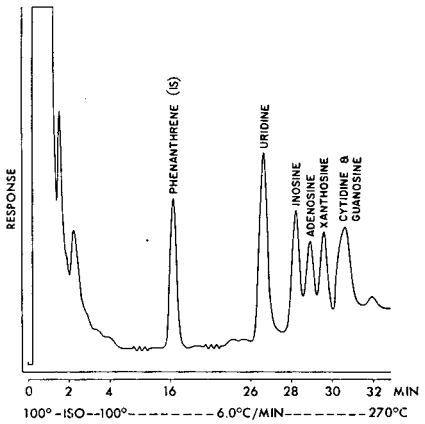


Fig. 5. GLC chromatogram of ribonucleosides.

Chromatographic separation of the derivatized bases using the HMDS–TMCS method was excellent and was equivalent to the separation achieved with the derivatives prepared with BSA. Only one peak for cytosine was observed for the HMDS–TMCS method as had been previously reported[11]. However, the molar response values relative to phenanthrene were considerably lower when using HMDS–TMCS, except for adenine, than those obtained using BSA. The results of this comparison are given in Table III. It is apparent from the differences in relative molar response,

TABLE III

COMPARISON OF HMDS–TMCS AND BSA SILYLATION METHODS

Base	*Relative molar response*[a] *HMDS–TMCS*[b]	*BSA*[c]	*% Increase with BSA*
Uracil	0.60 ± 0.03	0.67	11.7
Thymine	0.55 ± 0.02	0.68	23.6
Cytosine	0.43 ± 0.02	0.54[e]	25.6
Adenine	0.61 ± 0.03	0,60, 0.62[d]	not significant
Guanine	0.56 ± 0.03	0.72	28.6

[a] Relative molar response to phenanthrene as I.S.
[b] Average of two independent runs.
[c] Values obtained from Table I.
[d] RMR for adenine reconfirmed three months later.
[e] Silylation at room temperature.

that in general, the percent silylation or yield of derivative is greater using BSA than with HMDS–TMCS. Yield of derivative is one of the most important single factors affecting the precision and accuracy of a gas chromatographic method of quantitative analysis. A low yield could result in poor precision and severely limit the usefulness of the method. Even though the BSA method results in two peaks for cytosine, the relative area of the first of the two peaks was reproducible under the selected conditions and a linear calibration curve was obtained. This point of a single peak

for cytosine is the only discernible advantage of the HMDS–TMCS method. The method using BSA has the advantages of speed, simplicity, and higher yield of derivative. Therefore, it is a superior method of derivatization.

(8) Effect of ammonium chloride on base silylation with BSA

HASHIZUME AND SASAKI[15] reported a method for the GLC determination of orthophosphoric acid as the TMS derivative. They used this method for the analysis of orthophosphate obtained from perchloric acid hydrolysates of nucleotides. In their procedure the perchloric acid hydrolysate was first made alkaline with potassium hydroxide and then neutralized with hydrochloric acid. In the next step, concentrated ammonium hydroxide was added and the resulting mixture was evaporated to dryness at 100°. The purpose was to precipitate the perchlorate anion as the potassium salt, and to convert the tripotassium phosphate to the respective ammonium salt. The ammonium salt was then directly trimethylsilylated using hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS) in pyridine, without the removal of potassium perchlorate, ammonium and potassium chlorides, etc.

The described procedure[15] was investigated in our laboratory for the determination of base ratios in yeast RNA, except that BSA was substituted as the silylation reagent. BSA was previously demonstrated to be a superior silylating reagent. Initial studies showed that this method was not satisfactory due to the presence of the TMS-orthophosphate, which partially overlapped the TMS uracil peak. Also additional interfering peaks for the TMS derivatives of ribose and its degradation products were present. However, one very interesting observation was made. Silylation with BSA normally yields two derivatives for cytosine, but in this case, only a single peak for cytosine was observed. Ammonium chloride is produced in the previously described reaction sequence when ammonium hydroxide is added to the sample containing chloride ions and also in the reaction when HMDS–TMCS is used. Therefore, this observation suggested an investigation of the effect of NH_4Cl on trimethylsilylation with BSA.

Seven mixtures containing *ca.* 1 mg each of cytosine and phenanthrene (I.S.) were weighed into 16 × 75 mm screw top culture tubes. Approximately 0, 10, 25, 50, 75, 100, and 200 mg amounts of NH_4Cl were added. Anhydrous acetonitrile (1.2 ml), 0.4 ml BSA, and an 8 × 15 mm teflon covered magnetic stirring bar were added to each tube. The tubes were tightly capped with teflon lined caps, heated at 150° for 45 min, and then cooled to room temperature. These samples were analyzed by GLC.

The same experiment was repeated for the bases, uracil, thymine, adenine, and guanine, except only four samples of the bases, containing *ca.* 0, 10, 50, and 100 mg of NH_4Cl were analyzed.

The data from this investigation on the effect of NH_4Cl showed that a level of 10 mg of NH_4Cl per 1 mg of cytosine caused the second peak for cytosine to be decreased to 5 % of the first peak. If more than 10 mg of NH_4Cl were added the second peak for cytosine was absent. The NH_4Cl effect on the silylation of the five main purine and pyrimidine bases is graphically shown in Fig. 6. An important observation is that a significant increase in the relative response of cytosine, adenine, and guanine resulted on the addition of 10 mg of NH_4Cl. However, the amount of NH_4Cl present in the reaction for each mg of base is very important, as the $RMR_{B./Phen.}$ for adenine

and guanine were decreased by *ca.* 50 and 80%, respectively, in the presence of 50 mg of NH_4Cl.

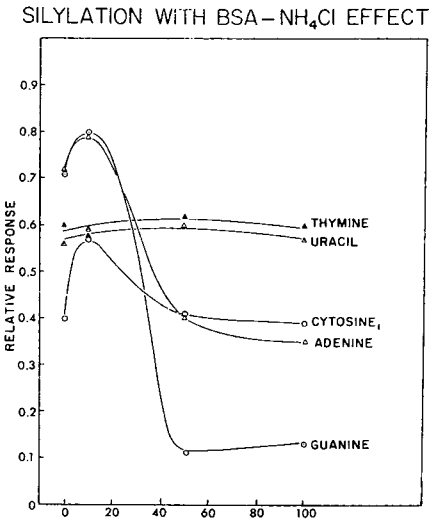


Fig. 6. mg of NH_4Cl/mg of Base.

Several other salts were also investigated to further define this effect. These included ammonium phosphate ($NH_4H_2PO_4$), potassium phosphate (KH_2PO_4), and sodium chloride. The phosphate salts were selected because of their presence in the hydrolysates of RNA and DNA prepared by the procedure of HASHIZUME AND SASAKI[15]. Sodium chloride was selected because of its assumed non-reactivity since both of the phosphate salts would enter into the silylation reaction. Similar results were obtained for potassium and ammonium phosphates as for NH_4Cl. At K and NH_4 phosphate salt levels of 10 mg/mg of cytosine, an enhanced response for cytosine was observed. At the 50 mg level only a very small cytosine peak and a very large TMS phosphate peak were present in the sample with $NH_4H_2PO_4$. This is explained by the large excess of $NH_4H_2PO_4$ reacting with the BSA, thus leaving only a small amount of BSA reagent for derivatization of cytosine. Also, a decrease in the RMR of cytosine was observed in the presence of 50 mg of KH_2PO_4 but not of the magnitude as for $NH_4H_2PO_4$. This is presumably due to the less reactivity of KH_2PO_4 with BSA as compared to $NH_4H_2PO_4$. Both the ammonium and potassium phosphate salts resulted in the loss of the second peak for cytosine at the 50 mg level. The effect of NaCl on the relative response of cytosine at the 50 mg/mg level was negligible and caused no loss of the second cytosine peak.

The second peak normally present on silylation of cytosine with BSA can be eliminated by adding 8–15 mg of NH_4Cl per mg of base. Further, the RMR of adenine and guanine is significantly enhanced on using an amount of NH_4Cl up to 15 mg. The effect of NH_4Cl on the silylation of uracil and thymine was negligible and has no apparent advantage or disadvantage in their derivatization. However, the fact that the amount of NH_4Cl present in the reaction is very critical with cytosine, adenine, and especially guanine, makes its use questionable since the successful quantitation

of the bases has previously been demonstrated, even though cytosine yields two derivatives under those conditions.

(9) *Evaluation of GLC column support phases for the TMS bases*

Chromatographic difficulties were encountered in reproducibly preparing the SE-30 column on High Performance Chromosorb G and necessitated the treatment of the prepared columns with a silylating reagent (Silyl 8) to obtain satisfactory response for the bases. Thus, to improve chromatographic precision and separation, a GLC column evaluation study was made on the following columns:

(1) 4 w/w % SE-30 on 80–100 mesh, High Performance Chromosorb G (previously used column for the TMS bases);

(2) 4 w/w % SE-30 on 80–100 mesh acid-washed Chromosorb G, heat treated for 15 h at 550°;

(3) 0.2 w/w % SE-30 on 100–120 mesh Corning Glass Beads (Type 0201);

(4) 8 w/w % SE-30 on 100–120 mesh Supelcoport (Chromosorb W type).

All columns were 1 m × 4 mm I.D. pyrex glass U-tubes. Columns Nos. 1 and 2 were preconditioned for 16 h at 275° with a carrier gas (N_2) flow rate of *ca.* 75 ml/min. Column No. 3 was preconditioned similarly except at 230°, because of the low loading percentage necessary for the glass bead column. Column No. 4 was also conditioned at 230° because both columns (3 and 4) were in the same chromatographic oven. The columns were then checked with a standard TMS base mixture.

The Supelcoport column required no silylation pretreatment and was found to be superior to the 4 w/w % SE-30 on 80–100 mesh, High Performance Chromosorb G column that had been used routinely for the preceding investigations. The columns of 4 w/w % SE-30 on acid-washed, heat treated Chromosorb G and the 0.2 w/w % SE-30 glass bead column were found to be totally unacceptable. The need for the higher percentage (8 w/w %) loading of the liquid phase on the Supelcoport column was to provide an approximately equal liquid phase film thickness compared to the 4 w/w % loading on the Chromosorb G columns. Supelcoport has approximately twice as much surface area per unit weight. The Supelcoport column was used for all subsequent investigations.

(10) *Comparative study of silylation reagents.*

The increased acceptance of the trimethylsilyl derivative in analytical GLC has brought about the development and introduction of new silylation reagents and also modifications of existing silylation mixtures. One of these new reagents, bis(trimethylsilyl)trifluoroacetamide (BSTFA), was synthesized in our laboratories[13] and has proven especially useful for derivatization of amino acids. Also, a reagent mixture of catalytic amounts of TMCS in BSA has been reported to have advantages for certain classes of compounds. This is explainable by the fact that as TMCS reacts with a replaceable hydrogen, HCl is formed which could have a catalytic effect on the silylation reaction. Due to the demonstrated usefulness of these reagents, it was necessary to evaluate and compare them with the presently used BSA reagent for the silylation of purine and pyrimidine bases.

Eight mixtures containing *ca.* 1.0 mg each of uracil, cytosine, adenine, guanine, and phenanthrene (I.S.) were accurately weighed into the previously described pyrex culture tubes. Four different silylation reagent solutions were used each at a 100

molar excess to total bases, and two independent samples were analyzed with each reagent. The solutions were:

(1) BSA–acetonitrile, 1.5:4.5 ml;
(2) TMCS (1 v/v %) in BSA–acetonitrile, 1.5:4.5 ml;
(3) TMCS (0.1 v/v %) in BSA–acetonitrile, 1.5:4.5 ml;
(4) BSTFA–acetonitrile (1:2.15 v/v) 1.9/4.1 ml*.

After the reagents and a stirring bar were added (previously described), the reaction tubes were tightly capped and heated at 150° for 45 min, then cooled to room temperature and the reaction mixtures analyzed by GLC using the normal operating conditions.

In regard to analytical derivatization yield there was no particular advantage or disadvantage in using BSTFA, or TMCS in BSA, in place of the previously used BSA as the silylating reagent since the $RMR_{B./Phen.}$ values obtained for the four different silylating reagent solutions were in good agreement (Table IV). This demonstrated that these silylating reagents were essentially equivalent under the reaction conditions used. The only discernible advantage of any of the reagents compared to BSA was that the chromatograms obtained with BSTFA showed a better base line with fewer small extraneous peaks. Also the injection peak was smaller due to the greater volatility and lower detector response of BSTFA and its by-product, N-trimethylsilyltrifluoroacetamide (MSTFA).

The major advantage of using BSTFA over BSA was not shown by the chromatograms but results from the complete miscibility of BSTFA with acetonitrile. With BSA, the required ratio of solvent for miscibility is 3:1 v/v, acetonitrile–BSA. Also it has been previously demonstrated that a 100 molar excess of BSA to total bases is required for optimum derivatization. Thus, the concentration of the bases in solution is limited. BSTFA does not present these limitations and therefore different sample weights of bases and volumes of solvent can be used. This permits one to analyze on a micro scale as the sample solution can be concentrated to a smaller volume.

(11) Application of BSA silylation to the analysis of yeast RNA

A major objective of this research was the application of the developed method of analysis of nucleic acid components to biological materials. Originally there were a number of possible approaches to the problem. One such approach involved the hydrolysis of the nucleic acid material with 1 *N* HCl to obtain the purine bases and pyrimidine nucleotides. Our investigations to this time indicated that the purine and pyrimidine bases could be more successfully analyzed by GLC than the nucleosides or nucleotides. The analysis of the larger, more complex nucleoside and nucleotide molecules would present chromatographic and quantitative derivatization problems. Thus, our research was oriented toward complete hydrolysis of the nucleic acids to the bases, followed by derivatization and chromatography.

As early as 1951 it was shown by MARSHAK AND VOGEL[16] that a concentrated perchloric acid hydrolysis of RNA or DNA would quantitatively yield the free purine

* With BSA–acetonitrile, the 1:3 v/v ratio is necessary for complete miscibility. However, this is not the case with BSTFA as it is miscible in all proportions with CH_3CN. Therefore, the volume ratio was changed for BSTFA so that the total final volume (6.0 ml) would be constant and the concentration of bases would be the same in all samples and experiments, and 100 molar excess of BSTFA.

TABLE IV

A COMPARATIVE STUDY OF SILYLATION REAGENTS

Silylation reagent	$RMR_{B./Phen.}$[a]					
	Uracil		*Average*	*Cytosine*$_1$		*Average*
BSA	0.60	0.60	0.60	0.41	0.43	0.42
1 v/v % TMCS in BSA	0.61	0.62	0.61	0.47	0.43	0.45
0.1 v/v % TMCS in BSA	0.63	0.62	0.62	0.43	0.42	0.42
BSTFA	0.63	0.63	0.63	0.42	0.36	0.39
	Adenine		*Average*	*Guanine*		*Average*
BSA	0.57	0.57	0.57	0.69	0.69	0.69
1 v/v % TMCS in BSA	0.56	0.59	0.58	0.72	0.71	0.72
0.1 v/v % TMCS in BSA	0.58	0.59	0.59	0.73	0.71	0.72
BSTFA	0.57	0.56	0.57	0.72	0.69	0.70

[a] All samples silylated at 150° for 45 min; 100 molar excess of silylating reagent. Each value is a single determination on an independent sample. Molar responses relative to phenanthrene (I.S.).

and pyrimidine bases. Much later, 1966, MACGEE[7] used this hydrolysis procedure in conjunction with a short ion-exchange clean-up process, for removal of the released phosphoric acid, ribose, and the ribose degradation products, to give purified purine and pyrimidine bases. Subsequently the N-methyl derivatives were prepared with TMAH and the base ratios determined by GLC. This procedure, however, was not without complications. The method of derivatization employed was not satisfactory as it resulted in multiple derivatives of two bases, adenine and cytosine, and necessitated the selection of a single peak for each compound for calculations. For these reasons a better derivatization and GLC analysis method was needed.

Our previously described trimethylsilyl derivatization procedure offered the possibility of quantitative analysis, once the problems of hydrolysis and purification were solved.

The following analytical–chromatographic method represents a combination of our silylation derivatization reactions with a modification of MACGEE's perchloric acid hydrolysis of the nucleic acids and ion-exchange clean-up procedure.

Sample hydrolysis and preparation. A 5.0 mg sample of yeast RNA was weighed into a 16 × 75 mm culture tube and 0.5 ml of 70 % perchloric acid was added. The tube was capped and the sample heated at 100° for 40 min with occasional shaking. The sample was then cooled in an ice-bath and made alkaline by the dropwise addition of 1.0 ml of ice-cold 8 *N* KOH with agitation. After the alkaline sample was held in an ice-bath for ½ h, the insoluble potassium perchlorate was centrifuged and the supernatant removed. The precipitate was then washed three times with 0.5 ml volumes of ice-cold 1 *N* KOH. The original supernatant was combined with the three washes and held in an ice-bath until placed on the anion-exchange column.

Ion-exchange clean-up. An anion-exchange column of Dowex 1-x2 formate, 100–200 mesh, was prepared with a resin bed of 9 × 105 mm. The alkaline sample (pH ⩾ 13) was then placed on the anion-exchange column and the liquid level was allowed to drop to the resin level. Double distilled water (50 ml) was then passed through the column and discarded. Twenty (20) ml of 1.0 *M* formic acid eluent were then passed through the column. The first 7 ml were discarded and the next 13 ml

were collected in a 16 × 150 mm culture tube. The collected fraction was evaporated to approximately 2 ml on a 100° sand bath while being flushed with a stream of filtered N_2 gas. The sample was then taken to dryness by lyophilization.

Derivatization and GLC analysis. The dried sample containing the purified purine and pyrimidine bases was silylated at 150° for 45 min using a 100 molar excess of BSA and a 3:1 v/v ratio of acetonitrile–BSA. The sample was then cooled to room temperature and analyzed by GLC. The GLC column used was 8 w/w % SE-30 on Supelcoport.

A series of standard mixtures of the bases was carried through this total procedure (*i.e.*, hydrolysis, ion-exchange, derivatization, and GLC analysis). Phenanthrene (I.S.) was added after lyophilization just prior to derivatization. At the same time a series of standard mixtures containing phenanthrene were carried through the derivatization and chromatography steps only, and by comparison of the RMR data, the percent recovery was determined (Table V). Although the percent recovery was approximately 82%, the recovery of the four bases was equivalent (± 1.5%). This demonstrates a non-selective loss through the total procedure. The losses were probably incurred in the transfer step (centrifugation), and refinement of the manipulative techniques should improve the recovery.

Table VI gives the results obtained for the base ratio determination of purified yeast RNA. The values are for duplicate analyses on three independent samples and demonstrate excellent precision for base ratio analysis. This experiment was undertaken for precision studies only. Yeast RNA can vary considerably in its base ratios depending on its source[17], and therefore, the values cannot be checked against the literature. A typical chromatogram of the TMS bases from yeast RNA is shown in Fig. 7. Although phenanthrene was added to this sample, it is not necessary for base ratio determination. However, if the absolute amount of bases is desired, it is necessary to add an internal standard such as phenanthrene. Also, it is essential for base ratio analysis that standard mixtures be prepared and analyzed concurrently with the biological samples to normalize for response variations due to changing gas chromatographic column characteristics and/or instrumental changes.

TABLE V

RECOVERY OF BASES TAKEN THROUGH COMPLETE METHOD

Base		$RMR_{B./Phen.}$[a, b]	*Average*	*Recovery (%)*
Uracil	Procedure[c]	0.49 0.50 0.49	0.49	80.3
	Standard	0.61 0.60	0.61	
$Cytosine_1$	Procedure[c]	0.36 0.35 0.35	0.35	83.3
	Standard	0.42 0.42	0.42	
Adenine	Procedure[c]	0.50 0.51 0.51	0.51	80.9
	Standard	0.62 0.64	0.63	
Guanine	Procedure[c]	0.64 0.63 0.63	0.63	82.9
	Standard	0.76 0.77	0.76	

[a] Each value represents duplicate analyses of an independent sample.

[b] $RMR_{B./Phen.} = \frac{A_{B.}/moles_{B.}}{A_{I.S.}/moles_{I.S}}$

[c] Mixtures carried through complete method, hydrolysis, ion-exchange, derivatization, and chromatography.

TABLE VI

GLC DETERMINATION OF BASE RATIOS IN YEAST RNA

Base	*Mole ratio*[a]			*Average*
Uracil	0.95	0.94	1.00	0.96
Cytosine	1.06	1.04	1.05	1.05
Adenine	1.00	1.00	1.00	1.00
Guanine	1.20	1.19	1.21	1.20

Sigma range of 0.029–0.037 with an average rel. S.D. of 3.0%.

[a] Base ratios relative to adenine assigned 1.00. Each value represents duplicate analyses of an independent sample.

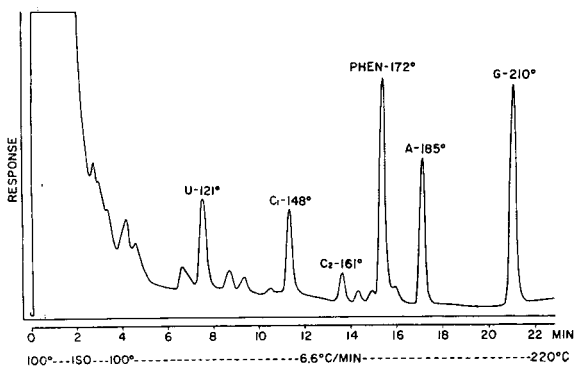


Fig. 7. Chromatogram of bases from yeast RNA. Column: 8 w/w% SE-30 on 100–120 mesh Supelcoport, 1 m × 4 mm I.D. pyrex glass. Each peak represents *ca.* 0.5 μg of base.

CONCLUSIONS

The major purpose of this investigation was to develop a quantitative gas–liquid chromatographic method of analysis for the components of nucleic acids (*i.e.*, purine and pyrimidine bases, nucleosides, or nucleotides), and further to apply this method to the analysis of biological material. Gas–liquid chromatography has the inherent advantages of speed, accuracy, and simplicity over other methods of analysis. However, before successful GLC of these compounds can be accomplished, they must first be converted to suitable volatile derivatives, as the compounds themselves are not sufficiently volatile.

The N-methyl derivatives of the purine and pyrimidine bases, prepared by thermal dissociation of their tetramethylammonium salts, as reported by MACGEE[6] were investigated and found to be unsuited for quantitative analysis due to the formation of multiple chromatographic peaks for cytosine, adenine, and guanine. However, investigation of the trimethylsilyl (TMS) derivatives of the bases demonstrated these derivatives to be far superior to the N-methyl compounds and well suited for the analysis of nucleic acid components, thus the TMS derivative was the derivative of choice.

A new silylating reagent, bis(trimethylsilyl)acetamide (BSA), was evaluated and found to be an excellent reagent for silylation of the bases. A procedure for the

quantitative analysis of the bases was developed. The optimum derivatization conditions were heating the bases in a closed tube at 150° for 45 min, with a 100 molar excess of BSA to total bases, and a 3:1 v/v acetonitrile–BSA ratio. Calibration curves for the five main bases (U, T, C, A, and G) were prepared and found to be linear over a sample weight range of 25–2000 μg of base. The relative standard deviations ranged from 1.1 % for uracil to 3.1 % for cytosine.

The minimum detectable amount (MDA) using the hydrogen flame ionization detector, was determined to be 3–5 $\times$ 10^{-9} g or *ca.* 3 $\times$ 10^{-11} moles of each base injected. The relative molar responses of the five main purine and pyrimidine bases and other bases, nucleosides, and nucleotides were determined relative to phenanthrene as internal standard. Both cytosine and 5-methyl cytosine exhibited two chromatographic peaks using the optimum derivatization conditions; however, these conditions were found to be necessary for the reproducible formation of the guanine TMS derivative, and the relative peak area remained constant under these conditions.

When the BSA method of derivatization was compared to the HMDS–TMCS method of SASAKI AND HASHIZUME[11], which was published during the course of this investigation, BSA was found to have the advantages of speed, simplicity, and most important, a higher yield of derivative. Therefore, it was shown to be a superior method of derivatization.

Ammonium chloride, when added to the BSA derivatization reaction, was found to have an effect on the silylation of the bases. At an ammonium chloride level of slightly greater than 10 mg per mg of base, the second peak for cytosine was not present and the relative response of the first peak was greater. Also, at a level of 10 mg of ammonium chloride per mg of base, the relative response of adenine and guanine were greater. However, a further small increase in the ammonium chloride concentration (*i.e.*, to 25 mg of NH_4Cl/mg of base) caused a very significant decrease in the response of adenine and guanine. The fact that the amount of NH_4Cl present was very critical with cytosine, adenine, and guanine, even though it showed no effect on uracil and thymine, makes its use questionable, since the successful quantitation of the bases in the absence of NH_4Cl has previously been demonstrated.

An evaluation of four GLC columns for the TMS bases showed that a column of 8 w/w % SE-30 on 100–120 mesh Supelcoport provided good resolution and stability, and was superior to the previously used column of 4 w/w % SE-30 on High Performance Chromosorb G.

A comparison was made of BSA as a silylation reagent with the new reagent, bis(trimethylsilyl)trifluoroacetamide (BSTFA), which was synthesized in our laboratory, and reagent solutions of 0.1 v/v % TMCS in BSA, and 1.0 v/v % TMCS in BSA. There were no significant differences in regard to analytical derivatization yield with any of these reagents. However, advantages of BSTFA include "cleaner" chromatograms with fewer extraneous peaks, and an important advantage arising from the complete miscibility of BSTFA in the solvent, acetonitrile. This latter advantage stems from the fact that silylation of the bases requires a 100 molar excess of BSA, and a required solvent–reagent ratio of 3:1 v/v acetonitrile–BSA for miscibility. Since BSTFA does not present these limitations, different sample weights of bases and volumes of solvents can be used. Also, recent research in our laboratory shows that the silylation time with BSTFA can be reduced to 15 min. This permits one to make analyses on a micro scale as the sample solution can be concentrated to a small

volume with sample integrity being maintained. From these studies it was concluded that BSTFA is the silylating reagent of choice.

The application of the developed method for purine and pyrimidine base analysis to the analysis of biological materials was accomplished using perchloric acid hydrolysis and anion-exchange removal of biological interferences prior to derivatization and GLC analysis. The combined hydrolysis and purification procedures were shown to give a recovery of the bases of *ca.* 82 % with the recovery of the four bases being equivalent (± 1.5 %), thus demonstrating a non-selective loss of individual bases. When the method was used for the analysis of purified yeast RNA, the base ratio determination of three independent samples demonstrated the excellent precision of the method with an average relative standard deviation of 3.0 %. Although the method has not been applied to analysis of microgram amounts of RNA and DNA, it should not prove difficult to accomplish with minor refinements of technique.

ACKNOWLEDGEMENTS

The authors wish to acknowledge Drs. WALTER A. AUE and DAVID L. STALLING for their suggestions and valuable comments, and Mr. DUANE LAKINGS for his excellent technical assistance throughout this investigation.

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CHROM. 3794

THE GAS CHROMATOGRAPHIC SEPARATION OF PAIRS OF ISOTOPIC MOLECULES BY MEANS OF POROUS POLYMER BEADS*

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(Received September 19th, 1968)

SUMMARY

The separation of various isotopic molecules with deuterium substitutions is reported.

Columns with porous polymer beads (Porapak Q) are employed. A comparison is made between this packing material and other adsorbents, as graphitized carbon black or silica gel. High separation factors are obtained. The working temperatures are usually higher with respect to the other materials, but the isotopic effects measured with the Porapak are more favourable for many of these separations.

INTRODUCTION

The outstanding properties of porous polymer beads as a fractionation medium, suggested its use as column packing for the separation of isotopic pairs. This investigation deals with the use of Porapak; this material has been used by CZUBRYT *et al.*[1] for the separation of CH_4 and CD_4 in a packed column. Several isotopic systems have been investigated, and it has been found that the separation factor on this material is quite high; separation in several cases could be realized by using classically packed columns.

EXPERIMENTAL

All chromatographic measurements at room temperature or higher have been made on a commercial apparatus (C. Erba, Milano). Measurements below 0°C have been carried out on a home-made gas chromatograph where accurate thermostating was realized by flowing methanol cooled with dry ice and acetone. Measurements at 143° and 113°K have been carried out by replacing methanol with pentane and isopentane cooled with liquid nitrogen.

Porapak Q (60–80 mesh) obtained from Waters Assoc., Framingham, Mass. (U.S.A.) has been used to pack the following columns.

(a) glass (1.5 m, 0.12 cm I.D.);

* Work carried out with financial assistance of the Consiglio Nazionale delle Ricerche.

(b) copper (4.3 m, 0.2 cm I.D.);
(c) copper (1.0 m, 0.15 cm I.D.);
(d) copper (2.5 m, 0.20 cm I.D.).

The following isotopic pairs have been examined: CH_4–CD_4, C_2H_2–C_2D_2, C_2H_4–C_2D_4, C_2H_6–C_2D_6, H_2S–D_2S, C_6H_6–C_6D_6, C_6H_{12}–C_6D_{12}, CH_3Cl–CD_3Cl, CH_3COCH_3–CD_3COCD_3, CH_3OH–CD_3OH.

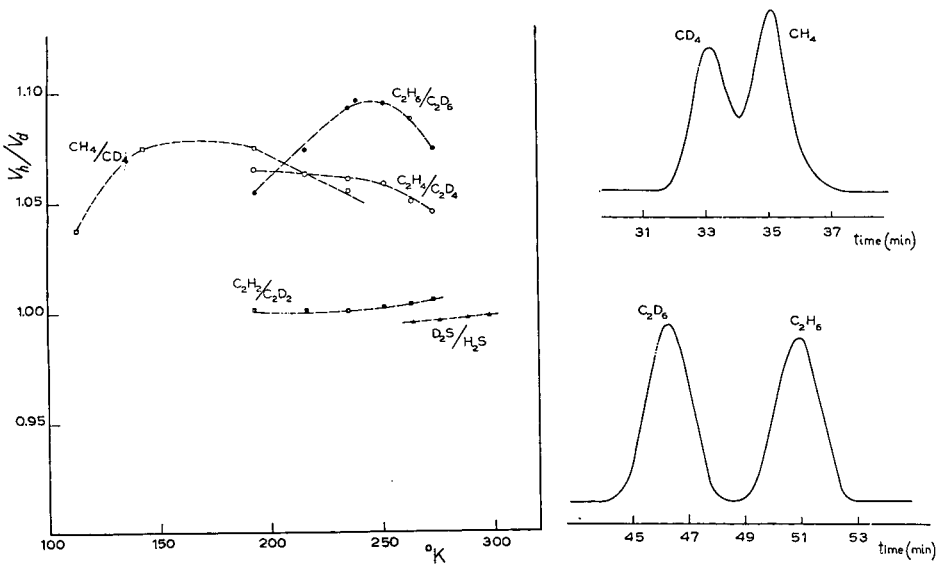

Fig. 1. Plots of the retention volumes ratio (V_h/V_d) *vs.* temperature for gas isotopic pairs.

Fig. 2. Chromatographic separation of CH_4–CD_4 and C_2H_6–C_2D_6 on a Porapak Q column (4.3 meter, 0.20 I.D.) CH_4–CD_4: T cal, 190°K; P_{N_2}, 1.5 atm; flow, 18.7 ml/min. C_2H_6–C_2D_6: T cal, 250°K; P_{N_2}, 1.75 atm; flow, 14.5 ml/min.

Nitrogen has been used as elution gas for all systems which were detected by a F.I.D.; hydrogen was used as a carrier gas in the study of H_2S–D_2S for which a microcell conductivity detector was employed.

RESULTS

Separation of gaseous compounds

The elution time of the investigated hydrocarbons on Porapak follows the order: methane, ethylene, acetylene, ethane. All isotopic pairs of hydrocarbons exhibit in the temperature range examined, a reverse isotopic effect, the heavier species being eluted first. To obtain the most favourable operating temperature, the separation factor has been measured in a fairly wide range and the results are plotted in Fig. 1. For the system CD_4–CH_4, the highest separation factor has been found between 140 and 200°K; below 140° it decreases much more than above 400°K. Fig. 2 shows the separation CD_4–CH_4 at 190°K; it has been obtained with a resolution factor (R) equal to 0.8 on column b which has only 4,500 theoretical plates for CH_4. A similar

column with 25,000 plates should separate the pair CH_4–CH_3D with the same resolution as the separation factor for this pair at the same temperature is 1.017.

A similar behaviour is exhibited by the pair C_2D_6–C_2H_6, which has been investigated in the temperature range 240–255°K; the separation factor, as indicated in Fig. 2, is better than for methane, the chromatogram being obtained at 250°K on the same column.

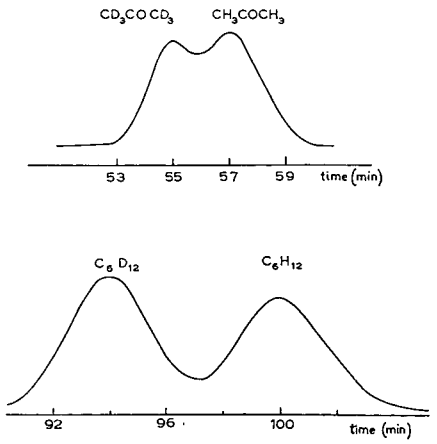

Fig. 3. Chromatographic separation of CH_3COCH_3–CD_3COCD_3 and C_6H_{12}–C_6D_{12} on a Porapak Q column (2.5 m 0.20 I.D.) CH_3COCH_3–CD_3COCD_3: T cal, 353°K; P_{N_2}, 0.95 atm; flow, 7.2 ml/min. C_6H_{12}–C_6D_{12}: T cal, 427°K; P_{N_2}, 0.6 atm; flow, 4.0 ml/min.

The separation of the pair C_2D_4–C_2H_4 is less affected by temperature; the trend should be the same, but the lower temperature range is difficult to investigate because of the very long retention time below 190°K.

The separation for the pair C_2D_2–C_2H_2 cannot be realized on Porapak Q as the separation factor is almost united and hardly affected by temperature. No peak separation has been obtained by operating on the pair H_2S–D_2S, but by performing separate chromatograms on the single isotopic species, a noticeable difference of retention time is measured and significant information is obtained. This pair exhibits a normal isotopic effect, the separation factor increasing by decreasing the temperature. Measurements have been limited to the temperature range 273–330°K using column c; at lower temperatures the peaks are strongly tailed and do not allow any satisfactory determination.

Separation of liquid compounds

A set of polar and non-polar isotopic pairs has been investigated. The operating temperature range (320°–450°K) is quite limited as the capacity ratio (K') strongly increases by decreasing the temperature; on the other hand, an increase of temperature decreases the separation factor; in several cases, however, a partial separation of the various systems has been realized. As an example, in Fig. 3 is shown the separation of a non-polar system (cyclohexane–deuterocyclohexane) and a polar system (acetone–deuteroacetone) performed on column d.

The results obtained for various liquid systems are expressed in terms of the

logarithm of the retention volumes ratio, log V_h/V_d, and are plotted *vs.* $1/T$ in Fig. 4. In the same graph have been reported the retention volume ratios obtained in GLC on stationary phases of different polarity (squalane, SE 30, triethyleneglycol)[2]. It seems that the interaction of most isotopic pairs on Porapak Q enhances the isotopic effect, measured in GLC. It has to be pointed out that measurements have been performed at higher temperatures where usually the isotopic effect is less relevant.

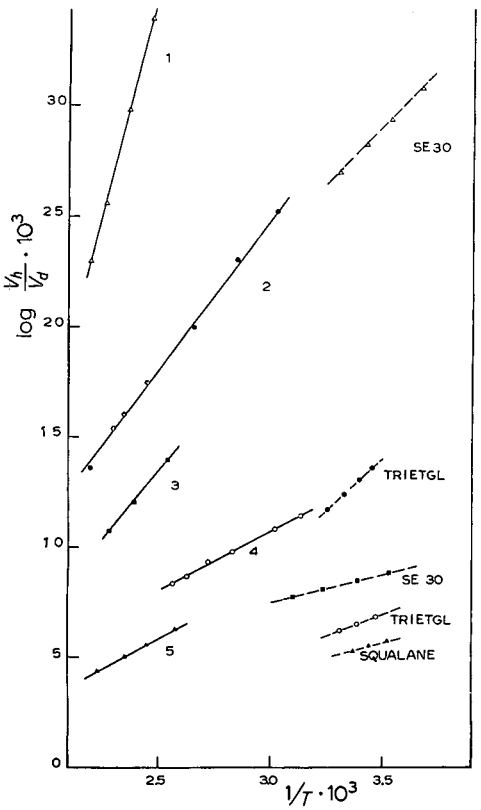

Fig. 4. Plots of logarithm $\times$ 10^3 of ratio of retention volumes *vs.* $1/T \cdot 10^3$ for liquid isotopic pairs on Porapak Q (continuous line) and by GLC (dotted line) on silicone oil, triethyleneglycol and squalane. $\triangle$ = C_6H_{12}–C_6D_{12}; $\bullet$ = CH_3COCH_3–CD_3COCD_3; $\blacksquare$ = C_6H_6–C_6D_6; $\circ$ = CH_3OH–CD_3OH; $\blacktriangle$ = $CHCl_3$–$CDCl_3$.

DISCUSSION

In Table I are collected the most favourable experimental separation factors on Porapak Q for various isotopic pairs at the indicated temperature, together with the separation factors obtained in adsorption gas chromatography on graphitized carbon black (GCB)[3], on etched glass (EG)[4] and on partition chromatography on squalane[5]. In all gaseous systems a higher separation factor has been measured; for liquid systems, the separation factor is usually favourable but it is obtained at a higher working temperature. The mechanism of gas chromatographic separations on

TABLE I

EXPERIMENTAL SEPARATION FACTORS

	Porapak Q	°K	*GCB*	°K	*EG*	°K	*Squalane*	°K
CH_4–CD_4	1.075	193	1.056	143	1.016	152		
C_2H_2–C_2D_2			1.070	77	1.075	77		
C_2H_4–C_2D_4	1.065	193	1.051	175	0.973	190		
C_2H_6–C_2D_6	1.095	251	1.087	175	1.043	164		
C_7H_8–C_7D_8	1.030	431	1.056	352	0.987	277	1.059	273
C_6H_6–C_6D_6	1.033	393	1.050	327	0.984	277	1.042	273
C_6H_{12}–C_6D_{12}	1.086	400					1.107	273

TABLE II

DIFFERENCES OF ENTHALPY AND ENTROPY CHANGES OF ISOTOPIC SYSTEMS

	$(\Delta H_{chr})_h - (\Delta H_{chr})_d$ *cal mol*$^{-1}$	$(\Delta S_{chr})_h - (\Delta S_{chr})_d$ *e.u* $\times 10^2$
C_6H_{12}–C_6D_{12}	−191.8	−32.1
C_6H_6–C_6D_6	− 73.1	− 8.6
CH_3COCH_3–CD_3COCD_3	− 62.6	− 7.1
C_2H_5OH–C_2D_5OH	− 29.7	−41.0
CH_3OH–CD_3OH	− 25.1	−24.0
$CHCl_3$–$CDCl_3$	− 22.8	− 3.0

microporous polymers is not yet elucidated; both partition and adsorption take place and the enhancement of the isotopic effect should be related to factors concerning chromatographic resolution connected to the mass transfer in the gas phase, in the mobile phase, and in the adsorption–desorption; all processes should, to a certain extent, be affected by isotopic substitution. To obtain some quantitative information on the magnitude of the isotopic effect, the differences of enthalpy and entropy changes of various isotopic pairs have been calculated for the systems where a linear relationship has been found between the logarithm of the ratio of the retention volumes and $1/T$. These values are collected in Table II. The enthalpy differences are, in most cases, much larger than the same values determined on other adsorption media.

As an adsorption medium, Porapak, being a copolymer between ethylvinylbenzene and divinylbenzene, can be considered mainly responsible for a non-specific interaction, as its molecule does not carry specific functional groups. It should be considered, however, that because of the presence of aromatic nuclei with easily polarizable electrons, a weak specific effect may also be expected, namely with molecules having lone electron pairs, π bonds or positively charged groups like protonic hydrogen atoms.

As it was expected, most isotopic pairs exhibit a reverse isotopic effect, due to a polarizability difference[6] since a non-specific interaction takes place. In the case of the isotopic pair H_2S–D_2S, a prevailing specific interaction takes place on Porapak because of the presence of protonic hydrogen atoms; this pair thus exhibits a normal isotopic effect. If, however, the C_6H_6–C_6D_6 is examined, which on silica exhibits a

normal isotopic effect, in spite of the presence of π bonds, a reverse isotopic effect is still observed. The specific interaction due to Porapak on the adsorption of an isotopic pair seems, therefore, to be very slight.

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CHROM. 3763

BEITRAG ZUR TRENNUNG VON SELTENERDEN DURCH EXTRAKTIONS-CHROMATOGRAPHIE MIT Di(2-ÄTHYLHEXYL)PHOSPHORSÄURE (HDEHP)

I. SILICAGEL ALS TRÄGERMATERIAL FÜR DIE STATIONÄRE PHASE*

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(Eingegangen am 28. August 1968)

SUMMARY

A contribution to the separation of rare earths by extraction chromatography with di(2-ethylhexyl)phosphoric acid (HDEHP). I. Silica gel as a support material for the stationary phase

Hydrophobised silica gel was used as support material for extraction chromatographic separations of rare earths with di(2-ethylhexyl)phosphoric acid (HDEHP). Columns with a capacity of 0.8 mequiv. per ml of column and a "height equivalent of one theoretical plate" of $H \leq 0.1$ mm for europium and gadolinium were obtained. H is independent of the type of silica gel when its mean pore diameter is ≥ 35 Å, and is also nearly independent of the amount of HDEHP used, as long as the silica gel only absorbs the dialkylphosphoric acid so that a dry powder is produced again after absorption. Examples of separation show the efficiency of the columns obtained.

EINLEITUNG

Bei der Analyse reinster Seltenerdpräparate ergibt sich häufig die Notwendigkeit einer Voranreicherung der Spurenverunreinigungen. Zu derartigen Makro–Mikrotrennungen, aber auch zur Bestimmung der radiochemischen Reinheit von Seltenerdpräparaten wird immer häufiger die Extraktionschromatographie unter Verwendung von Di(2-äthylhexyl)phosphorsäure (HDEHP) als Extraktionsmittel eingesetzt[1–5].

Voraussetzung guter Ergebnisse ist, dass eine leistungsfähige Trennsäule zur Verfügung steht. Das Trennvermögen der benutzten Kolonnen wird nicht nur von den relativ grossen Trennfaktoren, die mit Hilfe dieses Extraktionsmittels erzielt werden, bestimmt, sondern auch ganz entscheidend durch die kinetischen Verhält-

* Vorgetragen auf der radioanalytischen Konferenz in Starý Smokovec, 23.–26.4.1968.

** Die dieser Arbeit zugrundeliegenden experimentellen Untersuchungen wurden im Vereinigten Institut für Kernforschung in Dubna durchgeführt.

nisse innerhalb der Kolonnenpackung. Letztere hängen u.a. vom verwendeten Trägermaterial für die stationäre Phase ab. Von den bisher eingesetzten Trägermaterialien Polystyrol–Divinylbenzol[6], Aluminiumoxid[7], Teflon[8], Corvic[9], Cellulose[10], Kel-F[11], silikonisierte Kieselgur[12,13] und silikonisiertes Silicagel[14] wurden die besten Trennergebnisse mit den hydrophobierten Kieselsäurexerogelen erzielt.

Systematische Untersuchungen, optimale Arbeitsbedingungen zu finden, die von GROSSE-RUYKEN UND BOSHOLM[14] sowie SIEKIERSKI UND SOCHACKA[13,15] durchgeführt wurden, lieferten hinsichtlich der verwendbaren HDEHP-Menge unterschiedliche Ergebnisse. Ausserdem blieb die Frage offen, wie Materialeigenschaften des verwendeten Kieselsäuregels die Säulenqualität beeinflussen. Ziel vorliegender Untersuchung sollte sein, leistungsfähige Kolonnen hoher Kapazität zu erhalten, die auch den Einsatz von Makromengen Seltener Erden zur Lösung obengenannter Aufgaben gestatten.

EXPERIMENTELLES

Reagenzien

Wenn nicht besonders erwähnt, waren alle verwendeten Reagenzien vom Reinheitsgrad "zur Analyse".

Als Trägermaterial dienten verschiedene Silicagelsorten "für chromatographische Zwecke" von "Chimreaktivkomplekt" (UdSSR) sowie vom VEB "Feinchemie Eisenach". Dazu waren u.a. in Tabelle I zusammengestellte Daten gegeben.

TABELLE I

CHARAKTERISTIKA VERWENDETER SILICAGELSORTEN

r_{Por} = mittlerer Porenradius; V_{Por} = Porenvolumen; Poros. = Porosität; d_s = scheinbare Dichte; V_f = Feuchtigkeitskapazität in Gewichtsprozent bei relativer Luftfeuchtigkeit von 100%.

Sorte	*Oberfläche* (m^2/g)	r_{Por} (Å)	*Poros.* (%)	d_s (g/cm^3)	V_f (%)	V_{Por} (cm^3/g)
KSK Nr. 2	338	70	72.2	0.611	119	1.19
KSK Nr. 2.5	376	51.6	67.4	0.706	97.9	0.971
KSS Nr. 3	522	35.4	67.4	0.729	87.1	0.925
KSS Nr. 4	650	23.4	62.8	0.831	70.4	0.760
KSM-1 6s	624	11.6	44.1	1.218	34.8	0.362

Di(2-äthylhexyl)phosphorsäure (HDEHP) wurde nach PEPPARD *et al.*[16] gereinigt.

Trägerfreie radioaktive Europium- und Gadoliniumpräparate wurden aus Spallationsprodukten, die bei der Bestrahlung von Tantaltargets mit hochenergetischen Protonen entstehen, isoliert.

Säulenfüllung

Vorbereitung des Silicagels. Das Silicagel wurde gemahlen und durch Sedimentation fraktioniert. Verwendung fand der Anteil mit einer Korngrösse von 0.015 ± 0.004 mm. Die erhaltene Fraktion wurde 48 Std. bei 140° getrocknet und in einer gut verschlossenen Flasche nach dem Abkühlen portionsweise mit Dimethyldichlorsilan

(DDS) versetzt. Etwa 0.2 ml DDS/g Silicagel waren erforderlich, um eine ausreichende Hydrophobierung zu erreichen (bei Unterschuss wurden asymmetrische Elutionskurven und geringere Haltbarkeit der Säulenfüllungen beobachtet). Der Überschuss an DDS wurde durch dreistündiges Erwärmen auf 140° vertrieben.

Beladung des Silicagels mit HDEHP. Das hydrophobierte Silicagel wurde nach zwei verschiedenen Methoden mit HDEHP beladen.

(a) Direkte Beladung: Eine eingewogene Silicagelmenge wurde in einem Becherglas unter ständigem Rühren tropfenweise mit der jeweiligen HDEHP-Menge versetzt. Anschliessend wurde weiter durchmischt, bis wieder ein trockenes Pulver vorlag.

(b) Beladung aus verdünnter Lösung: Zu einer abgewogenen Menge hydrophobierten Silicagels wurde die notwendige Menge HDEHP mit soviel Chloroform zugesetzt, dass ein feuchtes Gemisch entstand. Anschliessend durchmischte man solange, bis das Lösungsmittel verdunstet war und sich wieder ein trockenes Pulver gebildet hatte. Die letzten Lösungsmittelreste liessen sich unter vermindertem Druck entfernen.

Füllung der Kolonnen. Auf Grund seiner stark hydrophoben Eigenschaften liess sich das beladene Silicagel nicht ohne weiteres mit Wasser benetzen. Deshalb wurde es in etwas 0.1 *N* HCl hineinzentrifugiert. Anschliessend wurde aufgeschlämmt. Am Silicagel anhaftende Luftbläschen konnten unter vermindertem Druck entfernt werden. Ein gut bereitetes Kolonnenmaterial muss körnig aussehen und darf keinerlei Flöckchenbildung zeigen.

Die Suspension des so vorbereiteten Sorbenten konnte wie ein gewöhnlicher Ionenaustauscher mit Hilfe eines Tropfers in die Trennsäule überführt werden. Verwendet wurde eine Glaskapillare von 2 mm Durchmesser und etwa 100 mm Länge, die mit einem Heizmantel versehen war.

Vor der ersten Beladung wurde die Kolonnenpackung durch Waschen mit 6 *N* HCl von Verunreinigungen befreit und durch Nachwaschen mit etwas 0.1 *N* HCl zur Beladung mit Seltenerdionen vorbereitet.

Arbeitsweise

Das Eu–Gd-Gemisch wurde, um eine möglichst schmale Beladungszone zu erhalten, in einigen Tropfen 0.1 *N* HCl auf die Kolonne gegeben. Nach dem Waschen mit einigen Tropfen 0.1 *N* HCl wurde der nicht mit Silicagel gefüllte Teil der Glaskapillare mit Elutionsmittel ausgespült. Anschliessend konnte mit der Elution begonnen werden. Die Flussrate betrug 1 Tropfen/Min, entsprechend 0.72 ml/cm^2·Min, die Temperatur 65°.

Das Eluat wurde tropfenweise auf Polyäthylenfoliestückchen von 25 × 25 cm^2 aufgefangen, zur Trockene eingedunstet und unter einem Fensterzählrohr ausgemessen.

Das freie Kolonnenvolumen V_0 liess sich ermitteln, indem man zu jedem Tropfen Eluat einen mit Methylorange angefärbten Tropfen Natronlauge mit einer Konzentration von $c/2$ (c—Konzentration des Elutionsmittels) gab. Nach Elution des Zwischenkornvolumens schlug die Farbe des Tropfens von Gelb nach Rot um.

Auswertung der Elutionskurven

Zur Beurteilung der Qualität einer Kolonnenfüllung wurde die "effektive Höhe eines theoretischen Bodens" H

$$H = \frac{l}{N} \tag{1}$$

(l = Länge der Kolonnenpackung, N = Anzahl der theoretischen Böden)

verwendet, die nach den von GLUECKAUF[17] für Ionenaustauscherharze entwickelten Vorstellungen aus einer dem Radius des Austauscherkornes proportionalen Mindesthöhe H_0 besteht, welche einen Zuwachs von H_T durch die begrenzte Geschwindigkeit des Stoffaustausches zwischen beiden Phasen sowie von H_L durch Diffusion in Längsrichtung der Kolonne erhält, so dass gilt:

$$H = H_0 + H_T + H_L \tag{2}$$

Die Anzahl der theoretischen Böden N berechnet sich aus den Elutionskurven gemäss Gleichung (3):

$$N_1 = 8 \ln 2 \left(\frac{V}{h}\right)^2 \tag{3}$$

(h = Breite des Peaks in seiner halben Höhe, V = Elutionsvolumen bis zum Peakmaximum)

Gleichung (3) liefert Werte für N_1, die vom Elutionsvolumen abhängig sind. Das erschwert, Angaben verschiedener Autoren über Bodenhöhen zu vergleichen. Deshalb wurde gewöhnlich der vom Elutionsvolumen unabhängige Ausdruck (Gleichung (4))[18] verwendet:

$$N_2 = 8 \ln 2 \frac{V(V - V_0)}{(h - h')^2} \tag{4}$$

(V_0 = freies Kolonnenvolumen, h' = Peakbreitenverzeichnung, graphisch aus der Abhängigkeit h von V zu ermitteln)

Wenn möglich, wurden die aus der Literatur entnommenen H_1-Werte in H_2-Werte umgerechnet. Dazu musste vorausgesetzt werden, dass benachbarte Lanthanide sich annähernd gleich verhalten.

ERGEBNISSE UND DISKUSSION

Einfluss der HDEHP-Beladung des Silicagels

Belädt man das Silicagel in direkter Weise (gemäss *a*) mit HDEHP, so nimmt bei den daraus hergestellten Kolonnen mit steigender spezifischer Beladung Q (ml HDEHP/g Silicagel) die theoretische Bodenhöhe zunächst allmählich ab, um nach Durchlaufen eines Minimums steil anzusteigen (siehe Tabelle II und Fig. 1). Das gilt sowohl für die Silicagelsorten KSK Nr. 2, KSK Nr. 2.5 und KSS Nr. 3 als auch, wie bereits GROSSE-RUYKEN UND BOSHOLM[14] fanden, für das Silicagel "Eisenach".

In Fig. 2 ist als Beispiel die Eu–Gd-Trennung beim Minimum der Bodenhöhe für KSK Nr. 2 (Q = 0.8 ml/g) angeführt.

Das Minimum der Bodenhöhe wird bei derjenigen HDEHP-Menge erhalten, die

vom Silicagel gerade noch aufgesaugt wird, so dass wieder ein völlig trockenes Pulver entsteht, welches auch in wässeriger Suspension noch körnig vorliegt. Bei höheren Q-Werten lassen sich grössere flöckchenhafte Aggregate beobachten.

Interessant ist, dass mit steigender spezifischer Beladung bis zum Minimum der Bodenhöhe hin das Produkt von $H \cdot Q$ konstant ist (siehe Fig. 1). Bei gegebener Säulenlänge ist also die Anzahl der theoretischen Böden der HDEHP-Menge direkt proportional. Das lässt sich erklären, wenn man annimmt, dass die Dialkylphosphor-

TABELLE II

ABHÄNGIGKEIT DER THEORETISCHEN BODENHÖHE VON DER SPEZIFISCHEN BELADUNG MIT HDEHP

Silicagel KSK Nr. 2; Kolonne 1.98 mm Durchmesser; 150 mg HDEHP–Silicagel-Gemisch; Elutionsmittel 0.62 bis 1.00 N HCl

Beladungsart	*Direkt*						*Aus $CHCl_3$-Lsg.*	
ml HDEHP/g Gel	*0.2*	*0.4*	*0.6*	*0.8*	*0.9*	*1.0*	*0.1*	*0.2*
Säulenlänge (mm)	100	92	85	80	74	64	105	100
N_{1Gd}	266	405	570	870	377	88	945	1000
N_2	260	405	655	823	397	76	1495	1420
H_2 (mm)	0.38	0.22	0.13	0.097	0.19	0.8	0.07	0.07

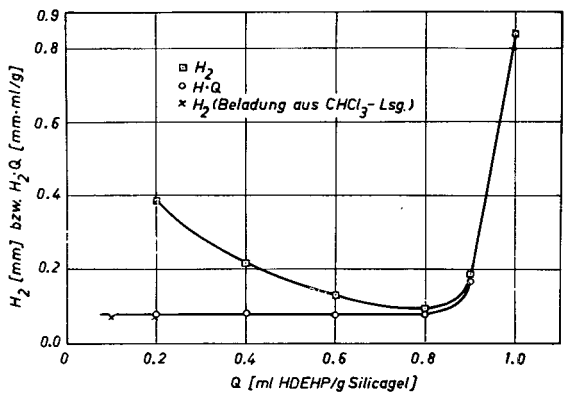

Fig. 1. Abhängigkeit der theoretischen Bodenhöhe von der spezifischen Beladung des Silicagels KSK Nr. 2 mit HDEHP (Versuchsbedingungen siehe Tabelle II).

säure beim Zutropfen zum Silicagel von den gerade in der Nähe der Eintropfstelle befindlichen Silicagelkörnern aufgesaugt wird. Wenn m dieser HDEHP-getränkten Teilchen, die nach dem Einschlämmen statistisch über die gesamte Kolonnenlänge verteilt sind, nun einen theoretischen Boden darstellen, wird bei Verdoppelung der HDEHP-Menge auch die Bodenzahl verdoppelt werden. Sind alle Poren aller Silicagelkörner mit HDEHP gefüllt, wird die maximale Bodenzahl erreicht. Bei weiterer Erhöhung der HDEHP-Menge findet die Flüssigkeit im Innern der Körnchen keinen Platz mehr und bedeckt die äussere Oberfläche. Dabei kommt es zum Zusammenkleben mehrerer Teilchen und somit effektiv durch Kornvergröberung zu einem Anwachsen der theoretischen Bodenhöhe und damit Sinken der Bodenzahl.

Aus Gesagtem folgt auch, dass, wenn es gelingt, die HDEHP gleichmässig über

alle Silicagelkörner zu verteilen, die Bodenhöhe mit steigender spezifischer Beladung Q zunächst annähernd konstant sein müsste, um nach Füllung aller Poren stark anzusteigen. Das wird tatsächlich durch Beladung aus verdünnter Lösung (gemäss *b*) erreicht (vgl. Fig. 1 und Tabelle II).

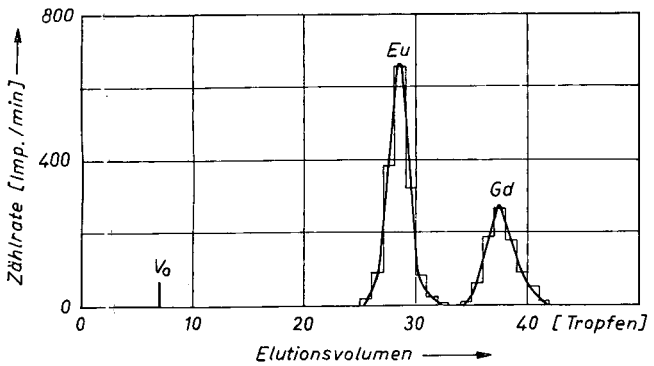

Fig. 2. Eu–Gd-Trennung. Silicagel KSK Nr. 2; Q = 0.8 ml HDEHP/g; Kolonne: 1.98 mm Durchmesser × 80 mm mit 150 mg HDEHP-Silicagelgemisch; Elutionsmittel 1.00 N HCl; Flussrate 1 Tropfen/Min; Temperatur 65°.

Letztere Beobachtung machten auch SOCHACKA UND SIEKIERSKI[13] bei Verwendung von Kieselgur als Trägermaterial. Die unterschiedlichen Ergebnisse in den Arbeiten Ref. 13 und 14 sind also nicht durch die verschiedenen Trägermaterialien, sondern durch unterschiedliche Beladungstechniken bedingt.

Bezeichnen wir die maximale HDEHP-Menge, bei der noch minimale Bodenhöhen erzielt werden, als "optimale" HDEHP-Menge O und berücksichtigen, dass 1 ml HDEHP 3.0 Milliäquivalente austauschbarer Wasserstoffionen enthält, so erhält man in Tabelle III aufgeführte Kapazitäten für Säulen aus verschiedenen Silicagelsorten.

TABELLE III

OPTIMALKAPAZITÄTEN FÜR VERSCHIEDENE TRÄGERMATERIALIEN

Trägermaterial	*Korngrösse* (*μm*)	*O* *ml HDEHP/ml Säulenfüllung*	*Kapazität* *mVal/ml Säulenfüllung*	H_2 (*mm*)	*Lit.*
Silicagel:					
KSK Nr. 2	15	0.27	0.81	0.1	diese
"Eisenach"	15	0.24	0.72	0.1	Arbeit
KSK Nr. 2.5	15	0.21	0.63	0.1	
KSS Nr. 3	15	0.20	0.60	0.1	
KSS Nr. 4	15	0.18*	0.54*	1.0	
KSM-1 6s	15	0.09*	0.27*	1.0	
"Hyflo Supercel"	15	0.11	0.33	0.3	13
"Corvic"	120	0.08**	0.24**	2	9
Teflon	1	0.3**	0.9**	2	8

* Kein Optimum.
** Keine Optimierungsversuche.

Das grösste Aufnahmevermögen besitzen die grossporigen Silicagelsorten. Die optimale HDEHP-Menge für Kieselgur ist über zweimal geringer als für Silicagel. Mit Teflon erreicht man zwar eine relativ hohe Kapazität, muss sich aber mit nur sehr grossen Bodenhöhen zufriedengeben.

Einfluss der Eigenschaften des verwendeten Silicagels

Die Tatsache, dass sich die Bodenhöhe bei steigender spezifischer Beladung bis zur optimalen Beladung relativ wenig ändert, lässt bereits vermuten, dass die Grösse der inneren Oberfläche eines porösen Trägermaterials keinen entscheidenden Einfluss auf die Trennleistung der Kolonne haben kann, obwohl unter Annahme einer gleichmässigen Verteilung der organischen Phase auf der Oberfläche die Grösse der Kontaktfläche mit der mobilen Phase bei steigendem Q abnehmen und schliesslich bei gefüllten Poren gleich der äusseren Oberfläche des Silicagelkornes sein müsste.

Versuche mit Silicagelsorten verschiedener innerer Oberflächen bestätigten diese Annahme. Wie aus Tabelle IV hervorgeht, ergeben sich für KSK Nr. 2, KSK Nr. 2.5 und KSS Nr. 3 bei $Q = 0.1$ ml/g innerhalb der Fehlergrenzen gleichwertige Bodenhöhen, während KSM-1 6s schlechte Werte liefert.

TABELLE IV

ABHÄNGIGKEIT DER BODENHÖHE VON DER SILICAGELSORTE

Kolonne 1.98 mm Durchmesser; 150 mg Silicagel mit 0.015 ml HDEHP; Beladung aus $CHCl_3$; Trennung Ce–Eu–Gd; Elutionsmittel 0.60 N HCl.

Silicagelsorte	*KSK Nr. 2*	*KSK Nr. 2.5*	*KSS Nr. 3*	*KSS Nr. 4*	*KSM-1 6s*
Säulenlänge (mm)	105	95	79	70	55
N_2	1490	1280	1110	73	59
H_2 (mm)	0.070	0.074	0.071	0.96	0.93
Oberfläche (m^2/g)	338	376	522	650	624
Porenvolumen (cm^3/g)	1.19	0.971	0.925	0.760	0.362
Mittl. Porenradius (Å)	70	51.6	35.4	23.4	11.6

Ein herausfallendes Verhalten zeigt KSS Nr. 4. Bei geringen Q-Werten werden Ce, Eu und Gd in einem einzigen, sehr breiten Peak eluiert, ohne getrennt zu werden. Erst bei Erhöhung der HDEHP-Menge auf 0.4 ml/g ist eine normale, wenn auch schlechte Trennung zu beobachten.

Der Vergleich der beobachteten Bodenhöhen mit den gegebenen Charakteristika der Silicagelsorten ergibt, dass sowohl die innere Oberfläche als auch der Porenradius bis herab zu 35 Å keinen Einfluss auf die Bodenhöhe haben.

Das Verhalten von KSS Nr. 4 lässt sich deuten, wenn man annimmt, dass sein mittlerer Porenradius ($\leqslant$ 23 Å, da durch Silikonisierung der Oberfläche verringert) etwa der Grösse der extrahierten $Ln[H(DEHP)_2]_3$-Molekeln entspricht, so dass deren ungehinderte Diffusion innerhalb der Pore erschwert ist. Infolgedessen kann sich keine Gleichgewichtsverteilung einstellen. Bei der Elution wird dann jeweils nur das am Porenausgang befindliche Seltenerdion unabhängig von den tiefer befindlichen Ionen an die wässerige Phase abgegeben. Dadurch erfolgt keine Trennung. Ist die HDEHP-Menge dagegen so gross, dass nicht nur die Poren ganz ausgefüllt sind, sondern auch ein merklicher Anteil der Dialkylphosphorsäure sich auf der äusseren

Oberfläche des Silicagelkornes befindet, kann in diesem Anteil der normale Austausch vonstatten gehen, und es erfolgt eine, wenn auch unvollständige Trennung der Elemente.

Beim silikonisierten Silicagel KSM-1 6s sind die Poren so klein, dass der Seltenerdkomplex nicht mehr hineingelangen kann und der Trennvorgang nur auf der äusseren Oberfläche abläuft. Die grossen Bodenhöhen sind auf Kornvergröberung durch Zusammenkleben mehrerer Teilchen zurückzuführen.

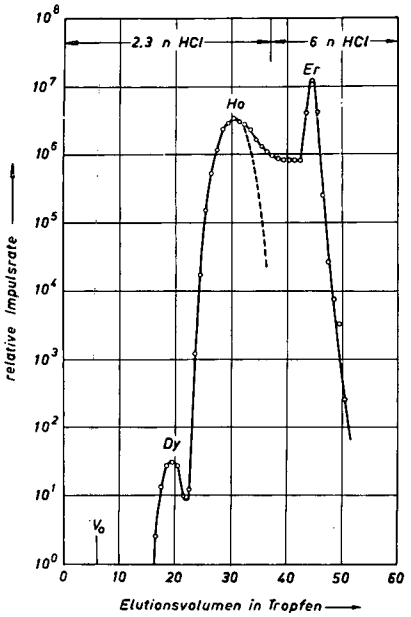

Fig. 3. Abtrennung der radioaktiven Tochterprodukte aus einem Gemisch kurzlebiger, neutronendefiziter Erbiumnuklide. Kolonne 2 mm Durchmesser × 65 mm; Temperatur 65°; Flussrate 1 Tropfen/Min).

Unter Berücksichtigung der Ergebnisse des vorhergehenden Abschnittes erlauben die durchgeführten Versuche den Schluss, dass die HDEHP-Schichtdicke (verschiedener Füllungsgrad der Poren) die Bodenhöhe nicht wesentlich beeinflusst. Dagegen führen nennenswerte HDEHP-Mengen auf der äusseren Oberfläche des Kornes durch Zusammenkleben mehrerer Teilchen zu einer effektiven Kornvergröberung und damit auch zu einer Verminderung der Kontaktfläche zwischen mobiler und stationärer Phase. Beides bewirkt eine Vergrösserung der Bodenhöhe (daher auch die grossen Bodenhöhen für nichtporöse Trägermaterialien (vgl. Tabelle III). Diese Ergebnisse werden verständlich, wenn man berücksichtigt, dass die Geschwindigkeit des Phasendurchtritts für Seltenerdionen etwa um zwei Grössenordnungen geringer ist als ihre Diffusionsgeschwindigkeit innerhalb der Phasen selbst[19].

Aus der relativen Unempfindlichkeit der Bodenhöhe gegenüber der HDEHP-Menge bis zur optimalen Beladung hin folgt ferner, dass die eingesetzte Dialkylphosphorsäure nicht gleichmässig über die gesamte Oberfläche verteilt ist, sondern die Poren lediglich zu einem bestimmten Grade füllt. Als Kontaktfläche zwischen

mobiler und stationärer Phase steht also im wesentlichen die Summe der Querschnitte der an der Kornoberfläche befindlichen Poren zur Verfügung. Sie wird umso grösser, desto kleiner der Korndurchmesser ist.

Bei der Auswahl eines Trägermaterials für die Extraktionschromatographie von Seltenerden mit HDEHP sind also poröse Stoffe vorzuziehen, wobei praktisch nur zwei Grössen zu beachten sind. Ein nicht zu kleiner Porenradius ($\geq$ 35 Å) ist notwendig, damit die kinetischen Vorgänge des Extraktionsprozesses ungestört ablaufen können, und eine möglichst grosse Porosität gestattet es, Kolonnen hoher Kapazität zu erhalten. Während Kolonnen mit Optimalkapazität durch direktes Zutropfen von HDEHP zum Silicagel hergestellt werden können, empfiehlt sich für Säulenfüllungen geringerer Kapazität die Beladung aus verdünnter Lösung.

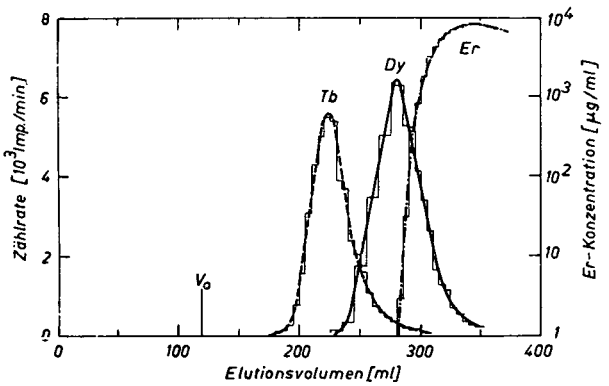

Fig. 4. Isolierung von Spurenverunreinigungen aus 2 g Erbium. Kolonne 26 mm Durchmesser × 410 mm; 100 g Silicagel "Eisenach" mit 60 ml HDEHP; Elutionsmittel 2.5 *N* HCl; Flussrate 0.8 ml/cm²Min; Temperatur 40°.

Die Leistungsfähigkeit der hergestellten HDEHP-Säulen sei an folgenden Beispielen belegt:

Für den Nachweis einer kurzlebigen Komponente in einem Gemisch neutronendefiziter Erbiumnuklide sollten die Dysprosium-Tochteraktivitäten abgetrennt werden. Wie Fig. 3 zeigt, gelang die Abtrennung des Dy aus einem über 10^6-fachen Überschuss der Nachbarelemente. Zu berücksichtigen ist hier noch, dass infolge veränderter Beladungstechnik die Länge der Beladungszone bereits etwa 15 % der Gesamtlänge der Kolonne betrug. Die scheinbar schlechte Ho–Er-Trennung ist auf die ständige Nachbildung von radioaktivem Holmium aus Erbium zu erklären.

Durch die gestrichelte Linie ist der theoretische Kurvenverlauf, wie er für reines Holmium in Modellversuchen erhalten wurde, angegeben.

In einem anderen Fall gelang es, aus einem Präparat neutronendefiziter Samariumnuklide die Neodym- bzw. Promethium-Tochternuklide nach 7 bzw. 12 Min zu isolieren[4].

Die hohe Kapazität der Säulen gestattet auch den Einsatz von Makromengen Seltener Erden.

Fig. 4 zeigt die Isolierung von radioaktiv indizierten Spurenverunreinigungen aus 2 g Erbium. Innerhalb einer Stunde können 44 % der Dysprosium- und 78 % der Terbiumspuren sowie alle leichteren Seltenerden mit noch grösserer Ausbeute erhalten

werden. Die benutzten Kolonnen arbeiten sehr gut reproduzierbar, so dass auf diese Weise Serienanalysen reinster Yttriumpräparate zur Produktionsüberwachung durchgeführt werden[5].

DANK

Abschliessend sei dem Autor gestattet, Herrn Kandidat der chemischen Wissenschaften W. A. CHALKIN und Herrn Dozent Dr.rer.nat.habil. H. GROSSE-RUYKEN für die grosszügige Förderung der experimentellen Untersuchungen und wertvolle Diskussionen zu danken. Herrn ZOY GYN SIK bin ich für die Durchführung zahlreicher Experimente verbunden.

ZUSAMMENFASSUNG

Versuche zur Verwendung von Silicagel als Trägermaterial für die extraktionschromatographische Trennung von Seltenerden mit Di(2-äthylhexyl)phosphorsäure (HDEHP) ergaben Kolonnen mit einer Kapazität von 0.8 mVal/ml Kolonnenfüllung und einer theoretischen Bodenhöhe von $H \leq 0.1$ mm. H ist nahezu unabhängig sowohl von der Silicagelsorte, wenn ihr mittlerer Porenradius $\geqslant 35$ Å ist, als auch von der verwendeten HDEHP-Menge, solange wie die Dialkylphosphorsäure noch vom Silicagel aufgesaugt wird, so dass wieder ein trockenes Pulver entsteht. Trennbeispiele demonstrieren die Leistungsfähigkeit der erhaltenen Kolonnen.

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CHROM. 3803

QUANTITATIVE ANALYSIS OF POLYNITROAROMATIC COMPOUNDS IN COMPLEX MIXTURES BY COMBINATION THIN-LAYER CHROMATOGRAPHY AND VISIBLE SPECTROMETRY

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(Received September 20th, 1968)

SUMMARY

A quantitative method of analysis for polynitroaromatic compounds in complex mixtures by combination thin-layer chromatography and visible spectrometry is described. Examples of the utility of the method for thermal decomposition studies and product analysis in complex reaction mixtures are presented.

INTRODUCTION

Although reasonable success has been achieved for the quantitative thin-layer chromatographic analyses of certain aliphatic nitro compounds in explosive mixtures by the spot area and weight method[1], spot densitometry[2], combination TLC and U.V. methods[3,4], relatively little has been done in the field of the polynitroaromatic compounds. An excellent summary of the thin-layer chromatography of explosives has recently been presented by KIRCHNER[5]. A spot area and weight method[6] for the analyses of 1,3-diamino-2,4,6-trinitrobenzene and 2,2',4,4',6,6'-hexanitrostilbene[7] after subjection to both thermal heating and nuclear irradiation has only recently been described. This latter method involved visualizing the developed zones with methanolic potassium hydroxide and photographing the resulting red spots with transparency film. In order to obtain the desired areas, the transparencies were projected on graph paper, traced, and finally measured with a compensating polar planimeter. The method depends strongly on excellent control of experimental conditions, is somewhat time consuming, but has been shown to have an accuracy within 5 %. Ideally, in terms of both speed and accuracy, one would desire a quantitative method of analysis based on the TLC separation of the desired polynitroaromatic compound, followed by physical or chemical analysis of the pure compound.

A method has been recently developed by GLOVER AND KAYSER[8] for the quantitative spectrophotometric analysis of polynitroaromatic compounds as their "Meisenheimer" complexes in ethylenediamine (EDA)–dimethylsulfoxide (DMSO) solutions. The method is especially suitable for microgram quantities of materials which can be applied on a single TLC plate, and provides a relatively rapid means for the quantita-

tive separation and analysis of polynitroaromatic compounds occurring in complex mixtures. We wish to report this procedure and illustrate its usefulness in several applications.

EXPERIMENTAL

Preparation of thin-layer acid-washed plates

Since considerable blanks in the visible were obtained from the DMSO extractions of the Silica Gel G support and subsequent treatment with EDA, it was necessary to prepare special, acid-washed plates according to the following. Thirty grams of Silica Gel G, according to Stahl, containing 13 % $CaSO_4$ (Brinkman Inst., Co.) was thoroughly mixed with 200 ml of acetone containing 2 ml concentrated HCl for 10 min. The slurry was suction-filtered on a Buchner funnel, and the filter cake washed with five 30 ml portions of acetone, sucked dry, and dried overnight at 100°. Thirty grams of this acid-washed Silica Gel G, when slurried with 50 ml distilled water, made approximately ten 10 × 20 × 0.03 cm coated TLC plates with a Camag applicator. Plates were dried for 1 h at 125°, and stored in a closed container. Negligible blanks were obtained with this specially prepared Silica Gel G.

Developing solvents

A mixed solvent system consisting of toluene–benzene–*n*-pentane–acetone in the ratio 40:40:20:5, by volume, was used for the development of all the polynitroaromatic compounds studied.

Visualizing reagent

Developed zones containing the polynitroaromatic compounds were visualized and located by spraying the TLC plate with a mixture of DMSO and EDA, (1:1) by volume (see Table I).

TABLE I

R_F VALUES AND COLORS OF VARIOUS POLYNITROAROMATIC COMPOUNDS ON SILICA GFL G PLATES VISUALIZED WITH DMSO–EDA

Compound	*R_F value*	*Color*
1,3,5-Trinitrobenzene	0.75	Red
3,5-Dinitroiodobenzene[9]	0.85	Purple
2,2′,4,4′-Tetranitrobiphenyl[10]	0.36	Violet
2,2′,6,6′-Tetranitrobiphenyl[11]	0.60	Blue-purple
3,3′,5,5′-Tetranitrobiphenyl[12]	0.62	Purple
3′,5′,2,4,6-Pentanitrobiphenyl[a]	0.54	Red

[a] Prepared by Dr. HOWARD E. RUSKIE of this Laboratory from 3,5-dinitrobromobenzene and picryl chloride in a mixed Ullman reaction.

Analytical procedure

About 0.1 g of the polynitroaromatic compound to be analyzed was accurately weighed, dissolved in acetone, and transferred into a 25 ml volumetric flask. Ten to thirty microliter aliquots of these solutions were spotted on a 10 × 20 × 0.03 cm acid-washed, Silica Gel G TLC plate in a series of two to six separate spots with the aid of a plastic spotting guide. A Hamilton 50 μl (No. 705) or 5 μl (No. 7005) syringe

was used to measure accurately the aliquots. The usual analytical care was exercised in filling and applying the aliquots on the TLC plate. For convenience in locating the developed zones, a "guide" spot of the polynitroaromatic compound to be determined was spotted separately at the origin at the same time. The spotted plate was allowed to air dry for about 1 min, then developed by an ascending technique in a 16 × 21 × 26 cm glass, rectangular developing chamber fitted with a ground glass cover.

At the completion of the chromatogram, or when the solvent front had traveled a distance of between 10 and 14 cm, the plate was removed from the developing chamber and allowed to air dry in a vacuum hood for about 10 min. The sample side of the TLC plate was then completely covered with a clean glass plate, while the "guide" side was left exposed. A small line was drawn in the silica gel at the edge of the protecting cover plate to prevent contamination of the sample side with spray reagent. Visualization of the "guide" spot was accomplished by spraying the exposed area with EDA–DMSO (1:1, by volume). The protecting cover plate was removed, the zone containing the polynitroaromatic compound marked, and scraped off the plate with a clean flat spatula into a small 5–10 ml beaker prior to extraction with DMSO.

The silica gel containing the polynitroaromatic compound was extracted at least four times with DMSO in 0.5 to 1.0 ml portions. For repetitive extractions and analyses, it was most convenient to suction filter the DMSO extracts from the silica gel through a 2 ml, medium porosity, sintered glass funnel directly into a calibrated, 10 ml, glass test tube (Fig. 1). After the necessary amount of EDA was added, the total volume was adjusted to 10 ml with DMSO, and the solution concentration was determined spectrophotometrically in the visible (Table II). Absorbancy readings were made with a Beckman spectrophotometer, model DU, using quartz cells with a 1 cm light path.

The EDA–DMSO solutions of 1,3,5-trinitrobenzene, 3,5-dinitroiodobenzene, 3,3′,5,5′-tetranitrobiphenyl and 3′,5′,2,4,6-pentanitrobiphenyl were stable for periods up to a half hour, while solutions of 2,2′,6,6′-tetranitrobiphenyl were stable for periods up to 5 min. Extrapolation of the straight line resulting from a plot of absorbance of the 2,2′,4,4′-tetranitrobiphenyl complex in EDA–DMSO solution *versus* time, to time zero, was necessary for the quantitative analysis of this compound. Beer's law was found to be valid over the entire concentration range investigated, 0.7×10^{-5} *M* to 4.0×10^{-5} *M* for all compounds.

TABLE II

SPECTRAL DATA. VISIBLE MAXIMA AND EXTINCTION COEFFICIENTS OF POLYNITROAROMATIC "MEISENHEIMER" COMPLEXES IN DMSO–EDA SOLUTIONS

Compound	*Milliliters EDA/10 ml solution*	*Max. in mμ; (ε)*
1,3,5-Trinitrobenzene	0.1	540 (15,500); 455 (29,100)
3,5-Dinitroiodobenzene	1.0	535 (24,800); 363 (15,450)
2,2′,4,4′-Tetranitrobiphenyl	2.0	545 (23,800); 355 (10,700)
2,2′,6,6′-Tetranitrobiphenyl	5.0	550 (20,100); 350 (10,800)
3,3′,5,5′-Tetranitrobiphenyl	1.0	550 (19,900); 450 (13,400)
3′,5′,2,4,6-Pentanitrobiphenyl	0.1	555 (13,600); 455 (29,100)

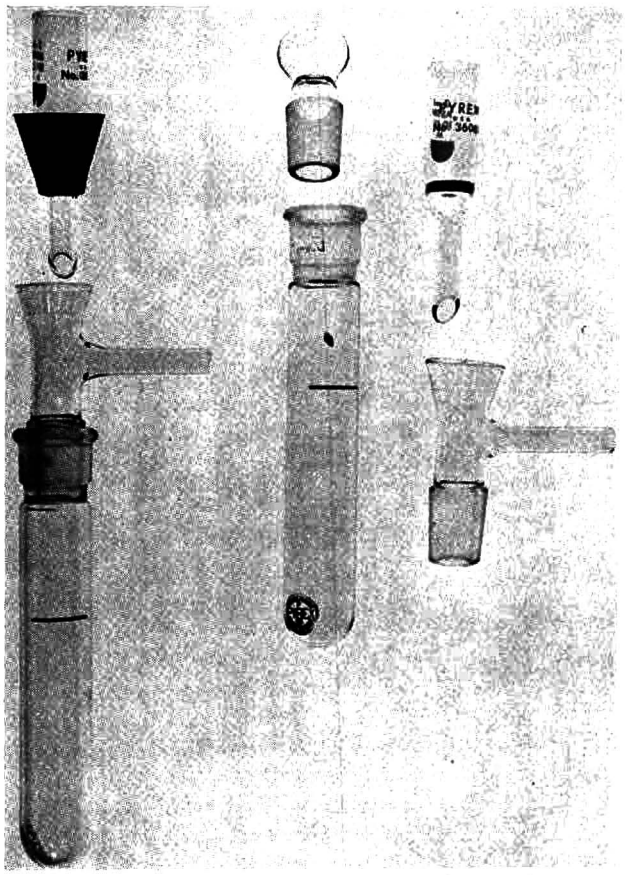


Fig. 1. Small-scale filtration apparatus for TLC extractions.

TABLE III

THIN-LAYER CHROMATOGRAPHIC–VISIBLE SPECTROPHOTOMETRIC ANALYSES OF KNOWN QUANTITIES OF POLYNITROAROMATIC COMPOUNDS

	Micrograms applied[a]		*Micrograms found*[b]	
	Plate 1	*Plate 2*	*Plate 1*	*Plate 2*
Single compounds				
1,3,5-Trinitrobenzene	100.9	100.9	99.2	99.2
3,3′,5,5′-Tetranitrobiphenyl	83.6	125.4	85.5	126.6
2,2′,6,6′-Tetranitrobiphenyl	130.6	87.2	130.2	87.0
2,2′,4,4′-Tetranitrobiphenyl	62.8	125.6	62.2	123.2
Mixture				
1,3,5-Trinitrobenzene	100.9	60.5	98.2	59.2
3′,5′,2,4,6-Pentanitrobiphenyl	53.3	32.0	51.8	30.4
2,2′,6,6′-Tetranitrobiphenyl	101.8	61.1	97.8	57.8

[a] Stock acetone solutions contained approximately 0.1 g of compound accurately weighed into a 25 ml volumetric flask.

[b] After development and extraction of silica gel zone containing compound.

RESULTS AND DISCUSSION

Analyses of single compounds and synthetic mixtures

To test the accuracy and validity of the thin-layer analytical procedure for the determination of polynitroaromatic compounds, analyses were performed as outlined on solutions of known concentrations for both single compounds and a three-component mixture. The results are shown in Table III. To obtain maximum resolution and avoid tailing of sample, each spot applied was made no larger than 5 μl and contained no more than 20 μg of compound. Routinely, the extinction coefficients occurring at maxima between 535 mμ and 555 mμ were used for the analyses (Table II), while those maxima between 350 and 455 mμ provided additional identification and verification. As Table III shows, the accuracy is 1–2 % for single compounds and 2–5 % for a three-component mixture analyzed simultaneously on a single plate.

APPLICATIONS

Thermal degradation of explosives

The thermal degradation of explosives has most commonly been assessed by total gas evolution[13]. Since the decomposition processes forming these gases are understandably extremely complex, perhaps a more reliable measure of stability would be an analysis of residual explosive after a specified heat treatment. The thermal degradation of 2,2',6,6'-tetranitrobiphenyl (m.p. 219–220°) in the melt at 260° is illustrative (Table IV). Samples of 2,2',6,6'-tetranitrobiphenyl were weighed into 10 ml pyrex glass tubes which were evacuated and sealed prior to heating. Analysis of residual 2,2',6,6'-tetranitrobiphenyl was accomplished by dissolving or extracting the entire contents of the opened tube with acetone and quantitatively transferring to a 25 ml volumetric flask. Aliquots of this solution were spotted on TLC plates, the plates developed, and zones containing the chromatographed 2,2',6,6'-tetranitrobiphenyl extracted into 10 ml volumetric flasks and analyzed spectrophotometrically. The results obtained (Table IV) indicate that the 2,2',6,6'-tetranitrobiphenyl decomposed almost linearly at a rate of 0.9 %/h with an average deviation of 0.1 %/h as a neat liquid at 260° to about 95 % decomposition.

TABLE IV

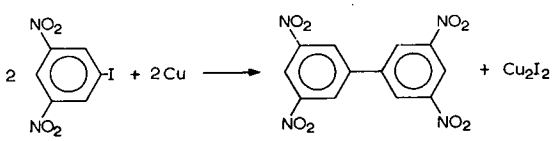
THERMAL DECOMPOSITION OF 2,2',6,6'-TETRANITROBIPHENYL AS A NEAT LIQUID AT 260°

Initial tetranitrobiphenyl (g)	*Heating period (h)*	*Residual tetranitrobiphenyl (g)*[a]	
		Analysis 1	*Analysis 2*
0.1046	8.03	0.0980	0.0968
0.1061	15.42	0.0897	0.0850
0.1040	21.03	0.0840	0.0828
0.1045	68.50	0.0427	0.0449
0.1026	100.00	0.0161	0.0182
0.1039	138.20	0.0051	0.0055

[a] Calculated from: absorbancy/ε × 10 ml/aliquot (ml) × 25 ml/1000 ml × mol.wt tetranitrobiphenyl.

Analyses of a complex reaction mixture. Ullman synthesis of 3,3′,5,5′-tetranitrobiphenyl

The research synthetic organic chemist would often like to be able to analyze a difficultly separable reaction mixture for the desired product without gross isolation of product. Not only is isolation of product time consuming, but more important, the isolation is never complete. How much is actually isolated often depends largely on the chemist's patience and technique. The Ullman synthesis of 3,3′,5,5′-tetranitrobiphenyl serves as another example of the utility of the TLC–spectrophotometric method of analysis for polynitroaromatic compounds.



Twenty grams (0.0670 moles) of 3,5-dinitroiodobenzene and 12.0 (0.189 moles) Cu (U.S. Bronze Powder Incorp., Venus Brand, Natural Copper Fine, No. 44-F) in 150 ml dry nitrobenzene were placed in a three-necked, round-bottom flask equipped with a mechanical stirrer, reflux condenser, and thermometer. The mixture was heated for a total of 2.5 h at 208°, and appeared muddy, black-brown at the end of this period. After filtration of the inorganic salts, excess copper, and washing the filter cake with methanol, 200 ml of a dark-brown filtrate was obtained. Qualitative TLC indicated that the filtrate contained at least ten materials including the desired 3,3′,5,5′-tetranitrobiphenyl and some unreacted 3,5-dinitroiodobenzene. Simultaneous analyses of the 3,3′,5,5′-tetranitrobiphenyl and the 3,5-dinitroiodobenzene were accomplished as follows. A 2 ml aliquot of the dark-brown filtrate was diluted to 10 ml with acetone, and 3 ml of this solution was further diluted to 10 ml. Then, 15 μl and 30 μl aliquots of this final solution were spotted on two thin-layer plates and developed with benzene–toluene–*n*-pentane (50:10:5, by volume). The location, isolation, extraction, and spectrophotometric analyses of the zones containing the 3,3′,5,5′-tetranitrobiphenyl and the 3,5-dinitroiodobenzene were accomplished by the general analytical procedure already given. The results of these analyses indicated the formation of 5.35 $\pm$ 0.01 g (0.0160 moles) of the 3,3′,5,5′-tetranitrobiphenyl and 2.34 $\pm$ 0.03 g (0.00796 moles) of the starting 3,5-dinitroiodobenzene was still left unreacted.

On the basis of these TLC–spectrophotometric analyses, a 53 % yield of the 3,3′,5,5′-tetranitrobiphenyl was actually formed under the conditions of the experiment and was based on the amount of 3,5-dinitroiodobenzene reacted. But, in the normal synthetic procedure after steam distillation, solvent extraction, and several carbon treatments followed by two recrystallizations, only 2.0 g (0.0060 moles) of the 3,3′,5,5′-tetranitrobiphenyl was actually isolated and represented a 20 % yield on the same basis. A 13 % yield has been reported[12] for the formation of the 3,3′,5,5′-tetranitrobiphenyl from 3,5-dinitroiodobenzene in the absence of solvent. These results clearly indicate the greater efficiency of separation by TLC as compared to conventional techniques.

ACKNOWLEDGEMENTS

The authors wish to express their sincere appreciation to the members of the Advanced Chemistry Division for their many helpful suggestions and especially to D. J. GLOVER, J. C. DACONS and J. M. ROSEN. Thanks are also due to B. O. WILKERSON, who carefully prepared the thin-layer chromatographic plates, and to J. D. UPTON, JR., who helped design and made the filtration apparatus.

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CHROM. 3790

CHROMATOGRAPHY OF ISOMERIC PEROXYBENZOIC ACIDS

II. THE THIN-LAYER CHROMATOGRAPHIC SEPARATION MECHANISM OF ISOMERIC MONO- AND DISUBSTITUTED PEROXYBENZOIC ACIDS ON VARIOUS POLYAMIDE AND CELLULOSE ADSORBENTS

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SUMMARY

The migration behaviour of several mono- and disubstituted peroxybenzoic acids has been studied on seven thin-layer chromatographic adsorbents. Some effects of the molecular structure of the peroxy acids on their chromatographic behaviour and the interactions between the stationary or the mobile phase and peroxy acids are discussed.

INTRODUCTION

In connection with our research on organic peroxy acids and related materials, we have recently shown that positional isomerism of substituted peroxybenzoic acids could have an appreciable effect on the mobilities of such isomers on paper chromatography[1]. In an attempt to gain further insight into the factors involved in these variations between positional isomers, we have investigated the migration behaviour as well as the nature of hydrogen bonding in the TLC of a series of isomeric mono- and disubstituted peroxybenzoic acids on a variety of thin-layer adsorbents.

EXPERIMENTAL

The mono- and disubstituted peroxybenzoic acids were synthesized by oxidizing small portions of parent aromatic acids with 95 % hydrogen peroxide in methanesulphonic acid according to the method of SILBERT *et al.*[2]. The products were purified by recrystallization from a mixture of hexane and diethyl ether, and in a few cases, pure compounds were obtained by sublimation *in vacuo*. Purity and identity of the individual isomers was checked by the usual physical and chemical methods.

MATERIALS AND METHODS

The following commercial products (obtained from Macherey-Nagel & Co., Düren, Germany) were used as adsorbents: MN-Cellulose Powder 300 HR, MN-Cellulose Powder 300 Ac/*ca.* 10 %, MN-Cellulose Powder 300 Ac/*ca.* 20 %, MN-Cellulose Powder 300 Ac/*ca.* 30 %, MN-Cellulose Powder 300 Ac/*ca.* 40 %, MN-Polyamide TLC 6/6, MN-Polyamide TLC 6 and MN-Polyamide TLC 11.

Thin-layer plates (5 × 20 cm and 20 × 20 cm) were prepared by one of the procedures outlined below:

(A) Acetylated cellulose adsorbents were applied as a slurry prepared as follows: 15 g of acetylated cellulose (for five plates) was mixed with a few ml of 95 % ethanol in a mortar, and 60 ml of ethanol were added with stirring in an electric mixer for approx. 1 min. The slurry obtained was applied to the plates in the usual way, using Desaga equipment. The plates were dried in air for 24 h before use.

(B) 15 g of Polyamide MN-6 and Polyamide MN-6/6 were homogenized with about 65 ml of water for 1 min in an electric mixer. Polyamide MN-11 was homogenized with 55 ml of methanol, and the slurry was applied to the plates, which were dried in air for 24 h before use.

The samples were applied as dilute (about 4–5 %) solutions in ethyl acetate, in quantities of 20 μg, by means of a micropipette. After applying samples, the plates were allowed to stand in air for half an hour before chromatography. The solvents were commercial products "for chromatography." The plates were developed in well-sealed glass tanks saturated with solvent vapours of a composition suitable for the TLC of the compounds being examined. When the solvent front had reached the 10-cm line on the layer—the time of development was between 20 and 30 min—the plates were removed and the spots located with an aqueous acetic acid solution of potassium iodide or with an aqueous acetic acid solution of *p*-aminodimethylaniline hydrochloride[1].

The temperature around the chromatographic systems was 21° ± 1°, and the relative air humidity 50–60 %.

RESULTS AND DISCUSSION

The TLC data for various isomeric mono- and disubstituted peroxybenzoic acids on seven different polyamide and cellulose adsorbents are summarized in Table I.

Thin-layer chromatography on Silica Gel G

Attempts have been made to obtain some thin-layer chromatographic data concerning the peroxy acids examined on silica layers (Silica Gel G, according to STAHL, Merck). Unfortunately, partial or complete decomposition was observed during the migration, and this was even more pronounced when developing solvents did not contain acetic acid. On the other hand, we noticed previously[1] that the same peroxy acids are stable during chromatography on acetylated and non-acetylated paper. On activated silica layers, the time for decomposition was about 10 min after application, on air-dried silica layers 20 min. The impregnation of silica gel with dimethylformamide enhances the stability for 30 min. Better results were obtained by lowering the temperature around the chromatographic system to 6°. The peroxy

TABLE I

R_F VALUES × 100 OF MONO- AND DISUBSTITUTED PEROXYBENZOIC ACIDS ON VARIOUS CELLULOSE AND POLYAMIDE ADSORBENTS

Substituted peroxybenzoic acid	*Stationary*[a] *and mobile phases*[b]											
	1	*2*	*3*		*4*		*5*	*6*		*7*		*8*
	A	*A*	*A*	*B*	*A*	*B*	*A*	*A*	*B*	*A*	*B*	*A*
2-Nitro-4-chloro-		26	27		17		13	9	34	12	40	10
2-Nitro-5-chloro-		25	29	62	16	44	11	7	31	10	38	8
2-Chloro-5-nitro-		28	22	58	17		12	11	37	14	43	11
4-Chloro-3-nitro-	76	41	37		24	49	18	14	43	16	48	14
2-Nitro-5-methyl-	69	22	21	60	14	45	10	4	36	6	42	8
4-Nitro-3-methyl-	85	50	43	70	33	56	28	21	52	24	56	21
2,4-Dichloro-	92	75	67	72	53	57	42	33	51	36	55	28
2,5-Dichloro-	91	74	65	64	45	58	37	22	48	31	51	21
3,4-Dichloro-	96	81	76	73	59	59	54	35	57	43	59	30
o-Chloro-	91	69	61	62	40	51	36	25	46	29	49	25
m-Chloro-	93	77	68	73	53	63	48	39	52	41	58	30
p-Chloro-	97	81	73	80	54	65	47		56	39	57	33
o-Nitro-	68	20	18	55	9	44	5	5	26	6	31	5
m-Nitro-	84	40	37	68	20	51	16	12	39	16	46	12
p-Nitro-	71	32	32	63	18	48	16	10	35	15	44	11
m-Fluoro-		75	71	78	46	61	43	34	51	41	55	30
p-Fluoro-		74	68	73	45	60	45	40	54	45	58	31
m-Bromo-		80	77		53			37	56	42	57	29

[a] Stationary phases: (1) MN-Cellulose Powder 300 HR; (2) MN-Cellulose Powder 300 Ac/*ca.* 10%; (3) MN-Cellulose Powder 300 Ac/*ca.* 20%; (4) MN-Cellulose Powder 300 Ac/*ca.* 30%; (5) MN-Cellulose Powder 300 Ac/*ca.* 40%; (6) MN-Polyamide TLC 6; (7) MN-Polyamide TLC 6/6; and (8) MN-Polyamide TLC 11 (Macherey-Nagel).

[b] Mobile phases: (A) carbon tetrachloride–acetic acid (10:1); (B) chlorobenzene–acetic acid (10:1).

acids are partly or completely decomposed on silica gel, presumably by the action of the inorganic binders ($CaSO_4$). This explanation could be supported by our results (obtained on Eastman-Kodak K 301R2 plates with polyvinyl alcohol as a binder), which showed that no decomposition or very slow decomposition was observed during the chromatographic separation on these plates of the peroxy acids examined.

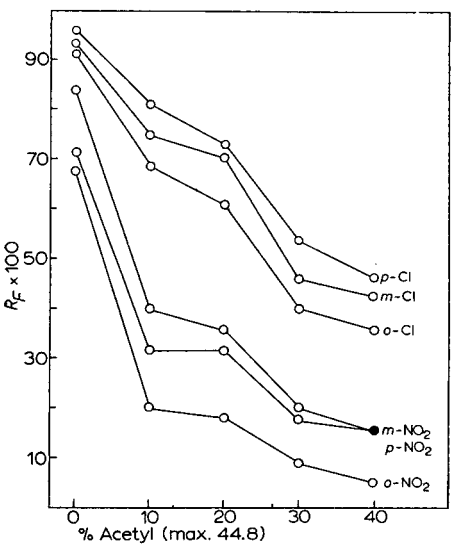
Another possible explanation of the observed phenomena would be similar to that apparent in the reaction of unsaturated fatty acids on Silica Gel G, where the energy-rich activated sites (non-solvatized silanol groups which are very weakly hydrogen bonded if at all) catalyse the decomposition[3]. If polyvinyl alcohol is used as a binder, these sites are occupied by the binder, which enables the formation of hydrogen bonds. The same experimental observations were made in our preliminary study of the decomposition kinetics of the peroxy acids examined using ethyl acetate as a solvent, in the presence of small amounts of both kinds of adsorbent. A more detailed investigation of this phenomenon is under way.

Thin-layer chromatography on acetylated celluloses

In general it is possible to use two different systems of developing solvents relative to the stationary phase on acetylated cellulose: (1) on a less polar mobild phase than acetylated cellulose or (2) on a more polar mobile phase. In order to fine

mobile phases which are less polar than acetylated cellulose, mixtures of different solvents (carbon tetrachloride, petroleum ether, aromatic hydrocarbons plus different quantities of acetic acid) were tested. On acetylated celluloses, the best results were obtained by using the combinations of carbon tetrachloride–acetic acid (10:1) and chlorobenzene–acetic acid (10:1). On non-acetylated cellulose the best separation was achieved by using the mobile phase light petroleum (b.p. 40–70°)–acetic acid (10:1). The results obtained by using mobile phases which are more polar than acetylated cellulose were not satisfactory. Small differences in polarity of isomeric peroxybenzoic acids are more pronounced in non-polar media.

The comparison of R_F values on various acetylated celluloses shows the progressive decrease when we go from the non-acetylated cellulose to cellulose thin layers of an increasing degree of acetylation (Figs. 1 and 2).



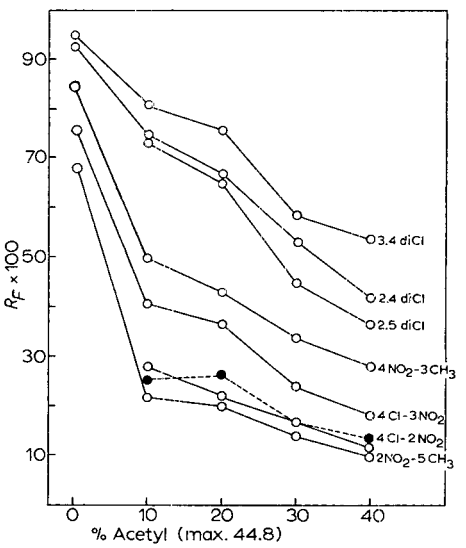


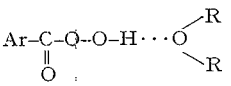
Fig. 1. Plot of R_F values of some monosubstituted peroxybenzoic acids on variously acetylated cellulose layers against the degree of acetylation.

Fig. 2. Plot of R_F values of some disubstituted peroxybenzoic acids on variously acetylated cellulose layers against the degree of acetylation.

The differences in mobilities of isomeric mono- and disubstituted peroxybenzoic acids on various acetylated celluloses are most probably the result of the following two factors: (1) the distribution between the water phase (up to 11 % in the case of non-acetylated cellulose and somewhat lower in acetylated celluloses) and the mobile phase; and (2) the formation of intermolecular hydrogen bonds between the molecules of peroxy acids and relatively basic oxygen atoms of the hydroxy, ether and ester groups of the cellulose.

Our I.R. investigation[4,5] indicates that aromatic peroxy acids appear in solvents which are not capable of forming hydrogen bonds as intramolecularly hydrogen-bonded monomers. Solvents and systems with basic oxygen, on the other hand, are

capable of forming intermolecularly hydrogen-bonded adducts by combining a molecule of the peroxy acids with a molecule of the Lewis base, according to the following scheme:



It is reasonable to assume that the influence of the first factor could be important, especially on non-acetylated cellulose and on celluloses of a low degree of acetylation. On the other hand, the second factor is more apparent with highly acetylated celluloses. The increasing migration of substituted peroxybenzoic acids on non-acetylated cellulose layers in comparison with that on acetylated ones, could be explained by the low solubility of these peroxy acids in water as compared to that in the mobile phase. The distribution coefficient between the mobile phase and the stationary phase is large. By increasing the degree of acetylation, the hydrophobicity is increased and therefore, the distribution coefficient becomes smaller. Although the shapes of the curves obtained by plotting the R_F values against the degree of acetylation are roughly hyperbolic (Figs. 1 and 2), there are small deviations from the ideal hyperbolic shape, especially between the 10 % and 20 % acetylated cellulose layers. These deviations are possibly due to a slight difference between the declared and actual degrees of acetylation of commercial adsorbents. In fact, however, this is probably not the most important factor, since the deviations due to the type of substituent are considerably greater. An additional possible explanation of this phenomenon could be the occurrence of simultaneous mechanisms of distribution and/or binding of peroxy acids on these layers, which are not dependent in the same manner on the degree of acetylation. The suggestion of hydrogen bonding in which the peroxy acid group or a nitro group acts as the proton acceptor, while the adsorbent acts as donor, could be plausible.

In Fig. 3 are plotted the moisture contents of variously acetylated celluloses, as well as the R_F values of one peroxy acid (*m*-nitroperoxybenzoic acid) against the degree of acetylation. The differences in the shapes of the two curves allow us to conclude that the moisture content in acetylated celluloses is not the predominant cause of decreasing mobility.

Thin-layer chromatography on various polyamides

Graphic presentation of the TLC migration patterns of mono- and disubstituted peroxybenzoic acids on different polyamides is given in Fig. 4.

The question of a correlation of R_F values obtained cannot be answered directly. There are somewhat greater differences in the pattern on the polyamide adsorbents than in that on the cellulose layers. Thus, the R_F values are greatest on Polyamide 6/6 and lowest on Polyamide 11, although the dielectric constant of Polyamide 6 is highest, and that of Polyamide 11 lowest. The apparent discrepancy between the R_F values on polyamide adsorbents and the values of dielectric constants does not provide a rational explanation for the above-mentioned phenomena. Still, we would like to mention the intensity of tailing on various polyamide layers examined. The observed tailing was directly proportional to the dielectric constant of the polyamide adsorbent. It appears that the formation of hydrogen bonds between the peroxy acids and the polyamide plays an important role in the TLC separation of peroxy

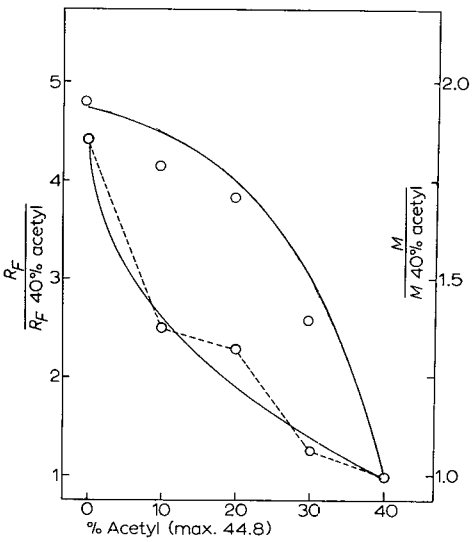


Fig. 3. Plot of R_F values of *m*-nitroperoxybenzoic acid on variously acetylated cellulose layers and moisture content of acetylated cellulose adsorbents against the degree of acetylation.

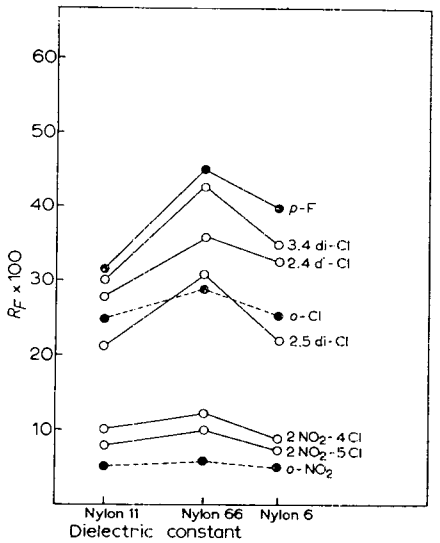
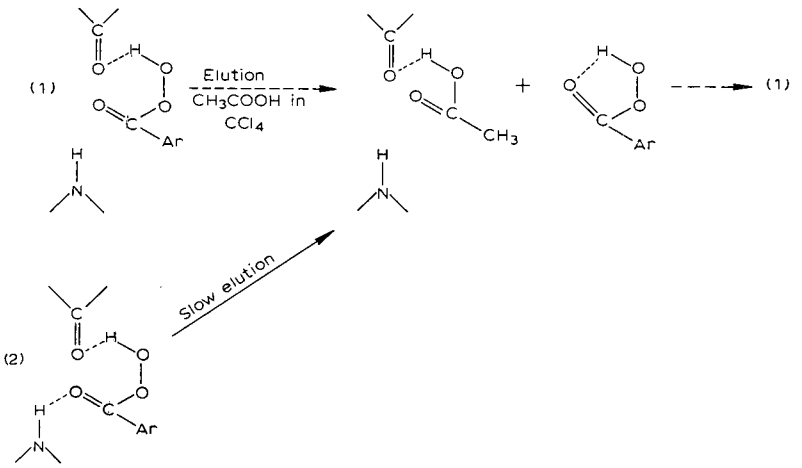


Fig. 4. Plot of R_F values of some mono- and disubstituted peroxybenzoic acids on various polyamide layers against the approximate trend of increasing dielectric constant of the same adsorbents.

acids on these adsorbents. Apparent irreversibility of such intermolecular hydrogen bond formation is quite in keeping with the tailing observed which was greatest on Polyamide 6 and smallest on Polyamide 11. We believe that the chromatographic separation of the peroxy acids examined on polyamide adsorbents is not connected with the formation of new types of products which would be formed by a reaction between peroxy acids and polyamides. The main factor in this separation is the formation of partially reversible intermolecular hydrogen bonds. The fact that developing systems such as acetic acid, dimethylformamide, and alcohol-containing systems do produce well defined spots without tailing, whereas there is heavy tailing with the acetone–acetic acid–water (1:1:3) system, provides additional support for the above-mentioned conclusion. In addition, the structure of the polyamide can also influence the formation of hydrogen bonding between the peroxy acid and the adsorbent. In ideal crystalline polyamide, all carbonyl groups are blocked with hydrogen bonds to parallel NH groups. In a commercially available polyamide, in powder form, less than 50 % is crystalline[6]. Besides, in Polyamide 6, nearly 40 % of the NH groups are free[7], which is also in accordance with the intensity of tailing observed. In Polyamide 11 and Polyamide 6/6, the number of free NH groups is much smaller. The results of the TLC of substituted peroxybenzoic acids on polyamide layers described above led us to the conclusion that during the separation, we are observing partially reversible formation of hydrogen bonds between the molecule of peroxy acid and polyamide. In the case of phenols[8] there exists the possibility of formation of only one hydrogen bond between the carbonyl oxygen atom of the polyamide and

the hydrogen atom of the phenolic group. With peroxy acids, the formation of one as well as two hydrogen bonds could be expected, according to the following scheme:



The formation of both types of hydrogen bond is conditioned by a rather favourable steric configuration of a peroxycarboxyl group which is not planar but somewhat skewed[9,10]. It is our assumption that appearance of the tailing is a result of the formation of double hydrogen bonds. The mobile phase (acetic acid in carbon tetrachloride or chlorobenzene) is not capable of simultaneously taking over both hydrogen bonds.

ACKNOWLEDGEMENT

The authors wish to thank the Boris Kidrič Fund for financial support of this research.

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CHROM. 3780

DETECTION OF PETROLEUM OIL DILUENTS IN COAL TAR CREOSOTE BY THIN-LAYER CHROMATOGRAPHY

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(Received September 11th, 1968)

SUMMARY

A thin-layer chromatographic method is described for the detection of four types of petroleum oils used as diluents in creosote. These oils vary widely in aromatic and paraffinic content. The presence of these oils can be demonstrated down to a level of 0.25 % in creosote. The indicator, α-cyclodextrin followed by iodine fumes, should be useful in other applications where differentiation between aromatic compounds and hydrocarbons is desired.

INTRODUCTION

In the United States, high temperature-type coal tar creosote is the most widely used material for the preservation of wood used for heavy construction. For land uses, such as for railroad crossties, blends of creosote with as much as 50 % byproduct petroleum fuel oils are recognized in American Wood-Preservers' Association (AWPA) Specifications and higher ratios of petroleum are used by some railroad companies. For marine uses, however, the effectiveness of creosote is reduced sharply by the presence of petroleum[1]. An important objective of specifications for marine-grade creosote is to restrict the presence of petroleum to the lowest practical limit. It is recognized that, in a commercial treating plant where a charge of marine piling treated with "pure" creosote may follow a charge of crossties treated with a creosote–petroleum blend, some contamination of the marine-grade creosote by oil is unavoidable. At present, there is no agreement as to what constitutes a reasonable tolerance.

Several methods for the determination of petroleum in creosote have been reported. Results from the distillation and specific gravity test[2] vary with the nature of the petroleum. Several methods have been based on differences in the solubility of aromatic and saturated compounds[3]. The U.S. Naval Civil Engineering Laboratory described a test by column chromatography[4]. Infrared techniques have been reported[5,6]. For one reason or another, none of these methods have come into extensive use, especially with creosotes containing low levels of petroleum oil. For use at treating

* Maintained at Madison, Wis. (U.S.A.), in cooperation with the University of Wisconsin.

plants, the most desirable method would be one that could obtain results quickly and be conducted with moderately priced equipment.

Since creosote is composed of a wide variety of compounds, perhaps 500, and contains liquid and solid aromatic hydrocarbons, we felt that thin-layer techniques could be used to simplify the separation process by holding the aromatics back and permitting a fraction of the petroleum additive to be positioned nearer a selected solvent front.

EXPERIMENTAL

Materials

A medium-residue creosote that had been carefully analyzed by standard American Wood-Preservers' Association methods and used in trials of additional test methods was selected as a typical commercial creosote.

Four petroleum oils were selected for blending with creosote. Two were heavy fuel oils of the types commonly used in blends for treating crossties. One was relatively low and the other very high in aromatic hydrocarbons. The other two petroleum oils were distillates that are used in preparing pentachlorophenol solutions for treating products such as poles and lumber having higher cleanliness requirements than crossties. These two oils differed widely in their content of hydrocarbons. Solutions were prepared by weight to contain 0.25, 0.5, 1, and 2 % petroleum diluent in the creosote.

30 g of Silica Gel H (Brinkman Instruments, Inc., Westbury, N.Y.) powder were shaken with 70 ml of water and spread on standard 20 cm square glass plates to give a layer 25 μ thick.

The developing solvent was Chromatoquality reagent *n*-hexane (Matheson, Coleman & Bell, East Rutherford, N.J., CQ 5093).

All samples were diluted with reagent-grade toluene for spotting.

Indicators were α-cyclodextrin (Applied Science Laboratories, Inc., State College, Pa.)[7] followed by iodine fumes obtained by passing air through a 150 mm drying tube which contained iodine crystals.

1 μl microcap pipettes were used.

Procedure

50 mg samples were weighed and diluted with 1 ml of reagent-grade toluene. The air-dried plates were activated by heating for 1 h at 105°. The diluted samples were spotted about 20 mm from the bottom of the plates. To reduce the size of the spots, each sample was spotted five times in the same place with the 1 μl pipettes to give a sample loading of 5 μl. Samples were: creosote alone; creosote containing 0.25, 0.5, 1, and 2 % petroleum diluent; and diluent alone. Plates were developed in glass chromatography jars with *n*-hexane as the solvent. The solvent was allowed to ascend to within 1 in. of the top of the plate. At 25°, development time was less than 1 h.

After development, the plates were sprayed with α-cyclodextrin. The plates were then allowed to air dry and fumed with iodine. A white spot against a brownish-purple background appeared near the solvent front for the samples containing the petroleum oils. The spots for the aromatic compounds in the creosote were brown.

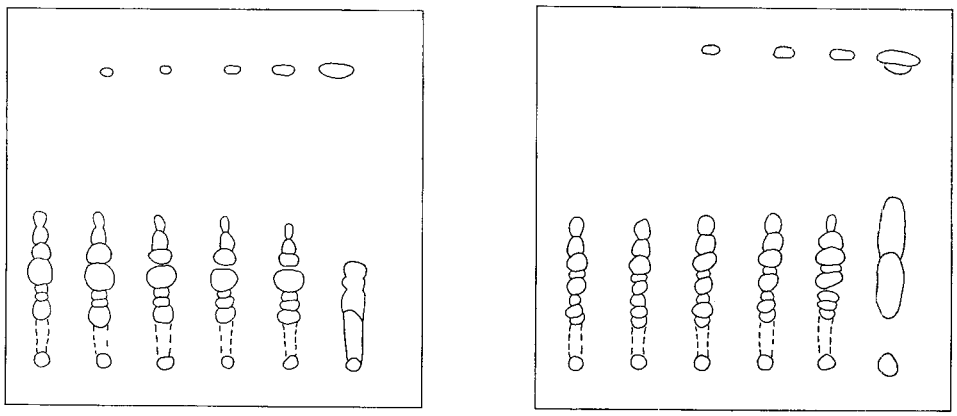

Fig. 1. Thin-layer chromatogram of creosote and creosote containing petroleum diluent. Activated Silica Gel H plate. Solvent: *n*-hexane. Indicator: α-cyclodextrin followed by iodine fumes. Left to right: creosote alone; creosote containing 0.25, 0.5, 1, and 2 % diluent; and diluent alone. 50 mg of each sample diluted with 1 ml of toluene for spotting. 5 μl spotted in each case. Diluent: high aromatic heavy fuel oil.

Fig. 2. Thin-layer chromatogram of creosote and creosote containing petroleum diluent. Activated Silica Gel H plate. Solvent: *n*-hexane. Indicator: α-cyclodextrin followed by iodine fumes. Left to right: creosote alone; creosote containing 0.25, 0.5, 1, and 2 % diluent; and diluent alone. 50 mg of each sample diluted with 1 ml of toluene for spotting. 5 μl spotted in each case. Diluent: high aromatic distillation pentachlorophenol carrier.

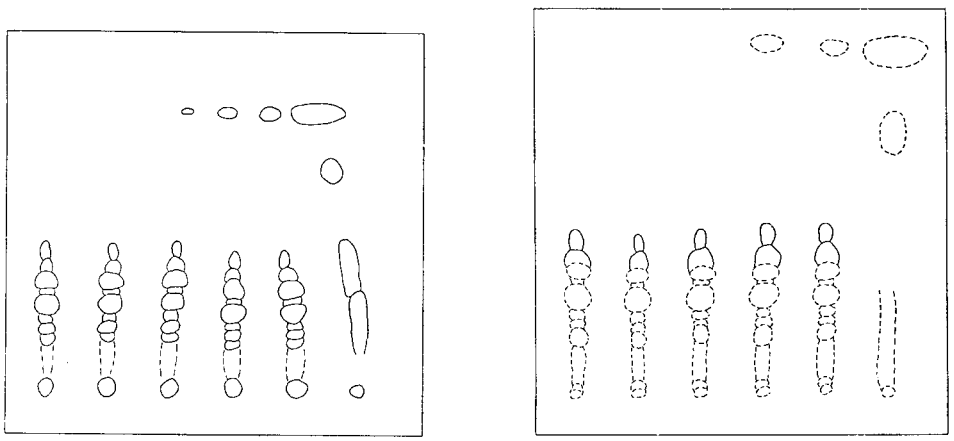

Fig. 3. Thin-layer chromatogram of creosote and creosote containing petroleum diluent. Activated Silica Gel H plate. Solvent: *n*-hexane. Indicator: α-cyclodextrin followed by iodine fumes. Left to right: creosote alone; creosote containing 0.25, 0.5, 1, and 2 % diluent; and diluent alone. 50 mg of each sample diluted with 1 ml of toluene for spotting. 5 μl spotted in each case. Diluent: P-9 type heavy pentachlorophenol carrier; high aromatic.

Fig. 4. Thin-layer chromatogram of creosote and creosote containing petroleum diluent. Activated Silica Gel H plate. Solvent: *n*-hexane. Indicator: α-cyclodextrin followed by iodine fumes. Left to right: creosote alone; creosote containing 0.25, 0.5, 1, and 2 % diluent; and diluent alone. 50 mg of each sample diluted with 1 ml of toluene for spotting. 5 μl spotted in each case. Diluent: low-aromatic heavy fuel oil.

RESULTS AND DISCUSSION

Results are shown in Figs. 1 through 4. Under the conditions used, the petroleum could always be detected at the 1 % level and, in most cases, at the 0.5 or even 0.25 % level. By increasing the sample size, *e.g.*, by using 0.10 g diluted with solvent to 1 ml, the detection limit can be reduced to one-half the above limits.

A number of thin layer coatings and solvent systems were used, but best results were obtained with the system described here. Activation of the plates improved the separation between the aromatic fractions and the straight-chain groups. α-Cyclodextrin complexes with the hydrocarbons, becomes less reactive, and is not colored by the iodine. The spots containing the hydrocarbons appear as white spots on a light brownish-purple background. This indicator should have application in other situations where aromatic groups and straight-chain hydrocarbons are involved. Apparently all of the blending oils, both high aromatic and paraffinic types, contain enough of the hydrocarbons to make the method useful for the detection of these oils.

The method is simple and requires a small amount of equipment. A complete test requires less than 2 h. It can be adapted for use in the field. For use in treating plants where the types of oil being used are known, known solutions may be prepared for spotting adjacent to the unknowns, and concentrations can be altered to meet particular needs of the plant.

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CHROM. 3768

SEPARATION OF POLYPHOSPHATES BY ANION EXCHANGE THIN LAYER CHROMATOGRAPHY

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(First received July 22nd; revised manuscript received August 19th, 1968)

SUMMARY

A rapid and simple anion exchange thin layer chromatographic technique for the separation of polyphosphates is described. The method allows separation of several linear and cyclical phosphates by one-dimensional development of thin layer plates and also permits estimation of the approximate chain lengths of the components of polyphosphate glasses.

INTRODUCTION

There has been considerable interest in the formation, degradation and characterization of polyphosphates in biological[1] and nonbiological systems[2]. Methods for estimating polyphosphates in biological systems depend upon analysis of polyphosphate as orthophosphate after strong acid hydrolyis. After extraction of polyphosphates from cells, attempts are often made to free the extracts of organic phosphates by removing nucleic acids and nucleotides by adsorption to charcoal[3]. Alternatively, polyphosphate is precipitated as the salt of a heavy metal[4].

In biological as well as nonbiological systems attempts have been made to resolve mixtures of polyphosphates, especially by paper partition chromatography. These techniques require long development times and often two-dimensional elutions[5–8]. Paper chromatography has been used to follow the kinetics of polyphosphate hydrolysis[2] and has been useful in resolving mixtures of polyphosphates of widely divergent chain lengths[9]. Partial separation of the components of a polyphosphate mixture using molecular-sieve column chromatography has been reported[10]. Anion exchange column chromatography has been used with success in separating polyphosphates up to chain length of about 15 (ref. 11).

There are several reports of partition chromatography upon various types of thin layers. RÖSSEL[12] achieved good separations of linear polyphosphates of up to eight phosphorus atoms per molecule. Cyclical phosphates were most readily separated from linear phosphates by two-dimensional development. At nearly the same time

AURENGE, DEGEORGES AND NORMAND[13] reported good separations of linear polyphosphates of up to eight phosphorus atoms per molecule. Several of the oligomeric components of Graham's salt were separated. Cyclical polyphosphates were separated using developing solutions which were unable to separate linear polyphosphates. CLESCERI AND LEE[14] separated ortho- from pyrophosphate by partition chromatography on thin layers. Good separations of linear and cyclical polyphosphates were achieved using ascending and circular thin layer techniques[15]. Others have reported partition chromatography on thin layers to be variously successful[16,17]. Recently, BERGER, MEYNIEL AND PETIT[18] reported successful separation of ortho-, pyro- and tripolyphosphate on ion exchange Biorex 5 (Cl^-) thin layers.

This report describes a rapid and simple anion exchange thin layer chromatographic technique for the separation of polyphosphates. The method allows simultaneous chromatography of large numbers of samples and estimation of the approximate chain lengths of components of polyphosphate mixtures.

EXPERIMENTAL

Polyethyleneimine (PEI) impregnated micro-crystalline cellulose (Avicel) coated thin layer plates (250 μ thickness) were employed (Analtech Inc., Wilmington, Del.). Plates were stored at 4°. As suggested by RANDERATH[19], lines were scratched in the thin layers, about 1.0 mm wide, extending from just below the origin to the bottom edge of the plate. Then the plates were predeveloped in distilled water and dried at room temperature.

Aqueous solutions of 2.5 mg/ml of various sodium salts of phosphates were spotted such that 0.20–0.25 μg of the salt was applied to the thin layer. The plates were placed in covered battery jars (20 × 8.5 × 21 cm) containing 300 ml of the following concentrations of LiCl: 0.05 *M*, 0.10 *M*, 0.30 *M*, 0.40 *M*, 0.50 *M*, 1.0 *M*, 1.6 *M*, or 3.0 *M*. The solvent front was allowed to advance about 10 cm past the origin at room temperature (22–25°). This required about 30 min. The plates were removed from the jars and allowed to air dry.

The location of phosphorus-containing spots was revealed after spraying with the reagent of HANES AND ISHERWOOD[20]. Freshly sprayed plates were placed horizontally upon a heavy glass slab in an oven preheated to 150° until the thin layer dried. In this way polyphosphate hydrolysis was accelerated while the diffusion of spots was minimized. It was often necessary to repeat the spraying and drying procedure when plates had been developed with higher concentrations of LiCl. After reduction of phosphomolybdate to molybdenum blue under ultraviolet light[21], plates were exposed to NH_3 vapors. Blue spots on a white background were readily marked. The color does not appreciably fade with time.

The following sodium salts of phosphate compounds were used as standards. Dibasic sodium phosphate (Na_2HPO_4), and sodium pyrophosphate ($Na_4P_2O_7 \cdot 10\ H_2O$) were analytical reagent grade and standard items of commerce. So-called sodium "hexametaphosphate" glass was supplied by the Monsanto Company, St. Louis, Mo. Highly purified samples of sodium trimetaphosphate ($Na_3P_3O_9$), sodium tripolyphosphate ($Na_5P_3O_{10} \cdot 6\ H_2O$), sodium tetrametaphosphate ($Na_4P_4O_{12} \cdot 4\ H_2O$), sodium hexametaphosphate ($Na_6P_6O_{18}$), and four sodium phosphate glasses having mean chain lengths of 5, 10, 20 and 174, respectively, and having the general empirical

formula $Na_{n+2}P_nO_{3n+1}$, were supplied through the generosity of Dr. E. J. GRIFFITH, Inorganic Research Dept., Monsanto Company, St. Louis, Mo.

RESULTS AND DISCUSSION

Fig. 1 is a typical chromatogram of the various phosphate salts developed by 0.30 *M* LiCl. Table I gives the R_F values of the sodium salts of ortho-, pyro-, tripoly, trimeta-, tetrameta- and hexametaphosphates when PEI plates were developed by concentrations of LiCl ranging from 0.05–0.50 *M*. Best separations were obtained with concentrations of 0.10–0.30 *M* LiCl. At 0.50 *M* or greater LiCl concentrations, all of the highly purified polyphosphates tested demonstrated R_F values approximating that of orthophosphate. When PEI plates were developed in distilled water, none of the phosphates moved from the origin. When Avicel plates, not impregnated with PEI, were spotted and developed in distilled water, all of the phosphates migrated with the solvent front. Thus the chromatographic behavior of the various phosphate salts

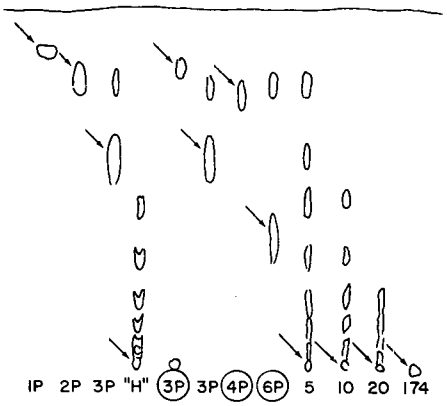

Fig. 1. Tracing of chromatogram of sodium salts of polyphosphates upon PEI impregnated Avicel thin layer plate developed with 0.3 *M* LiCl. Sodium salts of orthophosphate (1P), pyrophosphate (2P), tripolyphosphate (3P), so-called "hexametaphosphate" glass ("H"), trimetaphosphate ((3P)), tetrametaphosphate ((4P)), and hexametaphosphate ((6P)) as well as glasses having mean chain lengths of 5, 10, 20 and 174 were chromatographed. Arrows point to the developed spots which are of greatest color intensity.

TABLE I

R_F VALUES OF HIGHLY PURIFIED PHOSPHATE SALTS WHEN PEI IMPREGNATED AVICEL THIN LAYER PLATES WERE DEVELOPED IN VARIOUS MOLAR CONCENTRATIONS OF LiCl

Phosphate salt	*Molar concentration of LiCl*					
	0.05	*0.10*	*0.20*	*0.30*	*0.40*	*0.50*
Orthophosphate	0.58	0.75	0.84	0.85	0.88	0.90
Pyrophosphate	0.09	0.24	0.60	0.79	0.85	0.89
Tripolyphosphate	0.05	0.09	0.30	0.60	0.76	0.87
Trimetaphosphate	0.28	0.52	0.74	0.84	0.87	0.91
Tetrametaphosphate	0.06	0.20	0.50	0.79	0.84	0.89
Hexametaphosphate	0.00	0.05	0.15	0.39	0.59	0.83

was in accord with their anionic character and the anion-exchange quality of the thin layer plate rather than being a reflection of the relative solubility of the salts in aqueous solution.

Chromatograms developed with 0.05–0.40 *M* LiCl revealed that tripolyphosphate and hexametaphosphate samples contained an appreciable contaminant having an R_F value consistent with its identification as pyrophosphate. The hexametaphosphate salt also contained a contaminant not mobilized by 0.10 *M* LiCl while the trimetaphosphate salt contained a contaminant which could not be mobilized by even the highest concentration of LiCl tested (3.0 *M*).

All phosphate residues of a cyclic polymer should be anionically equivalent. If the chromatographic behavior of cyclical polyphosphates is merely a function of the number of phosphate residues per molecule, then a plot of R_M, the log $(1/R_F - 1)$, *vs.* the number of P atoms per molecule for an homologous series ought to yield a straight line[22,23]. Such a plot (Fig. 2) shows that for the cyclical (a) and linear (b) phosphates no families of unequivocally straight lines are developed when various phosphates are chromatographed at different LiCl concentrations. Inability to accurately estimate very low R_F values may, however, produce large errors in R_M computation and obscure true linearity of some lines.

Fig. 3 gives the results of chromatographic analysis of four phosphate glasses whose mean chain length are 5, 10, 20 and 174, respectively, and of so-called "hexametaphosphate" glass, by five different concentrations of LiCl developing solution.

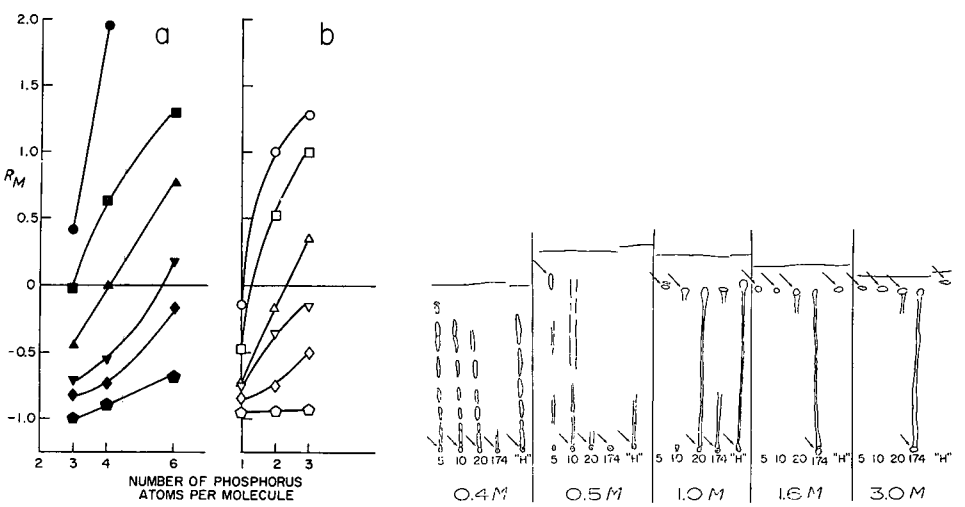

Fig. 2. Plot of R_M, the log $(1/R_F - 1)$, as a function of the number of phosphorus atoms per molecule at different concentrations of LiCl developing solution. In graph a the chromatographic behavior of trimeta-, tetrameta- and hexametaphosphate salts is presented using 0.05 *M* (●), 0.10 *M* (■), 0.20 *M* (▲), 0.30 *M* (▼), 0.40 *M* (◆) and 0.50 *M* (⬟) LiCl developing solution. In graph b the chromatographic behavior of ortho-, pyro- and tripolyphosphate salts is presented using 0.05 *M* (○), 0.10 *M* (□), 0.20 *M* (△), 0.30 *M* (▽), 0.40 *M* (◇) and 0.50 *M* (⬠) LiCl developing solution.

Fig. 3. Tracing of montage of chromatograms of polyphosphate glasses having mean chain lengths 5, 10, 20 and 174 and of so-called "hexametaphosphate" glass ("H"). The behavior in the following LiCl concentrations is shown: 0.4 *M*, 0.5 *M*, 1.0 *M*, 1.6 *M* and 3.0 *M*. Arrows point to the spots which have greatest color intensity.

Each of these phosphate glasses is a mixture of polyphosphates. Sometimes only a streak of molybdenum blue color could be discerned on the chromatogram; however, if greater amounts of phosphate glass were spotted, many intense blue spots could be delineated within the streak. Obviously, there are large numbers of components of these glasses.

It is possible to separate populations of polyphosphates of similar chain length. Fig. 3 shows that the major portion of a polyphosphate glass of mean chain length of 5 residues is not mobilized from the origin unless the chromatogram is developed with at least 0.50 *M* LiCl. Similarly, the major portion of a polyphosphate glass with a mean residue number of 10 is not mobilized unless 1.0 *M* LiCl is used. Mobilization of the major portion of a polyphosphate glass with a mean chain length of 20, and of the "hexametaphosphate" glass, requires development with at least 1.6 *M* LiCl, while the major portion of a polyphosphate glass with a mean chain length of 174 is not mobilized from the origin, even with 3.0 *M* LiCl. Hence, the relative chain length of an unknown polyphosphate can be estimated by development with concentrations of LiCl sufficient to mobilize a polyphosphate of known mean chain length but insufficient to mobilize a polyphosphate of greater mean chain length.

The behavior of polyphosphates upon these chromatograms appears to be a function of the negative charge which they bear, the anion exchange quality of PEI and the concentration (activity) of Cl^- in the developing solution. One cannot, however, completely exclude the influence of other factors upon chromatographic behavior.

We have found this chromatographic technique to be useful in characterizing the chain length of polyphosphates made by a microorganism. The method allows ready separation of inorganic polyphosphates from phosphate-containing organic compounds. It avoids the problems of non-differential adsorption of nucleotides and nucleic acids to charcoal and of incomplete precipitation of polyphosphates by heavy metals. When cells are prelabeled with ^{14}C, and then incubated with ^{32}P-orthophosphate, extracted in dilute alkali[24], and the extracts neutralized and chromatographed, one readily observes differential rates of migration of ^{14}C and ^{32}P radioactivity with various concentrations of LiCl developing solution. This behavior establishes the existence and relative chain length of biosynthetic polyphosphates. The amounts of newly formed polyphosphate are readily quantitated by evaluation of ^{32}P radioactivity.

ACKNOWLEDGEMENT

The authors wish to express their thanks to Dr. ESTELLE R. RAMEY, Department of Physiology and Biophysics, Schools of Medicine and Dentistry, Georgetown University, Washington, D.C. for helpful discussions during the course of this work.

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Notes

CHROM. 3782

Adventitious trimethylsilylation during combined gas–liquid chromatography–mass spectrometry

Combined gas–liquid chromatography–mass spectrometry (GLC–MS) is now recognized as a powerful method for the identification of a variety of substances from both natural and synthetic sources[1–12]. A technique which does not harbor pitfalls for the unwary practitioner is most rare, however, and combined GLC–MS offers no exception. For example, FOLTZ *et al.*[13] recently reported that they obtained the mass spectrum of trimethylfluorosilane when a sample of trimethylchlorosilane was analyzed on their instrument. The transformation was evidently caused by the presence of a fluorocarbon contaminant in the coupling system between the gas chromatograph and the molecular separator (which preferentially removes the carrier gas from the column effluent before it enters the mass spectrometer). We wish to report another anomalous finding, one which appears to be column related—trimethylsilylation of phenols during analysis using a GLC system which had previously come in contact with *bis*-trimethylsilylacetamide (BSA).

Experimental

All experiments were carried out with an LKB Model 9000 gas chromatograph–mass spectrometer equipped with the stainless steel molecular separator system of Ryhage[1,2]. Unless otherwise specified the mass spectrum of an eluted compound (5–10 μg samples were employed) was obtained at the time of maximum peak height on the chromatogram (scan time 6 sec). Gas chromatography conditions: 4 ft. × 3 mm. I.D. glass spiral or "cobra" column[14]; 5 % F-60 (DC-560), a non-polar methylpolysiloxane containing a few percent of *p*-chlorophenyl groups (Dow-Corning) on 80–100 mesh acid-washed and silanized[15] Gas-Chrom P; column, 210°; flash heater, 240°; molecular separator, 245°; helium carrier gas 30 ml/min. The mass spectrometer operating conditions were as follows: ion source, 290°; accelerating voltage, 3.5 kV; electron energy, 70 eV.

Results and discussion

A phenolic compound, the structure of which was "known" with considerable certainty (based on synthetic route, infrared, ultraviolet and NMR spectra) was submitted for combined GLC–MS analysis. The assumed molecular weight of this substance was 294, and it was expected that a signal for the molecular ion would be found at the appropriate *m/e*. The mass spectrum did contain a signal at this position, but there was a considerably stronger signal (3 times) at *m/e* 366 which appeared to be the molecular ion. The difference between 294 and 366 is 72 mass units, a number well known to those who work with trimethylsilyl (TMSi) derivatives—it is the difference in molecular weight between an alcohol and the corresponding TMSi ether. When a sample of the compound was allowed to react with BSA (a powerful trimethyl-

silylation reagent[16]) and then applied to the instrument the mass spectrum obtained showed no signal at *m/e* 294, and that at *m/e* 366 was the base peak (most abundant ion). This was clearly the spectrum of the TMSi ether derivative. (The phenol and the TMSi ether exhibit very similar retention behavior; the retention time of the latter is only 1.03 times that of the parent compound.) Determination of the mass spectrum of the phenol via a direct inlet or probe technique (*i.e.*, the sample was not introduced into the ion source through the gas chromatography system), however, produced a spectrum entirely compatible with the proposed structure, with no suggestion of trimethylsilylation. Comparison of the three spectra disclosed that the one resulting from analysis of the phenol using the combination technique was actually composite of the two "pure" spectra, and the inescapable conclusion was that this spectrum represents a mixture of phenol and TMSi ether. A plausible explanation for this "on column" derivatization is given below.

For several days previous to these observations, and actually until about an hour prior to combined GLC–MS analysis of the phenol, numerous such analyses were carried out on substances (not related to the compounds used in this study) dissolved in BSA. This liquid was used both as solvent and reagent, a not uncommon practice[17–19]. We assumed that sufficient BSA remained in the gas chromatography system to effect partial trimethylsilylation of the phenol as it passed through the column. (Deliberate "on column" derivative formation has been studied by ANDERS AND MANNERING[20].) To test this hypothesis, the flash heater and column temperatures were increased 20° above normal operating values for 15 h (overnight) and the carrier gas flow rate maintained at 30 ml/min in an attempt to "condition" the system and remove the source of TMSi groups. Samples of the phenol were then run by the combination technique under the normal operating conditions immediately before and 15 min after the application of 10 μl of BSA to the column (with the dual inlet valve, located between the molecular separator and the mass spectrometer, closed). The mass spectrum of the sample run prior to this BSA-treatment of the column was again a composite; however, it exhibited *m/e* values of 294 and 366 in the intensity ratio of 3:1, respectively, in marked contrast to the relative intensities of these signals observed before the "baking out" of the column. (One should not assume that the 294/366 amplitude ratio accurately represents the relative proportion of parent phenol and TMSi ether in the mixture, but it is a convenient approximation.) The mass spectrum of the eluted peak resulting from analysis of the phenol after application of BSA to the column was very similar to the composite spectrum first obtained for this compound, with a 294/366 ratio of 1:2.

In order to investigate this phenomenon further, the overnight column deactivation treatment was repeated and tested with the phenolic steroid estrone. The mass spectrum obtained from the mid-point of this peak indicated the presence of both estrone and its TMSi ether, but the signal at *m/e* 270 (molecular ion of estrone) was ten times the intensity of that of *m/e* 342, the molecular ion of estrone TMSi ether. Although an authentic mixture of estrone and its TMSi ether was resolved into two peaks (relative retention times of 1.0 and 1.1, respectively) a second peak was not observed in the initial analysis of estrone. Analysis of estrone 15 min after the application of 10 μl of BSA to the column produced a chromatogram in which the single peak gave mass spectrometric evidence for the presence of both estrone and its TMSi ether. Mass spectra of the ascending, center, and descending portions of the peak were taken.

Each of the spectra was a composite of those of authentic estrone and estrone TMSi ether; the 270/342 amplitude ratios for the three spectra were 5:1 for the front, 3:1 for the middle, and 1:1 for the rear of the peak. One must conclude that estrone TMSi ether is formed *in situ* during the analysis of the parent steroid*.

Demonstration of the presence of the TMSi ether in the front portion of the peak and the fact that two gas chromatographic peaks are not seen suggests that the functional group alteration does not occur "instantaneously" at the top of the column, but probably continuously as the estrone moves through the column. This effect may persist even after "conditioning" of the column system, and the conclusion to be drawn is that partial "on column" trimethylsilylation is a definite possibility when phenolic compounds are analyzed on a column previously exposed to BSA. Although BSA may be considered to be "innocuous" under certain conditions[21], this is certainly not always true. A more detailed investigation of this effect is beyond the scope of this note. We hope we have drawn attention to an interesting but potentially misleading phenomenon.

Acknowledgement

It is a pleasure to acknowledge the contributions of Dr. G. ALBERS-SCHÖNBERG to certain aspects of this paper.

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Received September 12th, 1968

* When androstane-3β-ol-17-one was employed as a test compound in the same manner as estrone only a very slight conversion was noted for this non-phenolic alcohol.

CHROM. 3787

The use of silver nitrate impregnated silica gel layers in the separation of monoterpene hydrocarbons

Silver nitrate impregnation of silica gel and other adsorbent layers has been used by a number of workers to separate unsaturated compounds. This technique has been especially useful in the field of lipids[1-3] where it has been used to separate *cis*- and *trans*-isomers and other compounds according to their number of double bonds. Recently[4,5], more interest has been shown in the separation of positionally isomeric unsaturated compounds.

In the field of essential oil and resin constituents, a number of workers have used the technique to aid them in generally difficult separations. A list of these is given in Table I. In this study the technique is used to determine the best impregnation for the separation of monoterpene hydrocarbons.

TABLE I

SILVER NITRATE THIN-LAYER CHROMATOGRAPHY OF ESSENTIAL OIL AND RESIN CONSTITUENTS

Type of compounds analyzed	*Percentage of silver nitrate impregnation*	*Reference*
Sesquiterpene hydrocarbons	15	7
Resin acid esters and related terpenes	12.5	8
Resin acid methyl esters	20	9
Sesquiterpene alcohols	10	10
Sesquiterpene hydrocarbons	12.5	11
Oxygenated terpenes	15	12
Sesquiterpene hydrocarbons	15	13
General essential oil constituents	8	14
Terpene and sesquiterpene alcohols	3	15
Terpene hydrocarbons	12.5	16
Allyl and propenyl phenol ethers	12.5	17
General essential oil constituents	6	18

Method

The slurries were prepared using 40 g of Silica Gel G (E. Merck) and 80 cc of a series of aqueous solutions of reagent grade silver nitrate (B.D.H.). The strength of the silver nitrate solutions used were 0, 6.25 %, 12.5 %, 18.75 % and 25.0 % respectively. Each set of layers was prepared using the Desaga equipment as described by LAWRENCE[6]. The layers were activated in a drying oven at 100–110° for 1 h and then stored in a desiccator over phosphorus pentoxide until used. It was found that differences in R_F value were within experimental error for the three day lifetime of the layers. (It is general procedure used by the author that all activated plates are given a three-day lifetime.)

The monoterpenes were spotted in 1 microlitre quantities with the aid of a 10 μl syringe (Hamilton) from 10 % (v/v) hexane solutions. All separations were carried out in a standard chromatographic tank containing a filter-paper wick liner using benzene as the eluent. For detection purposes a 10 % chloroform solution of antimony pentachloride proved to be the most useful reagent tried. The plates were

all developed for a standard 15 cm and R_F measurements were made from the most intense part of the detected spot. As a number of runs were made on each compound the $R_F \times 100$ values were recorded as a range rather than a single value.

Results and conclusions

The results obtained from this study (see Table II) show that the R_F value of a monoterpene hydrocarbon varies with percentage impregnation of silver nitrate, and from these results some interesting findings follow:

(1) The R_F value of a monoterpene hydrocarbon can be predicted within the range 0–25 % impregnation from a graphical representation of the results (see Table III).

(2) Certain separations which are difficult to achieve with gas chromatography can be readily carried out with $AgNO_3$-impregnated TLC, *e.g.* the separation of sabinene from β-pinene.

Now, the usefulness of the above separation stems from the ability of un-

TABLE II

THE EFFECT OF SILVER NITRATE IMPREGNATION ON THIN-LAYER CHROMATOGRAPHIC SEPARATIONS OF MONOTERPENES

Benzene was the eluent used in all cases.

Compounds	*Percentage impregnation of silver nitrate*				
	Zero	*6.25*	*12.5*	*18.75*	*25.0*
p-Cymene	72.5–73.5*	72.5–73.5	72.5–73.5	66.5–67.5	60.5–61.5
δ-3-Carene	71.5–72.5	71.5–72.5	71.5–72.5	65.5–66.5	59.5–60.5
α-Pinene	70.5–71.5	70.5–71.5	70.5–71.5	64.5–65.5	58.5–59.5
Terpinolene	70.5–71.5	70.5–71.5	67.5–68.5	60.5–61.5	52.5–53.5
γ-Terpinene	70.5–71.5	70.5–71.5	65.5–66.5	57.5–58.5	49.5–50.5
allo-Ocimene	69.5–70.5	68.5–69.5	66.5–67.5	62.5–63.5	56.5–57.5
Camphene	67.5–68.5	58.5–59.5	54.5–55.5	52.5–53.5	50.5–51.5
α-Terpinene	69.5–70.5	57.5–58.5	50.5–51.5	46.5–47.5	42.5–43.5
β-Pinene	69.5–70.5	57.5–58.5	49.5–50.5	45.5–46.5	41.5–42.5
α-Phellandrene	70.5–71.5	57.5–58.5	48.5–49.5	43.5–44.5	39.5–40.5
Limonene	71.5–72.5	51.5–52.5	40.5–41.5	34.5–35.5	30.5–31.5
Ocimene	70.5–71.5	41.5–42.5	34.5–35.5	31.5–32.5	29.5–30.5
Myrcene	70.5–71.5	39.5–40.5	32.5–33.5	29.5–30.5	27.5–28.5
Sabinene	70.5–71.5	37.5–38.5	29.5–30.5	26.5–27.5	24.5–25.5

* Refers to $R_F \times 100$.

TABLE III

THE PREDICTED VERSUS THE EXPERIMENTAL $R_F \times 100$ VALUES OF SOME MONOTERPENES ON A 3.125 % SILVER NITRATE IMPREGNATED LAYER USING BENZENE AS THE ELUENT

Compounds	*Predicted $R_F \times 100$ value*	*Experimental value*
Limonene	61.5–62.5	62.5
α-Phellandrene	62.5–63.5	63.0
α-Terpinene	69.5–70.5	70.5
Ocimene	51.5–52.5	52.0
δ-3-Carene	71.5–72.5	72.0
Myrcene	49.5–50.5	50.5

saturated compounds to form π-complexes with the silver ion. Considering this fact, some possible conclusions can be drawn from these results.

(1) Cyclic terpenes with single internal double bonds do not readily form π-complexes.

(2) Cyclic or acyclic terpenes with two non-terminal double bonds do not readily form π-complexes unless the double bonds are *cis* conjugated.

(3) Cyclic or acyclic terpenes with exocyclic or terminal double bonds do form π-complexes.

Recently, NANO AND MARTELLI[17] obtained a concurrent conclusion in their study of the separation of allylic and propenylic derivatives of benzene and cyclohexene. They found that only allylic isomers readily formed π-complexes and were thus able to carry out clean separations of the isomeric pairs studied. Hence, it can be seen that this impregnation technique is very useful in the separation of unsaturated compounds, however, for maximum separation the percentage impregnation is dependent on the types of compounds to be separated.

Acknowledgements

The author would like to acknowledge the financial assistance of the National Research Council via an Industrial Research Assistantship Grant coded 'Spices 807'; also the avid interest and support of Stange Canada and Lawry's Foods of Canada.

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Received September 17th, 1968

CHROM. 3783

Thin layer chromatography of some degradation products of organophosphorus insecticides

This note describes a thin layer chromatographic system, and two modifications of spray reagents for separating and visualising degradation products of certain organophosphorus insecticides.

Chromatography

Acetonitrile–water mixtures provide a simple system for the separation of degradation products of dialkyl aryl phosphorothionates on Silica Gel G layers. The R_F values are very dependent upon the water content. Good separations are obtainable with technical acetonitrile and water in the ratio of 88:12, v/v. A 10 cm development is achieved in 10 min, an advantage when speed is of importance. The silica gel layers

TABLE I

R_F VALUES OF SOME COMPOUNDS IN THE SYSTEM ACETONITRILE–WATER (88:12)

Compound	R_F
Inorganic phosphate	0.0
Inorganic phosphorothionate	0.01
Methyl phosphate	0.02
Methyl phosphorothionate	0.15
Dimethyl phosphate	0.05
Dimethyl phosphorothionate	0.33
Ethyl phosphorothionate	0.16
Diethyl phosphorothionate	0.40
Ethyl ethylphosphonate	0.10
Ethyl ethylphosphonothionate	0.30
Phenyl phosphate	0.00
Phenyl phosphorothionate	0.70
Methyl phenyl phosphorothionate	0.30
Diphenyl phosphate	0.50
4-Bromo-2,5-dichlorophenyl phosphorothionate	0.75
Methyl-4-bromo-2,5-dichlorophenyl phosphate	0.39
Methyl-4-bromo-2,5-dichlorophenyl phosphorothionate	0.59
Ethyl-4-bromo-2,5-dichlorophenyl phosphorothionate	0.46
4-Nitrophenyl phosphate	0.0
4-Nitrophenyl phosphorothionate	0.75
Methyl-4-nitrophenyl phosphate	0.28
Methyl-4-nitrophenyl phosphorothionate	0.37
4-Nitro-3-methylphenyl phosphorothionate	0.70
Methyl-4-nitro-3-methylphenyl phosphate	0.27
Methyl-4-nitro-methylphenyl phosphorothionate	0.50
2,4,5-Trichlorophenyl ethylphosphonate	0.27
2,4,5-Trichlorophenyl ethylphosphonothionate	0.70

should be activated at 100° for 30 min when complete resolution of the compounds with high R_F values is required. The R_F values for some substances are listed in Table I. The system is especially suitable for the separation of O-methyl-O-4-bromo-2,5-dichlorophenyl phosphorothionate (desmethylbromophos) and the corresponding

monoaryl phosphorothionate (bisdesmethyl bromophos). These substances have nearly identical R_F values in the butanol–acetic acid–water systems commonly used for separation of mono- and diesters of phosphoric and phosphorothionic acid.

Spray reagents

BRAITHWAITE[1] reported that the use of DQC, (2,6-dibromobenzoquinone-4-chloroimide) in cyclohexane as a spray reagent for thin layer detection of phosphorothionates, as described by MENN *et al.*[2], works better on layers when HCl is included in the gel. This was also the experience in our laboratory. Use of acetic acid instead of cyclohexane as a solvent for DQC, had the same effect as that described by BRAITHWAITE. 1% DQC in acetic acid was therefore used as spray reagent. This modification is especially favourable with monoalkyl phosphorothionates. They (*e.g.* monomethyl and monoethyl phosphorothionate, and alkyl aryl phosphorothionates) appear as yellow spots which develop instantly. The limit of detection is about 0.2–0.5 μg. Dialkyl phosphorothionates and ethyl, ethylphosphonothionate and aryl, ethylphosphonothionates as well as monoaryl phosphorothionates yield red colours, with full colour development after heating at 100° for 5 min. The limits of detection are about 0.1 μg.

JUNGNICKEL[3] developed a very sensitive method for the detection of phosphorus substances on paper chromatograms. With a modification which provides for the destruction of the compounds, his method is useful for thin layer detection of degradation products of insecticides. A suitable destruction method consists in the combination of heating and U.V.-irradiation. The layers are sprayed very lightly with 1% w/v ammonium molybdate in 0.1 *M* HCl, with 5% v/v of 60% w/v $HClO_4$ added, and then heated directly with the flame of a Thuringer or Bunsen burner, the flame being directed downward upon the gel side of the plate for 2 min. If the plates tend to shatter, because of the quality of the glass, they can be placed in an oven at 200° for an hour, instead. The layers are then exposed to strong U.V.-light for 30 min. Finally, they are sprayed with JUNGNICKEL's reagent No. 2. Crystal violet and Brilliant green provide the highest sensitivity, while Iodine green provides low sensitivity.

Compared to the HANES AND ISHERWOOD method[4], this modification of JUNGNICKEL's method gives a higher sensitivity and is more easily accomplished.

Acknowledgement

This work was supported by the Norwegian Council of Agriculture.

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Received September 16th, 1968

CHROM. 3799

Wool cortical cells: a support for thin-layer chromatography

Unlike cellulose and its derivatives, which have found diverse application, wool keratin is a naturally occurring polymer with potential as a chromatographic support which has been largely neglected. This is notable in view of the well known uses of a variety of chemically related materials of both natural and synthetic origin. Thus, columns of methyl-esterified albumin adsorbed on Kieselguhr[1,2], various proteins deposited on cellulose[3], silk fibroin[4], and of poly-L-lysine on Kieselguhr[5], have found application in the fractionation of biological materials. Various synthetic polyamides[6,7] (nylon or perlon type) have been used in column and thin layer fractionation procedures: polyacrylamides have been developed for molecular sieve chromatography[8].

Wool in fibrous form has been shown to effect partial resolution of racemic mixtures[9] by a process of stereoselective adsorption. As fabric[10] or felt[11] discs packed into columns, it has been utilized in studies of dye migration. However, wool fibres, even when chopped, are not especially convenient or effective for use in chromatographic procedures, no doubt because of their relatively small specific surface.

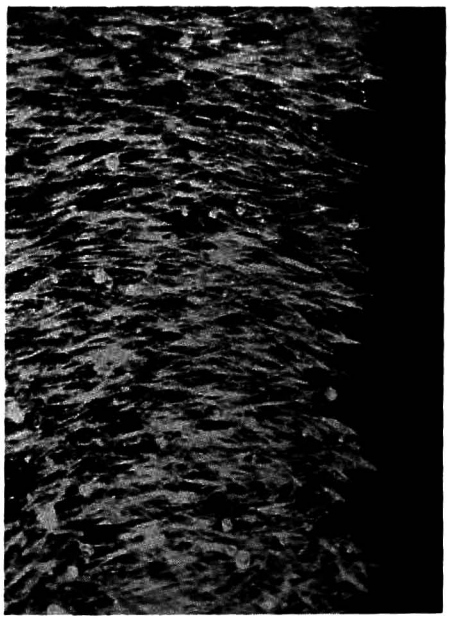
In view of interest in the use of proteins as chromatographic supports we wish to report the successful use of wool cortical cells[12] for this purpose. Cortical cells are readily isolated from fibres by enzymatic hydrolysis. In this work they were prepared by papain digestion of scoured 64's quality wool fibres in the presence of sodium hydrogen sulfite[13]. The cells were deposited on glass plates by doctoring a slurry* to form coherent films quite suitable for thin-layer chromatography. It was observed that layers formed by this means were characterized by the orientation of a large proportion of the component cells in the direction of application (see Fig. 1). These films also exhibited strong internal cohesion and rigid adherence to glass surfaces even in the absence of binder.

Cortical cell films were rather hydrophobic, nevertheless, satisfactory migration rates in the direction normal to the cell orientation have been obtained with both aqueous and non-aqueous irrigants in both the ascending and descending modes. Acid and alkaline chromogenic reagents have been used for detection purposes by spraying or by total immersion. The incorporation of a fluorescence indicator into the layer has permitted detection of substances having absorption in the ultraviolet region.

Initially, to demonstrate the viability of chromatography on thin keratin layers we wish to describe the migration of typical disperse dyes taken from the azo and anthraquinone classes. Replicate observations were made on plates prepared from three separate wool digests. All dyes migrated reproducibly as discrete coloured spots (Fig. 2) with pyridine–water (1:3, v/v) as irrigant in the ascending mode. The R_F values given in Table I are arithmetic means; all experimental observations are embraced by the limits quoted. The examples cited demonstrate that a wide range of R_F values is possible under suitable conditions.

Preliminary experiments have shown that keratin layers are well suited to the study of ionic dyes (including reactive dyes) and other textile auxiliaries. Clearly,

* We use the apparatus of C. Desaga, G.m.b.H., Haupstrasse 60, Heidelberg, Germany.



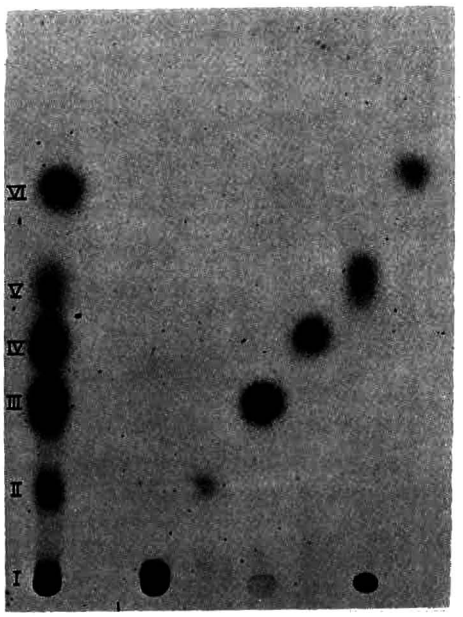


Fig. 1. Photomicrograph (125 ×) of edge of cortical cell layer formed on glass. Individual cells have dimensions of approximately 4 × 100 μ (ref. 12).

Fig. 2. Chromatography of azo dyes on a thin layer of wool cortical cells (see Table I).

TABLE I

C.I. Disperse[18] *dye No.*	*Fig. 2 key*	*Name*	R_F *value*
		Azobenzene	
Yellow 7	I	4-Phenylazo-4′-hydroxy-3′-methyl-	0.03 ± 0.01
Black 2	V	4,4′-Diamino-3′-methoxy-6′-methyl-	0.47 ± 0.02
		4-Nitroazobenzene	
Red 13	II	2-Chloro-4′-(N-ethyl-N-β-hydroxyethyl)amino-	0.18 ± 0.02
Orange 5	III	2,6-Dichloro-4′-(N-methyl-N-β-hydroxyethyl)-amino-	0.29 ± 0.02
Red 1	IV	4′-(N-Ethyl-N-β-hydroxyethyl)amino-	0.38 ± 0.02
Red 17	VI	4′-(N,N-Bis-β-hydroxyethyl)amino-2′-methyl-	0.61 ± 0.03
		Anthraquinone	
Red 15	—	1-Amino-4-hydroxy-	0.40 ± 0.02
Orange 11	—	1-Amino-2-methyl-	0.45 ± 0.03
Blue 14	—	1,4-Di-N-methylamino-	0.50 ± 0.03
Blue 1	—	1,4,5,8-Tetraamino-	0.53 ± 0.03
Violet 4	—	1-Amino-4-N-methylamino-	0.57 ± 0.02
Blue 23	—	1,4-Di-(N-β-hydroxyethyl)amino-	0.76 ± 0.03

however, they have a wider potential—particularly where the amphoteric nature of the substrate may be exploited or where advantage may be taken of interactions with the reactive amino, carboxyl and thiol groups[14,15] or with the hydrophobic side chains[16,17]. Studies on layer and liquid chromatography of other classes of compounds are in progress.

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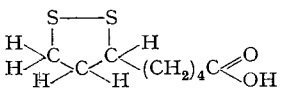
Received September 23rd, 1968

CHROM. 3798

A quick TLC test for detection of mercaptan groups in the presence of other types of sulfur functional groups

In our study of the effect of solvent on the photolysis of α-lipoic acid (LA),



TLC was used both analytically and preparatively. Iodine was used as the visualizing agent. It was observed in the initial runs that the spots due to the presence of LA turned brown immediately but those due to some of the photolysis products turned white. After continued exposure to the iodine, these spots, which had bleached the tan background, eventually became the same dark brown as the LA. Although this phenomenon was evident using analytical TLC, it was very striking on the preparative plates when comparatively large amounts of photolysis products were present. On identification of the photolysis products and on running reference compounds which contained the functional groups present in the products (—COOH, —S—S—, —S—H), it was found that the bleaching action was due to the presence of sulfhydryl groups. Apparently these groups reduced the iodine to iodide ion.

$$2\,RSH + I_2 \rightarrow R{-}S{-}S{-}R + 2I^- + 2H^+$$

Only after all the sulfhydryl was oxidized, could the disulfide which was formed complex with the iodine to produce the familiar dark brown spots or bands.

Therefore, this phenomenon can be used as a quick, qualitative test for the presence of sulfhydryl groups, even in the presence of disulfide and carboxyl groups. This technique can be used in biochemical and histological procedures where it is important to detect small amounts of sulfhydryl.

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Received September 27th, 1968

CHROM. 3801

The thin-layer and column chromatographic separation of some inorganic anions on microcrystalline cellulose

More than six thousand papers have now been published on the subject of thin-layer chromatography (TLC) in the last eight years[1]. The growth and acceptance of inorganic TLC has been noted by the recent review of LEDERER[2]. Much of the work in inorganic TLC, however, has been done on an absorbent of silica gel or alumina[3]. These separations range from the early simple ion separations of Seiler to the separation of some geometric (*cis–trans*) isomers of cobalt, platinum and palladium[4].

Work in our laboratory has shown that microcrystalline cellulose (Avicel) can be successfully used as an adsorbent in the separation of common inorganic cations and several geometrical isomers[5,6]. Its application to the synthesis of some substituted β-trichloroborazines has also been noted[7]. The data obtained in these studies indicate that TLC using microcrystalline cellulose is an analog of paper chromatography.

The present work was undertaken to study the chromatographic behavior of 27 inorganic anions using microcrystalline cellulose as the adsorbent. Both thin-layer and column chromatographic separations are reported.

Experimental

Preparation of the plates. The adsorbent was prepared by blending 75 g of microcrystalline cellulose (technical grade, Avicel Sales Division, FMC Corp., Marcus Hook, Pa.) with 227 ml of distilled water in a Waring blender for 15 sec at low speed. The mixture was then pumped on for 2 min by means of a water aspirator and gently shaken to remove the air bubbles. The mixture was spread on 20 × 5 cm glass plates to a thickness of 0.75 mm, allowed to dry overnight and stored in a sodium hydroxide desiccator until ready for use. This mixture prepared 40 plates.

Mixing time and the amount of water used may vary depending on the lot of microcrystalline cellulose. It is our experience that the most uniform plates are best prepared from a rather fluid Avicel slurry. This is a departure from the plate preparation technique originally suggested by WOLFROM *et al.*[8] and which we reported in our previous paper[5].

Anion preparation and application. The various anions were prepared in 1.0 % (w/v) aqueous solutions from their available sodium or potassium reagent grade salts. All mixtures of the anion solutions were applied to the microcrystalline coated glass by using capillary pipettes and were allowed to dry before insertion into the chromatographic tanks.

Solvent systems. Reagent-grade chemicals were used to make the various solvent systems. These systems were made up to a total volume of 50 or 100 ml. The development time varied from 20 to 70 min.

Detection. Table I summarizes the data of the different detection techniques and reagents used. All of the reagents were applied by a spraying technique. For a multiple detection Table I lists the order of application.

Limits of detection. Ten microliters of successively more dilute solutions of each anion was applied to a TLC plate, developed and detected. In all cases at least 10^{-2} mg of each anion was detectable.

TABLE I

INORGANIC ANION MIXTURES, SOLVENT SYSTEMS, AND R_F VALUES BY THIN-LAYER CHROMATOGRAPHY USING MICROCRYSTALLINE CELLULOSE

No.	*Anion mixture*	*Solvent system (% by volume)*	*Detection*	R_F *value*
1	BO_3^-	Acetone–ethyl acetate–water (3:1:1)	$AgNO_3$, DCF[a]	0.02
	F^-			0.11
	Cl^-			0.38
	Br^-			0.58
	I^-			0.93
2	F^-	Acetone–water (4:1)	$AgNO_3$, DCF	0.04
	Cl^-			0.15
	Br^-			0.27
	I^-			0.56
3	$Fe(CN)_6^{4-}$	Acetone–ethyl acetate–water (6:1:3)	$FeCl_3$	0.17
	$Fe(CN)_6^{3-}$		$FeCl_2$	0.67
	SCN^-		$FeCl_3$	0.97
4	BrO_2^-	Ethanol–water–1.5 *M* NH_3 (6:2:1)	10% KI in 2 *N* HCl	0.00
	IO_3^-			0.36
	BrO_3^-			0.73
	ClO_3^-			0.90
5	PO_4^{3-}	Methanol–butanol–water (2:1:1)	$AgNO_3$	0.20
	$S_2O_3^{2-}$			0.36
	$Cr_2O_7^{2-}$			0.75
6	$HAsO_4^{2-}$	Acetone–water–ethyl acetoacetate (6:1:3)	DCF, $AgNO_3$	0.00
	AsO_2^-			0.03
	SO_3^{2-}			0.05
	NO_2^-			0.12
7	$C_2O_4^{2-}$	Methanol–formic acid (100 ml:10 drops)	DCF, $AgNO_3$, kojic acid, *o*-coumaric acid	0.00
	CO_3^{2-}			0.54
8	CO_3^{2-}	2-Butanol–4 *N* HCl (4:1)	Kojic acid, *o*-coumaric acid	0.09
	SO_4^{2-}		DCF, Laurent's acid, heat	0.59
	NO_3^-		DCF, Laurent's acid, heat	0.81
9	$S_2O_5^{2-}$	Butanol–2-propanol–1.5 *M* NH_3 (1:2:3)	$AgNO_3$, DCF	0.42
	$S_2O_8^{2-}$		10% KI in 2 *N* HCl	0.75
10	CN^-	Methanol–ethylenediamine (100 ml:10 drops)	DCF, $AgNO_3$	0.15
	S^{2-}			0.35

[a] Dichlorofluorescein.

Column preparation. Standard burets fitted with a porous glass frit were filled to a length of 10 in. with microcrystalline cellulose. A 0.5 ml sample of a 1.0% (w/v) solution of the anions was used. A 0.25 in. of sand on top of the column prevented stirring of the adsorbent as the solvent was added. This column gave a flow rate of 12–15 drops per min and was satisfactory for the separation of mixtures 1 and 3 (Table I). Two milliliter fractions from each column were collected, spotted on TLC plates, and analyzed.

Dielectric studies. A Sargent model V oscillometer was used to measure the dielectric constant of various solvent mixtures of acetone, ethyl acetate and water

TABLE II

A COMPARISON OF R_F VALUE, DIELECTRIC CONSTANT, AND DEGREE OF SEPARATION OF $Fe(CN)_6^{4-}$, $Fe(CN)_6^{3-}$ AND SCN^-

Solvent ratio[a]	*Degree of separation*[b]	ε_i[c]	*Average R_F value*		
			$Fe(CN)_6^{4-}$	$Fe(CN)_6^{3-}$	SCN^-
50:20:30	excellent	31	0.21	0.60	0.94
50:30:20	good	28	0.03	0.19	0.82
60:10:30	excellent	39	0.17	0.67	0.97
60:20:20	excellent	31	0.02	0.14	0.85
60:30:10	none	21	0.00	0.00	0.55
70:10:20	good	33	0.00	0.13	0.93
70:20:10	fair	24	0.00	0.02	0.70
20:8 :80	none	60	1.00	1.00	1.00
0:80:20	none	13	0.00	0.00	0.00

[a] Mixtures of acetone, ethyl acetate and water.

[b] A predetermined judgment as to the type of separation considering the spreading of the band and differences in R_F values.

[c] At 25.2°.

used in the separation of $Fe(CN)_6^{4-}$, $Fe(CN)_6^{3-}$ and SCN^-. Table II summarizes the data for the correlation between dielectric constant and degree of separation.

Results and discussion

By using the appropriate solvent system, a separation of the anion groups listed in Table I was established. All solvent systems are novel except system No. 2, which has been reported by BARK *et al.*[9] R_F values were taken from an average of at least ten readings.

Plots of R_F value *vs.* percent ethyl acetate for solvent system No. 1, containing 50, 60, 70 and 80 % acetone, indicated that an increase in the percent of ethyl acetate decreases the R_F value of all four anions. Because of the high R_F value of the fluoride ion in system No. 1 as compared to system No. 2, the BO_3^- ion was also included in this separation. The $B_4O_7^{2-}$ ion gave the same R_F value in system No. 1 as the BO_3^- ion.

A large variation in the composition of solvent system No. 3 separated this anion group, however a large amount of spreading occurred. The mixture reported in Table I gave a spread to only 0.04 R_F units per anion.

Of the large number of detecting reagents tried for anion system No. 4 potassium iodide was the most reliable. It gave an instantaneous brown spot for IO_3^-. The bromate ion (BrO_3^-) gave a brown spot about 20 sec later and the ClO_3^- appeared as a light tan color in about 15 min.

No particular difficulties were encountered in separating anion group No. 5.

The use of U.V. light was most helpful in detecting the members of anion group No. 6. Using DCF the spots of $HAsO_4^{2-}$, SO_3^{2-} and NO_2^- were tan, pink and red, respectively and $AgNO_3$ gave a pink color to AsO_2^-. Under U.V. light, the AsO_2^- spot was yellow and the SO_3^{2-} spot became lighter in color.

Attempts to include other anions into system No. 7 resulted in spreading of the $C_2O_4^{2-}$ band. In the absence of these anions the red band of $C_2O_4^{2-}$ gave an R_F value of 0.00 with a small amount of spreading to R_F 0.02.

The detecting reagents of anion system No. 8 proved to be non-reliable in a wide variety of attempted solvent systems. The SO_4^{2-} and NO_3^- anions were best detected by spraying with DCF and Laurent's acid and heating the plates for a few minutes at 110°. A black and light tan band appeared in SO_4^{2-} and NO_3^-, respectively.

In solvent system No. 9 silver nitrate and DCF gave a visible pink to $S_2O_5^{2-}$, which later turned to reddish brown. $S_2O_8^{2-}$ formed a distinct visible brown on a yellow background with potassium iodide.

A visible dark gray and dark brown detected the CN^- and S^{2-} of system No. 10.

Anion mixtures No. 2 and 3 were successfully separated on a chromatographic column. Decreasing the column length from 10 to 6 in. yielded overlapping of I^-, Br^- and Cl^- fractions in the first 2-ml sample.

A comparison of the dielectric constant of solvent system No. 3 before and after a TLC separation of $Fe(CN)_6^{4-}$, $Fe(CN)_6^{3-}$ and SCN^- showed no significant change in the gross dielectric constant. It is to be noted from the various mixtures of solvent system No. 3 used that the degree of separation ranged from no separation to excellent. Those mixtures having a dielectric constant in the range of 31–39 units gave the best separation. An increase or decrease in the amount of ethyl acetate or acetone in the mixture caused a considerable change in the dielectric constant and this effect resulted in either no separation or excessive band spreading.

A correlation between dielectric constant and degree of separation of the halide ions, using solvent system No. 1, indicated that excellent separations were obtained with dielectric constants of the solvent mixture in the range of 26–31 and good separation from 28–39.

Conclusions

The data obtained in this study indicate that microcrystalline cellulose can be successfully used to separate a large number of inorganic anions. The method described has been applied to both thin-layer and column chromatography. The TLC plates gave a remarkably hard surface which stands up in a variety of solvents.

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Received September 23rd, 1968

Book Review

Amino Acid Determination, Methods and Techniques, edited by S. BLACKBURN, Marcel Dekker Inc., New York, and Edward Arnold Ltd., London, 1968, 271 pp., price £5.10.0.

The book deals with the determination of amino acids, while techniques used in sequence analysis of peptides and proteins are not treated. Dr. BLACKBURN has written several excellent chapters on column chromatography of amino acids, in which a great many recent advances (*e.g.* ultra-micro and accelerated techniques), besides the classical methods, are discussed. The chapters on hydrolytic procedures, spectrophotometric methods and miscellaneous procedures are very well written and very important from the practical point of view. However, the reviewer was unable to find STEGEMANN's well known technique of hydroxyproline estimation, although this procedure is probably the best one.

It is perhaps a matter of opinion, but the reviewer feels that paper chromatography of amino acids (which is treated in a really ridiculous manner) and thin-layer chromatography (which is not even mentioned for amino acids, DNP- and PTH-derivatives) should have been discussed in this book. Especially, TLC has found wide application in the last years (there are over 500 publications on TLC in amino acid and peptide chemistry), and seems to be superior to all other chromatographic techniques when very rapid information on the amino acid content of small molecules is required. On the other hand, "future techniques" (gas chromatography and mass spectrometry) are described, but perhaps a little too briefly.

On the whole, however, DR. BLACKBURN has done an excellent job and the book can be recommended to anyone interested in amino acid analysis.

Robapharm A.G., Basel 6, Switzerland GYÖRGY PATAKI

News

Meetings

FIFTH INTERNATIONAL MEETING OF FORENSIC SCIENCES

The International Association of Forensic Sciences announces the 5th International Meeting of Forensic Sciences, Toronto, Ontario, Canada from June 5th to 11th, 1969.

At this meeting, which is being organized under the auspices of the Canadian Society of Forensic Sciences, papers will be presented in the fields of Forensic Medicine, Pathology, Toxicology, Psychiatry, Criminalistics and Document Examination. The language of the Meeting will be English.

Speakers are requested to submit the titles of their papers by December 1st., and abstracts, not exceeding 400 words, by March 1st., 1969. Any enquiries about the convention can be made to Mr. D. M. LUCAS, M.Sc., President of the Association, 8 Jarvis Street, Toronto 2, Ontario, Canada.

A series of lectures describing recent advances in the use of mass spectrometry in conjunction with other analytical techniques and with automatic data acquisition systems, was held on Wednesday 26th June in the Main Lecture Theatre, Department of Mechanical Engineering, Imperial College, London S.W. 7. Demonstrations were given throughout 26, 27, 28th June in the Chemistry Department, Imperial College. The lectures were given by the staff of the Scientific Apparatus Division of GEC-AEI Electronics, Urmston, Manchester.

Appointments

In ihrer Versammlung am 31. März 1968 haben die Mitglieder der GDCh-Fachgruppe KUNSTSTOFFE UND KAUTSCHUK der Gesellschaft Deutscher Chemiker beschlossen, den Namen ihrer Fachgruppe zu ändern in GDCh-Fachgruppe MAKROMOLEKULARE CHEMIE.

Der Antrag, der von einigen Mitgliedern gestellt worden war, wurde damit begründet, dass die Bezeichnung Kunststoffe und Kautschuk die Fachgruppe offensichtlich als eine technisch-industrielle Interessengruppe klassifiziert. Damit werde aber in der Namensgebung, die ja programmatisch sein sollte, das Interesse der Fachgruppe an der akademischen und auch an der industriellen Grundlagenforschung nicht genügend gewürdigt. Ziel der Fachgruppe als Abteilung der Gesellschaft Deutscher Chemiker sollte aber gemäss § 2 ihrer Satzung der Zusammenschluss von Fachkollegen mit gleichen Interessen und eine bewusste Förderung der Grundlagenforschung sein. Dieser Zielsetzung wird der neue Name besser gerecht.

Der Vorstand der Gesellschaft Deutscher Chemiker hat der Namensänderung zugestimmt.

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Errata

J. Chromatog., 37 (1968) 162–171.

Page 166, legend to Fig. 1, "Heat treatment at 370° after silanisation" should read "Heat treatment-silanisation at 370°".

J. Chromatog., 38 (1968) 145–147.

Page 147, Lit. 3: "*J. Anal. Chem.*" should read "*Z. Anal. Chem.*".

J. Chromatog., 38 (1968) 189–199.

Page 189 and running heads of pages 190–198, to the name of the author "J. M. Vergnaud" should be added the name of "M. Fatscher".