JOURNAL
of
CHROMATOGRAPHY

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VOL. 51 1970

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SPECIAL ISSUE

SECOND INTERNATIONAL SYMPOSIUM ON THE UTILISATION OF GAS CHROMATOGRAPHY AND ASSOCIATED METHODS IN THE PETROLEUM AND PETROCHEMICAL INDUSTRIES

Bratislava (Czechoslovakia), December 3–6, 1969

Edited by J. JANÁK (Brno)

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PREFACE

The Second International Symposium on the Utilisation of Gas Chromatography and Associated Methods in the Petroleum and Petrochemical Industries was held in Bratislava (Czechoslovakia) on December 3–6, 1969 under the sponsorship of the Analytical Section of the Czechoslovak Scientific and Technical Society at the SLOVNAFT National Enterprise in Bratislava and the Institute of Instrumental Analytical Chemistry of the Czechoslovak Academy of Sciences in Brno. At this Symposium 120 specialists from 7 countries took part. The official language was English; Czech and Slovak papers were interpreted simultaneously.

The reports and papers presented (22 in total) covered the following three fields: (i) identification of hydrocarbons by gas chromatography; (ii) precision and accuracy of gas chromatographic analysis; and (iii) new applications and materials.

Introductory talks were delivered by Prof. A. I. M. KEULEMANS (Technical University, Eindhoven, The Netherlands) dealing with methods of hydrocarbon identification by gas chromatography, and by Prof. J. F. K. HUBER (University of Amsterdam, The Netherlands) concerning the problems of theory and techniques of the high-efficiency liquid chromatography. The concluding lecture was given by Dr. C. VIDAL-MADJAR (École Polytechnique, Paris, France) who referred to new separations of geometric isomers by gas–solid capillary gas chromatography. These reports were of an informal character and therefore they were not presented for publication.

This issue is a collection of the papers presented at the Symposium. The only papers omitted are those where the information was of limited, local, importance and those which were in print, mainly in this journal, at the time of the Symposium.

JAROSLAV JANÁK

CHROM. 4710

HIGH PRECISION MEASUREMENT OF SPECIFIC RETENTION VOLUMES

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SUMMARY

A statistical analysis has been performed of the errors incident to the individual experimental parameters necessary for expressing the specific retention volume. A gas chromatograph, in which the working conditions can be controlled with a high accuracy, has been constructed. When using packed columns in this apparatus, the precision of a single measurement corresponds to a coefficient of variation of about 0.2 %; with capillary columns the coefficient amounted to about 0.3 %. The width of the confidence interval of the V_g obtained in two chromatographic runs was about $\pm$ 3 ml/g, which corresponds to a resolving power of about 45,000 theoretical plates at a given relative retention. The reliability attained renders the V_g values useful for calculating the excess thermodynamic properties.

INTRODUCTION

The physico-chemical as well as analytical significance of gas chromatographic retention data have been recognized since the very beginning of gas chromatography[1]. However, the problem of the reliability of measuring retention data has only lately come to the fore as a matter of interest. There are roughly three trends in this field, relating to the schools of KEULEMANS, CRUICKSHANK and GUIOCHON. The objectives of the above trends have been, in the main, the use of precise retention data for fine correlation[2–10] and structure elucidation[11–16], calculation of thermodynamic properties[17–23], and precise measurement of the detector response[24].

A common feature of all the aspects of the problem is the need for a specially designed gas chromatograph, as both the quality and the arrangement of the individual components in conventional analytical gas chromatographs are inconvenient for the above purposes.

The most important gas chromatographic retention characteristic is the specific retention volume, V_g. However, the necessity of specifying and measuring the great number of parameters needed to determine the V_g presents difficulties in the precise and, particularly, the accurate measurement of this retention quantity. From this viewpoint, the determination of the V_g is much more of a problem than the determination of the retention time or relative retention data.

The aim of the present paper is to carry out a detailed analysis of the errors associated with the experimental parameters necessary for calculating the specific retention volume, and to construct a gas chromatograph which will allow measurements with a reliability of at least one order higher than has been attained with commercially available instruments.

THEORETICAL

According to the respective definitions[25], the specific retention volume can be expressed through use of experimental parameters by

$$V_g = \frac{(l_R - l_0)v_f P_f 273 j}{r w P_2 T_f} \tag{1}$$

where l_R and l_0 are the distances from the start line of the substance in question and of a nonsorbed component, respectively, as measured in the chromatogram, r is the chart speed, v_f is the carrier gas volume flow rate as measured under a pressure P_f and temperature T_f in the flowmeter, P_2 is the column outlet pressure, w is the weight of the effective sorbent in the column, and j is the JAMES–MARTIN factor[26], given by $j = 3\,P_2(P_1^2 - P_2^2)/2(P_1^3 - P_2^3)$, where P_1 is the column inlet pressure.

As it is hardly possible to define a standard value of the specific retention volume, it is difficult to check the accuracy of V_g values measured directly. Therefore, attention has been given to the problem of eliminating all the possible systematic errors. The accuracy of the data obtained has been checked[27] by comparing the thermodynamic properties calculated from the V_g with those determined by the calorimetric method. The precision has been expressed by the standard deviation and demonstrated in terms of the resolving power of the apparatus; the data on precision express the repeatability of the measurements.

The estimation of the standard deviation (briefly standard deviation) of a V_g value, s_{Vg}, can be obtained from the standard deviations of the individual parameters. It follows from the rules of statistics[28] that

$$s_{Vg} = \left[\left(\frac{\partial V_g}{\partial l_R}\right)^2 s_{l_R}^2 + \left(\frac{\partial V_g}{\partial l_0}\right)^2 s_{l_0}^2 + \cdots + \left(\frac{\partial V_g}{\partial j}\right)^2 s_j^2\right]^{1/2} \tag{2}$$

where

$$s_j^2 = \left(\frac{\partial j}{\partial P_1}\right)^2 s_{P_1}^2 + \left(\frac{\partial j}{\partial P_2}\right)^2 s_{P_2}^2. \tag{3}$$

Relations (2) and (3) serve as guides in the analysis of errors, unless it is possible to exclude the latter by appropriate measures.

Analysis of the problem

The nature, as well as the magnitude, of the errors to be considered obviously depend on the design of the apparatus. In our arrangement, we have used a specially designed capillary flowmeter with an oblique manometric tube, placed ahead of the injection port. This type of flowmeter[29] yields continuous and pressure-independent information on the flow rate. The column inlet pressure was measured by a multiple U-tube mercury manometer[30,31] situated between the flowmeter and the

inlet port; the resultant mercury level difference, Δh_m, is given by $\Delta h_m = \sum_i \Delta h_{mi}$. The temperatures of the column, flowmeter, and other items were measured by mercury thermometers graduated by 0.1 °C and, in some cases, a Beckman thermometer was used.

The errors of the individual experimental parameters as well as the arrangements used for eliminating some of them are summarized below.

Potential sources of error	*Elimination or estimation of the errors*
Measurement of the carrier gas flow rate	
Changes of temperature	Thermostating of both the measuring capillary and manometric tube
Measuring the angle of positioning the manometric tube	Fixed positioning
Nonuniformity of the internal diameter of the manometric tube	Reading out from both arms of the tube
Readout of the level difference (Δh) in the manometric tube	$s\Delta_h = 0.35$ mm
Measurement of the column inlet pressure	
Changes of temperature	Expressing the mercury column length at 0° C
Parallax	Covering the rear parts of the manometric tubes with a mirror
Nonuniformity of the internal diameter of the manometric tubes	Reading out from all the arms
Deviation from the standard acceleration of gravity	Correction
Readout	$s\Delta_h = \sqrt{3\Delta h_i^2} = 0.24$ mm
Measurement of the atmospheric pressure (The measurement[32] was carried out by means of a conventional mercury barometer)	
Changes of temperature	Recalculation of the density of Hg at 0° C
Deviation from the standard acceleration of gravity	Correction
Readout (using a vernier)	$s_h = 0.1$ mm $\equiv 1.31 \times 10^{-4}$ atm.
Measurement of temperature (ref. 32)	
Errors in the original calibration	Recalibration
Difference in the conditions in calibration and measurements	Correction
Zero point depression	Avoiding the measurement of lower temperatures immediately after having measured high temperatures
Readout	$s_{temp.} = 0.05$° C

Measurement of the retention parameters

The net retention time, $t_R - t_0$, was determined from the respective $l_R - l_0$ and r. The peak maximum was defined as a half distance between the peak shoulders just below the apex, and the point so obtained was transferred to the zero line of the chromatogram. The standard deviation of the difference $l_R - l_0$, $s_{\Delta l}$, determined by the above method was $s_{\Delta l} = \sqrt{s_{lR}^2 + s_{l0}^2} = 0.28$ mm. The standard deviation of the chart speed was determined experimentally and amounted to 2×10^{-4} mm/sec (*cf.* Table I).

Determination of the weight of the stationary phase in the column

The accuracy and precision of determining the weight of sorbent in the column

is of basic importance in measuring V_g. In our measurement, the total weight of the column packing was determined as the difference obtained by weighing an appropriate amount of the packing before and after filling the column. The standard deviation of a single weighing was 0.01 g, so that the standard deviation of the determination of the weight of the column packing, $s_{w(p)}$, is 0.014 g.

Although the solid support had been thoroughly dried before coating it with the stationary phase and the solvent used in the coating procedure carefully evaporated, it was not possible to determine the amount of stationary phase simply from the weight and supposed composition of the packing, owing to undefined changes in the support and stationary liquid proportions during the procedure. Therefore, the true amount of the stationary phase was determined by extracting the stationary phase from a sample of the packing, evaporating the solvent, and weighing the residue. Under the given conditions, the standard deviation when determining the weight of the liquid in the packing by the above procedure, s_{ex}, was 0.0038 g. If $w(p)^*$ and $w(ex)^*$ are the weights of the packing sample and of its respective extract, respectively, the standard deviation of the weight of the stationary liquid in the column, s_w is given by $s_w = [s^2_{ex} + (w(ex)^*/w(p)^*)^2 s^2_{w(p)}]^{1/2}$ and amounts to 0.0052 g.

The above analysis of the problem served as the basis for designing the final concept of the chromatograph. Using this arrangement and hexane chromatographed on squalane at 50° C, the repeatability of the specific retention volume and of measurements of individual parameters was determined. The respective data are summarized in Table I.

TABLE I

STANDARD DEVIATIONS AND PERCENTAGE COEFFICIENTS OF VARIATION ESTIMATED THEORETICALLY FOR RATED VALUES OF THE EXPERIMENTAL PARAMETERS CORRESPONDING TO THE CHROMATOGRAPHY OF HEXANE ON SQUALANE AT 50° C

Variables	*Dimensions*	*Rated value*	*s(theor)*	*V*
Δl	mm	437.9	0.28	0.06
r	mm/sec	0.3339	2×10^{-4}	0.06
v_f	ml/sec	0.3535	2×10^{-4}	0.06
T_f	°K	303.15	0.06	0.02
$P_f = P_1$	atm	1.8360	2.237×10^{-4}	0.01
P_2	atm	0.9917	1.316×10^{-4}	0.01
j		0.68119	1.4×10^{-4}	0.02
w	g	3.4817	0.0052	0.15
T	°K	323.16	0.001	0.001
V_g	ml/g	151.30	0.284	0.19

EXPERIMENTAL

Apparatus

A flow diagram of the apparatus is shown in Fig. 1. The carrier gas as well as the H_2 and air were taken from storage cylinders (the controlling and measuring devices for the air and H_2 paths have not been indicated in the diagram). The pressure of the carrier gas was reduced to about 4 atm by a coarse pressure controller (1) after which the carrier gas enters a flow controlling unit. This unit comprises two high pre-

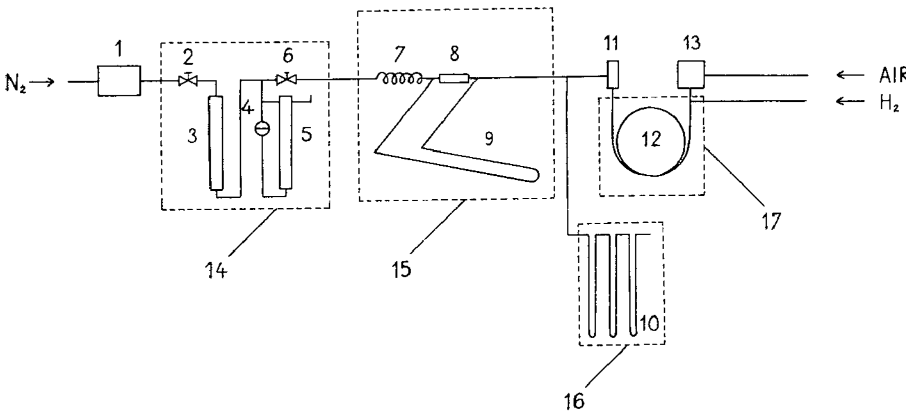

Fig. 1. Flow diagram of the gas chromatograph.

cision needle valves (2,6), a drying tube with molecular sieve 5A (3), and a Cartesian manostat (Hoke-Manostat Corp., Cresskill, N.J., U.S.A.) (4,5). The elements 2–6 were enclosed in a polystyrene-foam box (14) to protect the former against the effects of variations in the ambient temperature. It is evident from the diagram that the controls can function as a source of either constant pressure or flow.

The measuring part of the apparatus is represented by the capillary flowmeter (7, 8, 9) and multiple U-tube mercury manometer (10). Before entering the measuring capillary (8), carrier gas is tempered by passing it through a 2 m long copper capillary of 1 mm inner diameter (7). The pressure drop across the measuring capillary was measured by a 1 m long U-tube (9) filled with dinonylphthalate. The tube was situated in an oblique position, forming an angle of 15° with the horizontal. Both controlling and measuring capillaries as well as the manometric tube were kept at a constant temperature by a water ultrathermostat (15). The measuring capillaries are exchangeable in order to take measurements in various flow rate ranges, this permits for the measurement of various flow rates with an identical relative error. The capillaries were calibrated by means of a Mariotte flask.

The manometer (10) consisted of three 120 cm long U-tubes filled with mercury; the voids between the neighbouring Hg levels were filled with water. The individual tubes were interconnected by brass capillary joints attached to the glass by means of appropriate fittings with silicone rubber sealing rings. The whole manometer was bedded in a polystyrene-foam case (16).

The injection port (11) has been designed with regard to the need for minimizing the dead space while ensuring a sufficient flow-through cross section in order to prevent any appreciable pressure drop across the inlet port. This was achieved by using a special insert liner. The chromatographic columns (12) were stainless steel tubes, 120–190 cm in length and 4 mm I.D., coiled into helices of about 12 cm diameter. The columns were immersed in a glycerol thermostat (17) permitting a working temperature range of 30–150° C. Fine temperature control and vigorous stirring of the liquid ensured good temperature stability and homogeneity within the thermostat.

The set-up can also be used for work with capillary columns. For this purpose the lower part of the injection block can be provided with a fitting communicating

with a fine needle valve, thus forming an inlet splitter affording an adjustable split ratio. The column fitting on the detector side is the same as with packed columns, however, it is necessary that the capillary column outlet extends beyond the hydrogen intake.

A flame ionization detector (13) was used for detection, the signal being processed by means of a conventional amplifier and recorded by an EZ-3 recorder (Laboratory Equipment, n.e., Prague). The hydrogen and air flow rates were controlled, stabilized, and measured using fine needle valves and capillary flowmeters.

Procedures and materials

The performance of the apparatus was tested by chromatographing pentane, hexane, and benzene (B.D.H. Ltd., Great Britain) on a packed and a capillary column with squalane (Carlo Erba Co., Italy) as the stationary phase; and *m*- and *p*-xylene on a packed column with benzylbiphenyl (May & Becker Ltd., Great Britain) as the stationary phase. The purpose of the measurements was to carry out a comparison of the actual errors of V_g's obtained in replicate chromatographic runs with those predicted theoretically according to eqn. 2.

In the work with packed columns, about 25 wt. % of both squalane and benzylbiphenyl were used on Chromosorb W 60/80 mesh (Carlo Erba Co., Italy). Chromatography of the squalane on an SE-30 column showed that it contained 6.1 % of impurities mostly lower hydrocarbons.

After putting the instrument into operation and setting all the working conditions, the apparatus was left running for 3 h to ensure a steady state. The hydrocarbons were injected in the form of 0.1 % solutions in CS_2 with a 10 μl Hamilton syringe, each charge containing 1–2 μl of the solution and *ca.* 1 μl of CH_4 to obtain data on the dead volume.

In the work with the capillary column, the amount of stationary phase in the column was determined from the specific retention volume known from the measurements with packed columns, net retention volume, and the other experimental parameters (*cf.* eqn. 1). The carrier gas flow rate through the capillary column was measured with the help of a Mariotte flask.

RESULTS AND DISCUSSION

The specific retention volumes (arithmetic means of fifteen values) of pentane, hexane, and benzene on squalane, measured at different temperatures, along with the standard deviations of single measurements and the percentage coefficients of variation are summarized in Table II. The data at the bottom of the table refer to the measurements on the capillary column.

It is evident that the actual error in measuring the V_g agrees well with that estimated theoretically. Hence it follows that there were no essentially unpredicted sources of error in the measurements. A higher error ($V = 0.33$ %) can be noticed in the measurement with the capillary column; this is obviously due to a higher error in the measurement of the very low flow rates (10^{-3} ml/sec).

With the precision attained, the effects of impurities present in the stationary phase on the V_g measured became evident. It has been found that the amount of lower hydrocarbons in the squalane used as the stationary phase rose from the initial

TABLE II

V_g VALUES (ARITHMETIC MEANS OF 15–20 MEASUREMENTS) OF PENTANE, HEXANE, AND BENZENE ON SQUALANE AT VARIOUS TEMPERATURES, EXPERIMENTAL STANDARD DEVIATIONS OF SINGLE MEASUREMENTS OF V_g, AND THE CORRESPONDING PERCENTAGE COEFFICIENTS OF VARIATION

Packed column: column length and inner diameter 190 cm and 4 mm, respectively, weight of the column packing 13.5509 g (3.4817 g of squalane), carrier gas flow rate 0.33–0.41 ml/sec at a constant column inlet overpressure of 650 mm Hg; capillary column (last two lines): column length and inner diameter 60 m and 0.2 mm respectively, weight of the squalane 0.01157 g, carrier gas flow rate 3.8 × 10^{-3} ml/sec at an overpressure of 780 mm Hg, split ratio 1:1000.

Temperature (°*K*)	*Pentane*			*Hexane*			*Benzene*		
	$\bar{V}_g$	s_{V_g}	V	$\bar{V}_g$	s_{V_g}	V	$\bar{V}_g$	s_{V_g}	V
303.14	106.23	0.244	0.23	327.66	0.315	0.10	476.17	1.53	0.32
313.19	75.75	0.197	0.26	219.58	0.316	0.14	319.60	0.68	0.21
318.19	64.83	0.095	0.15	182.81	0.480	0.26	266.61	0.51	0.19
323.21	55.16	0.117	0.21	151.64	0.288	0.19	220.75	0.55	0.23
328.17	47.71	0.110	0.23	127.91	0.189	0.15	187.82	0.21	0.11
333.15	41.40	0.081	0.20	108.36	0.222	0.20	159.23	0.23	0.14
338.21	36.35	0.060	0.16	92.38	0.285	0.31	135.62	0.32	0.24
323.10	54.95	0.230	0.42				223.52	0.88	0.39
328.20				126.36	0.308	0.24	186.48	0.49	0.26

6.1 % to as much as 14.7 % upon heating the packing for some hours at 80°C, obviously to the detriment of the squalane content. This resulted in lowering the V_g values of pentane and hexane by 1.0 and 2.5 %, respectively, but the V_g of benzene increased by 1.5 %, in spite of carrying out the correction for loss of stationary phase owing to its volatilization. The above changes in retention are most likely due to changes in the activity coefficients, brought about by the changes in the composition of the stationary phase.

The V_g values of *m*- and *p*-xylene on benzylbiphenyl are in Table III. These data have been used to demonstrate the significance of the precision of V_g values with respect to using them for identification purposes. The separation efficiency of the column employed is apparently insufficient for the resolution of the above pair of substances when injected as a mixture. On the other hand, however, the difference in the V_g values obtained on chromatographing the individual components may or may not be decisive for distinguishing between the two substances. The reliability of identifying a substance from its specific retention volume is given by the reliability

TABLE III

V_g VALUES (MEAN VALUES OF *n* MEASUREMENTS) OF *m*- AND *p*-XYLENES ON BENZYLBIPHENYL AT 104.8 °C, STANDARD DEVIATIONS OF SINGLE MEASUREMENTS (s_{V_g}) AND THE MEAN, VALUES, $s_{\bar{V}_g}$, AND THE RESPECTIVE WIDTHS OF THE CONFIDENCE INTERVAL ($ts_{\bar{V}_g}$)

Column length and inner diameter 190 cm and 4 mm, respectively, weight of the column packing 10.4864 g (2.6216 g of benzylbiphenyl), carrier gas flow rate 0.42 ml/sec at a column inlet overpressure of 440 mm Hg.

Component	$\bar{V}_g$	s_{V_g}	V	$s_{\bar{V}_g}$	$ts_{\bar{V}_g}$	n
p-Xylene	216.77	0.635	0.30	0.1456	0.3059	19
m-Xylene	223.99	0.684	0.30	0.1529	0.3200	20

of the V_g measurement and can be expressed statistically. Namely, two values of V_g may be considered as different only if the difference exceeds the respective width of the confidence interval, *i.e.*, if the expression below holds[33].

$$|\overline{V}_{g2} - \overline{V}_{g1}| > t_\alpha s \sqrt{(1/n_2) + (1/n_1)} \tag{4}$$

where

$$s = \{[1/(n_1 + n_2 - 2)] [(n_2 - 1)s_2^2 + (n_1 - 1)s_1^2]\}^{1/2} \tag{5}$$

1 and 2 denote two different substances, t_α is the critical Student coefficient for the confidence level α and the given number of degrees of freedom, and n stands for the number of measurements. The symbol $\overline{V}_g$ signifies the arithmetic mean.

To illustrate the case in chromatographic terms, let us express the number of theoretical plates that would be necessary for perceptibly resolving two substances with V_g values differing from each other just by the width of a confidence interval. Using KAISER's method[34] for determining the number of theoretical plates N necessary to attain a degree of separation, v, it is possible to write

$$N = 2\left(\frac{V_{g2} + V_{g1} + 2V_{g0}}{V_{g2} - V_{g1}}\right)^2 \ln \frac{2}{1 - v} \tag{6}$$

where V_{g0} is the column dead volume. Taking account of eqns. 4, 5 and 6 and supposing that $v = 0.1$ is sufficient for perceptible resolution, it can be concluded that identification may be carried out with V_g values differing from each other by about ± 3 ml/g with only two chromatographic runs for each substance; this precision corresponds to a resolving power of more than 40,000 theoretical plates at a given V_{g2}/V_{g1} ratio and negligible V_{g0}. The number of theoretical plates used in the above conception obviously rises on increasing the number of measurements.

CONCLUSIONS

Judicious design of the gas chromatograph, careful calibration of all the measuring units, and accurate control of the working conditions have permitted the minimization of systematic errors in measuring the specific retention volume and have made it possible to measure V_g values with a precision corresponding to a coefficient of variation of about 0.2 %.

The actual standard deviation of the V_g values measured on an apparatus of the above type agreed well with the standard deviation estimated theoretically by statistical analysis of the individual experimental parameters. The reliability attained in measuring the V_g makes it possible to calculate excess thermodynamic functions from the V_g values successfully.

In the case of only two chromatographic runs with the substance under analysis, the width of the confidence interval of the respective V_g's corresponds to a resolution power equivalent to about 45,000 theoretical plates at a given relative retention.

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CHROM. 4711

THE APPLICATION OF PRECISION GAS CHROMATOGRAPHY TO THE IDENTIFICATION OF TYPES OF HYDROCARBONS

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SUMMARY

It appears that under precisely controlled conditions retention indices of apolar substances on apolar stationary phases can be reproduced to 0.03 units. This permits the accurate measurement of the temperature coefficients of the KOVÁTS index for different classes of hydrocarbons. In this way classes of compounds having similar ΔI values ($I_{polar} - I_{apolar}$) may be distinguished. This is of particular importance because of the lower precision of the index data on polar stationary phases.

INTRODUCTION

The importance of the characterization of volatile substances by accurate gas chromatographic retention data cannot be emphasized enough, particularly since the sample requirements differ by orders of magnitude from the amounts needed by spectroscopic techniques. The amount of information obtained increases if more accurate data become available (number of distinguishable peaks between two adjacent *n*-alkanes). Apart from an increase in precision identification can be enhanced by using different temperatures or stationary phases.

RETENTION INDICES ON TWO PHASES OF DIFFERENT POLARITY

The use of ΔI (the difference in the retention index on polar and apolar phases) has already been introduced by KOVÁTS[1] as an aid to recognizing types of compound. ΔI depends on both the polarity of the compounds and the polarity of the different stationary phases.

Retention indices of a number of hydrocarbons measured in our laboratory[2] on octadecene-1 and dimethylsulfolane at 25° are plotted in Fig. 1. Capillary columns of 50 m length and 0.25 mm I.D. were used. It should be noted that all the saturated

paraffins are scattered around the line $I_A = I_P$. The alkenes form groups of branching isomers on overlapping parallel lines (the Roofing tile effect according to WALRAVEN *et al.*[3]).

The scattering of point around isomer lines is real (not caused by inaccurate measurements) and gives a relationship between more detailed structure and retention behavior. The fine structure is demonstrated in Fig. 2 (WALRAVEN[3]).

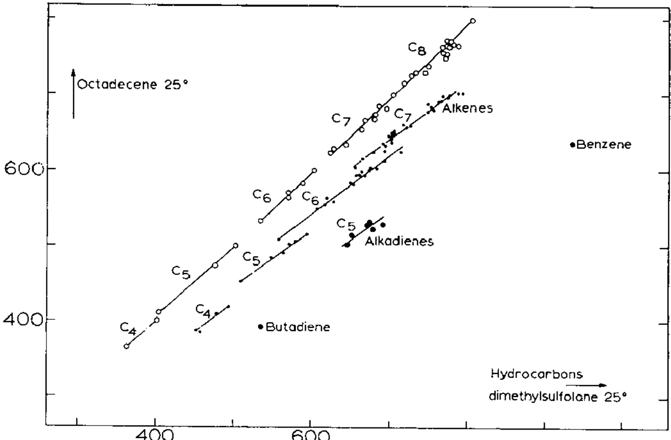


Fig. 1. Retention index plot on two phases of different polarity.

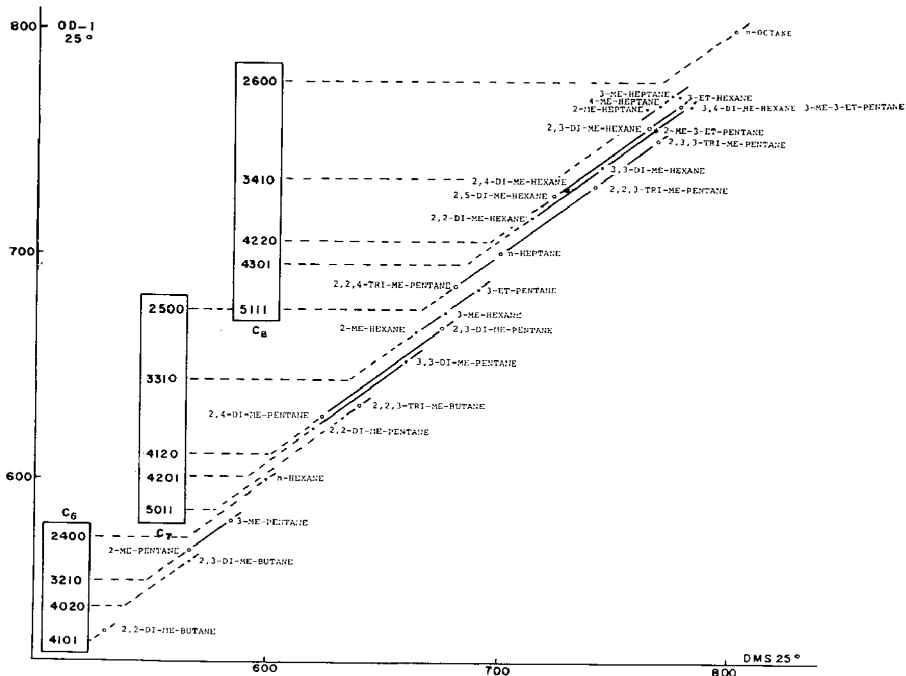


Fig. 2. Fine structure of retention index plot.

THE TEMPERATURE DEPENDENCE OF THE RETENTION INDEX

One of the reasons favoring the use of the retention index in the collection of retention data is its weak temperature dependence. However, if extreme care is taken in the measurements of retention indices, at two not too distant temperatures, the value of dI/dT appears to given some additional analytical information.

This offers promising possibilities[4], particularly in the analysis of complex mixtures of apolar substances (like petroleum fractions). With a small temperature change, peak inversion is unlikely to occur and dI/dT values are relatively easy to collect. The opposite is true for ΔI values, where quantisation of the peaks involved is essential for identification.

COLLECTION OF RETENTION DATA

For future work, sets of collected retention data will be essential for identification purposes. A review of the literature, however, reveals a lack of agreement between different sources. For instance $I_{\text{cyclohexane}}$ on squalane as the stationary phase at 100° shows a discrepancy of 60 units! On this basis KOVÁTS was right in not expecting decimal places in his index system.

The observed discrepancies may be due to imperfect instrumentation (inaccuracies in temperature and flow control). Other reasons are the use of not very well defined stationary phases, or chemical changes in the stationary phase during use. Adsorption on the solid support is also an important source of error, especially if polar substances are run on a non-polar column.

Nonlinearity in the chromatographic process, caused by overloading or adsorption, results in concentration dependant retention times. Therefore, retention times obtained from asymmetric peaks were discarded in our work and the measurements were repeated with smaller samples. Another important factor is that, for several reasons, shifting of peak maxima will occur in the case of incomplete resolution.

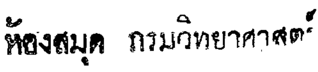
For many of the reasons listed below, the use of capillary columns is becoming more and more important. (High resolution, low concentrations, no solid support, sharp peaks, easier thermostating and flow control.)

PRECISE MEASUREMENT OF RETENTION INDICES

Instrument conditions:
Capillary column: 100 m, stainless steel, I.D. 0.25 mm
Stationary phase: squalane
Plate number $\simeq$ 300000
Temperature: 50–70° $\pm$ 0.05°
Pressure: 2 atm $\pm$ 0.002 atm
Sample size: $<$ 0.02 μg/component

Retention times are measured with a stop watch with an accuracy of 0.3 sec (The use of digital electronic integrators for this purpose appeared to be unsuccessful.) More accurate time measurements by means of an on-line process computer is in progress.

The retention time of an unretarded component must be accurately known for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the calculation of the KOVÁTS index as shown under the index definitions. With ionization detectors methane is usually used; however, methane is, to some extent, soluble in all stationary phases and thus a definite error is introduced in the calculations.

INDEX DEFINITIONS

KOVÁTS:

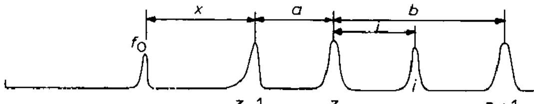
$$I = 100\,Z + \frac{\log \dfrac{i + a + x}{a + x}}{\log \dfrac{a + b + x}{a + x}} \cdot 100$$

$$\text{using } \log \frac{a + b + x}{a + x} = \log \frac{a + x}{x} \text{ yields:}$$

GROENENDIJK–WALRAVEN:

$$I = 100\,Z + \frac{\log (1 + i/a - i/b)}{\log b/a} \cdot 100$$

GROENENDIJK, VAN KEMENADE AND WALRAVEN of this laboratory have proposed a new definition of the retention index, utilizing the linear relationship that exists between the logarithms of the adjusted retention times and the number of carbon atoms in a homologous series of n-alkanes. They realize that this relationship is in fact non-linear, but *define* a linear plot between three alkanes $Z - 1$, Z, and $Z + 1$.



As may be seen from the index definitions, the two higher n-alkanes should bracket the component i, if I_i is to be calculated. However, following a suggestion of HALÁSZ, we succeeded in measuring t_0 directly with a flame ionization detector by introducing a relatively large sample of H_2 (or He). For this purpose the hydrogen flow to the FID must be diminished to about 60 % of its normal value.

Table I shows the retention data obtained by the different methods of t_0-estimation. It is clear that for capillary columns the use of methane gives a better approximation to the ideal value than does the use of extrapolation methods.
Using $t_{CH_4} = t_0$ retention indices of a number of hydrocarbons on squalane at 50–60° and 70° were measured.

Table II shows the repeatability on one instrument (over a period of three months) to be better than 0.03 I units!

Comparison of our data with similar work done by different workers[4] reveals deviations larger than can be explained by the confidence limits of the respective measurements. This can only partly be explained by systematic deviations in absolute temperatures or different methods in estimating t_0. In our opinion, the main factor contributing to the discrepancies in the literature data is the slight dependency of the retention times upon the sample size (peak asymmetry). A comparison of literature data is given in Table III. By definition the dI/dT for n-alkanes is zero.

As would be expected slightly branched hydrocarbons have only small values

TABLE I

RETENTION INDICES ON A SQUALANE CAPILLARY COLUMN AT 70° WITH DIFFERENT t_0

Solute	*t_0 measured*		*t_0 calculated*			*According to Groenendijk*
	CH_4	*H_2*	*$C_4C_5C_6$*	*$C_5C_6C_7$*	*$C_6C_7C_8$*	
1*t*2DiMeCPr	479.76	479.69	479.25	479.08	479.04	—
ECPr	511.53	511.51	511.38	511.33	511.31	511.38
22DiMeBu	538.46	538.41	538.13	538.02	537.98	538.13
112TriMeCPr	550.33	550.29	550.00	549.88	549.85	550.00
CPe	568.49	568.46	568.22	568.12	568.10	568.22
3MePe	585.14	585.12	584.99	584.94	584.92	584.99
ECBu	623.43	623.42	623.33	623.29	623.28	623.29
MeCPe	630.96	630.95	630.84	630.80	630.79	630.80
223TriMeBu	642.59	642.58	642.46	642.41	642.40	642.41
CHex	667.10	667.09	666.99	666.95	666.94	666.95
3MHex	676.94	676.93	676.86	676.82	676.81	676.82
1*t*2DiMeCPe	692.38	692.37	692.34	692.33	692.33	692.33
22DiMeHex	720.47	720.47	720.43	720.42	720.41	720.41
MeCHex	730.63	730.63	730.59	730.57	730.56	730.56
ECPe	737.49	737.49	737.44	737.42	737.42	737.42
223TriMePe	740.11	740.10	740.06	740.04	740.03	740.03
112TriMeCPe	767.88	767.87	767.83	767.82	767.81	767.81
3MeHept	772.92	772.91	772.88	772.86	772.86	772.86

TABLE II

REPEATABILITY OF MEASUREMENTS OVER A PERIOD OF THREE MONTHS

Operating conditions: column immersed in the silicone oil bath at 70° ± 0.05°; pressure: 2.00 atm; split ratio: 1:200, using Hamilton splitter inlet system; heated FID; sample: 0.1 μl liquid sample + 6 μl of methane and *n*-butane. Number of measurements: 6.

Compound	*a_{i,nC_6}*			*I*	
	Average	*σ*	*σ%*	*Average*	*σ*
n-Bu	0.1544	0.0002	0.15	400.00	0.00
1*t*2DiMeCPr	0.3307	0.0003	0.10	479.76	0.12
n-Pe	0.4012	0.0004	0.11	500.00	0.00
ECPr.	0.4458	0.0003	0.08	511.53	0.08
22DiMeBu	0.5701	0.0005	0.08	538.45	0.08
112TriMeCPr	0.6354	0.0003	0.04	550.33	0.03
CPe	0.7500	0.0003	0.05	568.49	0.04
3MePe	0.8731	0.0002	0.02	585.14	0.04
n-Hex	1.0000	0.0000	0.00	600.00	0.00
ECBu	1.2323	0.0006	0.05	623.43	0.04
MeCPe	1.3179	0.0005	0.04	630.96	0.03
CHex	1.8188	0.0007	0.04	667.10	0.02
3MeHex	1.9856	0.0006	0.03	676.94	0.01
1*t*2DiMeCP	2.2784	0.0008	0.04	692.38	0.01
n-Hep	2.4387	0.0010	0.04	700.00	0.00
22DiMeHex	2.9213	0.0012	0.04	720.47	0.01
MeCHex	3.1953	0.0013	0.04	730.63	0.01
ECPe	3.3946	0.0012	0.03	737.49	0.01
223TriMePe	3.4738	0.0020	0.04	740.11	0.01
112TriMeCPe	4.4380	0.0020	0.04	767.88	0.01
3MeHep	4.6396	0.0017	0.04	772.92	0.01
n-Oct	5.8916	0.0026	0.04	800.00	0.00
1*t*2DiMeCHex	6.2742	0.0027	0.04	806.21	0.00

TABLE III

COMPARISON OF OUR RESULTS WITH DATA GIVEN IN LITERATURE

Solute	*I-50*				
	Our results	TOURRES	HIVELY	KEMENADE	Δ_{max}
22DiMePr	412.30	411.7	412.6	—	0.9
2MeBu	475.32	474.0	474.9	—	1.3
22DiMeBu	536.81	536.6	537.1	—	0.5
CPe	565.74	565.7	566.5	—	0.8
2MePe	569.66	569.5	569.7	—	0.2
3MePe	584.24	584.0	584.6	—	0.6
22DiMePe	625.64	625.9	625.9	625.63	0.3
MeCPe	627.90	628.5	628.8	—	0.9
223TriMeBu	639.70	639.8	640.3	639.58	0.7
33DiMePe	658.85	658.9	659.2	658.72	0.5
CHe	666.62	666.5	666.9	666.58	0.4
11DiMeCPe	673.45	674.3	674.2	—	0.8
3MeHe	676.21	676.2	676.4	676.16	0.2

TABLE IV

VARIATION OF RETENTION INDICES WITH TEMPERATURE

Temperature: 25°. See also ref. 2

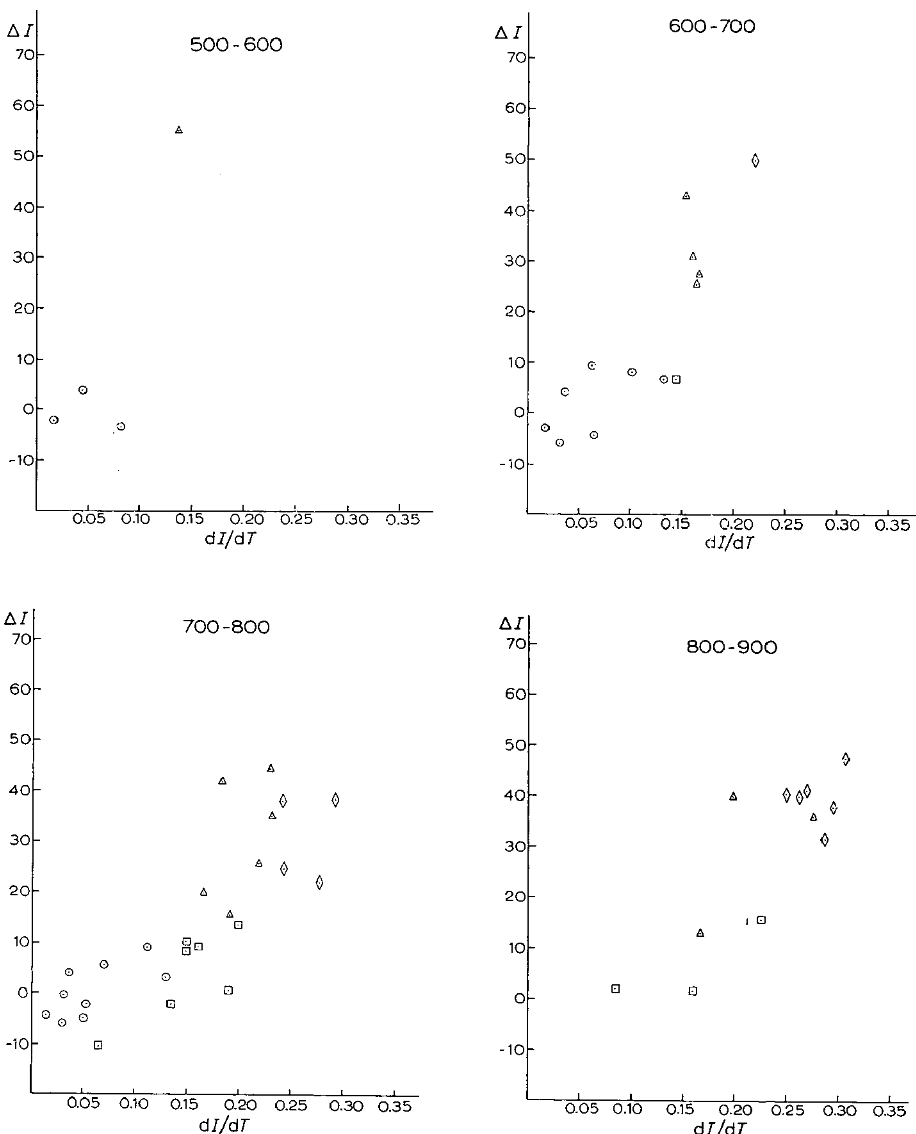
Solute	I_{50}	I_{70}	dI/dt	$\Delta I_{DMS-OCTAD}$
n-Bu	400.00	400.00	—	—
22DiMePr	412.30	412.91	0.030	−14.1
2MeBu	475.32	475.47	0.007	− 2.1
1*t*2DiMeCPr	479.20	479.76	0.028	—
n-Pe	500.00	500.00	—	—
ECPr	510.20	511.53	0.066	—
1*c*2DiMeCPr	515.01	516.15	0.057	—
22DiMeBu	536.81	538.45	0.082	− 3.3
112TriMeCPr	549.24	550.33	0.054	—
CPe	565.74	568.49	0.137	55.3
2MePe	569.66	570.01	0.017	− 2.1
3MePe	584.24	585.14	0.045	3.9
n-Hex	600.00	600.00	—	—
1122TetraMeCPr	619.85	621.75	0.095	—
ECBu	620.99	623.43	0.122	—
22DiMePe	625.64	626.95	0.065	− 4.2
MeCPe	627.90	630.96	0.153	42.9
223TriMeBu	639.70	642.59	0.144	6.8
33DiMePe	658.85	661.50	0.132	6.9
CHex	662.73	667.10	0.218	50.0
2MeHex	666.62	666.96	0.017	− 2.8
1*c*3DiMeCPe	682.65	685.92	0.163	25.8
1*t*3DiMeCPe	686.78	690.10	0.166	27.4
1*t*2DiMeCPe	689.20	692.38	0.159	31.1
n-Hep	700.00	700.00	—	—
22DiMeHex	719.44	720.47	0.051	− 5.0
113TriMeCPe	723.64	727.47	0.191	15.7
MeCHex	725.78	730.63	0.242	37.9
ECPe	733.83	737.49	0.183	42.1
223TriMePe	737.15	740.11	0.148	8.6

TABLE IV (*continued*)

Solute	I_{50}	I_{70}	*dI/dt*	$\Delta I_{DMS-OCTAT}$
1*t*2*c*3TriMeCPe	747.81	751.11	0.165	19.8
112TriMeCPe	763.24	767.88	0.232	35.2
2MeHep	764.86	765.16	0.015	− 4.4
3MeHep	772.29	772.92	0.031	0.3
1*c*2*t*4TriMeCPe	773.06	777.42	0.218	25.5
1*c*3DiMeCHex	785.04	789.89	0.242	24.6
11DiMeCHex	786.97	792.80	0.291	38.3
n-Oct	800.00	800.00	—	—
1*t*2DiMeCHex	801.80	807.54	0.287	31.4
1*c*2*c*3TriMeCPe	802.20	807.75	0.277	36.0
1*t*3DiMeCHex	805.61	810.84	0.261	39.7
223TriMeHex	821.56	824.33	0.138	—
1*c*2DiMeCHex	829.27	835.41	0.307	47.5
ECHex	834.28	839.66	0.269	40.9
113TriMeCHex	840.39	846.03	0.282	—
2233TetraMePe	852.11	857.61	0.257	—
n-Non	900.00	900.00	—	—
Tourres (ref. 4)				
24DiMePe			−0.032	− 5.8
23DiMePe			−0.101	8.1
3EtPe			−0.062	9.50
25DiMeHex			−0.030	− 6.0
24DiMeHex			−0.053	− 2.4
33DiMeHex			−0.130	3.2
234TriMePe			−0.150	10.2
23DiMeHex			−0.072	5.5
2Me3EtPe			−0.160	9.4
4MeHept			−0.027	− 0.4
34DiMeHex			−0.112	9.4
3Me3EtPe			−0.199	13.7
3EtHex			−0.037	4.4
2244TetraMePe			−0.190	0.5
225TriMeHex			−0.065	−10.3
224TriMeHex			−0.135	− 2.1
2234TetraMePe			−0.225	15.8
244TriMeHex			−0.160	1.6
235TriMeHex			−0.085	2.0
1*c*2DiMeCPe			−0.230	44.5
1*t*2*c*4TriMeCPe			−0.167	13.1
1*t*4DiMeCHex			−0.277	21.9
1Me1ECHex			−0.250	40.1
1*c*4DiMeCHex			−0.295	37.6
IsoPrCHex			0.265	38.3
PrCPe			0.198	40.0

of dI/dT. The numerical value of dI/dT increases with the degree of branching, the largest values are found for cyclic compounds (5 and 6 rings). HIVELY AND HINTON[5] correlated the magnitude of dI/dT to the increase in the minimum cross sectional area of the molecule. From dI/dT values only, it does not appear to be possible to distinguish highly branched paraffins and C_5 cyclic compounds. If, however, dI/dT for several components is plotted against ΔI values for the same components a distinction can be made because of the appreciably larger ΔI value for C_5 cyclic compounds. (This of course is in fact a three-dimensional plot.)

Values of dI/dT measured in our laboratory and some additional values of



Figs. 3–6. Plot of the temperature coefficient of the retention index (on squalane) against ΔI ($I_{\text{dimethylsulfolane}} - I_{\text{octadecene-1}}$). ⊙ = slightly branced paraffins; ⊡ = highly branched paraffins; △ = C_5 cyclic compounds; ◇ = C_6 cyclic compounds.

TOURRES[4] are collected in Table IV, together with ΔI data ($I_{\text{dimethylsulfolane}} - I_{\text{octadecene-1}}$ at 25°, see Ref. 2). The same data are presented in Figs. 3, 4, 5 and 6.

It can be concluded that this method offers the possibility of identifying different types of hydrocarbon in oil fractions, although further experiments have to be done, especially in the higher molecular weight range.

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CHROM. 4712

PRECISION OF GAS CHROMATOGRAPHIC RETENTION DATA IN THE MANUAL PROCESSING OF THE CHROMATOGRAM

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SUMMARY

Absolute and relative retention data and retention indices used for the qualitative characterization of substances analyzed in gas chromatography are not constant quantities, but quantities characterized by certain variances. A linear relationship has been found between the standard deviation of corrected absolute retention data and their magnitudes, the numerical expression of which can be looked upon as characteristic for the given type of substance analyzed, the stationary phase used and for the experimental conditions employed. Some questions concerning the calculation of the relative retention data and retention indices from absolute retention data are discussed on the basis of the above-mentioned empirical relation. It is possible to estimate, from the variance of absolute retention data obtained experimentally, the standard deviation of relative retention data and retention indices of the substances analyzed, the variances calculated being in a good agreement with those obtained experimentally. The standard deviation then characterizes, in a useful manner, the precision of the retention data of the substance analyzed, obtained under given conditions.

INTRODUCTION

The increasing number of published gas chromatographic data for substances analyzed on various stationary phases has brought forth a number of proposals for unifying the type of gas chromatographic retention data published. It has been suggested by the authors of "Recommendations for the Publication of Retention Data"[1] that the retention data should be published, for common analytical purposes, in the form of KovÁts indices I (ref. 2) whenever it is possible, or in the form of relative retention data R (ref. 3) related to *n*-nonane[4], either directly or by means of a secondary standard. The unification of the type of retention data published, the definition of the way they were obtained from the basic data measured, and a statement concerning the experimental conditions (the data are only given their full value on quotation of the latter)[1,5] were the first steps towards the utilization of the published retention data obtained from qualitative gas chromatography as a means of identifying the substances analyzed.

With the same purpose in view, some authors[1,6–8] evaluated the reproducibility of retention data measured for substances of different chemical character on various stationary phases, various instruments, and in different laboratories, and the results of these measurements were processed, to different extents, statistically.

ADLARD *et al.*[1] have recommended that the retention index I is used as a qualitative characteristic, as the percentage standard deviation displayed by retention indices of substances analyzed on squalane as a stationary phase were lower than those of relative retention data of the type R_{xN}, R_{x9}, and R_{xS} (related to a hydrocarbon with N and nine carbon atoms, and to a standard substance S, respectively).

The members of the Data Subcommittee of the GC Discussion Group[6] have measured the retention indices of an identical group of substances at different laboratories, and found a mean deviation of ± 1 unit on squalane and dinonylphthalate as stationary phases, and a mean deviation of ± 3 units on PEG 400. When PEG 400 coming from different batches is used, the mean deviation of the retention indices rose to ± 5 units. It was also found, from the same series of measurements, that the mean deviation of retention indices measured on polar stationary phases depends on the amount of sample injected. On the basis of reviewing retention indices measured on PEGA in "The Fatty Acids Ester — Hydrocarbon Correlation Trial", SWOBODA[7] recommended the use of secondary standards similar in chemical nature to the substances analyzed as a basis for a logarithmic scale to increase the precision of measuring the retention indices of polar substances.

A very detailed statistical treatment of the repeatability and reproducibility of retention indices as well as the respective results have been described in a paper by LOEWENGUTH[8]. He concludes by recommending that when publishing or using retention indices in qualitative analysis, the basic factors contributing to the variance of the retention indices should be taken into consideration, namely, the measurement of the retention time and column temperature control (the second factor plays a significant role particularly with substances showing higher $\Delta I/10$ values).

The list of papers giving more or less consistent data on the reproducibility of measuring retention data under conventional analytical conditions can be supplemented by papers devoted to the precision of measuring retention data at programmed temperatures[9,10]. In addition, data have been published on the precision of measuring retention indices under experimental conditions which have been arranged to give very precise measurements[11–14].

The aim of the present paper is to complete the existing information on the reproducibility of retention data measured under current analytical conditions by an analysis of the repeatibility of basic types of retention data measured under simplified conditions which eliminate such effects as the differences in the nature of solute and sorbents, chemical nonuniformity of the stationary phase, sample size, incomplete separation of the substances, and sorption activity of the support. The conclusions derived may assist in the correct handling of retention data in qualitative gas chromatography.

EXPERIMENTAL

The absolute retention data of basic types of hydrocarbons were measured on a Shimadzu GC-4A PTF apparatus, employing the variant with thermal conductivity

detection. 1.0 μl of a hydrocarbon mixture (n-alkanes: n-pentane, n-hexane, n-heptane, n-octane; alicyclic hydrocarbons: cyclopentane, cyclopentene, cyclohexane, cyclohexene; aromatic hydrocarbons: benzene, toluene, o-xylene, ethylbenzene) was repeatedly injected into a column, 2 m long and 0.4 cm I.D., packed with 25% squalane on Chromosorb W 60/80 mesh, over a period of 12 h. Hydrogen was used as the carrier gas at a flow rate of 40 ml/min. The column temperature and chart speed were 80° and 0.5 cm/min, respectively, and a single analysis took about 45 min. The retention distances, t_m, of peak maxima of the individual substances from the peak maximum of nonsorbed component (air) were measured by a rule graduated in millimeters. Mean values, $\bar{t}_m$, pertaining to the individual substances, were calculated. The calculated values of the standard deviations s_E may be looked upon, at the given number of measurements ($n = 15$), as a measure of precision of the absolute retention data, under the given conditions and experimental arrangements.

The values measured were analyzed by the usual statistical methods[15].

RESULTS AND DISCUSSION

Variance of absolute retention data

Absolute retention data are basic qualitative characteristics of the substances analyzed, from which various types of relative retention data are derived. Therefore, attention was given first to the variance of the absolute retention data of basic hydrocarbons which had been separated on squalane under conventional chromatographic conditions.

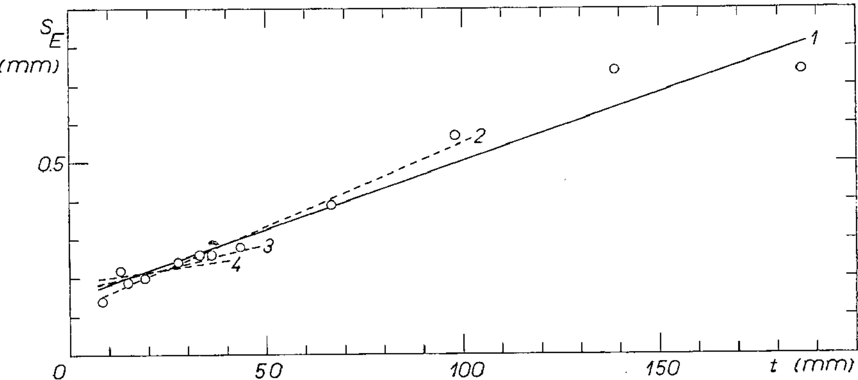

Fig. 1. The dependence of the standard deviations on the respective corrected absolute retention values of the substance analyzed. t is the corrected absolute retention value for the substance m (mm); s_E is the experimentally obtained estimation of the standard deviation of the value t (mm); the regression lines 1, 2, 3, and 4 have been calculated for 12, 10, 8, and 6 values of $[t, s_E]$, respectively.

It is evident from Fig. 1 that the magnitude of the absolute retention values of the substances analyzed is approximately linearly proportional to the respective standard deviation. Experimental data of the above type can be processed by the method of least squares[16], which simplifies further consideration or calculations with the measured data as well as the use of them. The linear regression of $[\bar{t}, s_E]$ values for twelve hydrocarbons gave the straight line

TABLE I

VARIANCE OF THE ABSOLUTE RETENTION VALUE t OF THE SUBSTANCES ANALYZED

(a) $\bar{t}$ is the mean of the corrected retention values t_j (in mm) of the substance m; s is the estimation of the standard deviation of the corrected absolute retention value of the substance m; s_t has been calculated from the relation (1a); $s_E = \{[1/(n-1)] \sum_{j=1}^{n} (t_j - \bar{t})^2\}^{1/2}$, $n = 15$; v is the coefficient of variation of the corrected absolute retention values of the substance m, expressed as a percentage, $v_t = 100(s_t/t)$, $v_E = 100(s_E/t)$. (b) $\bar{t}_D$ is the mean of the measured lengths t_D corresponding to the corrected absolute retention value of the substance m; s_D is the standard deviation of the measured lengths t_D; s_D^2 is the variance of the measured lengths. (c) $\bar{t}_M$ is the mean of the measured lengths t_M corresponding to the corrected absolute retention value of the substance m; s_M is the standard deviation of the measured lengths t_M; s_M^2 is the variance in determining the peak maximum, including the variance of the measured lengths, s_D^2. (d) F is the calculated value of the Snedecor criterion F; $F_1 = s_M^2/s_D^2$, $F_{crit} = 1.26$; $F_2 = s_m^2/s_D^2$, $F_{crit} = 1.74$; $F_3 = s_m^2/s_M^2$, $F_{crit} = 1.74$.

m	Ia						Ib			Ic			Id		
	$\bar{t}$ (mm)	s_E (mm)	v_E (%)	s_t (mm)	v_t (%)	s_t^2 (mm²)	$\bar{t}_D$ (mm)	s_D (mm)	$(t_D-\bar{t}_D)^2$ (mm²)	$\bar{t}_M$ (mm)	s_M (mm)	$(t_M-\bar{t}_M)^2$ (mm²)	F_1	F_2	F_3
1 *n*-Pentane	8.0	0.14	1.8	0.18	2.2	0.0324	8.4	0.10	0.15	8.2	0.05	0.04	3.12	9.81	3.15
2 Cyclopentene	12.8	0.22	1.7	0.19	1.5	0.0361	13.0	0.00	0.0	12.7	0.0	0.0		10.93	3.50
3 Cyclopentane	14.7	0.19	1.3	0.20	1.4	0.0400	15.0	0.00	0.0	14.9	0.13	0.24		12.12	3.88
4 *n*-Hexane	18.9	0.20	1.1	0.21	1.1	0.0441	19.0	0.00	0.0	19.0	0.11	0.18		13.36	4.28
5 Benzene	27.5	0.24	0.9	0.24	0.9	0.0576	28.0	0.00	0.0	27.5	0.08	0.08		17.43	5.59
6 Cyclohexane	33.8	0.26	0.8	0.27	0.8	0.0729	34.3	0.11	0.18	34.0	0.08	0.09		22.09	7.08
7 Cyclohexene	36.1	0.26	0.7	0.28	0.8	0.0784	36.6	0.10	0.13	36.3	0.08	0.09		23.76	7.61
8 *n*-Heptane	43.2	0.28	0.6	0.30	0.7	0.0900	43.3	0.05	0.04	43.0	0.05	0.04		27.27	8.74
9 Toluene	66.5	0.39	0.6	0.38	0.6	0.1444	66.7	0.00	0.0	66.5	0.05	0.04		43.75	14.02
10 *n*-Octane	98.1	0.57	0.6	0.50	0.5	0.2500	98.3	0.08	0.08	98.4	0.10	0.15		75.76	24.27
11 Ethylbenzene	138.8	0.72	0.5	0.64	0.5	0.4096	139.5	0.03	0.01	139.2	0.13	0.24		120.61	39.77
12 *o*-Xylene	186.0	0.72	0.4	0.81	0.4	0.6561	187.0	0.00	0.0	186.9	0.22	0.66		198.81	63.70
							$s_D^2 = 0.0033$			$s_M^2 = 0.0103$					

$$s = 0.15 + 0.0035t. \quad (1a)$$

If eqn. (1a) is rewritten in the form

$$s = a + bt \quad (1)$$

and rearranged to express the more illustrative coefficient of variation,

$$v = (a/t) + b \quad (1b)$$

it can be seen that the value of the coefficient of variation v decreases with rising value of t. With a sufficiently large t, the expression a/t would be expected to have no significant effect on the value of the coefficient of variation, and the latter will remain practically constant.

Thus, the value of the slope of regression line expressing the relation between absolute retention data and their estimated standard deviations represents the lowest value of the coefficient of variation of the absolute retention data measured under certain experimental conditions. If the regression line (eqn. 1) has been calculated from a sufficient number of points and if further measurements are carried out under identical conditions, then, after taking account of whether the chemical nature of the substance under analysis or some other factor might be the source of a substantial change in the variance of the retention data, it is possible to *calculate* from the relation (1a) the standard deviations of other substances, without carrying out the series of replicate injections usually necessary for calculating the standard deviation as a measure of the precision of the absolute retention values of the substance analyzed. If substances of a hydrocarbon nature are analyzed on a nonpolar phase, as has been the case in this work, the constants of the line (1) characterize, in a very useful way for the analyst, the instrument employed along with the experimental arrangements.

It is obvious that the linear regression of a different number of [t, s_E] points produces straight lines with different a and b constants. Unless the values of the standard deviation of the absolute retention data of the other substances are determined by extrapolation, the variability of the constants a and b is practically insignificant, which is shown objectively in Fig. 1 which shows the regression lines obtained with 6, 8, 10, and 12 [$\bar{t}$, s_E] points.

The measured t, s_E, v_E values as well as the calculated values of s_t, s_t^2, v_t pertaining to this part are quoted in Table Ia.

Analysis of the factors contributing to the resultant variance of absolute retention data

The value of s_t^2 represents the resultant variance of the absolute retention value t_m of the substance analyzed m. The effect of the individual factors which can, from the viewpoint of practical qualitative analysis, share in the ultimate value of the variance (*viz.* distance measurements, determination of the solute peak maxima, and the complex factor of the variability of working conditions) has been evaluated by analyzing the variance of experimental data in such a way that their variance is influenced by one particular factor at a time.

Analysis of the experimental data showed that the variance of the actual measurements of the retention distances was constant and independent of the absolute magnitude of the retention distance measured. The above variance, s_D^2, amounted to 0.0033 mm²; this value was determined by repeated measurements ($n = 15$), in an identical chromatogram, of the lengths t_D corresponding to the distances of peak

maxima of the individual substances from the peak of the nonsorbed substance, t_m. The results of the individual measurements served for calculating the mean $\bar{t}_D$ and the estimation of the standard deviation of the measurement of the respective length, s_D. As there was no apparent dependence between the values of $\bar{t}_D$ and s_D (Table Ib), the overall variance of measuring lengths, s_D^2, was calculated by

$$s_D^2 = \frac{1}{ij - 1} \sum_{i=1}^{15} \sum_{j=1}^{15} (t_{D_{ij}} - \bar{t}_{D_i})^2$$

The variance of the peak maximum determination for the substance under analysis was also constant and independent of the distance of peak maximum of the substance from the peak maximum of the nonsorbed component. Its value was $s_M^2 = 0.0103$, which was found by repeated determinations ($n = 15$), in an identical chromatogram, of the peak maxima of the substances and the measured distances t_M, corresponding to the replicate determinations of the peak maxima distances of the individual substances from the peak maximum of the nonsorbed component. The results of the measurements pertaining to each of the substances were processed to obtain the mean $\bar{t}_M$ and the respective standard deviation s_M of determining the peak maximum. It follows from the way the determinations were made that the variance s_M^2 also includes the variance of the measurements of length. There was no apparent dependence between the values of $\bar{t}_M$ and s_M (Table Ic); and the variance of the peak maximum, s_M^2, was calculated from:

$$s_M^2 = \frac{1}{ij - 1} \sum_{i=1}^{15} \sum_{j=1}^{15} (t_{M_{ij}} - \bar{t}_{M_i})^2$$

The part of the resultant variance, s_t^2, of absolute retention data which was not accounted for by determining the peak maximum of the substance analyzed and by measuring distances was held to be a measure of the variability of working conditions. Comparison of the variances s_D^2, s_M^2, and s_t^2 according to the Snedecor criterion (Table Id) showed that the only significant source of the variability of absolute retention data was a complex factor due to the variability of the working conditions. It may be assumed, that the variance of the retention data due to this factor is characteristic for the apparatus employed and for the conditions incident to the measurement.

A knowledge of the variances s_D^2, s_M^2, and s_t^2 is useful, from the practical point of view, in processing and evaluating the measured retention data for qualitative purposes, though it is evident that the conclusions based on comparing these variances depend on the actual values of the latter and, therefore, have no general applicability.

Variance of relative retention data

Relative retention data are easily available retention characteristics, and are very frequently used in gas chromatography practice. It has been assumed that the relating of the absolute retention data of substances analyzed to the retention datum of an appropriately chosen standard substance is sufficient to eliminate the effect of the variability of the basic working conditions, especially if the standard substance is present in every sample charge and if its retention time is close to that of the substance analyzed[1,3]. Thus, relative retention data have a more general applicability

compared with absolute retention data, and qualitatively characterize the substance analyzed on a given stationary phase at a given temperature.

As stated above, absolute retention data have associated with them the standard deviation s, the dependence of which on the magnitude of the absolute retention data measured is described by eqn. 1. The relative retention value r_m of a substance m is the quotient of the corrected absolute retention values of the analyzed (m) and standard (z) substances, t_m and t_z, respectively. Therefore, the relative retention data have to be looked upon as a function of two variables, t_m and t_z, and its standard deviation can be calculated according to the general formula[15]

$$s_r = \frac{1}{t_z}\left(s_{t_m}^2 + \frac{s_{t_z}^2 t_m^2}{t_z^2}\right)^{1/2} \qquad (2)$$

without carrying out replicate injections and calculations necessary to determine the standard deviation characterizing the precision of the relative retention data. This presumption was verified in the following way: The relative retention data for the hydrocarbons analyzed were calculated by relating the repeatedly measured absolute retention data of the individual hydrocarbons consecutively to n-pentane, n-hexane, n-heptane, and n-octane as internal standards. The fifteen values of the relative retention data of each of the hydrocarbons analyzed were processed to obtain the mean $\bar{r}_m$ and the respective estimations of variance, s_E^2. The subsequent calculation of the standard deviations of the relative retention data using eqn. 2 was carried out with the aid of the data concerning the variances s_m^2 and s_z^2 (the estimation of the standard deviations, s_E, for the relative retention data of the internal standards are obviously equal to zero; the estimations of standard deviation s_r for the standard substances have been calculated only formally for a case where $t_m \neq t_z$). The variances obtained experimentally are lower than the corresponding variances calculated by the derived relation (Table II).

The estimation of the standard deviations s_E and s_r illustrates the conception of the confidence interval ($\bar{r} - st$, $\bar{r} + st$) of relative retention data measured under certain experimental conditions (t denotes the Student coefficient in the interval of confidence).

Analysis of the factors contributing to the resultant variance of relative retention data

The value s_r^2 represents the resultant variance of the relative retention data of the substances analyzed. In addition to the factors influencing the resultant variance of absolute retention data, there are further factors which may contribute to the value of the resultant variance. In connection with this concept, the effect of the *selection* of a particular internal standard has been studied as well as the possibility of calculating relative retention data by relating the absolute retention data to an internal standard which was not injected simultaneously with the mixture of substances analyzed.

It has been recommended that a hydrocarbon or a substance having a chemical nature similar to that of the substance analyzed which elutes approximately in the middle of the chromatogram of components to be separated[1,3] should be chosen as the internal standard. The standard deviation s_r of the relative retention data

TABLE II

VARIANCE OF THE RELATIVE RETENTION VALUE r OF THE SUBSTANCES ANALYZED

$\bar{r}$ is the mean of the relative retention values r_j of the substance m; s^2 is the variance of the relative retention values of the substance m; s_r^2 has been calculated by the relation (2); $s_E^2 = [1/(n-1)] \times \sum_{j=1}^{n} (r_j - \bar{r})^2$, $n = 15$; v is the coefficient of variation of the relative retention values of the substance m, expressed as a percentage $v_r = 100(s_r/r)$, $v_E = 100(s_E/r)$; the internal standard was injected simultaneously with the substance analyzed.

m	*IIa*					*IIb*				
	Internal standard: *n-pentane*					*Internal standard*: *n-hexane*				
	$\bar{r}$	$s_E^2 \times 10^4$	v_E (%)	$s_r^2 \times 10^4$	v_r (%)	$\bar{r}$	$s_E^2 \times 10^4$	v_E (%)	$s_r^2 \times 10^4$	v_r (%)
1	1.00	0	0	10.13	3.2	0.425	0.558	1.8	1.188	2.5
2	1.59	7.14	1.7	18.60	2.7	0.674	0.620	1.2	1.589	1.9
3	1.83	12.1	1.9	23.34	2.6	0.775	0.404	0.8	1.877	1.8
4	2.36	18.4	1.8	35.15	2.5	1.00	0	0	2.56	1.6
5	3.42	32.2	1.7	68.82	2.4	1.45	0.775	0.6	4.41	1.4
6	4.20	56.4	1.8	101.8	2.4	1.78	1.57	0.7	6.25	1.4
7	4.49	64.6	1.8	115.3	2.4	1.91	2.50	0.8	6.76	1.4
8	5.37	92.9	1.8	161.7	2.4	2.28	1.93	0.6	9.00	1.3
9	8.28	234.0	1.9	372.4	2.3	3.52	6.50	0.7	19.4	1.3
10	12.2	371.0	1.6	800.3	2.3	5.18	19.5	0.8	39.7	1.2
11	17.3	857.0	1.7	1588	2.3	7.33	37.4	0.8	77.4	1.2
12	23.1	1450	1.6	2894	2.3	9.82	78.7	0.9	136.9	1.2

can be expressed more illustratively in the form of the coefficient of variation,

$$v_r = (v_m{}^2 + v_z{}^2)^{1/2}, \tag{2a}$$

and it can be seen from this that the coefficient of variation and, consequently, the standard deviation of relative retention data will both be smaller when the values of the coefficients of variation of the substance analyzed and the internal standard used are low. Since the value of coefficient of variation decreases with an increase in the value of the absolute retention data (*cf.* eqn. 1b), one should choose, in calculating relative retention data, from standards $z_1, z_2, \ldots, z_n$, for which $t_{z_1} < t_{z_2} < \ldots < t_{z_n}$ and $v_{z_1} > v_{z_2} > \ldots > v_{z_n}$, a standard which elutes so far from the starting point that the coefficient of variation of the former can no longer significantly influence the resultant value of the coefficient of variation of the relative retention data. Indeed, the values of calculated (v_r) as well as experimentally obtained (v_E) coefficients of variation decrease, in the series of the substances analyzed, with increasing retention distance of the standard used from the start (Table II), in accordance with eqn. 2a for calculating the coefficient of variation of relative retention data.

Hence, the resultant variance of the relative retention data is dependent on the selection of the internal standard and is lowest when the value of the coefficient of variation of the internal standard used is lowest. However, the choice of the internal standard does not influence the qualitative values of the relative retention data, as the values of retention indices calculated from relative retention data expressed with different internal standards do not differ materially from each other (Table IVb).

Comparison, by means of the t test, of the mean values of the relative retention

IIc					*IId*				
Internal standard: n-heptane					*Internal standard: n-octane*				
$\bar{r}$	$s_E^2 \times 10^4$	v_E (%)	$s_r^2 \times 10^4$	v_r (%)	$\bar{r}$	$s_E^2 \times 10^4$	v_E (%)	$s_r^2 \times 10^4$	v_r (%)
0.186	0.12	1.9	0.19	2.3	0.082	0.0183	1.6	0.0361	2.3
0.296	0.14	1.3	0.24	1.7	0.130	0.0314	1.4	0.0441	1.6
0.340	0.11	1.0	0.27	1.6	0.150	0.0207	1.0	0.0484	1.5
0.438	0.07	0.6	0.32	1.3	0.193	0.0286	0.9	0.0529	1.2
0.636	0.13	0.6	0.49	1.1	0.280	0.0279	0.6	0.0784	1.0
0.782	0.04	0.3	0.67	1.1	0.345	0.0364	0.6	0.1089	0.9
0.836	0.06	0.3	0.76	1.1	0.368	0.0350	0.5	0.1156	0.9
1.00	0	0	0.96	1.0	0.441	0.0543	0.5	0.1444	0.9
1.54	0.21	0.3	1.96	0.9	0.679	0.193	0.7	0.2704	0.8
2.27	1.31	0.5	4.00	0.9	1.00	0	0	0.4900	0.7
3.21	3.23	0.6	7.29	0.9	1.42	0.785	0.6	1.00	0.7
4.31	5.93	0.6	12.3	0.8	1.90	0.500	0.4	1.69	0.6

data determined by using an internal standard injected simultaneously with the mixture of substances to be analyzed (Table IIc) with those determined by using an internal standard injected after every charge (Table IIIa), or after the fourth and eleventh charges of the mixture (Table IIIb), employing the mean value of the internal standard absolute retention data (Table IIIc), shows that the relative retention data of the hydrocarbons analyzed are not influenced significantly by any of the above methods of calculation. The measured values of the variances s_E^2 of the relative retention data expressed in the above way (Table III) do not amount to the values of the calculated variances s_r^2 (Table II). Hence, if the resultant variance of the absolute retention data has been tested and if the value of the respective standard deviation is satisfactory for the purposes of qualitative analysis, it is not absolutely necessary to inject the internal standard simultaneously with the mixture of analyzed substances in order to calculate the relative retention data.

Variance of retention indices

Retention indices[2] qualitatively characterize the analyzed substance on a given stationary phase at a given temperature. In calculating the retention index of a substance m,

$$I_{\text{temp.}}^{\text{st. ph.}}(m) = 100 \frac{\log x_m - \log x_{z'n}}{\log x_{z'n+1} - \log x_{z'n}} + 100n \quad (3)$$

the retention parameter $x(x = V_g, t, r)$ of the substance analyzed is arranged so that it fits on the linear scale defined by the logarithms of the retention data of two

TABLE III

RELATIVE RETENTION DATA RELATED TO AN INTERNAL STANDARD NOT INJECTED SIMULTANEOUSLY WITH THE MIXTURE OF SUBSTANCES ANALYZED

The meaning of $\bar{r}$ and s_E^2 is the same as in Table II; t is the Student coefficient calculated from the relation

$$t = (\bar{r}_A - \bar{r}_B) \Big/ \left[\frac{s_A^2 + s_B^2}{n - 1}\right]^{1/2}, \; t_{crit.} = 2.05, \; n_A = n_B = 15;$$

the internal standard (n-heptane) was injected after every injection of the mixture (Table IIIa), after the fourth and eleventh injections of the mixture (Table IIIb), and, in the third case, the relative retention data have been calculated for the mean value $\bar{t}_z$ (Table IIIc).

m	*IIIa*			*IIIb*			*IIIc*		
	$\bar{r}$	$s_E^2 \times 10^4$	t	$\bar{r}$	$s_E^2 \times 10^4$	t	$\bar{r}$	$s_E^2 \times 10^4$	t
1	0.186	0.16	0	0.187	0.15	0.71	0.186	0.10	0
2	0.296	0.27	0	0.297	0.22	0.63	0.296	0.26	0
3	0.340	0.19	0	0.341	0.18	0.71	0.340	0.19	0
4	0.439	0.25	0.67	0.440	0.27	1.25	0.438	0.21	0
5	0.637	0.42	0.59	0.639	0.30	1.76	0.636	0.32	0
6	0.783	0.38	0.63	0.785	0.40	1.67	0.782	0.38	0
7	0.836	0.31	0	0.839	0.42	1.58	0.836	0.35	0
8	1.001	0.48	0.71	1.004	0.32	2.67	1.000	0.43	0
9	1.54	1.00	0	1.547	1.47	2.00	1.54	0.72	0
10	2.27	3.35	0	2.28	2.43	1.92	2.27	1.69	0
11	3.22	5.80	1.23	3.23	2.53	3.12	3.21	2.88	0
12	4.31	8.21	0	4.32	4.72	1.14	4.31	2.78	0

neighbouring n-alkanes z'_n and z'_{n+1}; with any temperature and stationary phase

$$I_{temp.}^{st.\,ph.}\,(n - C_nH_{2n+2}) = 100n \tag{3a}$$

Since retention data are measured with a standard deviation s_x under the given experimental conditions, the retention index has to be looked upon as a function of three variables, x_m, $x_{z'_n}$, and $x_{z'_{n+1}}$, so that its standard deviation is again characterized by the general formula[15]

$$s_{I_m} = \left[\frac{100 \log e}{\left(\log \frac{x_{z'_{n+1}}}{x_{z'_n}}\right)^2}\right] \left[\left(v_m \log \frac{x_{z'_{n+1}}}{x_{z'_n}}\right)^2 + \left(v_{z'_n} \log \frac{x_{z'_{n+1}}}{x_m}\right)^2 + \left(v_{z'_{n+1}} \log \frac{x_m}{x_{z'_n}}\right)^2\right]^{1/2} \tag{4}$$

It is evident from the above relation that the variance of retention indices is not constant, but depends on the variance of the retention parameters from which it has been calculated. The value of the variance of the retention index is the lower if the values of the coefficients of variation of the individual variables are smaller. This premise has been verified empirically, and the respective results are quoted in Table IV. The experimentally obtained variances s_E^2 of the retention indices calculated from absolute retention data are lower or do not differ significantly from the variances $s_{I_t}^2$ obtained from the eqn. 4. The coefficients of variation v_E and v decrease with increasing distance of the substance analyzed from the start point. (The retention indices of substances $m = 1$–9 were calculated by interpolation between two closest alkanes and the retention indices of substances $m = 11, 12$ were calculated by extrapolation from the nearest pair of n-alkanes. The estimation of the variances s_E^2 of

TABLE IV

VARIANCE OF THE RETENTION INDICES OF THE SUBSTANCES ANALYZED

(a) I_t is the retention index of substance m calculated from the absolute retention value t; s^2 is the variance of the retention index of substance m; s_{It}^2 was calculated by eqn. 4; $s_E^2 = [1/(n-1)] \sum_{j=1}^{n} (I_{t_j} - \bar{I}_t)^2$, $n = 15$; v is the percentage coefficient of variation of the retention index I_m of substance m, $v_{I_t} = 100(s_{I_t}/I_t)$, $v_E = 100(s_E/I_t)$. (b) I_r is the retention index of substance m calculated from the relative retention data; s is the standard deviation of the retention index I_r of substance m; s_{I_r} has been calculated by eqn. 4; the internal standard was injected simultaneously with the substances analyzed.

m	IVa							IVb							
								n-Pentane[a]		*n*-Hexane[a]		*n*-Heptane[a]		*n*-Octane[a]	
	$\bar{I}_t$	s_E^2	s_E	v_E (%)	s_{It}^2	s_{It}	v_{It} (%)	$\bar{I}_r$	s_{Ir}	$\bar{I}_r$	s_{Ir}	$\bar{I}_r$	s_{Ir}	$\bar{I}_r$	s_{Ir}
1	500	0	0	0	13.9	3.7	0.7	500	5.2	500	4.2	500	3.9	500	3.8
2	554.0	1.48	1.2	0.2	4.95	2.2	0.4	554.0	3.9	553.9	2.8	554.2	2.4	553.8	2.4
3	570.3	1.16	1.1	0.2	3.98	2.0	0.4	570.4	3.9	570.2	2.6	570.4	2.2	570.5	2.1
4	600	0	0	0	3.62	1.9	0.3	600	3.8	600	2.5	600	2.2	600	2.0
5	645.2	0.37	0.6	0.1	1.81	1.3	0.2	645.1	3.6	645.1	2.2	645.2	1.7	645.0	1.5
6	670.2	0.14	0.4	0.06	1.46	1.2	0.2	670.1	3.7	670.0	2.1	670.2	1.6	670.3	1.4
7	678.2	0.17	0.4	0.06	1.44	1.2	0.2	678.2	3.7	678.3	2.1	678.3	1.6	678.1	1.4
8	700	0	0	0	1.26	1.1	0.2	700	3.6	700	2.0	700	1.5	700	1.3
9	752.7	0.34	0.6	0.08	0.76	0.9	0.1	752.7	3.5	752.9	1.9	752.7	1.4	752.7	1.2
10	800	0	0	0	0.62	0.8	0.1	800	3.5	800	1.8	800	1.3	800	1.2
11	842.2	0.89	0.9	0.1	0.48	0.7	0.1	842.5	3.4	842.3	1.8	842.3	1.3	842.8	1.1
12	878.1	0.65	0.8	0.1	0.43	0.7	0.1	877.8	3.5	877.9	1.9	878.2	1.2	878.4	1.0

[a] Internal standard.

TABLE V

RETENTION INDICES CALCULATED BY EXTRAPOLATION

I and s_E^2 have the same meaning as in Table IVa; t is the Student coefficient calculated by

$$t = (\bar{I}_A - \bar{I}_B) \Big/ \left[\frac{s_A^2 + s_B^2}{n - 1}\right]^{1/2}, \; t_{\text{crit.}} = 2.05, \; n_A = n_B = 15;$$

the retention indices have been calculated by extrapolation from the pairs: n-pentane and n-hexane (Table Va); n-hexane and n-heptane (Table Vb); and n-heptane and n-octane (Table Vc).

m	Va			Vb			Vc		
	$\bar{I}$	s_E^2	t	$\bar{I}$	s_E^2	t	$\bar{I}$	s_E^2	t
1				496.2	5.66	5.98	494.9	9.39	6.24
2				552.2	2.12	3.55	551.3	3.40	4.57
3				569.1	1.11	2.99	568.4	2.56	3.68
4							599.4	0.82	2.48
5	643.6	1.85	4.01				644.9	0.74	1.06
6	667.7	2.75	5.51				670.0	0.16	1.37
7	675.4	3.79	5.26				678.1	0.17	0.64
8	696.4	4.88	6.09						
9	746.8	11.04	6.54	752.4	0.29	1.42			
10	792.1	20.86	6.48	799.4	0.78	2.54			

the retention indices of the n-alkanes used as a basis for the scale are obviously equal to zero; the values s_I^2 of the retention indices of the n-alkanes have been calculated from the values of $t_{z'n}$, $t_{z'n-1}$, and $t_{z'n+1}$.)

The values of standard deviation s_E and s_I illustrate the concept of the confidence interval ($\bar{I} - st$, $\bar{I} + st$) of the retention indices measured under certain experimental conditions.

Retention indices can be calculated from other data than absolute retention data in chromatographic practice. One often wants to compare different retention data for an identical substance, thus transforming the former into a universal scale of retention indices. A subsequent calculation of the retention indices (*cf.* eqn. 3) is simple when the retention data for all the necessary n-alkanes are known. If the precision of the retention data used in calculation is known, it is possible to obtain, employing eqn. 4, an idea of the precision of the additional retention indices calculated. If the different retention data of the substances analyzed have been obtained under really identical conditions, the retention indices calculated from them should agree well with each other.

Table IVb contains the retention indices of the hydrocarbons analyzed calculated from the relative retention data related, by turns, to n-pentane, n-hexane, n-heptane, and n-octane as internal standards. Their mean values are in good agreement with the mean retention indices calculated from the absolute retention data (see Table IVa). (The difference between the mean values of the retention indices calculated from absolute and relative retention data have been tested by the t-test. The calculated values of the Student coefficient were lower than the respective tabulated critical values.)

It is a different situation if the retention data of only some of the n-alkanes are available for calculating retention indices. In such cases, the retention indices of quite a variety of substances are calculated by extrapolation, often from a single pair

of neighbouring *n*-alkanes, or by interpolation between more distant *n*-alkanes. However, the linearity of the relation between the logarithms of the corrected retention data of the *n*-alkanes and their numbers of carbon atoms has not always been checked prior to the above calculation. The concepts published in the literature[17,18] on the linearity or nonlinearity of the above relation are different; it is obvious that retention indices suffer from bigger errors when calculated by extrapolation or by interpolation between more distant *n*-alkanes in limits where the relation between the logarithm of the retention data of the alkanes and their numbers of carbon atoms is nonlinear. Table V shows the retention indices of the hydrocarbons analyzed calculated by extrapolation from particular pairs of neighbouring *n*-alkanes (Table Va: *n*-pentane, *n*-hexane; Table Vb: *n*-hexane, *n*-heptane; Table Vc: *n*-heptane, *n*-octane) and the values of the calculated Student coefficients characterizing the significance of the difference between the values of the retention indices calculated by extrapolation and those obtained by interpolation between neighbouring *n*-alkanes (Table IVa).

CONCLUSIONS

The measurement of the absolute retention data has been carried out under conditions in which the data were not influenced by the amount of sample injected, sorption effects of the support, and different polarities of the solute and stationary phase. A linear relation, $s = a + bt$, has been found experimentally between the corrected absolute retention value t of the hydrocarbons analyzed and their standard deviation s_E, in which the constants a and b may be looked upon as characteristic for a given experimental arrangement and type of substance analyzed. From this viewpoint, relative retention data as well as retention indices of analyzed substances should be held as functions of variables characterized by certain variance. Their resultant variance can be found empirically (s_E^2); however, it can also be estimated (s_x^2) on the basis of the relation for calculating the standard deviation of a function. The calculated and empirically found variances of retention data agreed well with each other.

The fact that the retention value of the substance analyzed is characterized by a certain variance should not be neglected in processing measured data, determining and applying various types of correlation, and employing published retention data for the purposes of qualitative analysis; briefly, whenever the retention data are accepted and processed as a constant quantity. The author suggests that, in cases when it is expedient, the variance should be published along with the retention data for the experimental arrangement used, which would give some concept of the confidence interval for the published retention data.

ACKNOWLEDGEMENTS

Thanks are due to Drs. J. JANÁK and J. NOVÁK for their encouragement and interest in this work.

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CHROM. 4713

THE PHYSICO-CHEMICAL FUNDAMENTALS OF GAS CHROMATOGRAPHIC RETENTION ON POROUS POLYMER COLUMNS: PORAPAK Q AND T

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SUMMARY

In order to explain the retention behavior of organic adsorbates on porous polymer columns in gas chromatography an attempt was made to derive characteristic functional group incremental energies for the nonpolar Porapak Q and very polar Porapak T. The total adsorption energies of a series of characteristic organic compounds were calculated and the corresponding functional group values were derived using the incremental energy equations. It has been demonstrated that Porapak T exhibits a characteristic strong interaction with the oxygen atom in alcohols, ethers and ketones and very likely has the same intensity with the hydroxyl hydrogen. Porapak Q, on the other hand, shows repulsion or weak interaction with a hydroxyl, ether or ketone oxygen but strongly interacts with the hydroxyl hydrogen. The particularly high values of the CH_3 group incremental energies were calculated and discussed.

INTRODUCTION

Porous polymers, mostly crosslinked polystyrene copolymers, were originally developed for use in gel permeation chromatography, but their outstanding physical properties have made them a very popular column packing in gas chromatography. The main reason for their wide use as separating media in gas chromatography lies in their excellent performance in handling a great number of compounds which until now could not be analyzed at all or only with difficulty. Although organic in nature, porous polymers have been found to separate most of the inorganic gases very efficiently and, in addition, all classes and types of organic compounds. Out of nearly one hundred and fifty papers published in this field, almost half deal with the elution of pure inorganic, or mixtures of organic and inorganic compounds. A review of the literature data, however, shows very inconsistent information regarding elution from porous polymer columns. This deals with the possibility (or impossibility) of the elution of certain classes of compounds, reversed order of elution, trace analysis, column efficiency, etc. Regardless of these facts, porous polymers are undoubtedly superior

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to any known conventional column packing where the separation and trace analysis of glycols, polyols, amines and mixtures of organic with inorganic compounds is concerned.

Another major controversy concerning porous polymers is their mechanism of retention. In order to draw attention to their particular properties concerned in gas chromatographic separations HOLLIS[1] proposed the name gas gel chromatography for the technique, a term used earlier by LYSYJ AND NEWTON[2] when describing poly-trifluoromonochloroethylene column packings. It is a generally accepted idea that the retention mechanism on porous polymers is rather complex and that both adsorption and solution mechanisms are involved at least at higher temperatures. The rather limited experimental evidence presented so far tends to support this idea. At the present stage of research it is rather difficult to distinguish, conveniently, between the two phenomena, solution and adsorption taking place simultaneously. Some insight into the problem is offered, however, by investigation of the column dynamics[3], determination of the adsorption isotherms[4], and finally apparent adsorption–desorption times.

In this paper, however, another approach to explain the retention phenomena is discussed, energy changes in the organic adsorbates–porous polymers systems being considered. This way, the real nature of the retention is not ascertained, *i.e.*, whether it is, and to what extent, adsorption or solution, but the problem is investigated from the standpoint of molecular interactions regardless of at what physical state of the column packing these interactions are taking place. For the sake of convenience, however, and since porous polymers are very rigid macroporous beads of determined surface area, we are calling our treatment an investigation of the adsorptive properties of the porous polymers. This may not be quite correct but will certainly be a next best approximation. The treatment involves the gas chromatographic determination of the adsorption enthalpies of a series of characteristic organic compounds and the evaluation of functional group adsorption energy increments. Of sixteen commercially available porous polymers Porapak Q and Porapak T (Waters Associates) were chosen because of their characteristic properties. Porapak Q has a nonspecific, nonpolar surface, while Porapak T seems to be the most polar porous polymer available, capable of long retention of polar compounds, unlike Porapak Q, which shows quite low adsorptivity of polar and particularly oxygenated materials. These two representatives of the porous polymers were therefore chosen by reason of their surface characteristics rather than their wide applicability. Some other brands of porous polymers would show even better separation capabilities in some instances.

EXPERIMENTAL

Adsorption enthalpies were derived from the slope of a straight line log k' (capacity ratio) against $1/T_c$. Retention times were determined from the recorder chart paper and flow rates were measured with soap bubble flowmeter and adjusted to a rate of about 20 ml/min of molecular sieve dried helium (Matheson), which was used as carrier gas. Columns packed with Porapak Q and Porapak T were stainless steel (2 ft. long $\times$ 1/8 in. diameter fitted into a Hewlett-Packard Model 5754A gas chromatograph). The temperature was chosen so as to give reasonable retention

times of about 10 min, or more, and read using precision thermometers to the nearest 0.1°. Three to five groups of retention time measurements within a range of about 20° were carried out, each group containing at least five determinations. The slope of the straight line log k' against $1/T_c$ was derived by means of the least squares method.

Because of the tendency of porous polymers to exhibit tailing, even at low sample sizes, the amount of sample injected was kept as small as possible. Thermal conductivity detection (at the highest sensitivity setting) was applied by reason of convenient and frequent flowrate measurement. Sample sizes were actually of a "zero volume" representing the volume of liquid which penetrated by capillary action into the 1 μl Hamilton syringe when the very tip of the needle (with plunger positioned at zero) was dipped for one second into a liquid sample. The recorded peaks were broad but symmetrical, being very convenient for the retention measurements. The samples were commercial high purity chemicals used without further purification; *viz.* homologous series of C_1–C_4 *n*-alcohols, C_2–C_5 *n*-alkanes, isopropanol, *tert.*-butanol, acetone, diethyl ether, benzene, cyclohexane and water. Throughout the rest of the text all adsorption energies are expressed in −kcal/mole unless otherwise specified.

RESULTS AND DISCUSSION

In contrast to gas–liquid chromatography, the gas–solid chromatographic determination and treatment of the thermodynamic parameters seem to be more convenient. In practice, however, one usually meets one or several disadvantages with gas–solid chromatography, *e.g.*, nonlinearity (or a narrow range of linearity) of adsorption isotherms originating in the hydrodynamic and/or thermodynamic properties of the adsorbent surface, and pore size distribution. Furthermore, in order to derive meaningful adsorption thermodynamic data extremely small surface area coverages are of critical importance, in order to avoid excess lateral (adsorbate–adsorbate) interaction. Usually, the surface coverage should be kept below 0.1, though even at this low coverage unfavorable pore size distribution can produce unreasonably high results of the order of several kcal/mole of adsorption energy due to excess radial dispersion interactions within the narrow pores.

The adsorption energy of a molecule generally appears to be a much more important thermodynamic parameter than the entropy or free energy of adsorption, since theoretically and experimentally the entropy values are a less sensitive measure of molecular interactions[5]. On the other hand, like molecular polarizability or dipole moments, the heat of adsorption of a molecule is actually an integral of the "local" or incremental adsorption energy of each characteristic functional group. This applies regardless of the origin of the adsorption energy, whether it is a product of pure dispersion (nonspecific) interaction or electrostatic or induction interaction, and is usually denoted as a specific part of the adsorption energy[6]. Total adsorption energy is moreover the sum of dispersion (nonspecific), polarization and dipole interaction, and since the constants of molecular interactions are proportional to polarizability, the difference between total and nonspecific interaction would reveal the amount of specific interaction. It is necessary to point out that in nonspecific adsorption interaction the retention parameters definitely do not depend upon boiling points (being just a measure of adsorbate–adsorbate interaction), dipole moments, molecular size and weight but depend on molecular polarizability and indirectly on the stereochem-

istry of the adsorbate molecule. In specific interactions, involving adsorbents with the electrostatic field on the surface, the adsorbate's electron distribution (dipole moments) is overwhelmingly important.

In the explanation of retention behavior, or in fact molecular adsorbate–adsorbent interactions, particularly when the adsorbate appears to be a more or less complex organic molecule, it is very interesting to derive characteristic functional group adsorption energy increments, specific and nonspecific as well. Actually, the concept of incremental energy quantities in gas chromatography has been found very effective in explaining particular molecular interactions in both GLC[7,8] and GSC[9,10].

TABLE I

HEATS OF ADSORPTION, SPECIFIC INTERACTIONS EXCESS ADSORPTION HEATS AND POLARIZABILITY OF ORGANIC ADSORBATES ON PORAPAK Q AND T

Compound	$-\Delta H_a$ (kcal)		$\Delta(\Delta H_a)$ $T - Q$ (kcal)	ΔH_v (kcal)	$\Delta H_a - \Delta H_v$		Polarizability, α, $\times 10^{24}$ cm^3
	Porapak Q	Porapak T			Porapak Q	Porapak T	
Water	7.7	9.5	1.8	9.7	+2.0	+0.2	1.5
Methanol	8.7	10.8	2.1	8.5	0.2	2.3	3.9
Ethanol	10.3	12.4	2.1	9.3	1.0	3.1	5.6
n-Propanol	11.9	13.9	2.0	10.0	1.0	3.9	7.3
Isopropanol	10.2	13.2	3.0	9.7	0.5	3.5	8.4
n-Butanol	14.0	15.8	1.8	10.5	3.5	5.3	9.5
tert.-Butanol	11.5	12.9	1.4	9.6	1.9	3.3	—
Acetone	9.7	12.7	3.0	7.0	2.7	5.7	6.6
Diethyl ether	8.2	12.0	3.8	6.4	1.8	5.6	10.0
Cyclohexane	13.0	13.6	0.6	7.2	5.8	6.4	11.0
Benzene	12.9	14.2	1.3	7.4	5.5	6.8	10.4
Propane	8.7	7.7	+1.0	4.5	4.2	3.2	6.3
Butane	10.0	9.0	+1.0	5.3	4.7	3.7	8.2
n-Pentane	11.3	10.3	+1.0	6.2	5.1	4.1	10.0

The results of measurements of heats of adsorption on Porapak Q and T are presented in Table I. Since Porapak Q (a styrene–ethylvinylbenzene–divinylbenzene co-polymer) is nonpolar and therefore a *nonspecific* adsorbent, heats of adsorption derived for this adsorbent are considered as the quantities for a nonspecific interaction, in contrast to the generally higher values of the adsorption energy obtained for the polar Porapak T. As a result of the presence of benzene rings in the Porapak Q structure there is some possibility of weak specific interactions with the adsorbate molecules having local positive charges, *e.g.* alcohols. The structure of the really more interesting Porapak T has not yet been disclosed by the manufacturers, thus corresponding thermodynamic quantities have no real chemical interpretation. SAKODYNSKY[11] indicated the presence of a positive charge on the surface of Porapak T, thus giving an excess specific interaction energy term and making this porous polymer particularly suitable for the retention of oxygenated materials and compounds having free electron pairs (*e.g.* ethers and ketones) but rather insensitive towards compounds having π-bonds. Reading the difference, or the specific interaction energy from the third column in Table I, these conclusions are confirmed, further indicating the relatively low adsorption energy of isopropanol on Porapak Q, which is readily explain-

able in view of the stereochemistry of this compound. On the other hand, the value of the adsorption energy of isopropanol on Porapak T indicates that this interaction has suffered a much lower loss, if any at all. Thus the high specific interaction energy of isopropanol is more conveniently explained by the apparent loss in the nonspecific dispersion interaction, due to a diminution in the total number of electron orbitals involved in the adsorbate–adsorbent interaction. On the other hand, the higher specific energy of benzene compared to cyclohexane is due to the more polarizable π-electrons of the benzene ring.

The particularly notable decrease in the adsorption energy of *n*-alkanes on Porapak T is less explainable in view of the unknown chemical structure of this adsorbent, but this phenomenon otherwise completely corresponds to the adsorption behavior of alkanes on other adsorbents. For example the heat of adsorption of alkanes on silica gels (a specific adsorbent of the same general type as Porapak T — *electron acceptors*) is also lower than on a nonspecific adsorbent like graphitized carbon black[10]. Although it is an inorganic compound, water is included in this table because of the particular interest and low retention of water on Porapak Q columns. The value of 1.8 kcal for the specific interaction of water is about one third of an expected value for hydrogen bonding in liquid water. However, the Porapak Q–water system is another environment completely so that the total figure for the specific interaction fits well in the range of hydrogen bond energies. The large difference in the heats of absorption found for diethyl ether and acetone is plausible in view of the above discussion.

Although not of direct use here, the heats of vaporization of adsorbates are also tabulated and the excess heat of adsorption calculated. As mentioned earlier in this paper, the heat of vaporization has no connection with the adsorption phenomenon at low coverages but can be used just for comparison purposes here, although excess adsorption functions have been discussed in some detail recently[12]. The molecular polarizability values for each adsorbate are presented in Table I (last column); these are very important in further discussions on the adsorption phenomena. On the other hand very sparse and incomplete data exist in literature concerning thermodynamic measurements on porous polymers–organic adsorbate systems. GVOZDOVICH *et al.*[13] mentioned a heat of adsorption of H_2O on a home made styrene–divinylbenzene copolymer of about 6 kcal; this is lower than that for all lower molecular weight organic compounds. SAKODYNSKY[11] plotted the heats of adsorption for a series of alcohols and alkanes against polarizability but with no particular mention of what porous polymers were concerned and without tabulating pertinent data.

The usual plot of the adsorption energy against polarizability for the adsorbates mentioned is presented in Fig. 1, where data for both Porapaks and the difference T — Q is plotted. Converging straight lines for *n*-alcohols are obvious because of the slow diminution of specific interactions for higher members of the homologous series. In Fig. 2 a similar plot is presented for two homologous series of *n*-alcohols and *n*-alkanes, showing the particular properties and behavior of these compounds on both Porapaks.

A more realistic view on the nature of the molecular interactions in the process of adsorption is obtained by studying functional group adsorption energy increments. This concept is based upon the already discussed additivity of the potential energy terms of dispersion and electrostatic interaction[10]. Some of the equations for expressing

incremental energy quantities listed in ref. 10 were modified to suit the purpose of this work; rearranged equations are presented in Table II. Using the data on total adsorption energy presented in Table I, the values of the functional group energy increments can be derived, and are presented in Table III. For comparison purposes, the results obtained for a nonspecific adsorbent, graphitized carbon black (GCB)[10], are also included in Table III.

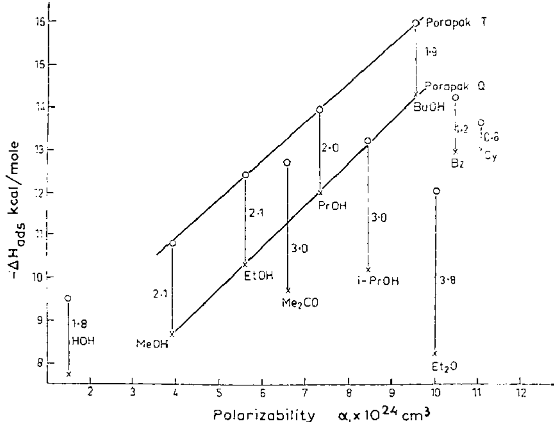

Fig. 1. The plot of the heats of adsorption against polarizability for various adsorbates on Porapak Q (×) and Porapak T (○).

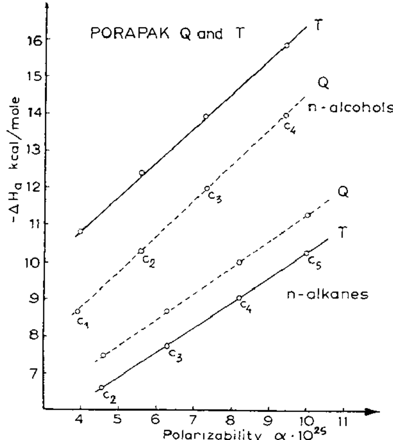

Fig. 2. The plot of the heats of adsorption against polarizability for a homologous series of n-alcohols and n-alkanes on Porapak Q (– – – –) and Porapak T (—)

As can be observed no mention of other characteristic functional groups, such as NH_2, COOH and CHO, has been made, since the elution of corresponding compounds from the porous polymers tested has stirred up considerable controversy. If the values in Table III are studied one can immediately notice a slight increase in the value for the CH_2-alcohol increment over that of the alkane methylenic group for both Porapaks, a fact which has not been recorded for other adsorbents. Furthermore,

a quite unexpected result is obtained for the methyl group increment showing a 2.4 and 1.9 kcal/mole increase over the value for the CH_2 increment for alkanes on Porapak Q and T, respectively. At present, there are insufficient data on the subject for a reasonable explanation; furthermore, it is worth mentioning that the CH_3 increment, even

TABLE II

ADSORPTION ENERGY INCREMENT EQUATIONS

ΔH_{CH_2} — alkanes C_n, alcohols
$\Delta H_{Cn} - \Delta H_{Cn-1}$
ΔH_{CH_3} — alkanes
$1/2[\Delta H_{Cn} - (n-2)\Delta H_{CH_2}]$
ΔH_O — ether
$\Delta H_{CnOCm} - \Delta H_{CnCm}$
ΔH_O — ketone
$\Delta H_{(CnCm)CO} - \Delta H_{CnCH_2Cm}$
ΔH_{OH} — alcohol
$\Delta H_{CnOH} - \Delta H_{Cn}$
ΔH_H — hydroxyl (water, alcohols)
$1/2(\Delta H_{H_2O} - \Delta H_{O\text{-ether}})$

on highly polar adsorbents (molecular sieves), usually has a value no higher than 0.3 kcal over that for the CH_2 value. Another interesting phenomenon is the slight endothermic specific (repulsion) interaction of the CH_3 group on polar Porapak T in comparison with Porapak Q. It is obvious from the foregoing that the CH_3 group behaves in a manner which is peculiar and not conveniently explainable in terms of an ordinary adsorption mechanism. We believe that here lies the origin of the specific properties of porous polymers demonstrated in the gas chromatographic retention of organic molecules, exhibiting presumed adsorption–solution effects. It is, however, interesting to mention here that the heat of vaporization energy increments for alkanes demonstrate similar trends, a CH_2 increment equals 0.8–0.9 kcal while the CH_3 amounts to about 1.9 kcal.

While the values of the incremental energies of the hydroxyl group of alcohols show the expected trend the value of a protonic hydrogen in OH groups is definitely very interesting, because of the rather high values on Porapak Q indicating that certain specific interaction mechanisms must have taken place. In other words, that the surface of Porapak Q is slightly negative or polarizable; this can be concluded from the foregoing discussion. A value of 3.3 kcal for the incremental energy of a

TABLE III

FUNCTIONAL GROUP ADSORPTION ENERGY INCREMENTS IN —KCAL/MOLE

Functional group	*Q*	*T*	*T – Q*	*GCB*[10]
CH_2-alcohol	1.6	1.6	0	1.6
CH_2-alkanes	1.3	1.3	0	1.6
CH_3-alkanes	3.7	3.2	+0.5	2.1
OH-alcohols	3.2–4	6.2–6.8	2.8–3.0	2.1
H-alcohols OH, H_2O	4.7	3.3	+1.4	0.7
O-ethers	+1.8	3.0	4.8	1.3
O-ketones	1.0	5.0	4.0	—

protonic hydrogen on Porapak T, in view of the positive electrostatic potential on its surface, is unexpectedly high, leaving approximately the same interaction energy for the oxygen atom of the hydroxyl group, a quantity which can be further identified as the increment of an ether oxygen. The endothermic incremental energy of the ether oxygen on Porapak Q seems to favor the assumption that there is a strong repulsion of either free electron pairs or negatively charged atoms or groups. These findings are partially in agreement with the adsorption energy values reported by SAKODYNSKY[11] who found a value of − 10.8 kcal for *n*-pentane and 10.4 kcal for diethyl ether on an unspecific porous polymer, presumably a styrene-divinylbenzene copolymer. In our case, the adsorption energy of diethyl ether on Porapak Q is even lower than that of propane (Table I).

The value for the incremental energy of a carbonyl oxygen (acetone) is higher by 2.8 kcal than that for the ether oxygen on Porapak Q and is obviously due to a completely different electronic environment and the possibility, under favorable conditions, of an eventual enolic interaction in this way obscures the true oxygen interaction potential. In any case, it is obvious that the high specific interaction of the oxygen atom in organic molecules (difference T — Q) is responsible for the favorable retention of these compounds on Porapak T. On the other hand, Porapak T seems to interact to some extent with the OH hydrogen, although to a smaller degree than Porapak Q. This result, as well as the strong repulsion of the ether oxygen indicates that Porapak Q is not a completely nonspecific adsorbent.

The above treatment illustrates the very interesting properties of porous polymers as column packings in gas chromatography. It can be demonstrated that both Porapak Q and T show characteristic interactions with specific functional groups, carrying an electric charge or free electron pairs. These preliminary results show great promise for further work on these interesting systems. The retention indices determined for a great number of compounds[14,15] show that very interesting molecular interactions are certainly taking place during the elution of a number of different compounds. In this laboratory an extensive investigation is under way covering a number of characteristic simple inorganic and organic compounds and the results will be published later.

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CHROM. 4714

THE USE OF A GAS CHROMATOGRAPH–MASS SPECTROMETER FOR THE ANALYSIS OF COMPLEX HYDROCARBON MIXTURES

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SUMMARY

In the present article several examples are given of the analysis of complex hydrocarbon mixtures by means of the combined gas chromatograph–mass spectrometer LKB-9000. Several methods, published in the literature or recommended as ASTM methods, were verified for group-type analysis on the mass spectrometric part of the apparatus. With fractions which can be divided into individual components by gas chromatography, the gas chromatograph–mass spectrometer enables the mass spectra of all the components to be determined without the necessity of isolating them from the mixture. The identification of seventeen individual hydrocarbons by one analysis of the fraction of aromatic hydrocarbons which have been isolated from a petroleum fraction boiling up to 200° was shown as an example. The high sensitivity of the LKB-9000 apparatus has also been utilized to obtain mass spectra of several hydrocarbons which have been unknown up to now and which have been prepared for this purpose in very low concentration by means of the methylene insertion reaction.

INTRODUCTION

The present development of the technology of petroleum processing makes demands upon the analytical control of processes which are well fulfilled by the modern methods of chromatography and spectrometry. The advantages of these methods consist in the minute sample amounts, relatively accurate results, elimination of subjective errors, and especially in a very short time of analysis.

While gas chromatographic methods are mainly applied to the analysis of substances in the light petroleum fractions at the present time mass spectrometry methods are chiefly used for group-type analyses which may be used equally for the light and heavy petroleum fractions. The direct linking of a mass spectrometer with a gas chromatograph (there has already been one on the market for several years) to give a single instrument represents one of the most perfect analytical possibilities, especially in the field of analysis of petroleum, petrochemical raw materials and products. This combination allows one to obtain mass spectra of all the components — and, hence, their identification in most cases — which the chromatographic column is able to separate, without their preliminary isolation from the mixture. With regard to the

possibility of using a capillary column with an order of resolving efficiency of about 100 000 theoretical plates, the apparatus just mentioned surpasses all kinds of analysis of complicated fractions used until now.

In the present paper we are presenting several examples of analyses of petroleum fractions and products, carried out on a Swedish gas chromatograph–mass-spectrometer LKB 9000, installed in the Technical University, Institute of Chemical Technology in Prague. These examples are selected in such a way as to demonstrate, as completely as possible, the wide applicability of the said instrument for analysis of complicated hydrocarbon mixtures.

APPARATUS

The apparatus consists of the gas chromatography part and of the mass spectrometer which is a single-focus Nier type apparatus with a 20 cm ion trajectory radius. Helium is used as carrier gas. The eluent from the chromatographic column is introduced into a separator where helium is separated from the components of the analyzed mixture; the latter enter directly to the ion source where they are ionized by an electron current. The ions so created pass through a magnetic field where their trajectories are bent in agreement with their dependence upon the m/e of the ion; they then pass through an amplifier to a recording system. The mass spectra are recorded on an oscillograph for a few seconds which even permits the scanning of the spectra of components eluted close together from the chromatographic column.

Of all the analytical problems we have solved by using the instrument described above, the first to be mentioned should be the group-type analysis of petroleum fractions. The published methods of mass spectrometry whereby the amounts of individual structural types of hydrocarbons are determined by means of their mass spectrum, were sequentially verified, using only the mass spectrometer part of the LKB-9000 apparatus. For these analyses, the samples of the fractions were dosed directly into the reservoir of the mass spectrometer without using the chromatographic column. First of all, the applicability of coefficients and the calculation methods derived for other types of instruments, and stated in the literature, was established for their application to the LKB-9000. The reproducibility for individual fractions was determined and the exactness of the results of the group-type analysis was further verified.

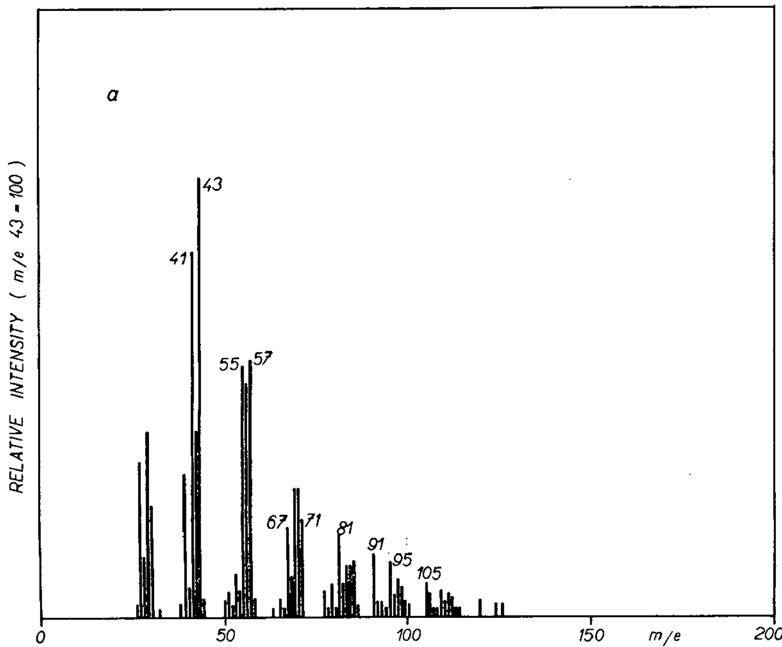
GROUP-TYPE ANALYSIS OF HYDROCARBON MIXTURES

The group-type analysis of complex hydrocarbon mixtures may be carried out practically in two ways: the fragment-peak method and the low-voltage method.

The fragment-peak method

This method utilizes the stable fragments with a mass lower than that of the primary molecule, and is based mainly upon the fact that the spectra of each structural group include some dominant fragment-peaks characteristic for each group which are not present, or in little amounts, in other groups.

The mass spectra of a petrol obtained by thermal cracking, and containing mainly paraffins and olefins, and those of the concentrate of aromatic hydrocarbons isolated from a petroleum fraction, are compared in Fig. 1. In the first spectrum the



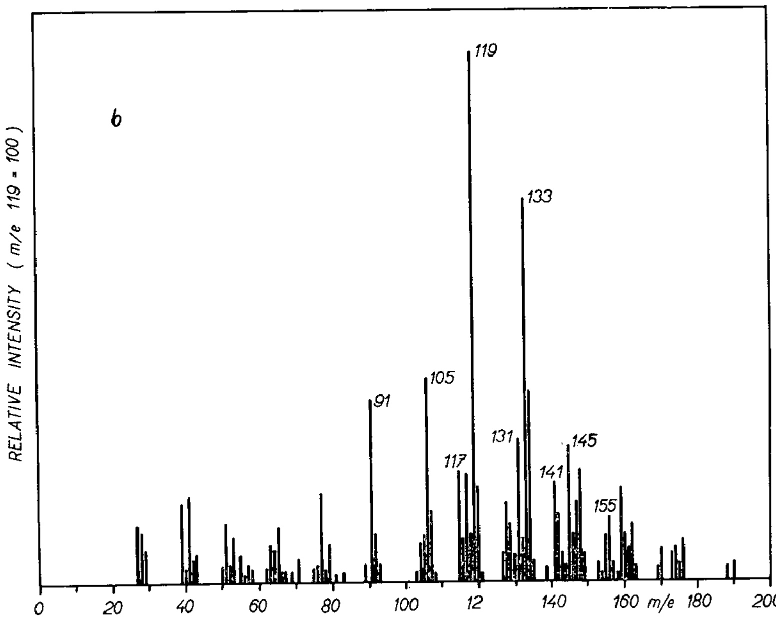


Fig. 1. Comparison of the mass spectra of petrol from thermal cracking (a) and aromatic concentrate (200–270°) from the kerosene fraction, obtained at 70 eV (b).

ions found at *m/e* 43, 57, 71, 85, belonging to the paraffins, the ions at *m/e* 41, 55, 69, 83, belonging to the monocycloparaffins and monoolefins, and the ions at *m/e* 67, 68, 81, 82, 95, 96, belonging to the dicycloparaffins, or diolefins, ultimately cycloolefins, are the dominant ones. In the spectrum of the aromatic hydrocarbon concentrate, the ions found at *m/e* 91, 105, 119, 133, belonging to the alkylbenzenes, the ions at *m/e* 117, 131, 145, 159, belonging to the indanes or tetralins ultimately, and the ions at *m/e* 128, 141, 155, 169, 183, belonging to the alkylnaphthalenes, are the most significant ones.

Each group of hydrocarbons in the mixture is then characterized by a summary of these dominant ions, and considered as a homogeneous component of the mixture, its content being calculated by solving a system of linear equations. It is necessary to know the calibration coefficients derived from the spectra of pure hydrocarbons for the purposes of calculation. These coefficients express the average values for isomers with a given molecular weight and depend upon the number of carbons in the molecule, and partly also upon the type of instrument used. Therefore, the application of this method requires a preliminary calibration of the apparatus to be used.

When using the usual mass spectrometers, possessing a resolution ability of about 1000, this method may be applied practically to any petroleum fraction, with the exception of the heavy residues. At the present time, we have already verified this method for the LKB-9000 instrument, for the fractions shown in Table I.

TABLE I

PETROLEUM FRACTIONS ANALYZED ON THE GAS CHROMATOGRAPH–MASS SPECTROMETER LKB-9000

Fractions	*Distillation limits*	*Number of hydrocarbon groups determined*
Petrol, low olefinic	95% distill. point 210°	7
Olefinic petrol	95% distill. point 210°	10
Kerosene	200–300°	8 + benzothiophenes
Middle distillates	200–350°	11
Heavy oils (saturated hydrocarbons)	average carbon number 16–32	8
Heavy aromatic concentrates	320–530°	9 + 3 thiophenic groups

TABLE II

COMPOSITION OF AN AROMATIC CONCENTRATE (200–270°) ISOLATED FROM THE KEROSENE FRACTION OF ROMASHKINO PETROLEUM

(Fragment-peak method[1] — 70 eV)

Hydrocarbon group	*Concentration in weight per cent*
Paraffins	0
Cycloparaffins non cond.	0.5
Cycloparaffins cond.	0.4
Alkylbenzenes	52.1
Indanes + tetralins	29.7
Alkylnaphthalenes	16.2
Acenaphthenes + diphenyls	1.1

When this method is used, the following hydrocarbon groups of light and middle petroleum fractions may be determined: paraffins; monocycloparaffins; dicycloparaffins; tricycloparaffins; alkylbenzenes; indanes + tetralins; indenes; alkylnaphthalenes; monoolefins; diolefins + cycloolefins + acetylenes; triolefins + cyclodiolefins; acenaphthenes + diphenyls; acenaphthylenes + fluorenes; triaromatic hydrocarbons.

As for the heavy petroleum distillates (oil fractions, high-boiling aromatic concentrates), in addition to some of the above groups, it is possible to determine other groups as well as follows: tetracycloparaffins; pentacycloparaffins; hexacycloparaffins; dinaphthenebenzenes; pyrenes; chrysenes; benzothiophenes; dibenzothiophenes; naphthobenzothiophenes.

As an example, the result of an analysis of an aromatic concentrate (boiling within the range of 200–270°) isolated from the kerosene fraction of Romashkino petroleum is shown in Table II.

The low-voltage method

This method is associated with the parent peaks obtained at a low energy of ionizing electrons. The method is used for analyses of mixtures containing hydrocarbon groups with characteristic parent ions (aromatics, olefins). Due to the absence of fragment ions, the spectrum is not so complicated, and for the computation of the composition itself, the system of linear equations is not used, which simplifies the calculation, especially in the case of narrow fractions. The simplification of the spectrum is apparent in Fig. 2, where the spectra of the liquid part obtained by petrol pyrolysis[3], and taken at 70 eV and 10 eV, are compared.

For application of this method, it is necessary to find out the coefficient of proportionality between the spectral image representing the parent ions and the concentration of each component in the analyzed mixture (so-called coefficients of sensitivity of the parent ions).

With respect to some hydrocarbon structures yielding parent ions with equal nominal values of m/e, and overlapping in the spectra, group-type analysis on the basis of parent peaks is limited to only seven hydrocarbon groups. The parent ions of the hydrocarbon type C_nH_{2n+z} have the same nominal value of m/e as the parent ions of the type $C_{n+1}H_{2(n+1)+(z-14)}$ — for example, indanes and pyrenes, or indenes and chrysenes. The differentiation of such couples is, therefore, possible only after an accurate determination of the m/e by a high resolution mass spectrometer.

The low-voltage method provides more data than the fragment-peak method, because it even permits the determination of the spectrum representing the individual components in their groups, in accordance with their molecular weights, in addition to the composition of each hydrocarbon group. The results of an analysis of the same aromatic concentrate obtained by the low-voltage method, are shown in Table III, as a comparison with the results shown in Table II.

The coefficients used for the calculation of the compositions of the fractions in both the fragment-peak and low-voltage method, depend upon a number of factors, and even upon the type of apparatus used. As we have not found in the accessible literature any statements about using the gas chromatograph–mass spectrometer LKB-9000 for group-type analysis of petroleum fractions and similar hydrocarbon mixtures, we have verified the feasibility of using the LKB-9000 for these analyses by various means, using the coefficients obtained with other instruments.

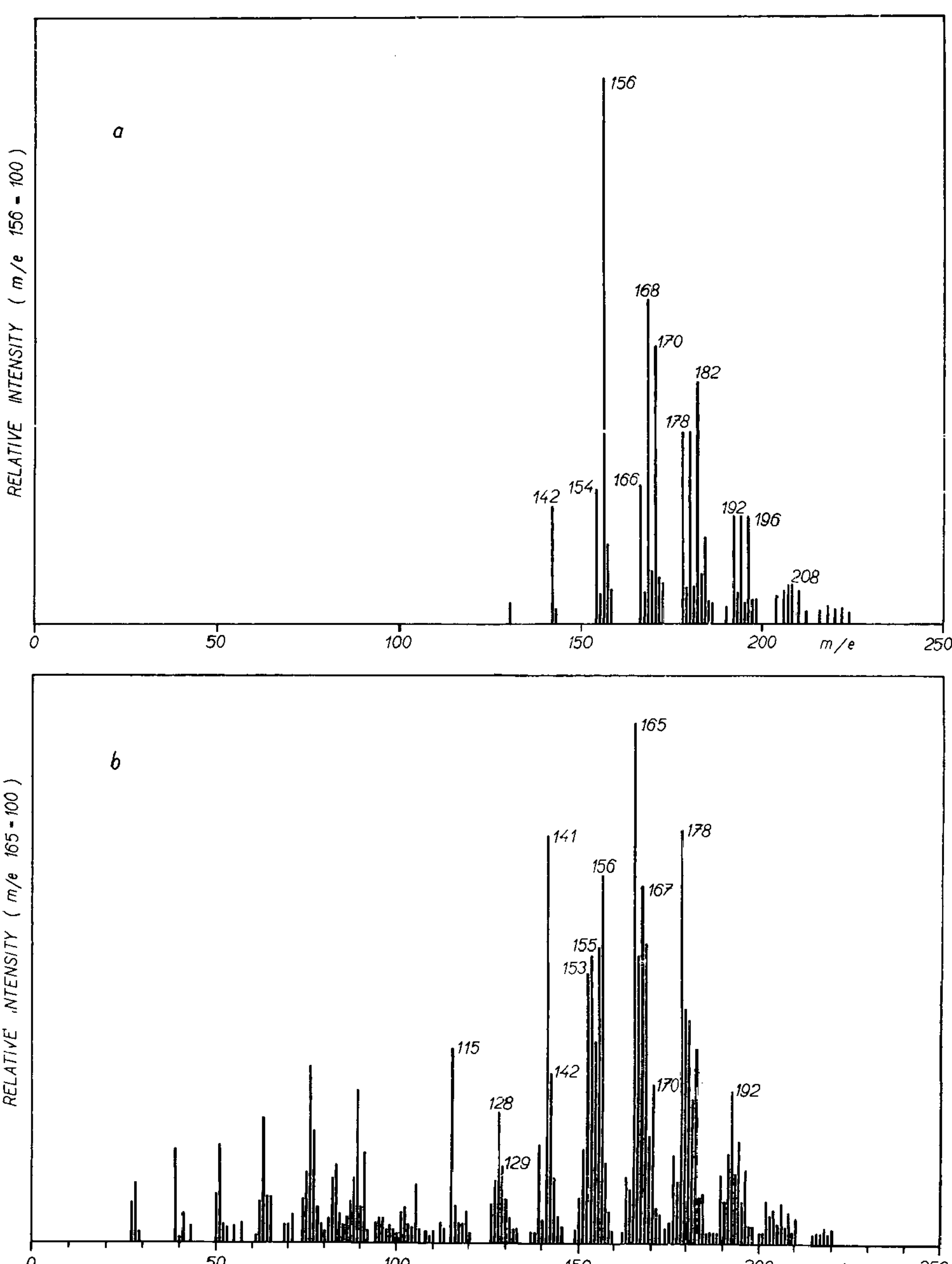


Fig. 2. Comparison of mass spectra of pyrolysis oil (250–350°) obtained at 10 eV (a) and 70 eV (b). (a) Parent ions of the dominant components (*m/e*): 142 = methylnaphthalenes; 154 = acenaphthene + diphenyl; 156 = alkylnaphthalenes C_{12}; 166 = fluorene + methylacenaphthylene; 168 = methylacenaphthenes + methyldiphenyls; 170 = alkylnaphthalenes C_{13}; 178 = anthracene + phenanthrene; 182 = alkylacenaphthenes + alkyldiphenyls C_{14}; 192 = methylanthracenes + methylphenanthrenes; 196 = alkylacenaphthenes + alkyldiphenyls C_{15}; 208 = alkylfluorenes + alkylacenaphthylenes C_{16}. (b) Characteristic ions for hydrocarbon groups (*m/e*): 91, 105, 119, 133 etc. = alkylbenzenes; 103, 117, 131 etc. = indanes + tetralins; 115, 129, 143 etc. = indenes + dihydronaphthalenes; 128 = naphthalene; 141, 142, 155, 156 etc. = alkylnaphthalenes; 153, 154, 167, 168 etc. = acenaphthenes + diphenyls; 151, 152, 165, 166 etc. = fluorenes + acenaphthylenes; 177, 178, 191, 192 etc. = anthracenes + phenanthrenes.

TABLE III

COMPOSITION OF AROMATIC CONCENTRATE (200–270°) ISOLATED FROM THE KEROSENE FRACTION OF ROMASHKINO PETROLEUM
(Low-voltage method[2] — 10 eV)

Number of carbons	*Concentration in weight per cent*				
	Alkylbenzenes	*Indanes + tetralins*	*Indenes + dihydro-naphthalenes*	*Alkyl-naphthalenes*	*Acenaph-thenes + diphenyls*
10	17.1	3.3	0.5	1.7	
11	13.7	7.0	0.4	4.4	
12	7.8	8.1	0.4	6.3	0.2
13	7.0	6.4	0.4	3.5	0.4
14	3.8	3.1	0.5	0.8	0.1
15	1.3	1.2	0.3	0.3	0
Total	50.7	29.1	2.5	17.0	0.7

TABLE IV

BLEND OF KEROSENE FRACTION AND AROMATIC CONCENTRATE

Hydrocarbon group	*Concentration in weight per cent*			
	Kerosene	*Aromatic concentrate*	*Blend of both*	
			Calculated	*Measured*
Paraffins	52.2	0	39.3	40.8
Cycloparaffins non cond.	23.1	0.5	17.4	16.4
Cycloparaffins cond.	9.9	0.4	7.4	7.8
Alkylbenzenes	11.3	52.1	21.5	21.5
Indanes + tetralins	3.4	29.7	9.7	9.6
Alkylnaphthalenes	0.1	16.2	4.0	3.6
Acenaphthenes + diphenyls	0	1.1	0.7	0.3

The repeatability of the results and the reproducibility of the measurements on the same sample upon two different instruments was established. The repeatability of four measurements on a petrol sample, independent of the time, was good, the average difference being not higher than 3 %, relatively. The reproducibility of the results for samples measured independently on two different apparatus, *e.g.* the LKB-9000 and the AEI type MS-2 at the Institut Français du Pétrole*, was also satisfactory, the differences of single measurements being not higher than 5% rel.

The method was also tested for the correct indication of changes in the composition of the fraction analyzed caused by the addition of a defined amount of another fraction which had previously been analyzed by the same method. In some cases, the results obtained by mass spectrometry were also compared with those of the gas chromatographic analysis. The results are shown in Tables IV and V.

* This measurement was made by the courtesy of Dr. BUZON.

The good results of the check tests enable us to use almost solely these mass spectrometric methods instead of the previously used methods of group-type and type analyses. For most of them we have already worked out a program for the

TABLE V

CONTENT OF SOME AROMATIC HYDROCARBONS IN PYROLYSIS OIL DETERMINED BY GAS CHROMATOGRAPHY AND MASS SPECTROMETRY (LOW VOLTAGE)

Hydrocarbons	*Concentration in weight per cent determined by*	
	Gas chromatography CHROM II	*Mass spectrometry LKB-9000*
Methylindenes	5.5	5.8
Naphthalene	24.4	22.8
Methylnaphthalenes	21.2	22.7
Acenaphthene + diphenyl	3.1	2.9
Fluorene	1.5	2.3
Anthracene + phenanthrene	2.0	1.7

required calculations on an automatic computer, so that the time needed for carrying out an analysis of a certan fraction is very much shorter compared with the previous methods. In addition, this method provides substantially more reliable information.

IDENTIFICATION OF INDIVIDUAL HYDROCARBONS IN MIXTURES

A further example of the usefulness of the gas chromatograph–mass spectrometer LKB-9000 is the identification of the main components in a fraction of aromatic hydrocarbons, boiling within the range of 135–200°, which was isolated from a kerosene fraction of the Romashkino petroleum by means of silica gel chromatography and reported earlier[4]. At that time, we had analyzed the aromatic fraction by gas chromatography only. In order to identify the main components, it was necessary to determine and compare the retention times of eighteen synthetic standard aromatic hydrocarbons in the range from C_8 to C_{10}, including all kinds of aromatics with a boiling point from 136 to 173°. When Apiezon L was used, some of the hydrocarbons had the same retention times, and in order to differentiate between them it was necessary to determine the retention times of the standards once again on a second column having a polar stationary phase (Bentone-34). Only after comparing the retention times of the main components of the fractions analyzed with those of standards on two stationary phases, was it possible to identify most of the components. However, we did not succeed in identifying some components with a higher boiling point (above 173°), as we did not possess all the standards needed in this range.

This time, during the testing of the gas chromatograph–mass spectrometer LKB-9000 apparatus, for the control and supplementation, we repeated the analysis of this fraction with a combined apparatus for gas chromatography and mass spectrometry. The chromatogram of the fraction analyzed, boiling within the range of 135–

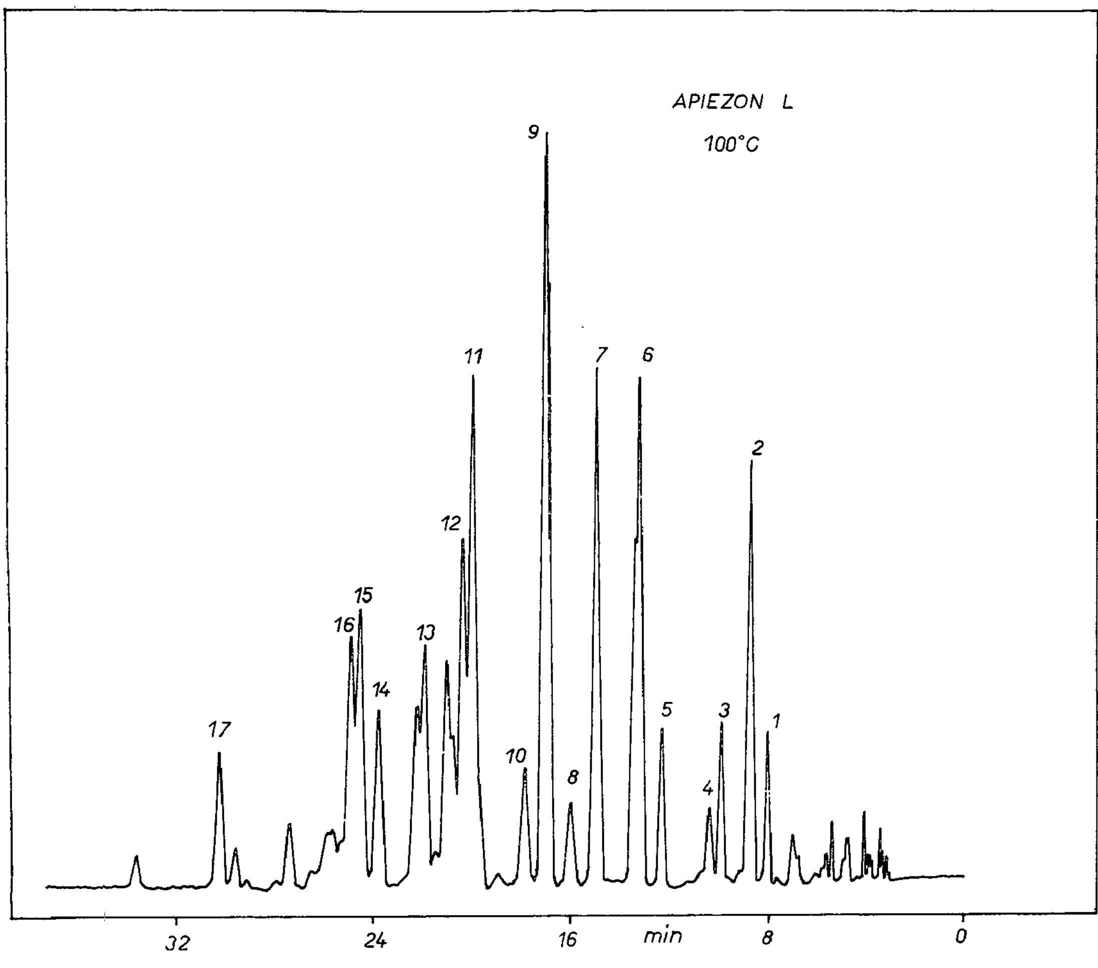


Fig. 3. Chromatogram of an aromatic concentrate (135–200°) isolated from the kerosene fraction of Romashkino petroleum.

200°, is shown in Fig. 3. The numbers 1–17 designate the main chromatographic peaks for which the mass spectra were measured in the same order as they left the chromatographic column. In this case, the identification was reduced to a comparison of seventeen mass spectra with the Spectrum Catalogue[5]. The results of the previous identification of the main components obtained by means of gas chromatography only are compared with those of their mass spectra provided by the LKB-9000 apparatus measurements, and are shown in Table VI. As may be seen, the basic structures of the components determined in accordance with the retention times and with the mass spectra, do agree in all cases. Mass spectrometry does not always differentiate exactly the isomers of aromatic hydrocarbons, for example xylenes, ethyltoluenes, trimethylbenzenes. On the other hand, it supplies more data about the higher boiling components when their identification by means of standards was not possible.

If several components elute in one chromatographic peak, the possibility of their identification by mass spectrometry is strictly limited. Peak 7, for example, apart from 1,3,5-trimethylbenzene contains about 20 % of *o*-ethyltoluene. This substance was incompletely identified by mass spectrometry as trimethylbenzene. In peak 9 three hydrocarbons (1,2,4-trimethylbenzene, isobutylbenzene and *sec.*-butylbenzene) were eluted simultaneously. In this case, where the components differ in molecular weight, it was possible by means of mass spectrometry to distinguish the presence of butylbenzene with a molecular weight of 134 apart from the trimethylbenzene.

TABLE VI

HYDROCARBONS DETERMINED IN AN AROMATIC CONCENTRATE (135–200°) FROM THE KEROSENE FRACTION OF ROMASHKINO PETROLEUM

Peak No.	*Gas chromatograph internal standards CHROM I*	*Gas chromatograph–mass spectrometer LKB-9000*
1	Ethylbenzene	Ethylbenzene
2	*p*-Xylene *m*-Xylene	Dimethylbenzene
3	*o*-Xylene	Dimethylbenzene
4	Isopropylbenzene	Isopropylbenzene
5	*n*-Propylbenzene	*n*-Propylbenzene
6	*m*-Ethyltoluene *p*-Ethyltoluene	Methylethylbenzene
7	1,3,5-Trimethylbenzene *o*-Ethyltoluene	Trimethylbenzene
8	*tert.*-Butylbenzene	Butylbenzene
9	1,2,4-Trimethylbenzene Isobutylbenzene *sec.*-Butylbenzene	Trimethylbenzene Butylbenzene
10	*p*-Cymene	Methylisopropylbenzene
11	1,2,3-Trimethylbenzene	Trimethylbenzene
12		Methylpropylbenzene
13		Dimethylethylbenzene
14		Dimethylethylbenzene
15		Dimethylethylbenzene
16		Dimethylethylbenzene
17		Tetramethylbenzene

From the comparison of both methods, it follows that the main advantage of the analysis of unknown mixtures by combined gas chromatography and mass spectrometry consists in the rapidity of the identification of the components, and also in the far larger independence of the necessity of standard substances.

As a final illustration of applicability of the gas chromatograph–mass spectrometer LKB-9000 to the solution of problems concerning the composition of petroleum, we would like to mention the mass spectra measurements on hydrocarbons prepared in very low concentrations by the methylene insertion method.

The method of methylene insertion was reported by DVORETZKY, RICHARDSON AND DURRETT[6] several years ago, and rapidly took its place in the field of gas chromatography where it is frequently used today. The method allows one to prepare a mixture of all methyl derivatives from any basic hydrocarbon, by the use of diazomethane.

Because of the necessity of working with a large surplus of the initial hydrocarbon, as compared with diazomethane, only about a 1 % solution of methyl derivatives is produced, while the ratio of the isomers present agrees with that of the C–H bonds in methyl-, methylene-, and methine-groups of the initial hydrocarbon. The low concentrations of the methyl derivatives prepared in the way described, however, permit the measurement of their retention times by gas chromatography, but the concentrations are too low for it to be possible to isolate single reaction products or measure their other properties.

The gas chromatograph–mass spectrometer provided a new opportunity for the application of this reaction. The sensitivity of the mass spectrometer in the LKB-9000 apparatus is such that it will measure the mass spectrum of 1 γ of a substance. This is sufficient for measuring even the mass spectra of the low concentrations of methylene insertion reaction products, after separating the particular methyl derivatives on a preliminary chromatographic column.

We have used this procedure for measuring the mass spectra of methyl derivatives of diamantane. Diamantane[7] is a crystalline pentacyclic naphthene with a high melting point, 237°, and with an empirical formula of $C_{14}H_{20}$, found in petroleum[8] like adamantane from which it is structurally derived. We have needed the mass spectra of methyldiamantanes for the identification of hydrocarbons which we have isolated from petroleum, together with the diamantane, since amongst them the presence of methyldiamantanes may be expected.

We prepared the methylene insertion products from a cyclopentane solution of diamantane by the use of diazomethane[9]. As expected, in addition to the unreacted diamantane, three methyldiamantanes in the ratio of 1:3:6 agreeing with that of different C–H bonds in diamantane, were found in the reaction product by gas chromatography.

The product of the methylene insertion was separated on a 50 m long squalane column in the LKB apparatus, and the mass spectra of diamantane and of all the three methyldiamantanes were measured. In order to eliminate the distortion of the mass spectra caused by traces of the stationary phase volatilizing from the chromatographic column, in the case of all the measured spectra, the background of the instrument was deducted, *i.e.* the mass spectrum obtained when only the pure carrier gas was passing through the column. For checking purposes, the mass spectra of the methyldiamantanes were even measured after their separation on a capillary column with a silicone coating. After the background was deducted (this was lower when the silicone column was used), spectra agreeing with those of the substances separated on a squalane column were obtained. Although the concentrations of the individual methyldiamantanes leaving the capillary column are only about 10^{-8} g, the mass spectra obtained were quite clear and easily reproducible. The mass spectra of methyldiamantanes are shown in Fig. 4. In these spectra the structure patterns of individual hydrocarbons (1-methyldiamantane, 4-methyldiamantane, 3-methyldiamantane) are also designated.

The spectra also show the different kinds of fragmentation of the methyldiamantanes. In derivatives having their methyl group bounded to the tertiary carbon of the diamantane skeleton (1-methyldiamantane and 4-methyldiamantane), the fragmented ion 187, corresponding to M minus CH_3, is dominant. On the other hand, in the spectra of 3-methyldiamantane with the methyl group bonded to a

secondary carbon, the dominant peak is that of the molecular ion 202. Here the fragment ion corresponding to the elimination of the methyl group is only the second of the most obvious ions.

These mass spectra, even when not as good as those measured by the usual way, give a clear picture of differences in the course of fragmentation of the methyldiamantanes, and are quite adequate for distinguishing individual isomers when identifying them in petroleum.

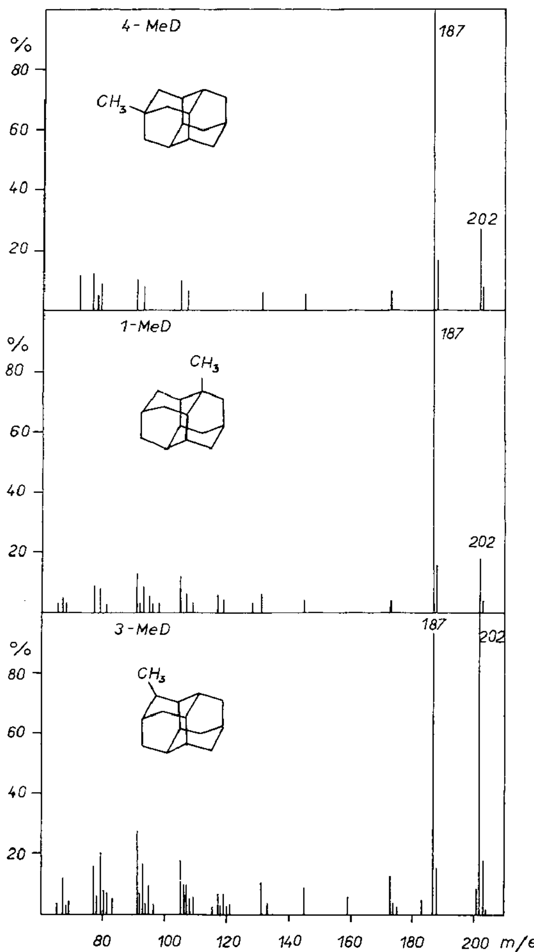


Fig. 4. Mass spectra of methyldiamantanes. (70 eV.)

The exploitation of methylene insertion in combination with the gas chromatograph–mass spectrometer provides a simple and, above all, rapid means of obtaining the mass spectra of even such methyl derivatives of hydrocarbons whose complete syntheses are either very laborious, or have not yet been reported.

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CHROM. 4715

CHARACTERIZATION OF POLYPROPYLENE BY PYROLYSIS GAS CHROMATOGRAPHY

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SUMMARY

A new method for the determination of polypropylene tacticity has been developed. The ratio established for the C_4 hydrocarbon content in the pyrolyzate permits the identification of the individual types of polypropylene, and the quantitative determination of the isotactic polypropylene content.

INTRODUCTION

Great potentiality is offered by pyrolysis gas chromatography for the quantitative determination of the microstructure of polymers. Our previous investigations on polyethylene have shown, that the microstructural differences can be determined with high precision when the requirements on reproducibility are met[1]. The published papers concerning the investigation of polypropylene by pyrolysis gas chromatography generally describe the identification of polypropylene specimens and the study of the degradation mechanism[2–19].

There has been some attempt to determine the stereoregularity of polypropylene by pyrolysis gas chromatography[20,23] but no results of this technique when applied as an analytical method for the identification and quantitative determination of polypropylene stereoregularity are given. The present work describes a simple pyrolysis gas chromatography method for the determination of polypropylene tacticity.

EXPERIMENTAL

The pyrolysis was carried out in a furnace type pyrolyzer[21] within the temperature range of 300–500°. A short precut column was used to prevent the entry of the heavier fragments of pyrolyzate into the chromatographic column[1]. The analyses of the light hydrocarbons were performed on a Perkin-Elmer Model 800 gas chromatograph under the following conditions:

Column: 2 m length, 6 mm O.D.; 5 % Carbowax 20 M on alumina 30–60 mesh pretreated with 50 % NaOH in methanol.

Carrier gas: He, 60 ml/min, 2.8 atm.

Sample weight: 0.5–1 mg.

Temperature program: 25–200° (2 min 25°, 2 min 50°), programming rate 5°/min.

The samples of polypropylene investigated were as follows:

Isotactic polypropylene: the insoluble part of a commercial type polypropylene (Moplen, Montecatini) which was dissolved in *n*-heptane.

Atactic polypropylene: the soluble part of the polymer when dissolved in propane.

Syndiotactic polypropylene SMC-77 with a syndiotacticity index of 1.8.

Blends of atactic and isotactic polypropylene prepared from solutions of atactic and isotactic polymers in xylene.

Polypropylene samples of the following different compositions:

No.	*Atactic polymer* (%)	*Stereoblocs* (%)
134	0.31	4.2
348	0.36	7.1
350	0.73	8.8

which were prepared in the Research Institute for Macromolecular Chemistry, Brno.

RESULTS

A typical pyrogram obtained under the above stated conditions is shown in Fig. 1.

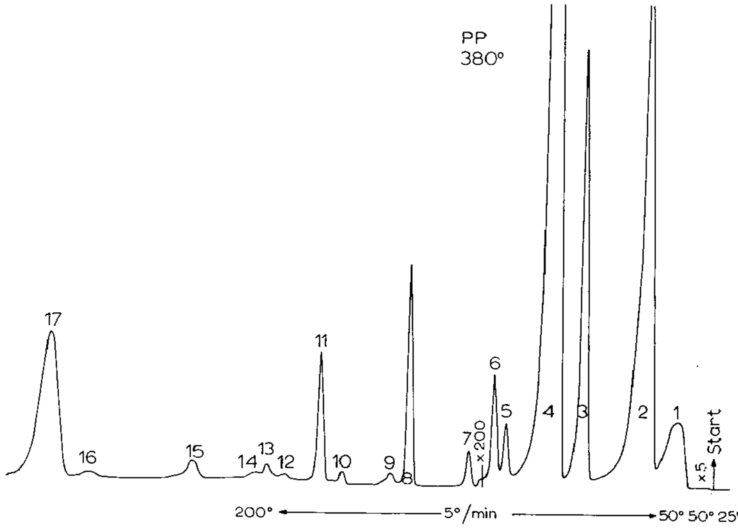

Fig. 1. Pyrogram of polypropylene. Peak No.: 1 = methane; 2 = ethane + ethylene; 3 = propane; 4 = propylene; 5 = isobutane; 6 = *n*-butane; 7 = butenes; 8 = *n*-pentane; 9 = pentenes; 10 = 3-methylpentane; 11 = hexenes; 12 = 2,4-dimethylpentane; 13 = 2-methylhexane; 14 = heptenes; 15 = 4-methylheptane; 16 = isononane; 17 = nonenes.

The lightest fraction of hydrocarbons originating from the pyrolysis of isotactic, atactic and syndiotactic polypropylene is shown in Fig. 2. The samples of isotactic polypropylene in relation to atactic and syndiotactic polypropylene showed a decidedly lower content of *n*-butane in the pyrolyzate. The characteristic relationships between isobutane and *n*-butane are evident for the individual polypropylene types. This relationship has been employed successfully for the identification of individual polypropylene types and for the determination of the isotactic polymer content in the polypropylene samples.

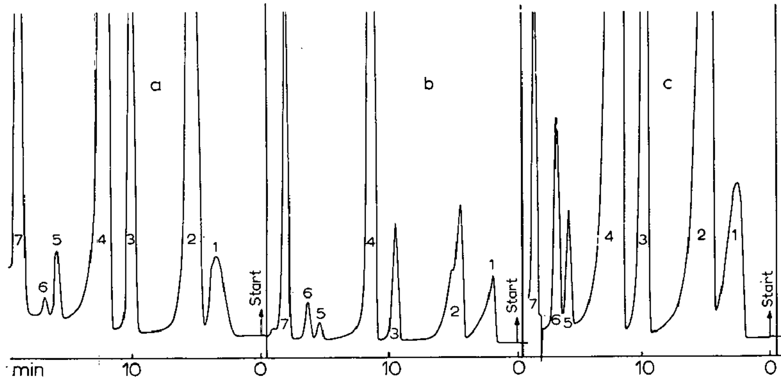

Fig. 2. Composition of the C_1–C_4 hydrocarbon fraction in the pyrolyzate of (a) isotactic, (b) syndiotactic and (c) atactic polypropylene. Peak No. 1 = methane; 2 = ethane + ethylene; 3 = propane; 4 = propylene; 5 = isobutane; 6 = *n*-butane; 7 = butenes.

TABLE I

THE RELATIONSHIP OF THE CHARACTERISTIC PEAK RATIO $isoC_4/nC_4$ AND THE PYROLYSIS TEMPERATURE FOR ISOTACTIC, SYNDIOTACTIC AND ATACTIC POLYPROPYLENE

	300°	*350°*	*420°*	*500°*	*550°*
i-PP	4.10	3.10	2.30	1.80	1.68
s-PP	0.24	0.37	0.60	0.75	0.87
a-PP	0.25	0.39	0.66	0.90	0.93

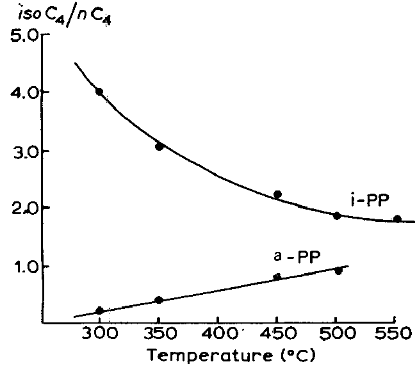

Fig. 3. Plot of $isoC_4/nC_4$ ratio *versus* temperature for isotactic and atactic polypropylene.

The established ratio of $isoC_4/nC_4$ was examined within the temperature range of 300–550° (see Table I).

It is of notable interest that not only do the differences between isotactic and both atactic and syndiotactic polypropylene become several times greater (Fig. 3) at lower temperatures, but that the existing differences in the $isoC_4/nC_4$ ratio between atactic and syndiotactic polypropylene become negligible (Table I). Likewise, no significant differences between stereoblocs and atactic polymer are noticeable.

From this it could easily be concluded that the quantitative determination of isotactic polypropylene in a mixture with atactic and syndiotactic would be much more convenient at lower pyrolysis temperatures.

However, some serious problems are encountered in performing the analysis at low temperatures. The slow degradation reactions result in broad overlapped peaks and prevent the precise measurement of the interesting peaks. Another problem is presented by the fact that in spite of the several times greater $isoC_4/nC_4$ ratio obtained with a low degradation temperature, the very small peaks obtained in the pyrograms, particularly in the case of isotactic polypropylene, strongly affect the precision and accuracy of results.

The difficulties in the chromatographic separation originating from slowly formed pyrolysis products can be avoided by a simple procedure: the inlet part of the chromatographic column is cooled in order to concentrate the pyrolysis products, and the dominant olefin components in the pyrolyzate are removed by a subtraction method[22].

However, we could not make use of the great advantage of high difference in the $isoC_4/nC_4$ ratios for isotactic and atactic polypropylene at 300°, because of the very small amount of pyrolyzate formed. A higher pyrolysis temperature had to be used and therefore, the results presented were obtained with a pyrolysis temperature of 380°.

The investigation with blends of known structure was carried out to establish the relationship between the *iso*- and *n*-butane content, and the sample composition. The results obtained point to the existence of a linear relationship between the $(isoC_4 - nC_4)/isoC_4$ ratio and the amount of isotactic polypropylene in the sample (Fig. 4).

Owing to the linear relationship between $(isoC_4 - nC_4)/isoC_4$ ratio and the sample

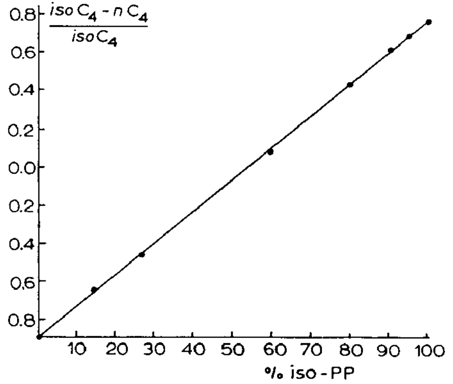

Fig. 4. Relationship between $(isoC_4 - nC_4)/isoC_4$ ratio and polypropylene blends composition.

composition, the procedure becomes very simple. In order to construct a calibration curve it is quite enough to determine the established ratio for pure isotactic and atactic polymer.

The agreement between the results obtained and the real blend composition is within $\pm 1.0\,\%$ of the isotactic polymer content. The maximum deviation in the results (Table II) does not exceed ± 0.020, which corresponds to $\pm 1.0\,\%$ of the isotactic portion in sample. The validity of the results was confirmed with the samples of polypropylene of known composition (Table III). On the basis of the results obtained

TABLE II

REPEATABILITY OF THE INDEX $(isoC_4 - nC_4)/nC_4$

Index value	*Mean*	*Deviation*	*Standard deviation*
0.875		0.000	
0.887		+0.012	
	0.875		±0.126
0.890		+0.015	
0.870		−0.005	
0.855		−0.020	

TABLE III

ANALYSIS OF POLYPROPYLENE SAMPLES OF KNOWN COMPOSITION

Sample No.	*% of isotactic polymer determined by*	
	Extraction	*PGC*
134	95.45	95.5
348	92.52	91.6
350	90.43	91.1

the pyrolysis gas chromatography procedure just described can be recommended as a new method for determination of the isotactic polypropylene content in polypropylene samples.

ACKNOWLEDGEMENTS

Thanks are due to Dr. GUIOCHON, École Polytechnique, Paris, Dr. MAJER, Ing. VILIM and Dr. OTTO, Institute of Macromolecular Chemistry, Brno, and Ing. TOMAŠIĆ, INA, Zagreb for supplying polypropylene specimens.

We also thank Dr. D. FLEŠ, INA-Institute, Zagreb, for his interest in this work.

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CHROM. 4716

GAS CHROMATOGRAPHY ON SOME SELF-ASSOCIATING OCTADECANE DERIVATIVES

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SUMMARY

The specific retention volumes of simple monofunctional organic compounds and lower alkanes were measured on different stationary phases. Octadecane derivatives were mainly used as stationary phases, but some other polar and nonpolar liquids were also used in order to obtain more information about the dependence of the specific retention values on the solute and the solvent structure. The purpose of this study was the determination of the influence of hydrogen bonding on the retention volumes and the determination of thermodynamic properties reflecting this influence. Systems showing an appreciable solvent self-association were of particular interest.

INTRODUCTION

GLC has been used in several instances as a convenient tool in the study of specific interactions in various systems[1–3]. The main advantage of the method is its simplicity and the speed with which results can be obtained if the phase is not appreciably self-associating. If the latter phenomenon cannot be negligible the method loses a great deal of its simplicity. However, there is still one possible approach to overcome this problem, that is by using several columns with different amounts of the polar stationary phase and proceeding with calculations neglecting the self-association. The results have to be extrapolated to a value for zero concentration. This method is accompanied by considerable experimental errors which obscure the results. In our work a rather different approach was used. We chose a model which can be described by certain equations, then compared the measured results with those predicted from the model.

METHOD

The following model was selected as a basis for the study of associating systems. Let us consider the system: nonpolar solvent–nonpolar and polar solute and polar solvent–nonpolar and polar solute. The nonpolar solvent is the reference one. Four specific retention volumes can be determined in this system:

$\bar{V}_g{}^{0d}$ .. $V_g{}^{0d}$ specific retention volume of nonpolar and polar solute in a polar solvent,

$\bar{V}_g{}^{0r}$ .. $V_g{}^{0r}$ specific retention volume of the same pair of solutes in a nonpolar reference solvent.

From this system we got our model after some restrictions. These restrictions are required by some of the calculations. In our work we used the approach of LANGER and co-workers which was discussed by MARTIRE[2].

They obtained the following equation:

$$\frac{\bar{\gamma}_2{}^r}{\bar{\gamma}_2{}^d} = \frac{e^{[Z \cdot N \cdot \varepsilon_{11}{}^r]\,(2RT)^{-1}}}{e^{[Z \cdot N \cdot \varepsilon_{11}{}^d]\,(2RT)^{-1}}} \tag{1}$$

where

N = Avogadro's number;

$\varepsilon_{11}{}^d$ and $\varepsilon_{11}{}^r$ = solvent–solvent pairwise potential energy of interaction in the reference solvent and in the polar one;

$\bar{\gamma}_2{}^d$ and $\bar{\gamma}_2{}^r$ = activity coefficients of nonpolar solute in the same pair of solvents.

Here we can see that for each nonpolar solute or for each solute which can interact only by the dispersion forces the ratio of the activity coefficients in two solvents of the same molecular shape and polarizability depends only on solvent–solvent pairwise potential energy and is independent of the solute. Because of this we can assume the above expression to be valid for an uncomplexed solute as well. This is true if all orientational effects are included in the complexation term and if all dipole-induced dipole interactions are small. For nonpolar solutes eqn. 1 holds in every case. This result was obtained by calculations made by MARTIRE[2] and serves as our model. For this model we require that:

the polarizabilities of the reference solvent and the polar solvent must be the same;

the molar volumes of the same pair of solvents must be the same;

the dipole-induced dipole interactions must be small;

only a 1:1 adduct is formed.

These requirements do not all hold for systems which were actually measured. In the case of octadecane derivatives not only a 1:1 adduct is formed since appreciable self-association occurs. Nevertheless, we can consider these systems in the same way as our model because all the equilibria which occur due to the association of solute–solvent and self-association are reversible. So the same probability can be given for each molecule of the solvent to be attached to the solute. It makes no difference whether the site of solute attachment is at the end or in the middele of the chain. But because of the solvent self-association the polarizability of the stationary phase changes and our model is not valid any more. For the other systems polarizabilities of reference and polar solvent differ so much that we certainly cannot expect eqn. 1 to be valid even if solvent self-association is not present.

In our work we also used the equations derived for the model for these systems. The differences between the results obtained and those predicted by the model should reveal the role played by the specific forces.

If the polarizabilities of two solvents are not the same then we can get eqn. 3 from eqn. 1 and from expression 2.

$$\varepsilon_{12} = \frac{3}{2} \cdot \frac{h}{a^6} \cdot \frac{\nu_1 \cdot \nu_2}{\nu_1 + \nu_2} \cdot \alpha_1 \cdot \alpha_2 \tag{2}$$

$$\frac{\bar{\gamma}_2{}^r}{\bar{\gamma}_2{}^d} = e^{\frac{Z \cdot N \cdot (1/2\varepsilon_{11}{}^r - \varepsilon_{12}{}^r + 1/2\varepsilon_{11}{}^d + \varepsilon_{12}{}^d)}{RT}} \tag{3}$$

where

a = distance between molecular centers;

α_1, α_2 = polarizabilities;

ν_1, ν_2 = frequencies of the electronic vibrators;

$\varepsilon_1, \varepsilon_2$ = solute-solvent pairwise potential energy of interaction.

From 1 and 3, if we write:

$$\varepsilon_{12} = c \cdot \alpha_1 \cdot \alpha_2 \qquad (4)$$

$$\left(c = \frac{3}{2} \cdot \frac{h}{a^6} \cdot \frac{\nu_1 \cdot \nu_2}{\nu_1 + \nu_2}\right)$$

we get:

$$\frac{\bar{\gamma}_2{}^r}{\bar{\gamma}_2{}^d} = c_1 \cdot e^{\frac{Z \cdot N \cdot c \cdot \alpha_2(\alpha_1{}^d - \alpha_1{}^r)}{RT}} \qquad (5)$$

where:

$$c_1 = e^{\frac{Z \cdot N \cdot (1/2\varepsilon_{1,1}{}^r - 1/2\varepsilon_{1,1}{}^d)}{RT}}$$

In this case the activity coefficient ratio is not independent of the solute. It depends on the polarizabilities of the solvents. Thus it cannot be the same for all nonpolar solutes and of course it cannot be the same for all uncomplexed solutes. This result will be discussed later. Now we can proceed with the calculations for our model. If an adduct between an electron-donor D and a proton-donor H is formed according to the next expression:

$$\mathrm{H} + \mathrm{D} \rightleftharpoons \mathrm{HD} \qquad (6)$$

we can write the equilibrium constant as follows:

$$K = \frac{a_{\mathrm{HD}}}{a_{\mathrm{D}} \cdot a_{\mathrm{H}}} \qquad (7)$$

where

a_{H} = activity of the proton-donor;

a_{D} = activity of the electron-donor;

a_{HD} = activity of the adduct.

Because we are dealing with infinitely dilute solutions and because the activity of the solvent does not change when the adduct HD is formed, we have:

$$K \cdot a_{\mathrm{D}} = K' = \frac{c_{\mathrm{HD}}}{c_{\mathrm{H}}} \qquad (8)$$

From eqns. 1 and 8 we get:

$$K' + 1 = \frac{V_g{}^{0d} \cdot \bar{V}_g{}^{0r}}{V_g{}^{0r} \cdot \bar{V}_g{}^{0d}} \qquad (9)$$

Thus the constant K' can be obtained by measuring four V_g values. K' is connected with the equilibrium constant K as follows:

$$K' = K \cdot \gamma \cdot c_{\mathrm{D}} \qquad (10)$$

DISCUSSION AND RESULTS

Our measurements were made in the temperature range 50–80° C. Lower aliphatic alcohols and alkanes were used as solvents. Stationary phases, in addition to the octadecane derivatives, were other liquids such as triethanolamine and squalane. The measure of how much a particular stationary phase is in agreement with the model regarding the reference solvent octadecane for the octadecane derivatives and squalane for the other phases is the ratio

$$\frac{\bar{V}_g^{0r}}{\bar{V}_g^{0d}} \text{ and } \frac{V_g^{0d}}{V_g^{0r}}$$

When $\alpha_1^r = \alpha_1^d$ then:

$$\frac{\bar{V}_g^{0r}}{\bar{V}_g^{0d}} = \frac{\bar{\gamma}_2^d}{\bar{\gamma}_2^r} C'' = c_1 \cdot C'' = \text{constant} \quad (11)$$

In the case when $\alpha_1^r \neq \alpha_1^d$, then $\bar{V}_g^{0r}/\bar{V}_g^{0d}$ is not independent of the solute and it is not constant for all nonpolar solutes. For the systems measured we get the values given below:

Stationary phase	*Solvent*		
	Hexane	*Heptane*	*Octane*
Octadecylamine	1.32	1.32	1.34
Octadecanol	1.39	1.80	1.88
Octadecyl cyanide	1.36	1.40	1.44
Triethanolamine	10.60	21.90	32.90
Diethylene glycol succinate	3.50	15.30	29.30
		$T = 70°$ C	
Octadecylamine	1.56	1.61	1.67
Octadecyl cyanide	1.37	1.45	1.57
		$T = 60°$ C	

Octadecane derivatives are compared with octadecane. If solvent self-association does not occur then the polarizability of each derivative is practically the same as for the reference solvent. When self-association occurs then the differences become notable. Octadecanol shows the strongest self-association and consequently the differences are here the largest. The other two derivatives show weaker association and thus the ratio is nearly constant. It is interesting to note that at 60° C this ratio is not constant any more.

With lower temperatures the association becomes stronger and the results for $\bar{V}_g^{0r}/\bar{V}_g^{0d}$ show this. This is reflected in Fig. 1.

Triethanolamine is a different case altogether. Here we have both effects: strong self-association and quite different polarizability between triethanolamine and the reference solvent squalane. As a result of this fact the differences in the ratio $\bar{V}_g^{0r}/\bar{V}_g^{0d}$ between homologs are very large.

Diethylene glycol succinate also has very different polarizability and the differences are large, although this solvent shows no self-association.

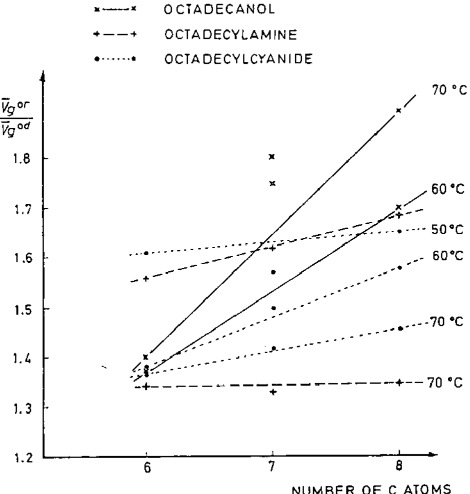

Fig. 1. The specific retention volume ratio as a function of C atom number.

In the case where $\alpha_1{}^r = \alpha_1{}^d$ we can write:

$$\frac{V_g{}^{0d}}{V_g{}^{0r}} = \frac{(K' + 1)\cdot\gamma_2{}^r\cdot M_r}{\gamma_2{}^d\cdot M_d} = \frac{\gamma_2{}^r}{\gamma_2{}^d}(K' + 1)C' = (K' + 1)\cdot\text{constant.} \qquad (12)$$

This ratio is dependent on K' only, *i.e.* on K. So it measures the specific interactions of the polar solute with the polar solvent. But when $\alpha_1{}^r \neq \alpha_1{}^d$ then the K' term in eqn. 12 is not constant and the difference between the polarizabilities of two solvents and the polarizability of the solute affects the results. If these dispersion forces are small we can expect that the relative order of these ratios will be in accordance with the general principles of acidity. For triethanolamine we get:

	Methanol	*Ethanol*	*1-Propanol*	*1-Butanol*	*1-Pentanol*
$V_g{}^{0d}/V_g{}^{0r}$	4.13	3.98	3.26	2.45	1.74
K'	92.3	89.0	72.6	54.9	38.8

The values decrease with the decreasing acidity of the alcohol. In this case the hydrogen bonds are very strong and dispersion forces have no effect on the relative order of the values for $V_g{}^{0d}/V_g{}^{0r}$.
For octadecanol we get:

	Methanol	*Ethanol*	*Propanol*	*Butanol*
$V_g{}^{0d}/V_g{}^{0r}$	2.57	5.04	5.48	5.82
K'	3.03	6.91	7.61	8.15

TABLE I

SPECIFIC RETENTION VOLUMES OF LOWER ALIPHATIC ALCOHOLS AND ALKANES ON DIFFERENT STATIONARY PHASES

	T(°C)	V_g^0 (*ml/g*)							
		Pentane	*Hexane*	*Heptane*	*Octane*	*Methanol*	*Ethanol*	*Propanol*	*Butanol*
Octadecane	50	105.85	263.45	634.40	1742.00	33.67	42.24	87.76	232.47
	60	68.33	167.40	423.22	1110.90	28.06	28.65	62.23	157.20
	70	55.46	130.42	303.33	733.80	24.00	22.40	44.98	105.60
Octadecanol	60	51.84	123.66	242.25	556.51	93.02	165.04	401.02	1046.80
	70	45.62	93.64	168.61	390.01	61.61	112.84	246.43	614.78
	80	37.17	72.04	120.51	262.65	44.50	74.04	157.44	369.50
Octadecylamine	60	47.88	107.73	263.34	664.16	99.18	167.04	444.60	1320.12
	70	42.38	98.12	229.83	549.31	64.00	113.10	255.80	678.40
	80	37.56	80.12	169.02	391.25	45.21	75.34	157.46	446.02
Octadecyl cyanide	50	69.06	164.01	405.70	1066.05	71.21	125.16	315.07	850.25
	60	58.91	122.03	290.35	709.05	50.50	88.37	227.23	547.04
	70	48.84	95.65	216.10	508.75	42.73	69.19	156.70	358.51
Triethanolamine	50	—	9.80	12.60	17.64	208.61	273.80	505.04	992.12
	60	—	6.96	8.35	11.13	141.45	172.20	301.84	564.57
	70	—	5.62	6.37	10.25	99.15	114.49	192.44	332.76
Diethylhexylsebacate	50	—	107.38	215.97	567.14	45.89	75.25	193.02	531.65
	60	—	91.53	139.43	342.20	33.64	49.41	117.91	304.92
	70	—	67.50	108.81	247.94	28.19	38.19	87.90	215.20
Diethylene glycol succinate	50	—	17.32	12.78	17.94	77.62	97.10	179.90	351.89
	60	—	17.32	9.74	14.31	61.79	73.06	128.76	243.52
	70	—	16.94	9.13	11.26	44.75	52.05	86.75	151.89
Squalane	50	—	100.76	259.04	687.94	33.67	51.68	112.69	294.06
	60	—	74.86	184.89	455.26	28.06	35.66	79.24	198.09
	70	—	59.72	139.04	329.65	24.00	28.93	59.17	135.76

TABLE II

$\bar{V}_g^{0r}/\bar{V}_g^{0d}$ RATIOS FOR LOWER ALKANES ON DIFFERENT STATIONARY PHASES

	T (°C)	$\bar{V}_g^{0r}/\bar{V}_g^{0d}$			
		Pentane	*Hexane*	*Heptane*	*Octane*
Octadecanol	60	1.318	1.354	1.747	1.691
	70	1.216	1.393	1.799	1.881
Octadecylamine	60	1.427	1.555	1.607	1.672
	70	1.308	1.329	1.320	1.336
Octadecyl cyanide	50	1.533	1.606	1.563	1.634
	60	1.159	1.372	1.488	1.566
	70	1.135	1.363	1.403	1.443
Triethanolamine	50	—	10.976	20.704	39.370
	60	—	10.526	24.119	49.100
	70	—	19.352	21.527	32.165

TABLE III

V_g^{0d}/V_g^{0r} RATIOS FOR LOWER ALIPHATIC ALCOHOLS ON DIFFERENT STATIONARY PHASES

	T (°C)	$\bar{V}_g^{0d}/\bar{V}_g^{0r}$			
		Methanol	*Ethanol*	*Propanol*	*Butanol*
Octadecanol	60	3.315	5.760	6.444	6.659
	70	2.567	5.037	5.478	5.821
Octadecylamine	60	3.534	5.830	7.144	8.397
	70	2.666	5.049	5.680	6.424
Octadecyl cyanide	50	2.963	2.963	3.590	3.657
	60	2.695	3.084	3.651	3.479
	70	2.529	3.088	3.483	3.395
Triethanolamine	50	6.659	5.154	4.543	3.110
	60	5.115	4.295	3.641	2.168
	70	4.133	3.277	3.149	2.612

TABLE IV

K' VALUES FOR LOWER ALIPHATIC ALCOHOLS ON OCTADECANE DERIVATIVES

	T (°C)	K'			
		Methanol	*Ethanol*	*Propanol*	*Butanol*
Octadecanol	60	4.062	7.801	8.846	9.174
	70	3.032	6.918	7.611	8.150
Octadecylamine	60	4.513	8.094	10.180	12.142
	70	2.524	5.674	6.508	7.498
Octadecyl cyanide	50	3.612	3.693	4.686	4.792
	60	2.762	3.305	4.096	3.856
	70	2.377	3.124	3.652	3.534

In the case of octadecanol the influence of the dispersion forces and of the other effects is so large that it completely changes the relative order. It is obvious that in this case the values calculated from the expression derived for the described model are far from the quantities like the equilibrium constant and others. Data are listed in Tables I–IV. For some system it would perhaps be possible to find some relationship between the values thus calculated and the true quantities.

EXPERIMENTAL

For this study, high accuracy in measuring specific retention volumes is required. In our work a Varian 1800 gas chromatograph with a flame ionization detector was used. Commercially available instruments of this kind keep the column temperature constant at best within $\pm 0.5°$ C or worse. For this reason most of our measurements were made in a home-made oil thermostat incorporated in the original chromatograph. The heaters for the original air thermostat were used to heat the oil bath and consequently a longer time is needed for the stabilization. Nevertheless quite good results were obtained. Temperature was constant within $\pm 0.02°$ C. Stainless steel columns 5 ft. × 1/8 in. I.D. were used. The detector signal was recorded on a Speedomax G, 1 mV, recorder. The temperatures of the detector and the injector block were the same as that of the column. Columns were prepared in the usual manner. Embacel 60–100 mesh was used as support and argon as the carrier gas. Reagents from Fluka AG were used without further purification. A 20 % loading was used in all cases.

The exact amount of liquid phase was determined by the combustion method. The solute sample sizes were between 0.02 and 0.1 μl. In this range the amount of solute has no effect on the retention time found. Peaks were symmetrical in most cases, exceptions being some polar solutes but the deformation was never large. For the determination of $V_g{}^0$ known methods were adopted[1,4].

Specific retention volumes were calculated from the expression given below:

$$V_g{}^0 = \frac{L \cdot F \cdot 273.2 \cdot [p_o - p_{H_2O}] \cdot \left[\left(\frac{p_i}{p_o}\right)^2 - 1\right] \cdot 3}{V \cdot W \cdot T_m \cdot p_o \cdot \left[\left(\frac{p_i}{p_o}\right)^3 - 1\right] \cdot 2} \qquad (13)$$

where

L = distance between solute peak maximum and starting point (cm);
V = recorder chart speed (cm/min);
F = carrier gas flow rate (ml/min);
W = amount of liquid phase (g);
T_m = flowmeter temperature (°K);
p_i = inlet pressure (mm Hg);
p_o = outlet (atmospheric) pressure (mm Hg).

ACKNOWLEDGEMENT

The authors are grateful to the Boris Kidrič and the Federal Funds for financial assistance.

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CHROM. 4717

OPEN TUBULAR COLUMN GAS CHROMATOGRAPHY OF DEHYDROGENATION PRODUCTS OF C_6–C_{10} *n*-ALKANES

SEPARATION AND IDENTIFICATION OF MIXTURES OF C_6–C_{10} STRAIGHT-CHAIN ALKANES, ALKENES AND AROMATICS

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SUMMARY

A gas chromatographic method for the analysis of dehydrogenation products of C_6–C_{10} alkanes has been developed. The optimum working temperatures for the separation on a 200 m squalane column were determined. The analysis of approx. sixty components in the products, at 115°, takes about 170 min.

The retention indices of straight-chain alkenes and aromatics, C_6–C_{10}, of the dehydrogenation products on squalane were measured at temperatures of 86 and 115°. The changes of retention indices with temperature were calculated on the basis of the data. It was found out that aromatic hydrocarbons exhibit the greatest change of $10 \cdot \delta I/\delta T = 2.0$–$2.9$, followed by the *cis*-alkenes (0.2–0.4), while the retention indices of the *trans*-alkenes vary much less. Thus by varying the column temperature it is possible to change the selectivity for the separation of aromatics, alkenes and alkanes side by side, and also to vary the selectivity of the *cis/trans* alkenes separation. These relationships have been used for the identification of products. The structural increments H^s were calculated from the retention indices determined. It was found that the structural increment in the region of C_6–C_{10} *n*-alkenes depends upon the number of C atoms in the molecule and the column temperature. These characteristic functions can be applied to the identification of higher molecular compounds than the C_{10} alkenes.

INTRODUCTION

Normal alkanes separated from petroleum fractions by means of molecular sieves are used as the raw material for catalytic dehydrogenation. The products of dehydrogenation can contain practically all theoretically possible straight-chain alkenes, aromatic hydrocarbons and amounts of unreacted *n*-alkanes. The analytical problem, besides the satisfactory separation of the constituents, concerns their identification, because standards are lacking for some of the aromatics as well as for many of the alkenes. Likewise the retention data published so far for this field are far from complete.

Squalane is used mostly as the stationary phase for the separation of hydrocarbons. The hydrocarbons are eluted from the squalane column approximately in

the sequence of their boiling points. This facilitates their identification. Since the boiling points of the isomeric alkenes, having the same number of carbon atoms in the molecule, are very close and it is also necessary to consider the presence of the aromatics and the residual *n*-alkanes, the demands on the resolving power of the column for the separation of the dehydrogenation products are rather heavy. The use of effective open tubular columns for the separation of these multicomponent mixtures is an inevitable condition, because small structural deviations are only manifested in measurable differences of retention data in the case of highly effective columns.

EXPERIMENTAL

The gas chromatograph Chrom-3, having a flame ionisation detector, was used. The capillary column of 200 m length and 0.2 mm inside diameter was coated with squalane by the dynamic method. The theoretical efficiency for ethylbenzene was found with an inlet pressure of N_2 carrier gas of 4 kp/cm², a column temperature of 115° and a capacity ratio $k = 1.4$ to be $n = 375\,000$ and the effective efficiency to be $N = 125\,000$ plates.

Complete retention indices on squalane for straight-chain alkenes have only been published up to alkenes having 8 C atoms in the molecule[1]. For C_9 and C_{10} alkenes the retention data are incomplete or they are quoted as structural increments, H^s (ref. 2).

The identification of C_6–C_8 alkenes was done by comparison of the retention indices determined with literature data. The straight-chain C_9 and C_{10} alkenes were determined from the structural increments found for C_6–C_8 alkenes. A polyethylene glycol column was used[3] for the identification of aromatics.

RESULTS AND DISCUSSION

Separation with a squalane column

The published retention data for squalane and straight-chain alkenes having the double bond between the 3 and 5 carbon atoms, show very near or equal values. In such cases a good gas chromatographic separation can only be performed with columns having maximum separation efficiency. It is for this reason that an open tubular column of 200 m length was used for the separation of the dehydrogenation products.

Even though squalane is considered as a nonpolar phase, the effect of column temperature upon the separation selectivity has been noticed. SANDERS AND MAYNARD[4] reported that at lower temperatures on a squalane column the retention times of the C_4–C_6 alkenes can increase and the retention time of benzene decreases with respect to the corresponding alkanes. HIVELY AND HINTON[5] demonstrated that the variations of the retention indices with temperature on squalane are functions of the minimum cross-sectional area of the molecule. These values indicate that a separation of the dehydrogenation products with a squalane column at diverse temperatures will be convenient.

The chromatograms of the dehydrogenation products, which were concentrated by the FIA method according to the amount of alkenes and aromatics, were determined at temperatures of 86° and 115° and are shown in Fig. 1. The problem of the separation of the aromatics, already identified with the polyethylene glycol column, from the alkenes and alkanes was first investigated.

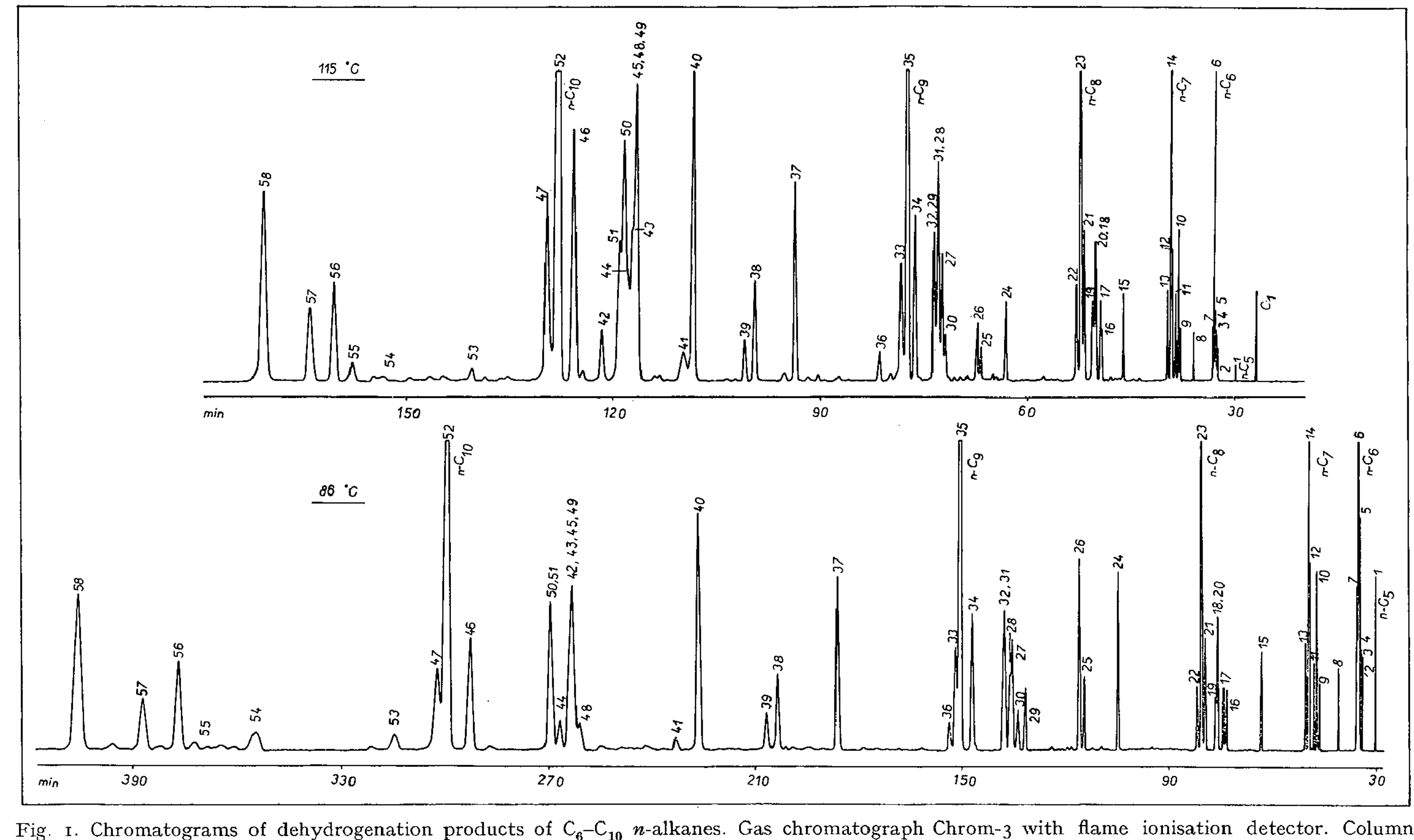

Fig. 1. Chromatograms of dehydrogenation products of C_6–C_{10} *n*-alkanes. Gas chromatograph Chrom-3 with flame ionisation detector. Column 200 m × 0.2 mm I.D. coated with squalane. Column temperature 86 and 115°. Sample size 1 μl, split 1/300. Carrier gas N_2, inlet pressure 4 kp/cm^2. Peaks, see Table I.

TABLE I

RETENTION INDICES OF STRAIGHT-CHAIN ALKENES AND C_6–C_{10} AROMATICS OF DEHYDROGENATION PRODUCTS DETERMINED ON A SQUALANE COLUMN AT 86 AND 115°

Peak number	*Component*	*Boiling point (°C)*	I^S_{86}	I^S_{115}	$10 \cdot \frac{\delta I^s}{\delta T}$
1	*n*-Pentane	36.07	500.0	500.0	0.0
2	1-Hexene	63.48	583.1	584.0	0.3
3	*trans*-3-Hexene	67.08	591.4	590.6	−0.3
4	*cis*-3-Hexene	66.45	593.0	593.7	0.2
5	*trans*-2-Hexene	67.88	596.7	596.4	−0.1
6	*n*-Hexane	68.74	600.0	600.0	0.0
7	*cis*-2-Hexene	68.89	604.6	605.4	0.2
8	Benzene	80.10	646.6	653.6	2.4
9	1-Heptene	93.64	682.8	683.5	0.2
10	*trans*-3-Heptene	95.67	687.5	687.4	0.0
11	*cis*-3-Heptene	95.75	691.7	692.3	0.2
12	*trans*-2-Heptene	97.95	698.7	698.7	0.0
13	*cis*-2-Heptene	98.41	704.3	705.1	0.3
14	*n*-Heptane	98.43	700.0	700.0	0.0
15	Toluene	110.63	754.2	760.7	2.2
16	1-Octene	121.28	782.3	782.9	0.2
17	*trans*-4-Octene	122.25	784.2	784.1	0.0
18	*cis*-4-Octene	122.54	787.9	788.6	0.2
19	*cis*-3-Octene	122.90	789.5	790.2	0.2
20	*trans*-3-Octene	123.30	788.4	788.0	−0.1
21	*trans*-2-Octene	125.00	797.7	797.3	−0.1
22	*cis*-2-Octene	125.64	802.8	803.6	0.3
23	*n*-Octane	125.66	800.0	800.0	0.0
24	Ethylbenzene	136.19	844.3	851.3	2.4
25	*p*-Xylene	138.35	858.1	864.9	2.3
26	*m*-Xylene	139.10	860.3	866.3	2.3
27	*trans*-4-Nonene	(144)	884.2	884.4	0.0
28	*cis*-4-Nonene	(144)	884.8	886.0	0.4
29	*o*-Xylene	144.41	880.1	888.1	2.7
30	1-Nonene	146.86	882.2	882.8	0.2
31	*trans*-3-Nonene	(147.9)	886.6	886.5	0.0
32	*cis*-3-Nonene	(147.9)	887.0	888.1	0.4
33	*cis*-2-Nonene	148.50	901.5	902.6	0.4
34	*trans*-2-Nonene	—	896.6	896.6	0.0
35	*n*-Nonane	150.80	900.0	900.0	0.0
36	Isopropylbenzene	152.39	903.5	910.8	2.5
37	*n*-Propylbenzene	159.22	932.6	940.2	2.6
38	*m*-Ethyltoluene	161.31	945.6	952.1	2.2
39	*p*-Ethyltoluene	161.99	948.0	955.1	2.4
40	*o*-Ethyltoluene	165.15	961.2	968.8	2.6
41	1,3,5-Trimethylbenzene	164.92	965.3	971.2	2.0
42	1,2,4-Trimethylbenzene	169.35	982.2	990.4	2.8
43	*cis*-4-Decene	(170.5)	982.2	983.4	0.4
44[a]	*trans*-4-Decene	(170.5)	982.2	982.7	0.2
45	1-Decene	170.57	982.2	982.7	0.2
46	*trans*-2-Decene	(170.6)	996.7	996.6	0.0
47	*cis*-2-Decene	(170.6)	1001.2	1002.2	0.3
48	*cis*-5-Decene	(170.7)	981.0	982.1	0.4
49[a]	*trans*-5-Decene	(171.3)	984.0	984.7	0.2
50	*trans*-3-Decene	(173.3)	985.4	985.5	0.0
51	*cis*-3-Decene	(173.3)	985.4	986.4	0.3
52	*n*-Decane	174.12	1000.0	1000.0	0.0
53	1,2,3-Trimethylbenzene	176.08	1007.5		

[a] Identified on the basis of dehydrogenation mechanism.

TABLE I (*continued*)

Peak number	*Component*	*Boiling point (°C)*	I^S_{86}	I^S_{115}	$10 \cdot \frac{\delta I^s}{\delta T}$
54	1,3-Diethylbenzene	181.10	1025.0		
55	1-*m*-3-*n*-Propylbenzene	181.80	1030.5	1037.1	2.3
56	*n*-Butylbenzene	183.27	1033.0	1040.0	2.4
57	1,2-Diethylbenzene	183.75	1036.0	1043.6	2.6
58	1-*m*-2-*n*-Propylbenzene	184.80	1042.0	1050.5	2.9
59	1,3-Dimethyl-4-ethylbenzene	188.41		1070.8	
60	*n*-Undecane	195.89	1100.0	1100.0	0.0

TABLE II

STRUCTURAL INCREMENTS H^s OF STRAIGHT-CHAIN C_6–C_{10} ALKENES ON SQUALANE AT TEMPERATURES OF 86 AND 115°

Component	*Structural increment H^s*		$10 \cdot \frac{\delta I^s}{\delta T}$
	86°	*115°*	
1-Hexene	−16.9	−16.0	0.3
1-Heptene	−17.2	−16.5	0.2
1-Octene	−17.7	−17.1	0.2
1-Nonene	−17.8	−17.2	0.2
1-Decene	−17.8	−17.3	0.2
trans-2-Hexene	− 3.3	− 3.6	−0.1
trans-2-Heptene	− 1.3	− 1.3	0.0
trans-2-Octene	− 2.3	− 2.7	−0.1
trans-2-Nonene	− 3.4	− 3.4	0.0
trans-2-Decene	− 3.3	− 3.3	0.0
cis-2-Hexene	4.7	5.4	0.2
cis-2-Heptene	4.3	5.1	0.3
cis-2-Octene	2.8	3.6	0.3
cis-2-Nonene	1.5	2.6	0.4
cis-2-Decene	1.2	2.2	0.3
trans-3-Hexene	− 8.6	− 9.4	−0.3
trans-3-Heptene	−12.5	−12.6	0.0
trans-3-Octene	−11.6	−12.0	−0.1
trans-3-Nonene	−13.4	−13.5	0.0
trans-3-Decene	−14.6	−14.5	0.0
cis-3-Hexene	− 7.0	− 6.3	0.2
cis-3-Heptene	− 8.3	− 7.7	0.2
cis-3-Octene	−10.5	− 9.8	0.2
cis-3-Nonene	−13.0	−11.9	0.4
cis-3-Decene	−14.6	−13.6	0.3
trans-4-Octene	−15.8	−15.9	0.0
trans-4-Nonene	−15.9	−15.6	0.1
trans-4-Decene	−17.8	−17.3	0.2
cis-4-Octene	−12.1	−11.4	0.2
cis-4-Nonene	−15.2	−14.0	0.4
cis-4-Decene	−17.8	−16.6	0.4
trans-5-Decene	−16.0	−15.3	0.2
cis-5-Decene	−19.0	−17.9	0.4

The aromatics benzene and toluene are separated in the temperature range investigated (86 and 115°) from the other constituents. The separation problems begin at *o*-xylene, which at a temperature of 86° elutes immediately before the nonenes. With an increase in the column temperature the retention of *o*-xylene is shifted into the nonenes and at a temperature of 115° it is eluted immediately behind *cis*-3-nonene. In the separation of aromatic C_9 hydrocarbons the effect of temperature is manifested in the separation of isopropylbenzene from *cis*-2-nonene, and possibly from *n*-nonane. Temperatures of 86° and higher are favourable for the separation. At 86°, of the remaining theoretically possible C_9 aromatics 1,2,4-trimethylbenzene, *sec.*-butylbenzene and isobutylbenzene can elute in the decene zone. By increasing the column temperature to 115° they will be shifted between *cis*-3-decene and *trans*-2-decene. The separation of C_{10} aromatics does not present a problem, because the dehydrogenated mixture does not contain undecenes.

The column temperature also affects the separation of the individual alkenes to a smaller extent, and sometimes their separation from the *n*-alkanes. Among the straight-chain C_6–C_9 alkenes the following two pairs, *trans*-3-octene + *cis*-4-octene and *trans*-3-nonene + *cis*-3-nonene, are not even partly separated, at a column temperature of 86°. In the case of the straight-chain decenes a common peak is observed for *trans*-3-decene + *cis*-3-decene and *trans*-4-decene + *cis*-4-decene + 1-decene. The separation of these unseparated alkenes can partly be achieved by adjustment of the column temperature. The separation of *cis*-4-octene from *trans*-3-octene was obtained at a column temperature below 65°; the separation of all nonenes, even in the presence of *o*-xylene, was obtained at a temperature of 92°, and at the temperature of 115° *cis*-3-decene was separated from *trans*-3-decene and *cis*-4-decene from the common peak for 1-decene + *trans*-4-decene + *cis*-5-decene. The only information which was not ascertained, out of the 33 theoretically possible straight-chain C_6–C_{10} alkenes, were data for 1-decene and *trans*-4-decene.

The problems of *n*-alkane separation from straight-chain alkenes on squalane are substantially those concerning the separation of *cis*- and *trans*-2-alkenes from the corresponding *n*-alkanes. The separation is affected by the working temperature of the column. Higher column temperatures are preferable for the separation of *cis*-2-alkenes from *n*-alkanes.

Identification of straight-chain alkenes

The retention indices were calculated out of the chromatograms determined for the dehydrogenation products at temperatures of 86 and 115°. Comparison of measured and tabulated data of the structural increments H^s and the variations of retention indices with temperature were used for the identification. The numbering of the identified chromatographic peaks, the boiling points, the retention indices and their variations with temperature are shown in Table I. Some interesting correlations can be deduced.

Effect of the carbon chain length upon the structural increment H^s

The structural increments H^s for the straight-chain C_6–C_8 alkenes, which had been calculated from the retention indices, indicated a certain dependence on the number of C atoms. Table II presents these stated values of the structural increments at temperatures of 86 and 115° for the individual groups of straight-chain alkenes.

A certain deviation is found for *trans*-2-hexene, *trans*-3-heptene and *trans*-4-octene.

The correlations found permit the determination of the values for the structural increments, and consequently the values of the retention indices for straight-chain alkenes having a higher number of C atoms per molecule than 10 can also be found.

Squalane column temperature: effect upon the retention index of straight-chain alkenes

From the chromatograms determined with the squalane column (Fig. 1) it can be seen that at different temperatures a number of different kinds of peaks were recorded and a shift of some peaks was also noticed. This result demonstrates the variation of the relative retention of some of the dehydrogenation products according to the column temperature. The corresponding variations of the retention indices with temperature for the various constituents are given in Table I. The greatest variations were found for the aromatics ($10 \cdot \delta I/\delta T = 2.0$–$2.9$). The values for *cis*-alkenes and 1-alkenes correspond approximately to one tenth of this value (*e.g.* 0.2–0.4). The retention indices of the *trans*-alkenes are practically temperature independent. The variation of the retention indices for the individual alkenes are given in Table II.

Considering that under the given conditions 0.6 index units are sufficient for the resolution of two constituents, by varying the column temperature by 20° it is possible to obtain the partial separation of *cis–trans* isomers, whose peaks generally overlap. The retention of *cis*-alkenes, relative to the corresponding *trans*-isomers, can be extended by raising the temperature of the squalane column. The temperature effect upon the separation selectivity for the *cis/trans* alkenes is demonstrated in Table III. The differences in the retention indices found by the subtraction of the indices for *trans*-alkenes from the *cis*-isomers are compared for alkenes having a simular position of the double bond.

TABLE III

DIFFERENCES IN THE RETENTION INDICES OF THE *cis*- AND *trans*-ALKENES AT TEMPERATURES OF 86 AND 115°

No. of carbon atoms	*2-Alkenes*		*3-Alkenes*		*4-Alkenes*		*5-Alkenes*	
	86°	*115°*	*86°*	*115°*	*86°*	*115°*	*86°*	*115°*
C_6	7.9	9.0	1.6	3.1	—	—	—	—
C_7	5.6	6.4	4.2	4.9	—	—	—	—
C_8	5.1	6.3	1.1	2.2	3.7	4.5	—	—
C_9	4.8	5.9	0.0	1.6	0.7	1.6	—	—
C_{10}	4.5	5.5	0.0	0.9	0.0	0.7	3.0	−2.6

The effect of temperature on the squalane column is even more expressive in the case where aromatics are to be separated together with alkenes. The variations of retention indices for the aromatics, compared with those of the straight-chain alkenes, are approximately ten times higher. That means that by varying the column temperature (at which the peaks for the alkenes and aromatics overlap) by about 2° or 3° a partial separation can be obtained.

In this way, the knowledge of the variations of the retention indices with temperature permits the calculation of the optimum temperature for the separation of the constituents of a mixture. It also serves for the identification of unknown components.

Dependence of straight-chain alkenes retention indices upon the boiling point

The comparison of the boiling points and the corresponding retention indices of straight-chain C_6–C_8 alkenes and alkanes on the squalane column indicates that at 40° the boiling point does not follow the retention index in the case of the following pairs: *cis*-3-hexene and *trans*-3-hexene, *cis*-2-heptene and *n*-heptane, *cis*-2-octene and *n*-octane. It is necessary to mention that at this temperature the other *cis*-alkenes are retained by the squalane column longer than would correspond to the differences between the boiling points of the *trans*-alkenes, or possibly of the *n*-alkanes. By raising the temperature of the column to 115° this difference increases. The retention indices of the aromatic C_6–C_{10} hydrocarbons at a temperature of 86° fulfil approximately the sequence of the boiling points of the other constituents in the dehydrogenation product. Raising the column temperature transfers the aromatics into the zone of higher boiling alkenes. At a lower temperature the opposite is valid.

The retention indices found and the known boiling points of straight-chain alkenes were used for verification of the Kováts equation defining the relationship between the retention index and the boiling point of the components ($\delta I = 5\delta t_b$). Good agreement was only obtained for geometrically similar *trans*–*trans* and *cis*–*cis* isomers (approx. about one unity of index). The equation was found to be more reliable with an increase of C atoms in the molecule.

The constant of proportionality calculated from the retention indices and corresponding boiling points indicates a certain dependence for C_6–C_8 alkenes. On the basis of this dependence and the known boiling points of 1-nonene and 1-decene, the boiling points for the remaining nonenes and decenes can be calculated; in Table I these are given in parentheses as approximate values. More details will be published in future papers.

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CHROM. 4718

GAS CHROMATOGRAPHY OF THE PRODUCTS FORMED BY THERMAL DECOMPOSITION OF "PYROLYSIS RESIN"

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SUMMARY

The subject of this study was to determine the amount and composition of the pyrolysis products formed by pyrolysis of so called "Pyrolysis Resin", which is a waste material formed during the cracking of light hydrocarbons to propylene. In the first part of this paper the pyrolysis products obtained at temperatures from 500° to 1000° in a molten metal bath were determined by direct chromatography. The products of thermal degradation consisted mostly of aromatic hydrocarbons. Benzene, toluene, xylene, indene and 2-methylindene were present in the amounts of about 1–2 %. Substantially larger amounts of naphthalene (about 20–25 %) were found at degradation temperatures of 900–1000°. The total weight of the pyrolysis procedure was determined over the same temperature range.

"Pyrolysis Resin" investigated here is a high molecular weight organic residue that is usually removed as an unidentified material after pyrolysis of light hydrocarbons to propylene.

INTRODUCTION

Many methods have already been described for carrying out pyrolysis–gas chromatography. Among these the most commonly used is the degradation of the substance by applying it to an electrically-heated spiral wire[1–5]. This method cannot be used for solid samples without special precautions. A number of devices have been designed for this purpose[6–10]. Most of them suffer from the unsatisfactory heat transfer to the sample and some from the necessity of interrupting the carrier gas flow before each experiment.

KARR *et al.*[11] used this technique to compare the chemical natures of the resinous fractions isolated from the low temperature tar formed by brown coals, caking and non-caking coals, and electrode pitch. GRILING[12] has studied the degradation products formed when different coals were heated in such a manner as to produce a steady rate of temperature rise. BRICTEUX[13] compared the pyrograms of exinite and vitrinite while HOLDEN AND ROBB[14] have heated coal samples directly in the ionisation chamber of a mass spectrometer.

DIRECT DETERMINATION OF PYROLYSIS PRODUCTS

The thermal decomposition of "Pyrolysis Resin" was carried out in a quartz pyrolyser that contained a molten tin bath. Pyrolysis was accomplished by dropping a small sample into a bath of molten tin kept at a constant temperature by an external electric heater. 2 mg of sample were carefully weighed beforehand into a hollow tin cylinder which was then closed by a small plug of the same metal. The cylinder had a height and diameter of 5 mm, the plug had a diameter of 4 mm and a height of 2 mm. Because of the high heat capacity of the bath and the rapid rate of heat transfer the sample container melted almost instantaneously.

The samples after insertion in the cylinders were fed to the pyrolyser by means of an automatic magazine which operated at certain preset times. The magazine was made from a block of polymethylmethacrylate (1, 2) with holes drilled in it as shown in Fig. 1. The samples in the cylinders were piled one above the other in the hollow tube (13) which was closed with a metal screw and packing (10). The carrier gas entered at (12) and purged the magazine containing the samples of the resin. The lowest sample cylinder in the pile sits in the hole (14) of the feeder rod (4) and was moved by the solenoid (3) to a position over the opening of tube (7), through which it fell into the metal bath of the pyrolyser. The rod can be adjusted with a screw (6). The next sample then moved into the hole (14) upon the return of the feeder rod which was actuated by spring (5). The magazine was connected to the pyrolyser by a piece of vacuum rubber tubing. Tube (7) was fastened to the feeder with a gland nut (8) and PVC seal (9).

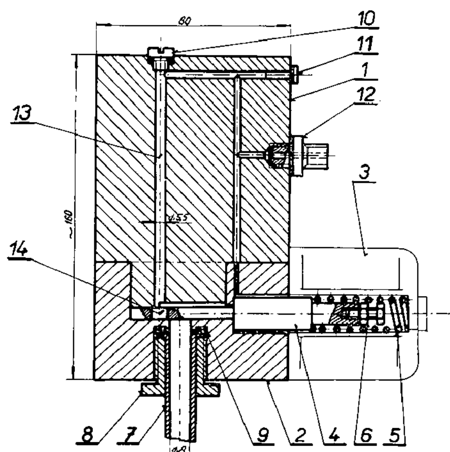

Fig. 1. Automatic magazine. 1, 2 = body made from polymethylmethacrylate; 3 = solenoid; 4 = iron core; 5 = spring; 6 = adjusting screw; 7 = sample outlet tube for connection to pyrolyser; 8 = gland nut; 9 = PVC packing; 10 = metal screw with packing; 11 = blanked-off opening; 12 = carrier gas inlet; 13 = hollow tube for holding sample cylinders; 14 = hole in feeder rod.

The pyrolyser (Fig. 2) was made of quartz. The samples fell from the sample magazine down tube (4) into the bath of molten tin (6), the temperature of which was measured by a thermocouple situated outside the wall of the pyrolyser (8). The pyrol-

ysis products were removed by the carrier gas from the pyrolyser through a capillary (7). The pyrolyser was heated externally by an electric heater and could be set to the desired temperature of the metal bath.

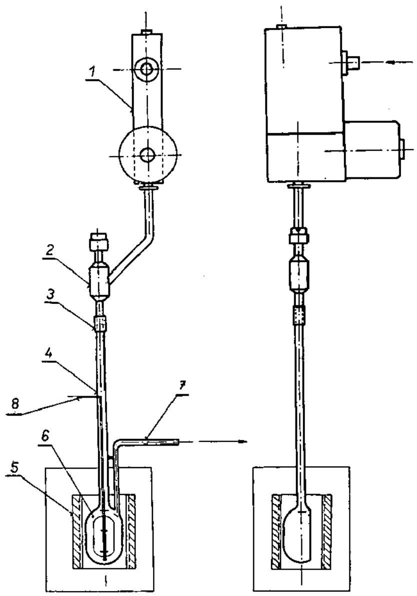

Fig. 2. Pyrolyser with automatic magazine attached. 1 = automatic magazine; 2 = union; 3 = rubber tubing; 4 = sample inlet; 5 = electric furnace; 6 = molten tin bath; 7 = carrier gas outlet; 8 = thermocouple.

The products of the thermal decomposition were analysed by a gas chromatograph Chrom II. The column was 4 m long, with a diameter of 6 mm, and packed with Celite 545 coated with 20 % of Apiezon L. The flow of carrier gas was maintained at its optimum value of 50 ml/min for nitrogen, 40 ml/min for hydrogen and 400 ml/min for air. The working temperature of the column was 168°. The temperatures of the tin bath were for individual runs 620, 700, 800, 850, 900, 950 and 1000°.

Qualitative evaluation of the results was carried out on the basis of comparing the elution times of the pyrolysis products with the elution times of various pure hydrocarbons. This was done by adsorbing the vapours of these pure hydrocarbons on the surface of a resin sample. A sample would then be kept for a definite time in a stoppered weighing bottle under the vapours of the hydrocarbon suspected of producing the particular peak of interest. On pyrolysis of the sample prepared in this way the adsorbed hydrocarbon caused a marked increase in the height of the peak in question if identical with the standard, the elution time being the same.

Celite 545 coated with 20 % of tricresylphosphate at 120° instead of Apiezon L was used for determination of the elution times for some important compounds.

The quantitative evaluation of the chromatographic peaks was carried out on the basis of calibration curves, which were obtained by dosing 1 % solutions of pure compounds in a solvent which did not interfere with the compounds used. The results are shown in Table I and Figs. 3 and 4. Only compounds with low boiling point (from

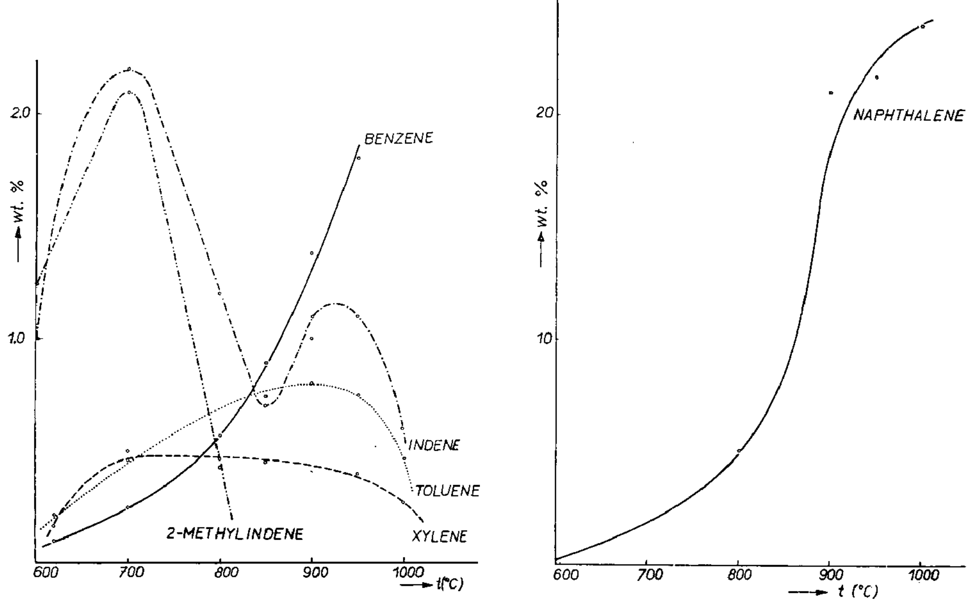

Fig. 3. Content of light aromatic hydrocarbons in pyrolysis gas.

Fig. 4. Content of naphthalene in pyrolysis gas.

benzene up to naphthalene) were determined by this method. The content of light aromatic hydrocarbons in the pyrolysis gas (Fig. 3) is relatively low and ranges from 1 to 2 %. A considerably higher concentration was found in the case of naphthalene (Fig. 4). The amount of naphthalene produced in the pyrolysis range of 900–1000° was about 25 % (with respect to the total weight of the resin).

TABLE I

DEPENDENCE OF THE YIELD OF PYROLYSIS PRODUCTS ON THE TEMPERATURE

Temperature of pyrolysis	*% benzene per g of resin*	*% toluene per g of resin*	*% xylene per g of resin*	*%indene per g of resin*	*% 2-methyl-indene per g of resin*	*%naph-thalene per g of resin*
620	0.1	0.22	0.33	1	1.25	1
700	0.26	0.46	0.55	2.2	2.1	2.2
800	0.38	0.56	0.45	1.2	0.43	5.2
850	0.78	0.74	0.45	0.7	—	8.5
900	1.38	0.8	0.4	1	—	21
950	1.8	0.75	0.4	1.1	—	21.6
1000	2.6	0.47	0.27	0.6	—	24.1

DETERMINATION OF THE TOTAL CONTENT OF THE SOLID, LIQUID AND GASEOUS PRODUCTS OBTAINED BY THE DECOMPOSITION OF PYROLYSIS RESIN AT DIFFERENT TEMPERATURES

The pyrolysis apparatus (Fig. 5) consisted of an electrically heated feeder (1), which was equipped with a capillary jet (7) on the lower, narrower end. The pyrolyser,

which was connected with the feeder by a joint, was equipped with an inlet tube for the carrier gas, a thermocouple well, and an outlet tube for the pyrolysis products. In order to increase the heat capacity, the reaction vessel was filled up to 1/3 of its height with small pieces of crashed quartz.

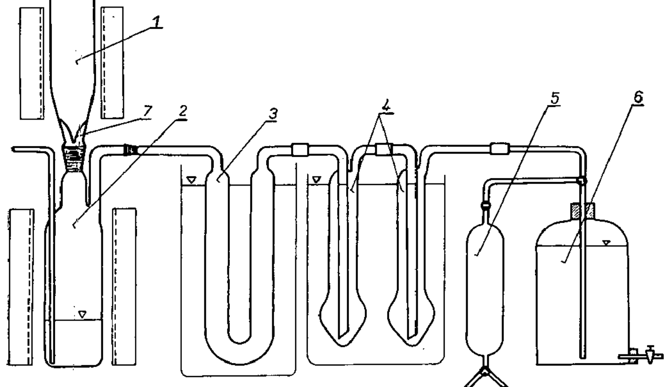

Fig. 5. Pyrolysis apparatus. 1 = Electrically heated feeder; 2 = pyrolyser; 3,4 = condensation vessels; 5 = gas sample vessel; 6 = store bottle; 7 = capillary jet; 8 = electric furnace; 9 = inlet of carrier gas; 10 = outlet of pyrolysis products.

The condensation vessels, the pyrolyser, and the feeder that contained the resin were weighed before the measurement. The apparatus was then set up according to Fig. 5, and purged with pure nitrogen. After removal of air from the pyrolyser and the condensation vessels, the flow of nitrogen was stopped and the tube sealed off. The condensation vessel (3) was cooled with ordinary water, vessels (4) were cooled in a bath of dry ice in methanol. The pyrolyser was heated to the desired decomposition temperature. After the temperature in the pyrolyser became steady, the vent on the side outlet of the store bottle (6) was opened, and the feeder was heated at the same time. The molten resin dropped into the pyrolyser at a rate of 1 drop per 2–3 sec.

The pyrolysis products formed condensed mostly in vessel (3); only a small amount of the light portions condensed in vessel (4). The amount of pyrolysis gas was equal to the amount of water that flowed out of the store bottle. A sample of the gas was taken into the vessel (5). The analysis was carried out on a gas chromatograph of the JANÁK type. Some of the pyrolysis products condensed on the cooler outlet tube of the pyrolyser during the decomposition. In order to remove these products, the inlet of carrier gas was opened and the store bottle was disconnected. The flow of the carrier gas was adjusted to 100 ml/min. The condensed products were transferred to the condensation vessels by this operation at the temperature used for 1 h. The individual parts of the apparatus were weighed after the run was over and total balance calculated. The results are presented in Table II and Fig. 6. The largest proportion of liquid products was obtained at temperatures of 500–600°; with higher temperatures than these the yield of the liquid products fell to 19 % at a temperature of 1000°. Simultaneously with the decrease of the amount of the liquid portion, the amount of the solid portion increased. The solid portion consisted of coke and soot. The formation of soot became observable at a temperature of 800° and formed a sub-

stantial part of the solid residue (about one half) at a temperature of 1000°. Similarly, the total amount of pyrolysis gas increased with increasing temperature and, consequently, the composition of the gas changed as well.

TABLE II

WHOLE-WEIGHT BALANCE OF PYROLYSIS RESIN

Temperature of pyrolysis	*Residue of pyrolysis (wt. %)*	*Liquids (wt. %)*	*Amount of gas (ml/g of resin)*	*Composition of gas*		
				H_2 (%)	CH_4 (%)	*Higher hydrocarbons* (%)
500	27	71	33	19.4	60	20.6
600	29	69.8	62	27	60	13
650	29.8	69	75	31.4	59	9.6
700	31	67.5	120	37	56	7
750	32.1	63.9	200	40	53.1	6.9
800	35	59.5	340	49.5	44.5	6
850	36	50.5	460	53	42	5
900	40	40	550	60	35.4	4.6
1000	63.7	19.1	1130	67.8	29.0	3.2

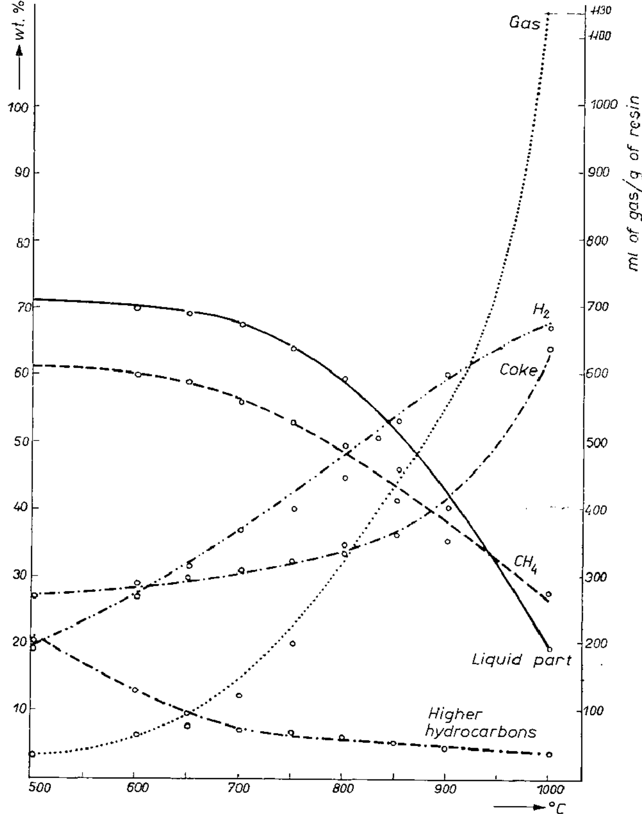

Fig. 6. Total yield of pyrolysis products.

In the range of temperatures 500–650°, the content of methane in the gas was about 60 %, only 27 % of methane was found at a temperature of 1000°. On the other hand, the concentration of hydrogen increased almost linearly with increasing temperature in the range of temperatures of 500° (about 20 % of hydrogen) to 1000° (about 68 % of hydrogen). Concentrations of both methane and hydrogen were roughly equal (about 47 % of each component) at temperatures close to 800°. The content of higher hydrocarbons decreased from 20.5 % at 500° to 3 % at 1000°.

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CHROM. 4719

CHROMATOGRAPHIC ANALYSIS OF PRIMARY LIGHT GASOLINE AND Pbi-H FRACTIONS OF ROMASHKINO CRUDE OIL

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SUMMARY

Both qualitative and quantitative analyses of the hydrocarbon components of straight-run and hydrogenated pyrolysis gasolines have been made by gas–liquid chromatography. Several different open tubular (capillary or Golay) columns with squalane, Ucon LB-550, or mannit-hexakis-cyanoethyl ether as stationary phase were used for the analysis. Chromatograms of the samples analysed on squalane are given, using temperature programming. The chromatographic peaks have been identified by the use of pure standards, prepared by different chemical methods, before the sample input and by purification of some components on a preparative gas chromatograph. The purified compounds were identified by infrared spectroscopy.

INTRODUCTION

In the beginning of our chromatographic analyses of light straight-run gasoline and hydrogenated pyrolysis gasoline, we had neither capillary columns, nor a sufficient assortment of pure standard compounds. We therefore decided to proceed in the following manner.

Samples were taken from the production plant, in our case the straight-run light gasoline as feed stock and the hydrogenated gasoline as a product of the ethylene plant, and separated by distillation into fractions according to the number of carbon atoms. Some pure compounds, used as standards, were obtained from these fractions by separation on a preparative gas–liquid chromatograph; they were then identified by their IR spectra.

In the case of the straight-run gasoline, we could assume the presence of saturated and cyclic hydrocarbons. On the other hand the presence of unsaturated, *i.e.* olefinic and polyolefinic or acetylenic compounds, could be excluded. Nevertheless, we tested our samples by shaking with concentrated sulphuric acid to confirm this. Since the composition of the straight-run gasoline is relatively simple, we resolved to identify the components of this mixture first. The chromatographic analysis of the hydrogenated pyrolysis gasoline was more complicated due to the presence of a lot of olefinic and diolefinic, as well as aromatic, hydrocarbons.

EXPERIMENTAL

The instruments used for the analyses were:

CHROM 11, an instrument of the National Interprise Laboratorní přístroje, Prague, equipped with a flame-ionisation detector and an open tubular column.

Model GT, Series 200 from Carlo Erba, a dual column, research class instrument, equipped with FID, temperature and gas-flow programming, both being used in the analyses described simultaneously.

TABLE I

ANALYTICAL CONDITIONS

A. Apparatus	GT Series 200, Carlo Erba
Detector	FID (flame ionisation detector)
Sample size	1 μl
Splitting ratio	1:220
Column	capillary, stainless steel 45 m × 0.2 mm I.D. with 8% by wt. of squalane in *n*-hexane
Temperature programming	from $-5°$ at the start up to *iso*C_5, then at 25° to benzene elution, then programming up to 90° at a rate of 2°/min
Carrier gas flow programming	at $-5°$ at the start 1.4 ml/min flow rate at 1.5 kp/cm² to *o*-xylene elution, then with an increment of 0.2 ml per min up to a flow rate of 4.26 ml/min at 2.6 kp/cm² pressure. The column was cooled according the method of SANDERS AND MAYNARD[10].
B. Apparatus	GT Series 200, Carlo Erba
Detector	FID
Sample size	0.5 μl
Column	capillary, stainless steel 45 × 0.2 mm I.D. with 10% by wt. of polypropylene glycol (Ucon LB-550 X)
Splitting ratio	1:100
Carrier gas	1 ml N_2/min at 1.5 kp/cm²

Fractovap Model P, Carlo Erba, Milan, a preparative gas chromatograph.

Unicam SP-200, a routine double-beam IR spectrophotometer.

The analytical conditions used in these instruments are listed in Table I.

Some of the standards used for identification were obtained from Lachema N.I., Brno, others were obtained by purification on the Fractovap P from synthetic samples prepared in our laboratories or from distillation fractions of hydrogenated or non-hydrogenated pyrolysis gasoline and straight-run light gasoline.

Squalane and Ucon LB-550 X — as liquid stationary phases for the capillary column — were obtained as gas chromatographic pure chemicals from Carlo Erba.

Analysis and identification

First the C_1–C_6 hydrocarbons present in the straight-run light gasoline were identified on classical columns packed with a carrier and stationary phase. In further work, we continued with capillary columns with squalane as stationary phase. The conditions can be seen in Table I.

The standards available were used for plotting the correlation between elution times and boiling points, for the calculation of the Kováts indices and relative retention times. The latter were compared with literature date of TOURRES[1] and DESTY

et al.[2]. The straight-run light gasoline so analysed was then used as a mixture of known saturated hydrocarbons for further identifications in the analysis of the hydrogenated pyrolysis gasoline, containing olefinic, diolefinic, and higher aromatic hydrocarbons. The identification of such a wide range of heterogenous hydrocarbons in hydrogenated pyrolysis gasoline in such a manner was not unambiguous over the entire range. It was necessary to separate the distillation fractions further by chemical methods, removing some components from the chromatographic spectrum by chemical reaction. Several fractions of hydrogenated pyrolysis gasoline were modified by the following operations:

(a) hydrogenation (for removing olefins, diolefins, acetylenes and styrenes);
(b) reaction with conc. sulphuric acid (for removing unsaturated and aromatic hydrocarbons);
(c) reaction with Ilosvay reagent (removal of acetylenes);
(d) reaction with maleic anhydride (removal of conjugated diolefins);
(e) adsorption on silica gel (removal of olefins and aromatics). This last mentioned method was not satisfactory with the silica gel used.

For the separations, known classical columns were used:

(1) *n*-Butyric ester of triethylene glycol on Chromosorb P, 60–80 mesh (EKM).
(2) Tricresylphosphate on Chromosorb P, 60–80 mesh (TKF).
(3) Squalane on Chromosorb P, 60–80 mesh.

The C_1–C_4 hydrocarbons were identified on the EKM column by comparison with injected pure gaseous hydrocarbons or calibration gases of known composition from the rich gas separation and pyrolysis gas separation plants (ethylene plant).

In further work, we continued on capillary columns and gradually used further pure compounds, which had not been available at the beginning of our investigations. For the identification of the non-aromatic components, a 45 m long stainless steel open tubular column was used wetted with squalane. The operational temperature was 40°. The method of identification of the hydrogenated pyrolysis gasoline components on the capillary columns was the same as in the case of the straight-run gasoline. We could make full use here of our analytical results on packed columns and from literature data published by MATSUKUMA[4] and HIVELY AND HINTON[5], containing Kováts indices of olefins and paraffins.

For the identification of aromatics in the straight-run light gasoline and in the hydrogenated pyrolysis gasoline, it was necessary to use several columns with stationary phases having different polarities. Squalane was used as a basic liquid phase, separating the analysed components of the mixtures approximately according to their boiling points. We used a 45 m long capillary column at the temp. 90°. The corresponding retention times were compared with the relative retention times given in the paper by MIYAKE *et al.*[6]. Using a more polar solvent, Ucon LB-550 X, and linear temperature programming from 60 to 130° at a rate of 1°/min the elution of alkenylaromatic hydrocarbons was retarded relative to alkylaromatic hydrocarbons having the same or similar boiling points. The relative retention times and the Kováts indices were compared with data given by BAUMANN AND CZICSERY[8] and MCTAGGART AND MORTIMER[9].

The elution data using Ucon LB-550 X were evaluated on the basis of the conclusions of WILLIS[7]. The di-tridecylphthalate used in this paper as stationary phase and Ucon LB-550 X have similar polarities. For confirmation of our opinion that the

hydrogenated pyrolysis gasoline under investigation contains also styrenes and indene, we used the strongly polar stationary phase mannit-hexakis-cyanoethyl ether, prepared in the Main Laboratory of Leuna-Werke, G.D.R. On this phase, *n*-decane is eluted before benzene and the elution times of styrenes and indene are appreciably retarded compared with those of the alkylaromatics.

We tried also *m*-bis-*m*-phenoxyphenoxybenzene as a stationary phase, suggested originally by WALKER AND AHLBERG[11] and recently used in several papers. Our results were good, but different from the results published.

In conclusion, we applied the chromatographic method of SANDERS AND MAYNARD[10] to our problems for the determination of C_3–C_{12} hydrocarbons in full-range motor gasoline.

We analysed the straight-run light gasoline and hydrogenated pyrolysis gasoline as a whole on a 45 m long stainless steel capillary column with squalane as liquid phase, with temperature programming from $-5°$ to $+90°$ and simultaneous carrier

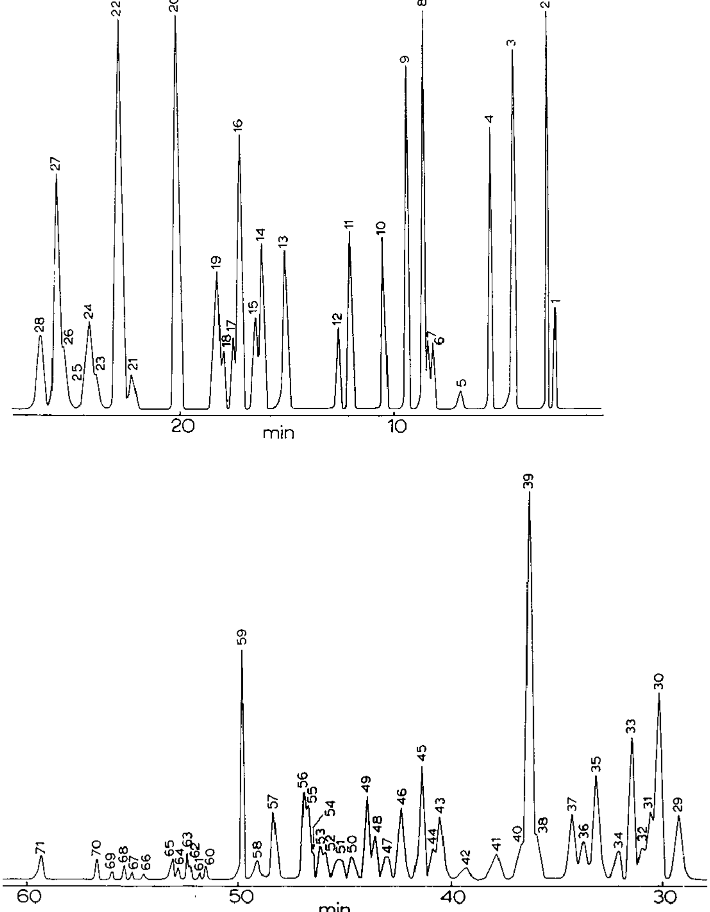

Fig. 1. Chromatogram of straight-run light gasoline on a capillary column with squalane. For the operational conditions see Table I. The identification of the peaks is given in Table II.

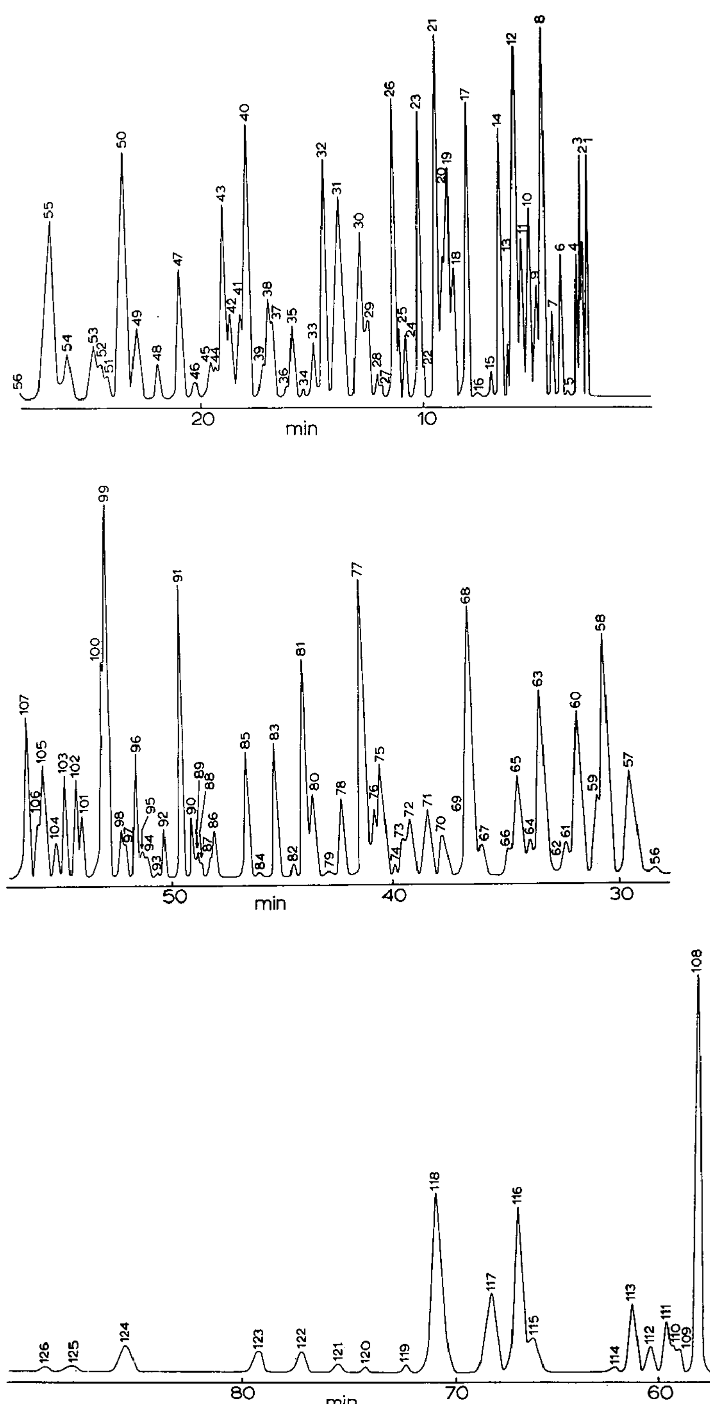

Fig. 2. Chromatogram of a hydrogenated pyrolysis gasoline on a capillary column with squalane. For the operational conditions see Table I. The identification of the peaks is given in Table III.

TABLE II

IDENTIFICATION OF CHROMATOGRAPHIC PEAKS SHOWN IN FIG. 1

Peak number		*Boiling point (°C)*	*% wt.*
1	Isobutane	−11.73	0.15
2	*n*-Butane	− 0.5	9.45
3	Isopentane	27.85	11.04
4	*n*-Pentane	36.07	16.42
5	2,2-Dimethylbutane	49.74	0.14
6	Cyclopentane	49.26	1.04
7	2,3-Dimethylbutane	57.99	1.39
8	2-Methylpentane	60.27	5.09
9	3-Methylpentane	63.28	5.51
10	*n*-Hexane	68.74	10.72
11	Methylcyclopentane	71.81	3.06
	2,2-Dimethylpentane	79.20	
12	Benzene	80.10	1.28
	2,4-Dimethylpentane	80.50	
13	Cyclohexane	80.74	1.83
14	2-Methylhexane	90.05	1.93
15	2,3-Dimethylpentane	89.78	1.03
	1,1-Dimethylcyclopentane	87.85	
16	3-Methylhexane	91.85	3.09
17	1-*cis*-3-Dimethylcyclopentane	91.73	0.89
18	1-*trans*-3-Dimethylcyclopentane	90.77	0.81
19	1-*trans*-2-Dimethylcyclopentane	91.87	1.71
	3-Ethylpentane	93.48	
20	*n*-Heptane	98.43	4.99
21	1-*cis*-2-Dimethylcyclopentane	99.57	0.18
22	Methylcyclohexane	100.93	2.47
	2,2-Dimethylhexane	106.84	
	1,1,3-Trimethylcyclopentane	106.89	
23	2,5-Dimethylhexane	109.10	0.20
	Ethylcyclopentane	103.47	
24	2,4-Dimethylhexane	109.43	0.74
25	2,2,3-Trimethylpentane	109.84	0.14
26	1,2,4-*cis*-Trimethylcyclopentane	109.29	0.43
27	Toluene	110.63	1.38
	3,3-Dimethylhexane	111.97	
28	2,3,4-Trimethylpentane	113.47	0.51
29	2,3,3-Trimethylpentane	114.76	0.43
	1,1,2-Trimethylcyclopentane	113.73	
30	2-Methylheptane	117.65	1.31
31	4-Methylheptane	117.71	0.45
32	3,4-Dimethylhexane	117.73	0.13
	1-*cis*-2-*trans*-4-Trimethylcyclopentane	116.73	
33	3-Ethylhexane	118.53	0.92
	3-Methylheptane	118.93	
	3-Methyl-3-ethylpentane	118.26	
34	1,1,3-*trans*-4-Tetramethylcyclopentane	121.6	0.12
	2,2,5-Trimethylhexane	124.08	
	1-*cis*-2-*cis*-4-Trimethylcyclopentane	118	
35	1-*cis*-3-Dimethylcyclohexane	120.09	0.73
	1,1-Dimethylcyclohexane	119.54	
36	1-Methyl-*trans*-3-ethylcyclopentane	120.8	0.19
37	2,2,4-Trimethylhexane	126.54	0.43
38	1-*trans*-2-Dimethylcyclohexane	123.42	0.21
	1-*cis*-2-*cis*-3-Trimethylcyclopentane	123.0	

TABLE II (*continued*)

Peak number	*Boiling point (°C)*	*% wt.*
39 *n*-Octane	125.67	2.32
40 1-*cis*-4-Dimethylcyclohexane	124.32	0.14
41 2,4,4-Trimethylhexane	130.65	0.05
42 2,3,5-Trimethylhexane	131.34	0.07
43 2,6-Dimethylheptane	135.21	0.31
1-*cis*-2-Dimethylcyclohexane	129.73	
44 *n*-propylcyclopentane	130.95	0.09
45 Ethylcyclohexane	131.78	0.61
Ethylbenzene	136.19	
46 3,3-Dimethylheptane	137.3	traces
47 1,1,3-Methylcyclohexane	136.63	0.39
48 2,2,3-Trimethylhexane	137.68	0.07
49 *p*-Xylene	138.35	0.21
50 *m*-Xylene	139.1	0.42
51 X_1		0.13
52 X_2		0.13
53 X_3		0.12
54 X_4		0.14
55 X_5		0.34
56 *o*-Xylene	144.41	0.29
57 X_6		0.18
58 X_7		0.03
59 Isopropylbenzene	152.39	0.66
n-Nonane	150.80	
60 *n*-Propylbenzene	159.22	0.04
61 X_8		0.02
62 X_9		0.06
63 X_{10}		0.12
64 1-Methyl-3-ethylbenzene	161.31	0.06
65 1-Methyl-4-ethylbenzene	161.99	0.09
66 X_{11}		0.02
67 1,3,5-Trimethylbenzene	164.72	0.06
68 X_{12}		0.04
69 X_{13}		0.03
70 1,2,4-Trimethylbenzene	169.35	0.09
71 1,2,3-Trimethylbenzene	176.08	0.09

gas flow programming. In this way, all the components were determined quantitatively.

An analysis of the straight-run light gasoline, made in the manner described, is shown in Figs. 1 and 2. In Tables II and III the separated components in the chromatograms mentioned and their amounts in the mixtures analysed are shown.

In the analysed straight-run light gasoline, 71 chromatographic peaks were found, 57 of which have been identified. The non-identified components are present in very low concentrations and they consist mainly of non-aromatic hydrocarbons boiling over 130°; the total amount of non-identified hydrocarbons is only 1.36 %.

In the hydrogenated pyrolysis gasoline, 126 substances were determined and 91 identified, the amount of non-identified hydrocarbons being 3.05 %. Taking into account the fact that some aromatics elute together with some aliphatic components on squalane, we repeated these analyses of hydrogenated pyrolysis and straight-run

TABLE III

IDENTIFICATION OF CHROMATOGRAPHIC PEAKS SHOWN IN FIG. 2

Peak number		*Boiling point (°C)*	*% wt.*
1	Isobutane	−11.73	0.76
2	Isobutylene	−6.9	
	Butene-1	−6.26	0.48
	1,3-Butadiene	−4.41	
3	*n*-Butane	−0.5	0.75
4	*trans*-2-Butene	0.88	0.45
5	Neopentane	9.5	0.03
6	*cis*-2-Butene	3.72	0.19
7	3-Methyl-1-butene	20.06	0.48
8	Isopentane	27.85	2.23
9	Pentene-1	29.97	0.64
10	2-Methyl-1-butene	31.16	1.18
11	2-Methyl-1,3-butadiene	34.07	0.91
12	*n*-Pentane	36.07	5.47
	trans-2-Pentene	36.35	
13	*cis*-2-Pentene	36.94	0.81
14	2-Methyl-2-butene	38.57	3.06
15	1,3-Pentadiene	42.03	
	1,3-*trans*-Cyclopentadiene	42.03	2.16
	3,3-Dimethyl-1-butene	41.24	
16	2,2-Dimethylbutane	49.74	0.04
17	Cyclopentene	44.24	0.17
18	3-Methyl-1-pentene	54.14	0.12
	4-Methyl-1-pentene	53.88	
19	Cyclopentane	49.26	
	4-Methyl-*cis*-2-pentene	56.30	0.54
	2,3-Dimethyl-1-butene	55.67	
20	2,3-Dimethylbutane	57.99	0.31
21	2-Methylpentane	60.27	2.28
22	2-Methyl-1-pentene	60.72	0.16
23	3-Methylpentane	63.28	
	2-Ethyl-1-butene	64.66	1.65
	Hexene-1	63.49	
24	*cis*-3-Hexene	66.47	0.1
	trans-3-Hexene	67.08	
25	3-Methyl-*cis*-2-pentene	67.7	
	2-Methyl-2-pentene	67.29	0.31
	3-Methylcyclopentene	65	
26	*n*-Hexane	68.74	3.78
	4,4-Dimethyl-1-pentene	72.49	
27	*trans*-2-Hexene	67.87	0.07
28	*cis*-2-Hexene	68.84	0.13
29	4,4-Dimethyl-*trans*-2-pentene	76.75	0.49
	3-Methyl-*trans*-2-pentene	70.44	
30	Methylcyclopentane	71.81	1.33
	3,3-Dimethyl-1-pentene	77.57	
31	Benzene	80.10	21.93
	2,4-Dimethylpentane	80.50	3.73
	4,4-Dimethyl-*cis*-2-pentene	80.42	
32	2,2,3-Trimethylbutane	80.88	1.78
	2,4-Dimethyl-1-pentene	81.64	
33	2,4-Dimethyl-2-pentene	83.26	
	3-Ethyl-1-pentene	84.11	0.41
	3-Methyl-1-hexene	84	

TABLE III (*continued*)

Peak number		*Boiling point* (°C)	*% wt.*
34	2-Methyl-*trans*-3-hexene	86	0.04
	5-Methyl-1-hexene	85.31	
35	Cyclohexane	80.74	0.61
	4-Methyl-*cis*-2-hexene	87.31	
36	4-Methyl-1-hexene	86.73	0.08
	4-Methyl-*trans*-2-hexene	87.56	
37	Cyclohexene	82.98	0.53
38	2-Methylhexane	90.05	0.89
	5-Methyl-*cis*-2-hexene	89.5	
39	2,3-Dimethylpentane	89.78	0.26
	3,4-Dimethyl-*cis*-2-pentene	87.99	
40	3-Methylhexane	91.85	1.14
41	2-Methyl-1-hexene	91.95	0.32
	3,4-Dimethyl-*trans*-2-pentene	90.5	
42	Heptene-1	93.64	0.36
	2-Ethyl-1-pentene	94	
43	3-Ethylpentane	93.48	0.81
	3-Methyl-*trans*-2-hexene	94	
44	2,2,4-Trimethylpentane	99.24	0.13
	trans-3-Heptene	95.67	
45	*cis*-3-Heptene	95.75	0.17
	3-Methyl-*cis*-3-hexene	95.33	
	2-Methyl-2-hexene	95.44	
	3-Methyl-*trans*-3-hexene	93.53	
46	3,5-Dimethyl-1-hexene	104	0.07
	3-Ethyl-2-pentene	96.01	
47	*n*-Heptane	98.43	2.37
	trans-2-Heptene	97.95	
48	3-Methyl-*cis*-2-hexene	94	0.05
	2,3-Dimethyl-2-pentene	97.40	
	cis-2-Heptene	98.5	
49	4,4-Dimethyl-1-hexene	107.2	0.09
	3,4,4-Trimethyl-1-pentene	104	
50	Methylcyclohexane	100.93	1.28
	cis-2,4-Dimethyl-3-hexene	109	
	2,2-Dimethylhexane	106.84	
51	2,4-Dimethyl-2-hexene	110.6	0.06
52	2,5-Dimethylhexane	109.1	0.15
	trans-2,4-Dimethyl-3-hexene	107.6	
53	2,4-Dimethylhexane	109.43	0.28
	3,3-Dimethyl-2-ethyl-1-butene	110	
54	3-Ethyl-1-hexene	110.3	0.25
55	Toluene	110.63	16.28
	3,3-Dimethylhexane	111.97	0.18
	2,4-Dimethyl-1-hexene	111.2	
56	3,3,4-Trimethylpentane	113.47	0.01
	trans-4,4-Dimethyl-2-hexene	106	
57	2,3,3-Trimethylpentane	114.76	0.34
58	2-Methylheptane	117.65	0.61
	2,3,4-Trimethyl-2-pentene	116.26	
59	4-Methylheptane	117.71	0.19
	trans-6-Methyl-2-heptene	118	
	2-*n*-Propyl-1-pentene	117.7	
60	3-Ethylhexane	118.53	0.46
	3-Methylheptane	118.93	
	3-Ethyl-3-hexene	116	
	cis-6-Methyl-2-heptene	118	

(*continued on page* 100)

TABLE III (*continued*)

Peak number		*Boiling point (°C)*	*% wt.*
61	2,2,5-Trimethylhexane	124.08	0.05
	cis-4-Methyl-3-heptene	122	
62	2-Ethyl-1-hexene	120	0.02
63	*trans*-3-Ethyl-2-hexene	121	0.51
	Octene-1	121.28	
64	*cis*-3,4-Dimethyl-3-hexene	122	0.07
65	2,3-Dimethyl-2-hexene	121.77	0.26
	2,2,4-Trimethylhexane	126.54	
66	*cis*-4-Octene	122.54	0.06
67	*trans*-2-Octene	125	0.15
68	(*n*-Octane)	125.67	1.21
	cis-2-Octene	125.64	
69	X_1		0.22
70	X_2		0.02
71	X_3		0.05
72	X_4		0.03
73	X_5		0.02
74	X_6		0.01
75	2,6-Dimethylheptene	135.21	0.13
76	*n*-Propylcyclopentane	130.95	0.05
77	Ethylbenzene	136.19	0.69
	Ethylcyclohexane	131.78	0.65
78	X_7		0.32
79	X_8		0.25
80	*p*-Xylene	138.35	0.86
81	*m*-Xylene	139.10	1.98
82	X_9		0.06
83	Styrene	145.14	1.11
84	X_{10}		0.25
85	*o*-Xylene	144.41	0.88
86	X_{11}		0.05
87	X_{12}		0.02
88	X_{13}		0.01
89	X_{14}		0.02
90	X_{15}		0.04
91	Isopropylbenzene	152.39	0.19
	(*n*-Nonane)	150.80	
92	X_{16}		0.05
93	X_{17}		0.01
94	X_{18}		traces
95	X_{19}		0.02
96	*n*-Propylbenzene	159.22	0.08
97	X_{20}		0.04
98	X_{21}		0.03
99	1-Methyl-3-ethylbenzene	161.31	0.29
100	1-Methyl-4-ethylbenzene	161.99	0.22
101	α-Methylstyrene	165.5	0.04
102	1-Methyl-2-ethylbenzene	165.15	0.07
103	1,3,5-Trimethylbenzene	164.71	0.08
104	*tert.*-Butylbenzene	169.12	0.02
105	*m*-Methylstyrene	171.6	0.14
106	*p*-Methylstyrene	172.77	traces
107	1,2,4-Trimethylbenzene	169.35	0.14
108	X_{22}		0.67
109	X_{23}		traces

TABLE III (*continued*)

Peak number		*Boiling point (°C)*	*% wt.*
110	1,2,3-Trimethylbenzene	176.08	0.01
	1-Methyl-4-isopropylbenzene	177.10	
111	1-Methyl-2-isopropylbenzene	178.15	0.04
	Indane	177.82	
	Indene	182.44	
112	X_{24}		0.01
113	X_{25}		0.07
114	X_{26}		traces
115	X_{27}		traces
116	X_{28}		0.22
117	X_{29}		0.09
118	X_{30}		0.27
119	X_{31}		0.01
120	X_{32}		0.01
121	X_{33}		0.02
122	X_{34}		0.04
123	X_{35}		0.03
124	X_{36}		0.08
125	X_{37}		0.01
126	X_{38}		0.01

gasolines on Ucon LB-550 X for quantitative evaluation; under these conditions these components were separated.

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PROPERTIES OF ACID-WASHED AND SILANIZED CZECHOSLOVAK SUPPORTS: CHROMATON N AND CHEZASORB

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SUMMARY

The separating efficiency as well as the adsorptive and catalytic properties of the new Czechoslovak acid-washed Chromaton N-AW and Chezasorb AW supports, as well as silanized Chromaton N-AW-DMCS, Chromaton N-AW-HMDS, and Chezasorb AW-HMDS supports for gas chromatography, were studied. The results were compared with data obtained in the same manner with untreated supports and functionally corresponding supports of the Chromosorb W and P types. The pore size distribution was measured in the acid-washed supports.

It has been found that the separating efficiency of Chromaton N type supports is similar to that of the Chromosorb W type. The separating efficiency of Chezasorb type supports is slightly better than that of Chromosorb P type products. The catalytic properties are the same with all mutually corresponding supports, but is considerably reduced by acid washing and silanization. The adsorptive properties are least with Chromaton N-AW-DMCS and Chromosorb W-AW-DMCS, are slightly greater with Chezasorb AW-HMDS and Chromaton N-AW-HMDS and are relatively greatest with Chromosorb W-AW-HMDS and Chromosorb P-AW-DMCS.

INTRODUCTION

In recent years, new Czechoslovak supports for gas chromatography have been developed; these supports, treated by acid washing and silanization, have appeared on the market this year. The physical and chemical characteristics of the untreated supports, as well as separating efficiency, and catalytic and adsorptive activity tests of treated supports, are described here. The results are compared with values measured in the same manner for untreated as well as functionally corresponding supports of the Chromosorb W and P types.

SUPPORTS BASED ON CHROMATON N

Chromaton N is a diatomaceous earth support which is white colored and according to OTTENSTEIN's classification[1] belongs to type II. It is obtained by calcinating physically and chemically purified diatomaceous earth (from the Borovany deposit) with an alkaline flux and, prior to the final step of production, working it up into spherical beads. For physical and chemical data concerning this support see Table I.

TABLE I

PHYSICAL AND CHEMICAL DATA OF CHROMATON N (see ref. 2)

Approximate chemical composition	SiO_2	93%
	Al_2O_3	3.3%
	Fe_2O_3	0.04%
	TiO_2	0.01%
	$CaO + MgO$	0.1%
	$Na_2O + K_2O$	3.4%
Specific surface	approximately 1 sq. m/g	
Bulk weight	0.235 g/cm^3	
Specific weight of the skeleton	2.3 g/cm^3	
pH of a 5% water suspension	9–10	
Capacity for stationary phases	20–25%	
Appearance	snow white regular spheres up to ellipsoids	
Particle size	7 varieties covering the scale from 0.1 mm to 0.63 mm	

The acid-washed support Chromaton N-AW is obtained by leaching Chromaton N with acid, washing and floating with water and further washing with alcohol. The alkalis are thus washed off the surface of the support and, consequently, the pH value of a 5 % water suspension is between 6 and 8. Fine powder particles which decrease the separating efficiency of the column and increase the column resistance to the carrier gas are removed from the support at the same time. The other properties of the support remain the same as those of untreated Chromaton N.

The silanized supports Chromaton N-AW-DMCS and Chromaton N-AW-HMDS are obtained by treating the acid-washed Chromaton N-AW with dimethyldichlorosilane (DMCS) or hexamethyldisilazane (HMDS) respectively; this treatment results mainly in removing the undesirable catalytic and the adsorptive activity of the support.

SUPPORTS BASED ON CHEZASORB

Chezasorb is a pink diatomaceous earth support which, according to OTTENSTEIN's classification[1], belongs to type I, *i.e.* the Firebrick type. It is obtained by calcinating chemically purified diatomaceous earth (the Kučlín deposit) with additives and crushing the mass formed. The mechanical strength of this support is far greater than that of Chromaton N. Physical and chemical data of this support are shown in Table II.

Acid-washed Chezasorb AW is obtained by leaching Chezasorb with acid, washing and floating with water to neutral reaction. The purpose of this treatment is not to change the surface reaction as it was with Chromaton N, but the removal of R_2O_3 type oxides showing catalytic and adsorptive activity. The leaching of a certain amount of ferric oxide from the surface is indicated by some fading of the initial pink coloring. In the flotation process undesirable powder particles are removed from the support, while the other properties are retained.

The silanized support, Chezasorb AW-HMDS, is obtained by treating Chezasorb AW with hexamethyldisilazane.

TABLE II

PHYSICAL AND CHEMICAL DATA OF CHEZASORB[3]

Approximate chemical composition	SiO_2	90–95%
	Al_2O_3	3.5%
	Fe_2O_3	1.5%
	TiO_2	0.09%
	CaO + Mg	O0.3–0.5%
	$Na_2O + K_2O$	0.5–1.0%
Specific surface	3.0 ± 0.5 sq. m/g	
Medium pore radius	0.5–0.9 μ	
Total pore volume	0.5–0.6 cm^3/g	
Bulk weight	0.6–0.7 g/cm^3	
Specific weight of the skeleton	approximately 1.9 g/cm^3	
pH of a 5% water suspension	6.0–7.4	
Capacity for stationary phases	up to 20%	
Appearance	pink particles of irregular shape	
Particle size	5 varieties covering the scale from 0.1 mm to 0.58 mm	

EXPERIMENTAL

The pore size distribution and the total pore volume of the supports were measured on a mercury pressure porosimeter in the Adsorption and Gas Chromatography Laboratory, Department of Chemistry, Lomonosov University, Moscow, U.S.S.R.

The separating efficiency was expressed by the number of theoretical plates N of an 800 mm column[4] for toluene and by the resolution R of an 800 mm column for *o*-xylene and *p*-xylene, where:

$$N = 16\left(\frac{V_R'}{w}\right)^2$$

and

$$R = \frac{2(V_{R2} - V_{R1})}{w_1 + w_2}$$

A glass column, 4 mm I.D., packed with the support under study and coated with 3 % Apiezon L was used for these measurements. The temperature used was 70°, the flow rate of the nitrogen carrier gas was 40 ml/min and 0.5 μl of a mixture consisting of benzene, toluene, *o*-xylene, and *p*-xylene was injected.

In addition, graphs of the Van Deemter functions for benzene and isopropyl ether were plotted, and from these plots the minimum height equivalents to a theoretical plate ($HETP_{min.}$) were obtained. The measurement was carried out using a metal column, 880 mm length and 0.2826 cm^2 cross section, packed with the support under study and coated with 15 % squalane; the temperature was 100° and the charge of benzene and isopropyl ether mixture 0.5 μl. In these measurements, it was arranged that approximately the same particle size of the supports to be compared was used: 0.16–0.20 mm for Chromaton N-AW-DMCS, 80–100 mesh for Chromosorb W-AW-DMCS, 0.20–0.25 mm for Chezasorb AW-HMDS, and 60–80 mesh for Chromosorb P-AW-DMCS.

The catalytic properties of the supports (K) were determined according to POSPÍCHAL[5] and expressed as the degree of catalytic decomposition of n-butanol on the uncoated support being studied, when it was heated to 290 ± 1° in a 110 × 4 mm metal column. The undecomposed n-butanol was separated from its decomposition products in an 800 × 4 mm glass column packed with Chromaton N coated with 10 % Tridox (tridecanol-polyethyleneoxide). The temperature of the separating column was 100°, the flow rate of the nitrogen carrier gas 25 ml/min and the sample quantity, 1 μl. The degree of decomposition was expressed in % and was determined by measuring the areas of the peaks of n-butanol and its decomposition products planimetrically. n-Butanol was injected repeatedly, till the area of the peaks compared did not change in the course of three tests following one after another.

The adsorptive properties of the supports were determined from the peak width of n-amyl alcohol ($S_1/_{10}$) measured at one tenth of its height. The measurement was carried out under the conditions described for the determination of N and R, 1 μl quantities of 20 % n-amyl alcohol solution in ether were injected and the shift speed of the chart was 12.5 mm/min.

The supports of Chromaton N and Chezasorb types examined were supplied by the Research Institute of Pure Chemicals, Lachema Natl. Corp., Brno, from current production and sale, while the supports of the Chromosorb W and P types were supplied by Becker, Delft (The Netherlands).

With the exception of the Van Deemter functions, for the measurement of which a Chrom II chromatograph with flame ionization detector (Laboratory Apparatus Natl. Corp.) was used, all other measurements were carried out with a Fractovap chromatograph, DACI model (Carlo Erba, Milan, Italy) with a flame ionization detector.

RESULTS AND DISCUSSION

The pore size distribution and total pore volume of the acid-washed supports are evident from Fig. 1. The total pore volume of Chromaton N-AW amounts to 1.34 cm³/g, while the value found for Chezasorb AW, 0.61 cm³/g, is slightly higher than

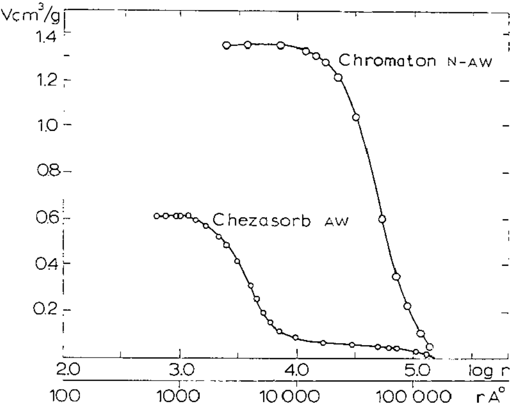

Fig. 1. Pore size distribution of Chromaton N-AW and Chezasorb AW.

the range indicated for an unwashed support by the manufacturer. The slope of both curves up to their horizontal portions and towards smaller pores suggests a narrow pore size distribution as well as the absence of undesirable micropores. The medium pore radius of Chromaton N-AW is about 4 μ, while the value found for Chezasorb AW, 0.4 μ, is somewhat lower than the range indicated for an untreated support by the manufacturer.

TABLE III

SEPARATING EFFICIENCIES, CATALYTIC AND ADSORPTIVE ACTIVITIES OF CHROMATON, CHEZASORB, AND CHROMOSORB SUPPORTS

Supports	*Particle size*	*N*	*R*	*K* (%)	$S_{1/10}$ (*mm*)
White supports, type II	(*mm*)				
Chromaton N	0.20–0.25	420	1.0	0.5	—
Chromaton N-AW	0.20–0.25	460	1.1	0.4	—
Chromaton N-AW-DMCS	0.16–0.20	490	1.1	0.2	3.2
Chromaton N-AW-HMDS	0.20–0.25	440	1.1	0.2	4.0
	(*mesh*)				
Chromosorb W	60–80	450	1.1	0.4	—
Chromosorb W-AW	60–80	460	1.1	0.3	—
Chromosorb W-AW-DMCS	80–100	430	1.1	0.2	3.2
Chromosorb W-AW-HMDS	60–80	420	1.0	0.2	11.5
Pink supports, type I	(*mm*)				
Chezasorb	0.10–0.20	750	1.3	28.1	—
Chezasorb AW	0.10–0.20	870	1.5	9.7	—
Chezasorb AW-HMDS	0.10–0.20	780	1.3	1.2	4.0
	(*mesh*)				
Chromosorb P	80–100	570	1.24	27.1	—
Chromosorb P-AW	80–100	670	1.5	12.6	—
Chromosorb P-AW-DMCS	60–80	750	1.3	0.47	10.0

Table III gives the separating efficiency of the new Czechoslovak supports and the Chromosorb products (Johns-Manville). The separating efficiency of Chromaton N type supports is similar to that of Chromosorb W products. When comparing the pink supports with one another, it must be remembered that these are commercial products and that the particle size does not correspond accurately. Though the range of the particle size of Chezasorb supports is broader than that of corresponding Chromosorb P products, the separating efficiency of the former is slightly better. Under column conditions, the separating efficiency of the pink supports is greater than that of the white products; this is due to their having approximately twice as great a specific surface and more than double bulk weight.

The separating efficiency can be compared more precisely according to the $HETP_{min.}$ values. The course of the Van Deemter function on Chromaton N-AW-DMCS and Chromosorb W-AW-DMCS can be seen in Fig. 2. The value of the minimum height equivalent for Chromaton N-AW-DMCS to a theoretical plate for benzene is 3.2 mm and 1.9 mm for isopropyl ether. The corresponding $HETP_{min.}$ value found for isopropyl ether on Chromosorb W-AW-DMCS is 2.3 mm. No conclusions regarding

superior properties for Chromaton N-AW-DMCS can be drawn from the difference between these values.

The course of Van Deemter function on Chezasorb AW-HMDS is similar (see Fig. 3), but the minima of the curves lie lower. A value of 0.90 mm $HETP_{min.}$ was obtained for benzene and 0.70 mm for isopropyl ether. The corresponding values of minimum height equivalent to a theoretical plate for isopropyl ether on Chromosorb P-AW-DMCS is 1.10 mm, suggesting a better separating efficiency with silanized Chezasorb.

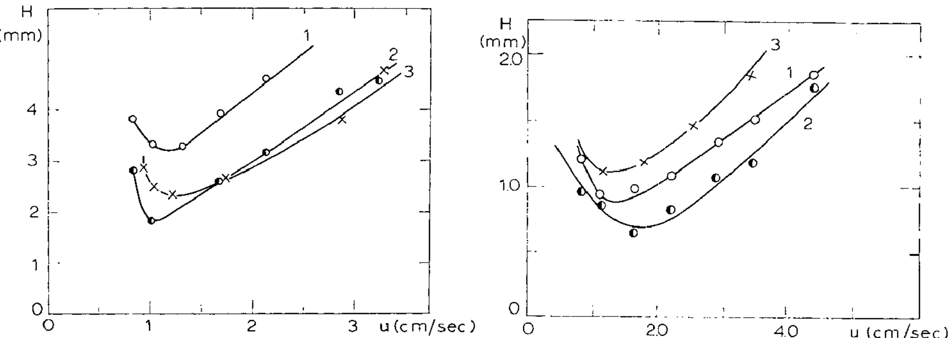

Fig. 2. Plot of the Van Deemter function for Chromaton N-AW-DMCS (1 = benzene; 2 = isopropyl ether) and for Chromosorb W-AW-DMCS (3 = isopropyl ether).

Fig. 3. Plot of the Van Deemter function for Chezasorb AW-HMDS (1 = benzene; 2 = isopropyl ether) and for Chromosorb P-AW-HMDS (3 = isopropyl ether).

It can be seen from Table III that under the conditions of the test, the white supports only show a very slight catalytic activity and that this is reduced to practically zero by silanization. The considerable degree of decomposition of *n*-butanol which takes place on the surface of untreated pink supports may be accounted for by ferric oxide and aluminium oxide, the removal of which from the surface of the supports by acid washing is accompanied by a considerable decrease in catalytic activity. This is further reduced by silanization and drops approximately to the level of the untreated white supports.

The test for adsorptive activity ($S_{1/10}$), in the modification applied by us, is only suitable for silanized supports, as with the other supports such a deformation of the *n*-amyl alcohol peak occurs that the results cannot be evaluated. From the results of measurements summed up in Table III, it may be concluded that the adsorptive activity is most reduced in the white supports treated with dimethyldichlorosilane. Chromaton N-AW-DMCS is equivalent to Chromosorb W-AW-DMCS, while Chromosorb W-AW-HMDS does not attain the level of Chromaton N-AW-HMDS. Thus, for white supports, dimethyldichlorosilane is a more efficient silanizing agent than hexamethyldisilazane, which is in good agreement with reports in literature[6,7]. The adsorptive activity of pink supports is in some degree higher than that of the white ones. Chezasorb-AW-HMDS appears to be better than Chromosorb P-AW-DMCS, as its adsorptive activity is reduced to a simular extent to that of Chromaton N-AW-HMDS.

Numerically expressed results may be documented in a more illustrative manner by the shape of *n*-amyl alcohol peaks on white (see Fig. 4) and pink supports (see Fig. 5).

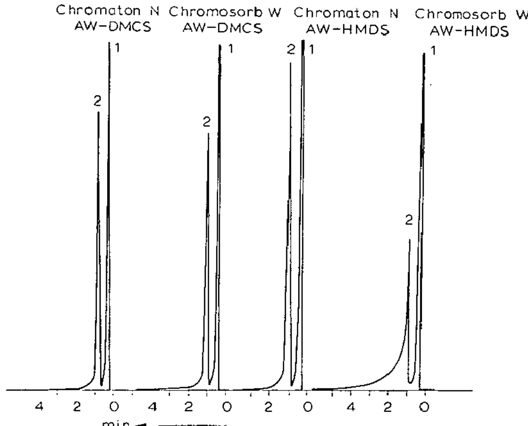

Fig. 4. Tailing of the peaks of *n*-amyl alcohol on silanized white supports. 1 = ether; 2 = *n*-amyl alcohol.

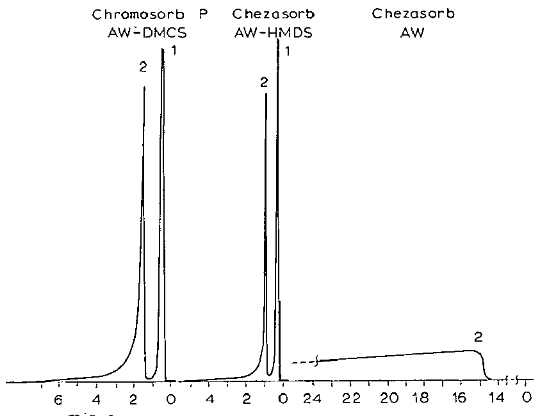

Fig. 5. Tailing of the peaks of *n*-amyl alcohol on silanized pink supports and on untreated Chezasorb AW. 1 = ether; 2 = *n*-amyl alcohol.

The remarkable effect resulting from silanization is also very clearly shown from the comparison with an untreated support in Fig. 5. If a more polar phase, di-*n*-decylphthalate (see Fig. 6), is used, the difference between a silanized and a non-silanized support is manifested rather by the longer elution times of alcohols on a non-silanized support than by the tailing of their peaks.

CONCLUSIONS

From the results of the measurements quoted and also from our experience we can conclude that treated supports of the Chromaton N and Chezasorb type are

mutually matching products, owing to their properties. It is recommended that white supports are used for the analyses of substances sensitive to catalytic decomposition or for the separation of polar, strongly adsorptive substances when the supports are coated with low amounts of non-polar or medium polar stationary phases. The properties of Chromaton N-AW-DMCS are most advantageous in this respect.

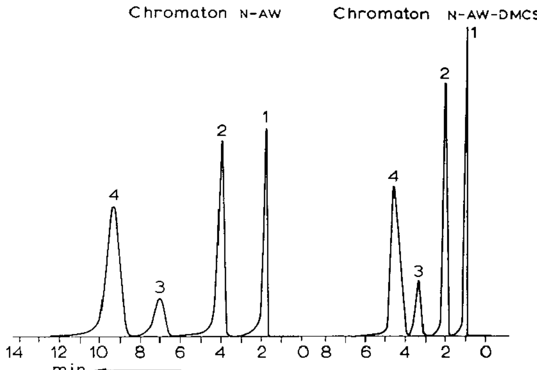

Fig. 6. Extension of elution times of alcohols in consequence of the adsorptive effect of the support. Coating 3% di-*n*-decylphthalate. 1 = propanol; 2 = *n*-butanol; 3 = isoamyl alcohol; 4 = *n*-amyl alcohol. T = 80°, flow rate N_2 = 40 ml/min.

Treated pink supports based on Chezasorb are preferable in all cases when maximum separating effect is to be attained. These supports are of particular use in the separation of non-polar and medium polar substances, which are not too sensitive to catalytic decomposition. Chezasorb AW-HMDS even permits the separation of strongly polar substances with low coating of the support with non-polar stationary phases.

Acid-washed and silanized supports based on Chromaton N and Chezasorb broaden the scale of applicability of basic supports, their properties being equivalent to those of acid-washed and silanized Chromosorb W and P products.

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CHROM. 4721

"SYNACHROM", A MACROPOROUS COPOLYMER, AS A SORPTION MATERIAL FOR GAS CHROMATOGRAPHY

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SUMMARY

A chromatographic sorbent called "Synachrom" based on a styrene–divinylbenzene bead copolymer has been developed. The chromatographic separation properties of this material were investigated for mixtures of gases and a number of other materials, for which the Kováts' elution indices were measured. The elution of several near boiling substances from different homologous series was investigated in order to elucidate the separation properties of "Synachrom" and it was found, that the elution order corresponds to the increasing value of the polarizability of substances. From this it is evident, that Synachrom behaves not only as an adsorbent but also as a liquid phase of non-polar character. The properties of Synachrom correspond to analogous types of unmodified polymer sorbents, which are available on the world market at present.

In recent years macroporous copolymers have been used frequently as packings for gas chromatography columns; especially copolymers of styrene with divinylbenzene or ethylvinylbenzene and divinylbenzene, respectively. They are usually prepared by means of pearl copolymerization; the preparation, and some of the properties of the bead polymer have been described by LLOYD AND ALFREY[1,2], the preparation being claimed by a number of patent specifications, including some from Czechoslovakia[3].

The use of these materials for gel permeation chromatography was described by MOORE[4], and by HOLLIS[5] who followed the work of the former, using, for the first time, macroporous copolymers of the styrene–divinylbenzene type for gas chromatography. At present the material is available from a number of firms (in particular Waters Associates), and is designated by different trade-marks, such as Porapak Q-PAR, Polypack etc. Further development was pursued especially by Waters Associates, who now offer further types of Porapak (S, R, T, N) which, again, are copolymers based on styrene and divinylbenzene with a certain quantity of a polar monomer, containing different groups, *e.g.* carboxylic groups. Another type of porous polymer based on polyphenylethers has been described by TRANCHANT[6].

As a result of earlier experience in the preparation of macroporous ion exchangers at the Research Institute for Synthetic Resins and Lacquers we tried to prepare an analogous type of styrene–divinylbenzene copolymer having suitable properties for use as a sorption material for gas chromatography. The bead copolymer was prepared by suspension copolymerization of styrene and divinylbenzene in the presence of an inert substance producing a porous structure and which was removed after the end of the polymerization. A three-dimensional both inter and intramolecular network is produced during polymerization. The microstructure of the bead copolymer is shown in Fig. 1. The structure and properties, *i.e.* swelling capacity, particle size, specific surface, distribution and diameter of pores, are characterized by:

(a) the type of the inert substance and its quantity;

(b) the quantity of divinylbenzene;

(c) the conditions of the pearl polymerization (temperature, polymerization time, suspension stabiliser, mixer revolutions, etc.).

The size of the specific surface of the copolymer, measured by the BET method, varies, according to the preparation conditions, within the limit of 10^2-10^3 m^2/g; with Synachrom the specific surface size was found to be 520 m^2/g. Using the thermal desorption method, a value of 620 m^2/g was found for the same sample. The volume of the inside pores per g of material was found to be 2.34 ml/g from the measured densities of helium (ρ = 1.038 g/ml). If one assumes that the pores have the form of infinite cylinders, the mean diameter of the pores can be calculated, which, in case of the given Synachrom sample, was 45 Å.

Another part of our work was devoted to the evaluation of the macroporous copolymer as the sorption packing for a chromatographic column. Like any solid

Fig. 1. Microstructure of Synachrom: a section under an electron microscope.

material with a large and rough surface the copolymer has the character of an adsorbent. Furthermore, especially when vapors of organic substances are separated, not only adsorption can occur, but also the molecules of the sorbate can penetrate into the structure of the copolymer, *i.e.* absorption, which depends upon the character

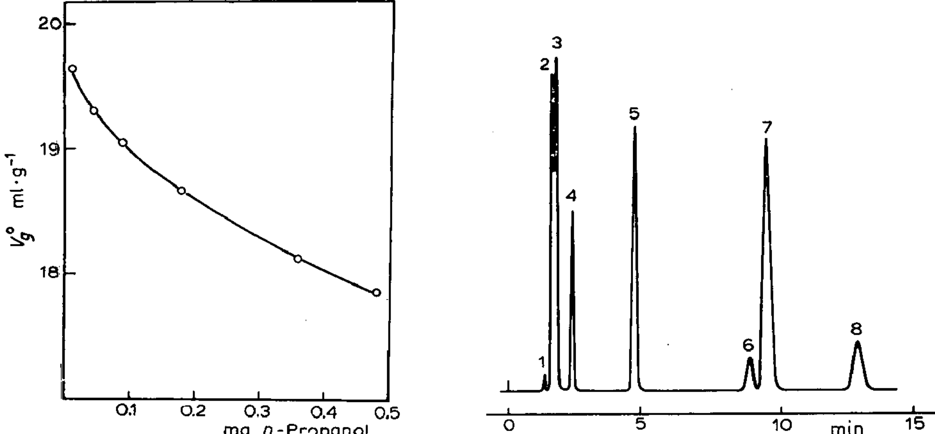

Fig. 2. Dependence of specific retention volume V_g^0 on the sample size of *n*-propanol.

Fig. 3. Fractovap GV (Carlo Erba), thermal conductivity detector, Synachrom 60/80 mesh, column 5 m × 2 mm, stainless steel, temperature 26°, carrier gas He, 25 ml/min. 1 = H_2; 2 = $N_2 + O_2$; 3 = CO; 4 = CH_4; 5 = CO_2; 6 = C_2H_4; 7 = C_2H_2; 8 = C_2H_6.

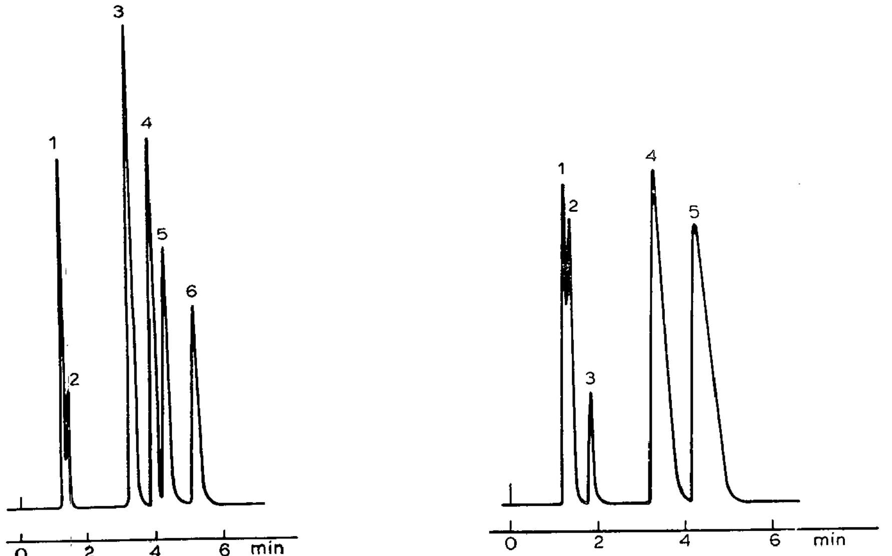

Fig. 4. Synachrom 60/80 mesh, column 5 m × 2 mm, stainless steel, temperature −78°, carrier gas He, 40 ml/min. 1 = H_2; 2 = Ne; 3 = N_2; 4 = O_2; 5 = Ar; 6 = CO.

Fig. 5. Synachrom 60/80 mesh, column 5 m × 2 mm, stainless steel, temperature 24°, carrier gas H_2, 27 ml/min 1 = N_2; 2 = CO; 3 = CH_4; 4 = CO_2; 5 = N_2O.

of the sorbate or on its relation to the sorbent. Consequently, the final separation effect of the copolymer is a result of combined adsorption and absorption.

The adsorption influence can be distinguished quite easily in the shape of the peaks especially of substances which due to their polarity do not diffuse into the skeleton, and are separated primarily on the adsorption principle. Their peaks are asymmetric to a certain extent which is an indication of a non-linear separation isotherm. In addition, the working temperature of the chromatographic column has to be about 50° higher than that of a conventional gas chromatography arrangement in order to obtain acceptable retention times. Last but not least the shape of the chromatographic peak and the position of the peak maximum depends upon the sample size. Fig. 2 shows the dependence of the specific elution volume on the sample size of *n*-propanol. An interesting example of the separation effect of Synachrom as compared with classical adsorbents such as active charcoal and silica gel, is the separation of C_2-hydrocarbons. Their separation together with other gases is illustrated in Fig. 3. Another example of a separation of a mixture of gases can be found in Figs. 4 and 5.

As mentioned above, the molecules of the sorbate penetrate into the copolymer structure during the absorption, so that the whole of the mass of the copolymer can behave like a liquid non-polar phase of a polyaromatic character. To compare the polarity of the packing, ROHRSCHNEIDER's[7] process was used, and the polarity was expressed as the difference of the Kováts' index for benzene on squalane and Synachrom at 150°. Teflon was used as solid support in the case of the column filled with squalane. From the value found $\Delta I_{Bz}^{Sq/Syn.} = -38$ it is evident that the copolymer behaves like a phase of even a lower polarity than squalane. Evidently we have here a non-polar column packing; the elution of individual substances will be determined first of all by means of the dispersion forces between the sorbent and the molecules of the substances to be separated, *i.e.*, by the total polarizability of the substances. In Table I a series of several substances with similar boiling points from various homologous series, along with indications of their boiling points, total polarizability α, and corresponding Kovats' indices is shown. It is evident from Table I that the order of elution is in agreement with the increasing values of the polarizability of substances. This phenomenon is still more obvious in strongly polarized substances like water, lower aliphatic acids, diols, etc. It is above all this very fact which has made polymeric column packings extremely popular. Their use means a substantial solution of troubles during the analysis of *e.g.* aqueous solutions, because water, as evident from Table II,

TABLE I

RELATION BETWEEN THE KOVÁTS' RETENTION INDICES AND THE TOTAL POLARIZABILITY OF SUBSTANCES

Compound	*B.p.* (°*C*)	$\alpha \cdot 10^{-24}$	*Kováts index*
Ethyl alcohol	78.4	5.06	412
Acrylonitrile	81.8	6.11	488
Methyl ethyl ketone	79.5	8.19	559
Ethyl acetate	76.8	8.82	582
1-Chlorobutane	77.8	10.09	612
Benzene	80.1	10.36	622
Cyclohexane	80.7	10.99	625

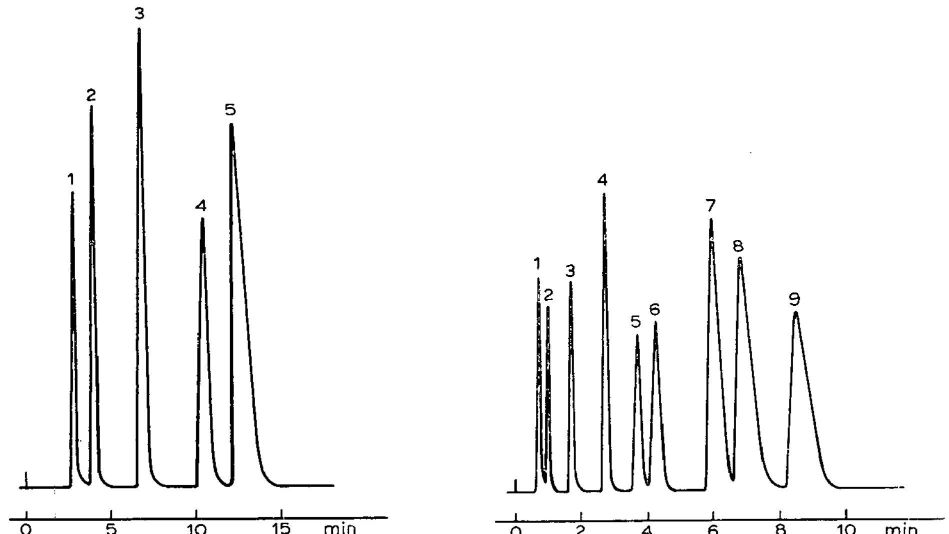

Fig. 6. Synachrom 80/100 mesh, column 1.5 m × 5 mm, glass, temperature 150°, carrier gas He, 60 ml/min. 1 = water; 2 = methyl alcohol; 3 = ethyl alcohol; 4 = acetone; 5 = ethyl ether.

Fig. 7. Synachrom 80/100 mesh, column 1.5 m × 5 mm, glass, temperature 150°, carrier gas He 50 ml/min. 1 = water; 2 = methyl alcohol; 3 = ethyl alcohol; 4 = isopropyl alcohol; 5 = propyl alcohol; 6 = *tert.*-butyl alcohol; 7 = *sec.*-butyl alcohol; 8 = isobutyl alcohol; 9 = butyl alcohol.

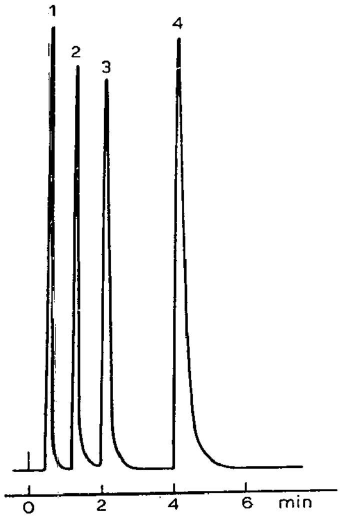

Fig. 8. Synachrom 80/100 mesh, column 1.5 m × 5 mm, glass, temperature 175°, carrier gas He, 60 ml/min. 1 = water; 2 = formic acid; 3 = acetic acid; 4 = propionic acid.

is eluted between ethane and propane in a relatively symmetrical peak. Examples of several separations are shown in Figs. 6–8, and the Kováts' indices of these and some other substances are given in Table II.

TABLE II

KOVÁTS' RETENTION INDICES ON SYNACHROM

Column conditions: Synachrom 60/80 mesh; Fractovap GV (Carlo Erba); Column 1.5 m × 5 mm, glass, temperature 150°; Thermal conductivity detector, 150 mA, temperature 200°; Carrier gas, He, 60 ml/min.

Compound	*B.p.* (°*C*)	*Kováts index*
Water	100.0	250
Methyl alcohol	64.7	319
Acetaldehyde	20.2	365
Ethyl alcohol	78.4	412
Formic acid	100.6	444
Acetonitrile	81.8	446
Acrolein	52.5	459
Acetone	56.5	468
Isopropyl alcohol	82.5	472
Dichloromethane	40.7	480
Acrylonitrile	78.5	488
Ethyl ether	34.6	493
Methyl acetate	56.9	494
Allyl chloride	44.6	495
Propyl alcohol	96.6	501
Cyclopentane	97.8	510
Acetic acid	118.1	526
tert.-Butyl alcohol	82.9	531
Vinyl acetate	72.3	551
Methyl ethyl ketone	79.5	559
Trichloromethane	61.3	569
sec.-Butyl alcohol	99.5	570
Tetrahydrofuran	66.0	572
2-Methylpentane	60.3	580
Ethyl acetate	76.8	582
Isobutyl alcohol	108.0	586
3-Methylpentane	63.3	595
1,2-Dichloroethane	82.4	595
Methylcyclopentane	71.8	604
Butyl alcohol	117.5	609
1-Chlorobutane	77.8	612
Carbon tetrachloride	76.7	618
Benzene	80.1	622
Cyclohexane	80.7	625
Propionic acid	141.1	631
Ethyleneglycol	197.3	641
Cyclohexene	83.0	643
Acrylic acid	141.1	645
Isopropyl acetate	88.9	655
Propyl acetate	101.6	682
Pyridine	115.2	699
Ethylcyclopentane	103.5	711
Toluene	110.6	721

Because of their frequently exceptional properties the macroporous copolymers have become a popular material not only for analysis of aqueous solutions, but also for trace analysis. A shift in the elution pattern of the trace impurities can be made possible in many cases whereby it elutes before the main component. Apart from this they are suitable not only for gas chromatography, but also as sorbents for thin-layer chromatography[8], and as column packing materials for liquid chromatography.

From the results quoted above it is evident, that Synachrom materials can be used successfully and added to the list of other macroporous materials used so far as sorbents in gas chromatography.

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CHROM. 4812

THE APPLICABILITY OF THE LINEAR FREE ENERGY RELATIONSHIPS FOR THE IDENTIFICATION OF SOME ALKYL AROMATIC HYDROCARBONS BY OPEN TUBULAR COLUMN GAS CHROMATOGRAPHY

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(Received May 8th, 1970)

SUMMARY

The validity of some relationships existing between the structure of C_6 to C_{10} alkyl aromatics and their retention data, found on capillary polyethylene glycol and squalane columns, is discussed. A new substitution constant was determined for the correlation of the activity coefficient ratio. A relationship between the linear correlation of free energies for the alkyl aromatics and the differences of the Kováts' indices found on two liquid phases is proposed. An empirical relationship existing between the Kováts' indices and the new substitution constant was determined. The validity of the correlations found was proved experimentally and used in the identification of some C_{11}-alkyl aromatics.

INTRODUCTION

The analysis of complex hydrocarbon mixtures has been facilitated by progress in open tubular gas chromatography. The identification of the chromatographic peaks found may be difficult, because not only are the necessary standards, but also the retention data, often missing or inaccessible.

The identification of the components separated by gas chromatography can be carried out either by a combination of gas chromatography with IR or mass spectrophotometry, or by the application of the correlations existing between retention data and the structure of the separated molecules[1,2]. KEULEMANS[3] studied the identification of aromatic hydrocarbons, after hydrogenation, on the basis of relationships between retention time and the structure of the cycloparaffins so formed. GRANT AND VAUGHAN[4] derived from the Clausius–Clapeyron equation and the Trouton law correlations for the boiling points and the logarithm of retention data. A linear relationship between the logarithm of retention time and saturation vapour pressure for alkyl aromatics was found by HOARE AND PURNELL[4]. The applicability of the Kováts' indices for the identification of C_6 to C_{10} hydrocarbons has been demonstrated in our preceding publications[5,6].

The scope of this work was the examination of the validity of relationships existing between the structure of C_6 to C_{10} dialkyl derivatives of aromatic hydrocarbons and their retention data, and if possible, to determine the possibility of using retention data calculations for some aromatics during the analysis of C_{11} alkyl aromatic hydrocarbons formed in *n*-undecane dehydrogenation.

THEORETICAL

The linear function of free energy was successfully applied in the investigation of relationships between the equilibrium or rate constants of similar chemical reactions and the structure of the reacting substances[7].

KARGER *et al.*[8,9,14] used gas chromatography to study the linear correlation of free energy with the structure of the substances separated. If the standard state for the vapour phase is an ideal gas and an infinitely dilute solution that for the liquid phase, then a relationship exists between the standard molar mixing free energy, during the change of structure from "s" into "x", and the separation factor α, *viz.*

$$\Delta(\Delta\bar{G}_s{}^0) = \Delta(\bar{G}_s{}^0)_x - \Delta(\bar{G}_s{}^0)_s = -RT \ln \alpha \tag{1}$$

The separation factor in gas chromatography can be expressed as:

$$\alpha = \frac{K_x}{K_s} = \frac{\gamma_s{}^\infty \cdot P_s{}^0}{\gamma_x{}^\infty \cdot P_x{}^0} = \frac{t'_{Rx}}{t'_{Rs}} \tag{2}$$

where K_s and K_x are the thermodynamic distribution coefficients, $\gamma_s{}^\infty$ and $\gamma_x{}^\infty$ the activity coefficients; $P_s{}^0$ and $P_x{}^0$ the saturation vapour pressures at the column temperature; and t'_{Rs} and t'_{Rx} are the retention times corrected for the retention time of the unabsorbed component[8,9,14]. The variation of the standard molar free energy of mixing can be expressed as:

$$\Delta\bar{G}_s{}^0 = \Delta\bar{G}_i{}^0 + \Delta\bar{G}_e{}^0 = RT \ln P^0 + RT \ln \gamma^\infty \tag{3}$$

where $\Delta\bar{G}_i{}^0$ is the standard free energy of the ideal solution; $\Delta\bar{G}_e{}^0$ is the excess partial mixing free energy expressed as[4,8,9,14]:

$$\Delta\bar{G}_e{}^0 = RT \ln \gamma^\infty \tag{4}$$

The variation of the standard excess molar energy of mixing can, for the change of structure of the substance from "s" to "x", be expressed by the equation[8]:

$$\Delta(\Delta\bar{G}_e{}^0) = (\Delta\bar{G}_e{}^0)_x - (\Delta\bar{G}_e{}^0)_s = -RT \ln\left(\frac{\gamma_s{}^\infty}{\gamma_x{}^\infty}\right) \tag{5}$$

Considering the mixing process in several phases (I, II ... N), the ratio of the variations of the standard excess molar energies for the transformation of the substance having structure "s" into structure "x" can be expressed as:

$$C_{\mathrm{I}} \ln\left(\frac{\gamma_s{}^\infty}{\gamma_x{}^\infty}\right)_{\mathrm{I}} = C_2 \ln\left(\frac{\gamma_s{}^\infty}{\gamma_x{}^\infty}\right)_{\mathrm{II}} = \ldots = C_N \ln\left(\frac{\gamma_s{}^\infty}{\gamma_x{}^\infty}\right)_N \tag{6}$$

Using the same method as Hammett for the derivation of the equation[7,10], we found that the equation for the linear correlation of standard molar free energies in gas chromatography is:

$$\log \left(\frac{\gamma_s^\infty}{\gamma_x^\infty}\right)_N = \varrho_N \cdot \sigma_C^* \tag{7}$$

where "s" belongs to the original substance and "x" to the derivative; ϱ_N is the constant characterizing the liquid phase; σ_C^* is the chromatographic substitution constant.

By means of regression analysis KARGER *et al.*[9] transformed the original σ values of Hammett into σ_C values. Correlating the logarithm of the ratio of activity coefficients of phenol derivatives and the substitution constants, he expressed eqn. 7 in the form:

$$\log \left(\frac{\gamma_s^\infty}{\gamma_x^\infty}\right)_N = \varrho_N \cdot \sigma_C + b_1 \tag{8}$$

where the term b_1 is a constant independent of the electron shifts in the molecule[9].

Eqn. 8 is only valid for the separation of phenols on polyester phases because the variation of the excess energy expresses, in the main, only the interactions between the liquid phase and the separated phenols (the formation of hydrogen bonds). On a non-polar phase (SE-30), which is only a weak proton acceptor, the values for σ_C are unsuitable and eqn. 8 is not valid. (The correlation coefficient in this case was $r = 0.61$ (ref. 9).) In the case where substituted anilines are separated by chromatography on polyester phases the correlation coefficient for eqn. 8 was also found to be insufficient (a value of $r = 0.89$ was found).

Since the experimental results showed that the correlation coefficient, according to eqn. 8, gives low values for the alkyl aromatics, a new suitable equation resulting from the variation of excess free energies was proposed.

EXPERIMENTAL

The open tubular columns (Chrom-3, Laboratorní přístroje N.E. Prague) were wetted by a dynamic method[11]. The samples were injected with a 1 μl Hamilton microsyringe. A mixture of C_6 to C_{10} aromatic hydrocarbons, separated from reformates and made up with standards prepared in the Research Institute of Petroleum and Hydrocarbon Gases, Bratislava, was used for the analysis. The retention times and the Kováts' retention indices were corrected for the retention time of air (Table I). The values of the vapour pressures were calculated by the Antoine equation (see

TABLE I

EXPERIMENTAL CONDITIONS

	Polyethylene glycol 400	*Squalane*[1]	*Squalane*[2]
Instrument	Perkin-Elmer F 11	Perkin-Elmer F 11	Chrom-3
Detector	Flame ionization	Flame ionization	Flame ionization
Column dimensions	45 m/0.2 mm	45 m/0.2 mm	200 m/0.2 mm
Column temperatures	60, 72, 82 °C	86, 92 °C	86; 115 °C
Carrier gas; inlet pressure	Nitrogen; 0.9–1.3 kp/cm²	Nitrogen; 1.3 kp/cm²	Nitrogen; 3–4 kp/cm²
Sample size; split	0.1–0.3 μl; 1/100	0.1–0.3 μl; 1/100	0.5 μl; 1/300
Column efficiency	n = 118000 TP; N = 93000 EP at k = 7.8 and t = 72 °C	n = 105000 TP; N = 80000 EP at k = 7.0 and t = 92 °C	n = 250000 TP; N = 190000 EP at k = 6.6 and t = 115 °C

ห้องสมุด กรมวิทยาศาสตร์

TABLE II

PEG = polyethylene glycol, SQ = squalane, t'_R = corrected retention time, I = Kováts' index.

Compound	PEG						SQ		$\log \gamma_t^\infty/\gamma_x^\infty$			
	60 °C		72 °C		82 °C		92 °C		60 °C	72 °C	82 °C	92 °C
	t'_R	I	t'_R	I	t'_R	I	t'_R	I				
Toluene	21.1	1061	19.9	1073	15	1079	35.0	758	—	—	—	—
1,2-Dimethylbenzene	55.0	1194	48.3	1208	35.2	1217	93.7	883	—	—	—	−0.04246
1,3-Dimethylbenzene	40.8	1153	36.1	1164	26.5	1172	80.0	863	−0.1629	−0.1664	−0.1966	−0.03808
1,4-Dimethylbenzene	39.0	1147	34.9	1158	25.9	1167	78.9	861	−0.1647	−0.1651	−0.1547	−0.03110
1-Methyl-2-ethylbenzene	89.8	1263	75.9	1267	54.8	1285	175.0	964	—	—	—	−0.06445
1-Methyl-3-ethylbenzene	70.9	1230	60.2	1240	43.4	1249	155.0	948	−0.2786	−0.2824	−0.2698	−0.06335
1-Methyl-4-ethylbenzene	69.8	1228	59.7	1229	43.2	1249	157.4	950	−0.2897	−0.2919	−0.2783	−0.06214
1-Methyl-2-*n*-propylbenzene	142.3	1329	118.1	1342	84.3	1353	324.8	1045	—	—	—	−0.09438
1-Methyl-3-*n*-propylbenzene	115.3	1299	95.6	1311	68.2	1320	296.0	1033	−0.4398	−0.4298	−0.4036	−0.09231
1-Methyl-4-*n*-propylbenzene	117.5	1300	97.0	1312	69.5	1323	309.0	1039	−0.4428	−0.4354	−0.4076	−0.08670
1-Methyl-2-isopropylbenzene	115.3	1299	95.6	1313	68.2	1320	260.8	1015	—	—	—	−0.07548
1-Methyl-3-isopropylbenzene	93.7	1268	78.0	1278	56.3	1289	234.0	1002	−0.3863	−0.3849	−0.3617	−0.07716
1-Methyl-4-isopropylbenzene	92.2	1270	76.8	1280	56.3	1290	248.0	1010	−0.4221	−0.4190	−0.3513	−0.07810

Table IV)[12]. The calculations were done on a digital Celatron Serie 2c computer (G.D.R.).

RESULTS AND DISCUSSION

As, in our experiments, we have used small amounts of sample and relatively low pressures, we can presume that the experimental conditions coincide with the standard states.

Twelve derivatives of methyl monoalkyl benzenes were used in the investigation of the influence of the alkyl substituent upon the thermodynamic properties of the aromatic hydrocarbons. The effect of the alkyl substituent on the properties was compared with regard to toluene, which after preliminary experiments was shown to be a more suitable standard than benzene.

The squalane phase used for the separation of the alkyl aromatics is less selective than polyethylene glycol (Table III), but it permits the study of *ortho*-derivatives which when using a polyethyleneglycol phase exhibited decided *ortho*-effects.

Correlation of the activity coefficients ratio and substituent constants

The logarithms of the relative activity coefficients of toluene and its monoalkyl derivatives (Table II) were calculated from the corrected retention times and the

TABLE III

s = standard deviation; r = correlation coefficient, n = number of components.

Column	*Temp. (°C)*	*Equation*	s	r	n
Squalane	92	$\log \gamma_t^\infty/\gamma_x^\infty = 0.3650 \cdot \sigma_c - 0.03390$	$8.30 \cdot 10^{-3}$	0.560	5
Polyethylene glycol 400	60	$\log \gamma_t^\infty/\gamma_x^\infty = 2.0245 \cdot \sigma_c - 0.1504$	$3.08 \cdot 10^{-2}$	0.936	5
	72	$\log \gamma_t^\infty/\gamma_x^\infty = 1.9913 \cdot \sigma_c - 0.1533$	$3.05 \cdot 10^{-2}$	0.964	5
	82	$\log \gamma_t^\infty/\gamma_x^\infty = 1.6284 \cdot \sigma_c - 0.1561$	$3.12 \cdot 10^{-2}$	0.946	5

vapor pressures calculated from eqn. 2. Table III gives the straight line equations, the correlation coefficients and the standard deviations. The straight line equations were found by the method of least squares, from eqn. 8, using the σ_C values obtained by KARGER and co-workers[9]. Because of the different types of interaction the values of σ_C obtained were not suitable in the case of aromatics separated on a squalane phase. The correlation coefficients obtained on a polyethylene glycol phase are more suitable. This can be partially explained by the formation of a donor–acceptor hydrogen bond (the formation of π-complex) (Table III).

On the basis of the experimental results we found eqn. 9 for the correlation of C_6 to C_{10} alkyl aromatics

$$\log \frac{\gamma_t^\infty}{\gamma_x^\infty} = \varrho_N \cdot \tau_T + b_2 \qquad (9)$$

which expresses the relationship between the alkyl aromatic structures and their excess free energy. The original value of σ_C was substituted by $\tau_T = \log (T_t/T_x)$; τ_T expresses the variations in the electron configuration of methyl-alkyl aromatics (x) according to the reference standard toluene (t); T is the boiling point in °K.

TABLE IV

σ_C = chromatographic substituent constant, $\tau_T = \log T_t/T_x$.

Compound	*Boiling point (°K)*	*Vapor pressures (mm Hg)*				σ_C	τ_T
		60 °C	*72 °C*	*82 °C*	*92 °C*		
Toluene	383.79	138.94	219.26	311.82	433.72	—	—
1,2-Dimethylbenzene	417.57	40.828	68.732	102.72	149.58	—	−0.03660
1,3-Dimethylbenzene	412.26	49.380	82.372	112.25	176.46	+0.01	−0.03111
1,4-Dimethylbenzene	411.51	51.445	85.472	126.458	182.395	−0.01	−0.03027
1-Methyl-2-ethylbenzene	438.31	18.838	32.992	50.820	76.088	—	−0.05767
1-Methyl-3-ethylbenzene	434.47	21.770	37.819	57.907	86.230	−0.05	−0.05389
1-Methyl-4-ethylbenzene	435.15	21.522	37.309	57.046	84.854	−0.09	−0.05454
1-Methyl-2-*n*-propylbenzene	457.96	8.264	15.266	24.470	37.985	—	−0.07672
1-Methyl-3-*n*-propylbenzene	454.96	9.235	16.963	27.075	41.867	—	−0.07387
1-Methyl-4-*n*-propylbenzene	456.46	9.000	16.503	26.308	40.635	—	−0.07530
1-Methyl-2-isopropylbenzene	451.31	11.414	20.609	32.466	49.593	—	−0.07037
1-Methyl-3-isopropylbenzene	448.30	12.854	23.051	36.125	54.922	−0.11	−0.06747
1-Methyl-4-isopropylbenzene	450.26	12.031	21.642	33.995	51.786	−0.13	−0.06936

The straight line equations, the standard deviations s and the correlation coefficients r, calculated using eqn. 9, are given in Table V. Comparison of Tables III and V shows that by using the values of τ_T instead of σ_C more exact correlations are obtained.

TABLE V

Column	*Temperature (°C)*	*Equation*	*s*	*r*	*n*
Squalane	92	$\log \gamma_t^\infty/\gamma_x^\infty = 0.9853 \cdot \tau_T + 0.002787$	$3.73 \cdot 10^{-3}$	0.9853	12
Polyethylene glycol 400	60	$\log \gamma_t^\infty/\gamma_x^\infty = 6.4180 \cdot \tau_T + 0.04230$	$1.63 \cdot 10^{-2}$	0.9917	8
	72	$\log \gamma_t^\infty/\gamma_x^\infty = 6.2090 \cdot \tau_T + 0.03191$	$1.39 \cdot 10^{-2}$	0.9940	8
	82	$\log \gamma_t^\infty/\gamma_x^\infty = 5.1326 \cdot \tau_T - 0.01055$	$1.75 \cdot 10^{-2}$	0.9854	8

The linear correlation of free energies and the structure of aromatic hydrocarbons, as given by eqns. 7–9, calls for very exact and reproducible retention times and correct values of vapor pressures at several temperatures[9,14].

Correlation of Kováts' indices on two phases

The following relationship[13] exists between the logarithm of the ratio of retention times and the difference of the Kováts' indices:

$$\log \frac{t'_{Rx}}{t'_{Rs}} = \frac{(I_x - I_s)}{100} \log \frac{t'_{Rn+1}}{t'_{Rn}} \tag{10}$$

where "s" is the standard substance and "x" the substance having the structure x; t'_{Rn+1}/t'_{Rn} is the separation factor of the n-alkanes on the phases considered.

From eqns. 1 and 2 for the variation of the standard free mixing energy caused by a structural change, it follows that:

$$\Delta(\Delta \bar{G}_s{}^0) = RT \ln \frac{t'_{Rx}}{t'_{Rs}} \tag{11}$$

The comparison of eqns. 10 and 11 with respect to eqn. 6 results, for the chromatographic process on two different phases, in eqn. 12

$$C_1 \cdot \Delta I_1 \cdot \log \alpha_1 = C_2 \cdot \Delta I_2 \cdot \log \alpha_2 = \ldots = C_N \cdot \Delta I_N \cdot \log \alpha_N \quad (12)$$

where $C_1 \ldots C_N$ are constants; the numbers 1 ... N define the liquid phases; $\alpha_1 \ldots \alpha_N$ are the separation factors of the n-alkanes; and $\Delta I = I_x - I_s$.

In the case where one of the phases is the standard phase and the separation factor for the n-alkane is in a certain section of the homologous series constant for this phase then eqn. 13 will be valid:

$$\Delta I_N = \varrho' \cdot \Delta I_1 \quad (13)$$

where ϱ' is slope characterizing the phase. From eqn. 12 follows that:

$$\varrho' = \frac{C_1 \cdot \log \alpha_1}{C_N \cdot \log \alpha_N} \quad (14)$$

After studying the differences of the Kováts' indices for toluene and monosubstituted alkyl toluenes on squalane and polyethylene glycol phases we found that eqn. 13 has to be corrected into the form:

$$(I_x - I_t)_{\text{PEG 400}} = \varrho'(I_x - I_t)_{\text{SQ}} + b_3 \quad (15)$$

The values for the alkyl derivatives of toluene, calculated by the least squares method from eqn. 15, are given in Table VI.

TABLE VI

Column	*Temp. (°C)*	*Equation*	*s*	*r*	*n*
Squalane	92	—	—	—	—
Polyethylene glycol 400	60	$(\Delta I)_{\text{PEG}} = 0.8434 \cdot (\Delta I)_{\text{SQ}} + 2.77$	4.25	0.998	8
	72	$(\Delta I)_{\text{PEG}} = 0.8455 \cdot (\Delta I)_{\text{SQ}} + 1.21$	4.50	0.997	8
	82	$(\Delta I)_{\text{PEG}} = 0.8558 \cdot (\Delta I)_{\text{SQ}} + 2.72$	4.19	0.998	8

As the variations of the retention indices with temperature on a polyethylene glycol phase are substantially higher than on a squalane phase[5,6] one can assume that a more exact control of thermostat temperature decreases the standard deviation s (Table VI). This problem will be the subject of our further work.

Correlation of the Kováts' indices and the substituent constants

The relationship existing between the Kováts' indices and the substituent constant τ_T was found by the least squares method to be given by

$$\log \frac{I_t}{I_x} = \varrho \cdot \tau_T + b_4 \quad (16)$$

The equations, the correlation coefficients and the standard deviations for monoalkyl substituted toluenes, calculated from eqn. 16 are given in Table VII.

The deviations of the Kováts' indices determined for aromatic hydrocarbons are given in Table VIII. They were calculated from three measurements on a 200 m

TABLE VII

Column	*Temp. (°C)*	*Equation*	*s*	*r*	*n*
Squalane	92	$\log I_T/I_x = 1.8071 \cdot \tau_T - 5.89 \cdot 10^{-5}$	$5.4 \cdot 10^{-4}$	0.995	12
Polyethylene glycol 400	60	$\log I_T/I_x = 1.1779 \cdot \tau_T + 9.69 \cdot 10^{-4}$	$1.5 \cdot 10^{-3}$	0.997	8
	72	$\log I_T/I_x = 1.1702 \cdot \tau_T + 15.94 \cdot 10^{-4}$	$1.7 \cdot 10^{-3}$	0.995	8
	82	$\log I_T/I_x = 1.1886 \cdot \tau_T + 4.10 \cdot 10^{-4}$	$4.8 \cdot 10^{-3}$	0.978	8

squalane column at 115°. The validity of the relationship in accordance with eqn. 16 was verified by recalculating the boiling points and by comparison of the values found with those tabulated (Table VIII).

TABLE VIII

Compound	$I_{115}^{SQ} \pm s$	$T_{b.p.}$ (°K)	ΔT
Toluene	761.1 ± 0.4	383.79	—
1,2-Dimethylbenzene	888.4 ± 0.1	417.57	± 0.2
1,3-Dimethylbenzene	867.1 ± 0.5	412.26	± 0.1
1,4-Dimethylbenzene	865.5 ± 0.6	411.51	± 0.3
1-Methyl-2-ethylbenzene	969.3 ± 0.3	438.31	± 0.1
1-Methyl-3-ethylbenzene	952.7 ± 0.4	430.47	± 0.4
1-Methyl-4-ethylbenzene	955.6 ± 0.3	435.15	± 0.3
1-Methyl-2-*n*-propylbenzene	1051.0 ± 0.3	457.96	± 0.4
1-Methyl-3-*n*-propylbenzene	1037.0 ± 0.4	454.96	± 0.2
1-Methyl-4-*n*-propylbenzene	1044.0 ± 0.2	456.46	± 0.2
1-Methyl-2-isopropylbenzene	1024.7 ± 0.2	451.31	± 0.6
1-Methyl-3-isopropylbenzene	1006.4 ± 0.1	448.30	± 0.6
1-Methyl-4-isopropylbenzene	1015.0 ± 0.1	450.26	± 0.6

Eqn. 16 was applied practically for the identification of aromatic compounds formed in the dehydrogenation of *n*-undecane. The results found so far confirm the validity of the suggested relationship for C_{11}-alkyl aromatics as well.

ACKNOWLEDGEMENTS

The authors thank Dr. J. JANÁK and Dr. J. NOVÁK (both of the Institute of Instrumental Analytical Chemistry, Czechoslovak Academy of Science, Brno) for many valuable discussions on the manuscript.

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CHROM. 4837

VARIATION OF PERFORMANCE OF POROUS POLYMER BEAD COLUMNS IN GAS CHROMATOGRAPHY

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(Received May 19th, 1970)

SUMMARY

A study has been made of the variation of performance of different batches and types of porous polymer bead columns for the analysis of water–alcohol mixtures. The effect of preconditioning of the columns, column wall material, and sample size on quantitative analysis, retention time, peak asymmetry, efficiency and resolution are reported.

INTRODUCTION

Synthetic porous polymer beads have been in widespread use for several years and form a valuable addition to the wide selection of traditional column packings. Porous polymer bead columns are particularly useful for the analysis of aqueous samples and those containing other highly polar compounds, since satisfactory elution profiles may be obtained in contrast to the gross distortions often encountered using conventional stationary phases and supports. HOLLIS AND HAYES[1], in an account of the use of porous polymer beads for the analysis of a variety of aqueous samples, state that little if any loss of water by adsorption occurs on the column packing, although no detailed quantitative data are presented. Adsorption may however occur on the column walls and HOLLIS AND HAYES stress the need to minimise bare metal surfaces. In the present work the effect of different column wall materials on the quantitative analysis of aqueous samples is reported. The effect of sample size on retention time has recently been discussed[2,3] and some data are presented herein. The thermal stability of a column and its ability to maintain a constant level of performance during use are important criteria in the choice of packing material. The stability of porous polymer columns at 200°, after conditioning at 225°, expressed in terms of relative retention time, has been reported by PALFRAMAN AND WALKER[4]. The effect of preconditioning of columns, on quantitative analysis, retention time, peak asymmetry, efficiency and resolution are now reported. HOLLIS[5] has stated that

by proper control of the synthesis of porous polymer beads one could expect to obtain a polymer which would be reproducible from batch to batch, and which would give good retention reproducibility. The present authors have carried out such an investigation in which water–alcohol mixtures were repeatedly analysed on different types and batches of porous polymer bead.

EXPERIMENTAL

All analyses were carried out on a Pye 104 chromatograph fitted with a Gow-Mac Minigade 625 gas density detector. Previous work[6] has shown that although the response of the gas density detector is predictable under some operating conditions, it is advisable to calibrate the device. This was carried out using a mass detector[7], connected in series with the gas density detector.

All columns were 4 ft. × ¼ in. O.D. and were thoroughly cleaned but not silanised prior to use. Column packing was sieved to 80–100 BS mesh. Columns of stainless steel were prepared from three different batches of Porapak Q, one batch of Porapak Q-S (silanised by manufacturers), and one of Chromosorb 102. In addition one glass and one teflon column were packed with the same batch of Porapak Q as one of the metal columns, to compare the contribution of the column wall materials. Details of the columns are given in Table I. The maximum operating temperatures recommended by the manufacturers are 300° for Porapak Q and 250° for Chromosorb 102.

TABLE I

COLUMN DETAILS

Column No.	*Material*	*I.D. (mm)*	*Packing*	*Batch No. of packing*[a]	*Weight of packing (g)*
1	S/steel	5.0	Porapak Q	1	7.9
2	glass	3.0	Porapak Q	1	4.6
3	teflon	4.5	Porapak Q	1	6.4
4	S/steel	5.0	Porapak Q	2	8.0
5	S/steel	5.0	Porapak QS	3	7.7
6	S/steel	5.0	Chromosorb 102	4	7.7
7	S/steel	5.0	Porapak Q	5	8.8

[a] Authors' assignation.

All packing material was white and fairly free flowing prior to conditioning except Porapak Q batch 5 which was yellow and did not flow freely. Each column was filled by forcing the packing into the coiled tubing under a slowly increasing pressure (0–30 p.s.i.g.), accompanied by gentle vibration. Columns were conditioned overnight at 150° in a stream of nitrogen, the packing adjusted to the same height in each column, and the columns plugged with silanised glass yarn. The following experiments were carried out on each of the seven columns. With a 10 μl syringe, 1 μl samples of water–alcohol mixtures were injected. Each sample was injected in triplicate and the injection septum was renewed after every 6th injection. No sample was kept for more than a few hours, and the same syringe was used throughout the

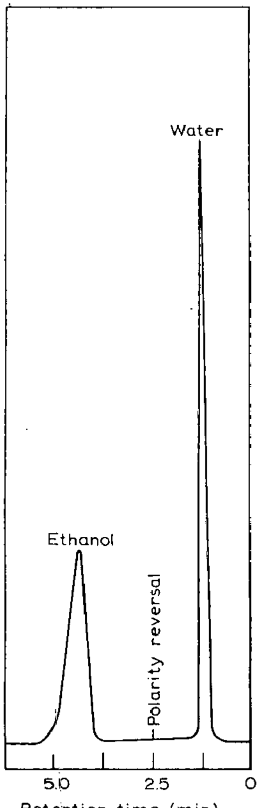


Fig. 1. Chromatogram of water–alcohol mixture.

TABLE II

OPERATING CONDITIONS

Apparatus:	Pye 104
Detector:	Minigade 625 gas density
Carrier gas:	Nitrogen
Carrier gas flow rate:	50 ml min^{-1}
Reference gas flow rate:	100 ml min^{-1}
Column temperature:	125°
Detector temperature:	130°
Detector filament current:	150 mA
Sample size:	1 μl

work. A chromatogram is shown in Fig. 1. Peak area measurements were made using a Kent Chromalog II integrator. The GC operating conditions are given in Table II.

RESULTS

From the chromatograms comparison between the various packings was made on the basis of the quantitative results, retention data, peak asymmetry, efficiency and resolution. The stability of the columns was studied by further conditioning followed

TABLE III

COMPARISON OF QUANTITATIVE RESULTS WITH VARIOUS COLUMNS AND COLUMN CONDITIONINGS

Conditioning		*% Water detected in sample ($\bar{x}$)*							*True % water*
Temp. (°C)	*Time (h)*	*1*[a]	*2*	*3*	*4*	*5*	*6*	*7*	*(x_0)*
150	16	36.17	35.75	35.33	35.91	36.60	35.33	31.17	35.73
220	12	37.22	36.13	36.62	37.12	36.56	37.00	37.79	36.47
275	12	36.23	36.38	36.64	36.11	36.32	36.52	39.10	36.50
275	336	33.42	32.83	—	33.85	34.30	32.80	26.15	33.33
22	336	33.50	35.88	—	33.79	32.49	30.08	34.49	33.38

[a] For key to column numbers, see Table I.

by a repetition of the experiments described above. A comparison of the quantitative results, expressed in terms of the percentage water detected, is given in Table III.

Due to the small differences in true percentage composition of the various samples, the effects of conditioning on the quantitative results are more readily compared in terms of the percentage bias of the results. Bias values are quoted in Table IV. The analyses were in reasonable agreement with the true values, using all columns except No. 7. There was no evident difference in adsorption losses between any of the packings or between the different column wall materials. Prolonged conditioning of the columns had no effect on the analytical results, although column No. 3 had to be discarded due to deterioration of the teflon. Preconditioning of column No. 4 with 500 μl of water had no effect on the quantitative results. Mean values are

TABLE IV

BIAS OF QUANTITATIVE RESULTS

Conditioning		*Percentage bias*[a]						
Temp. (°C)	*Time (h)*	*1*[b]	*2*	*3*	*4*	*5*	*6*	*7*
150	16	+1.23	+0.06	−1.12	+0.50	+2.43	−1.12	−12.76
220	12	+2.06	−0.93	+0.41	+1.78	+0.25	+1.45	+ 3.62
275	12	−0.74	−0.33	+0.38	−1.07	−0.49	+0.05	+ 7.12
275	336	+0.27	−1.50	—	+1.56	+2.91	−1.59	−21.54
22	336	+0.36	+7.30	—	+1.23	−2.67	−9.92	+ 3.33

[a] Percentage bias, defined as $[(\bar{x} - x_0)/x_0] \times 100$, where $\bar{x}$ = mean experimentally determined % composition, and x_0 = true % composition.
[b] For key to column numbers, see Table I.

TABLE V

EFFECT OF PRECONDITIONING WITH WATER

True % composition	*Experimentally determined % composition*	
	No pretreatment	*Water pretreatment*
36.50	36.11	36.28

given in Table V. Absolute and relative retention distances (ethanol–water) are given in Tables VI and VII respectively.

TABLE VI

ABSOLUTE RETENTION DISTANCES

Conditioning		*Retention distances of water and ethanol (mm)*						
Temp. (°C)	*Time (h)*	*1*[a]	*2*	*3*	*4*	*5*	*6*	*7*
150	16	13.5[b]	9.5	12.3	11.3	10.0	10.5	18.0
		56.7[c]	35.0	50.3	43.0	42.0	35.0	94.0
220	12	12.3	8.7	12.5	11.0	10.0	12.7	20.0
		54.7	32.7	54.7	44.7	41.7	38.5	93.0
275	12	12.2	8.0	12.0	10.8	10.0	11.7	18.0
		55.0	33.0	48.0	46.0	42.7	45.2	95.0
275	336	8.5	5.5	—	12.7	7.5	8.0	11.5
		40.0	26.0	—	59.0	33.5	35.0	63.0
22	336	8.0	6.0	—	11.0	8.0	8.5	11.5
		38.0	25.0	—	53.0	33.5	34.0	62.0

[a] For key to column numbers, see Table I.
[b] Retention distance of water.
[c] Retention distance of ethanol.

TABLE VII

RELATIVE RETENTION RATIOS

Conditioning		*Relative retention ethanol/water*						
Temp. (°C)	*Time (h)*	*1*[a]	*2*	*3*	*4*	*5*	*6*	*7*
150	16	4.20	3.68	4.09	3.81	4.20	3.33	5.22
220	12	4.45	3.76	4.38	4.06	4.17	3.03	4.65
275	12	4.51	4.13	4.00	4.26	4.27	3.86	5.28
275	336	4.71	4.72	—	4.64	4.47	4.37	5.48
22	336	4.75	4.17	—	4.82	4.18	4.00	5.38

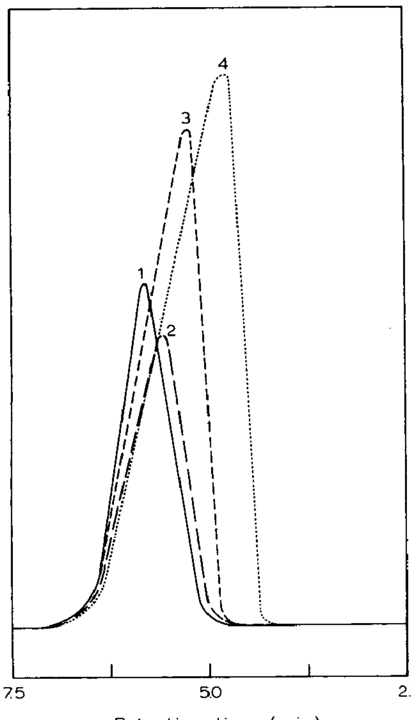
[a] For key to column numbers, see Table I.

Absolute retention distances varied with the extent of conditioning and changed significantly after prolonged treatment at 275°. In general relative retention ratios increased as conditioning progressed. Similar values for retention data were obtained for all columns except No. 7, for which the values were much greater. It has been reported in the literature[3] that retention distance (to the peak maximum) varies with sample size, and this is clearly demonstrated by the results given in Table VIII, which were obtained using column No. 1.

TABLE VIII

VARIATION OF RETENTION DISTANCE WITH SAMPLE SIZE

Compound	*Retention distances (mm)*			
	0.5 µl	*1.0 µl*	*2.0 µl*	*5.0 µl*
Water	14.0	13.5	13.5	12.2
Ethanol	57.8	56.7	54.2	49.2



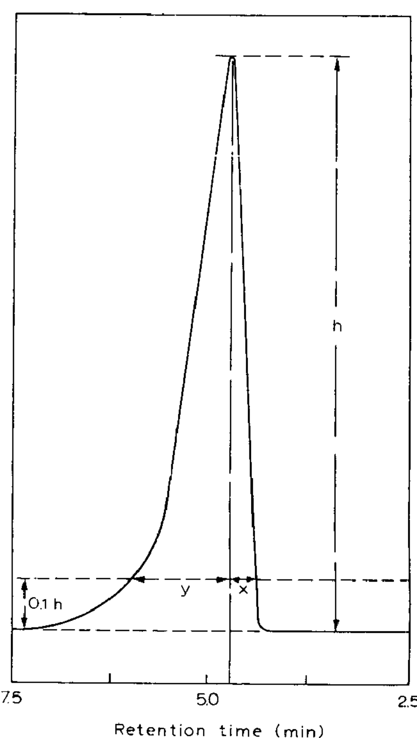


Fig. 2. Chromatogram showing effect of sample load on peak symmetry. 1 = 0.5 μl (attenuation ×2); 2 = 1.0 μl (attenuation ×5); 3 = 2.0 μl (attenuation ×5); 4 = 5.0 μl (attenuation ×10).

Fig. 3. Definition of peak asymmetry.

Fig. 2 shows four superimposed chromatograms obtained under identical conditions using sample sizes of 0.5–5.0 μl.

Mean values of the peak asymmetry (x/y values as defined in Fig. 3) of the alcohol peaks are given in Table IX. Comparison of the data shows that the most symmetrical peaks were obtained using Nos. 4 (unsilanised Porapak Q) and 5 (silanised Porapak Q). The remaining batches of Porapak Q and Chromosorb 102 gave somewhat

TABLE IX

PEAK ASYMMETRY

Conditioning		*Peak asymmetry*[a]						
Temp. (°C)	*Time (h)*	*1*[b]	*2*	*3*	*4*	*5*	*6*	*7*
150	16	0.49	0.44	0.40	0.67	0.75	0.50	0.47
220	12	0.48	0.50	0.33	0.62	0.64	0.42	0.53
275	12	0.50	0.39	0.24	0.71	0.73	0.50	0.60
275	336	0.49	0.54	—	0.56	0.80	0.55	—
22	336	0.55	0.59	—	0.62	0.73	0.56	0.53

[a] A symmetrical peak takes the value 1.00.
[b] For key to column numbers, see Table I.

poorer results. Peak asymmetry was not affected by thermal treatment except in the case of column No. 3, which was attributed to deterioration of the column walls. The results quoted in Table IX refer to a sample size of 1 μl. The changes in peak symmetry with sample size, for column No. 1, are given in Table X, and are illustrated in Fig. 2.

TABLE X

EFFECT OF SAMPLE SIZE ON PEAK ASYMMETRY

Sample size (μl)	0.5	1.0	2.0	5.0
Peak asymmetry	0.56	0.49	0.36	0.22

TABLE XI

COLUMN EFFICIENCY

Conditioning		*HETP (mm)*[a]						
Temp. (°C)	*Time (h)*	*1*[b]	*2*	*3*	*4*	*5*	*6*	*7*
150	16	2.85	3.62	3.65	2.56	1.33	2.73	3.19
220	12	3.27	3.42	3.15	3.01	1.54	1.79	6.33
275	12	4.09	4.34	4.02	3.43	2.56	5.97	14.2
275	336	7.50	5.77	—	3.54	3.54	7.22	26.2
22	336	5.24	4.77	—	6.72	5.35	5.42	16.4

[a] Using the expressions: $n = 5.545 \left(\frac{\text{retention distance}}{\text{peak width at } \frac{1}{2} \text{ height}}\right)^2$ and $\text{HETP} = \frac{\text{column length}}{n}$

[b] For key to column numbers, see Table I.

Column efficiencies were compared by calculating HETP values on the ethanol peaks. These are quoted in Table XI. Efficiencies are somewhat lower than those attainable using conventional coated supports. By far the most efficient column was No. 5 (silanised Porapak Q). Efficiency was not affected very much by conditioning up to 220° (except column No. 7), but deteriorated rapidly on conditioning at 275°. The efficiency of column No. 7 was particularly poor.

Values for the resolution[8] of the water and alcohol peaks are given in Table XII. Column No. 5 (silanised Porapak Q) gave by far the most satisfactory performance. Some deterioration in resolving power after conditioning occurred with all the columns.

TABLE XII

PEAK RESOLUTION

Conditioning		*Peak resolution*						
Temp. (°C)	*Time (h)*	*1*[a]	*2*	*3*	*4*	*5*	*6*	*7*
150	16	4.9	4.6	4.9	4.9	6.6	4.3	4.4
220	12	4.9	5.1	4.7	4.5	5.6	5.2	4.0
275	12	5.0	3.8	4.1	4.4	5.7	3.6	3.1
275	336	4.0	3.7	—	5.6	4.2	3.7	2.3
22	336	4.2	4.2	—	4.2	3.9	3.8	3.0

[a] For key to column numbers, see Table I.

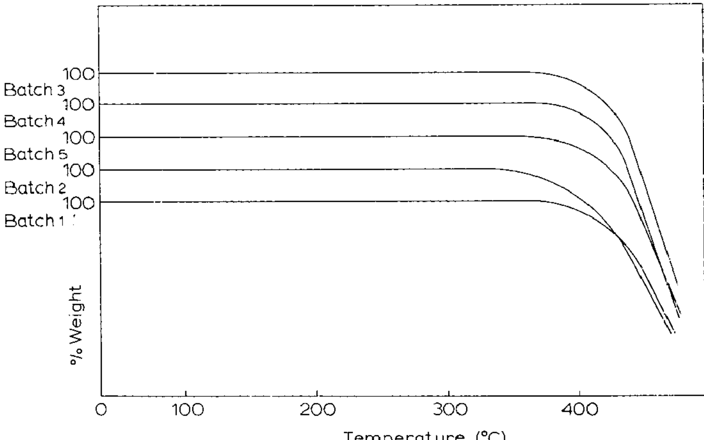


Fig. 4. Thermograms of unused porous polymer beads.

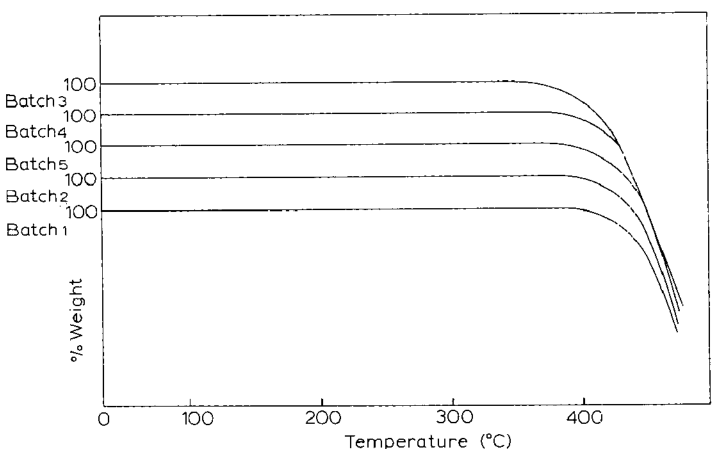


Fig. 5. Thermograms of used porous polymer beads.

TABLE XIII

WEIGHT LOSSES OF POROUS POLYMER BEADS AT 425°

Batch No.	*Percentage weight loss*	
	Before use	*After use*
1	15.6	5.9
2	26.6	6.7
3	10.9	16.4
4	12.2	8.7
5	11.8	6.9

The column packings were examined after completion of the experiments described above. All were found to be off-white except batch 5 which was light brown. Some coagulation of the beads had occurred. 10-mg samples of the beads, taken before and at the end of the experiments, were subjected to thermogravimetric analysis under nitrogen, covering the temperature range 50–475°. Similar thermograms (see Figs. 4 and 5) were obtained in all cases except that of batch 5 beads prior to treatment, which showed weight loss beginning at 250°. Percentage weight losses at 425° for all the samples are given in Table XIII.

CONCLUSIONS

The quantitative analysis of water–alcohol mixtures was found to be satisfactory using all but one of several different batches and types of porous polymer beads. Column wall material had no effect on the analyses. Retention times changed with the extent of column conditioning, and varied with sample size. Peak asymmetry was not affected by column conditioning, but varied with the different column packings and sample size. Column efficiencies and resolution varied with the packing material and deteriorated after prolonged conditioning. One particular batch of Porapak Q gave a very much poorer overall performance than any of the other packings. By far the most satisfactory performance was achieved using Porapak Q-S.

ACKNOWLEDGEMENTS

The authors acknowledge the contribution of Dr. B. FLAHERTY and Mr. D. J. HEYWOOD who carried out the thermogravimetric analyses. The Government Chemist is thanked for granting permission for this paper to be published.

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CHROM. 4854

DETERMINATION OF FORMIC ACID IN DILUTE AQUEOUS SOLUTION AND SEPARATION AND DETERMINATION OF ANILINE AND TOLUIDINE ISOMERS BY GAS CHROMATOGRAPHY OF FORMANILIDE AND FORMYL TOLUIDIDES

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(Received April 23rd, 1970)

SUMMARY

The determination of formic acid in various aqueous conditions (as free acid and as a salt in the presence of mineral or other carboxylic acids) by the gas chromatography of the formyl derivatives of aniline and *o*-, *m*- and *p*-toluidine is described. The analysis for these four amines and N-methyl aniline and N-methyl *p*-toluidine in aqueous and non-aqueous mixtures by the gas chromatography of either their formyl or their unsubstituted acetyl derivatives is also described. Columns of sodium dodecylbenzenesulphonate coated on Chromosorb G are used for the chromatography in both cases.

INTRODUCTION

Electro-organic synthesis has received a great deal of interest in recent years. One of the major problems associated with this type of study is the analysis for the organic compounds in aqueous media in the presence of supporting electrolytes which may be mineral acids and salts, or organic quarternary bases and salts. The present work deals with two examples of this situation:

(a) The quantitative determination of formic acid (present as free acid or as an alkali metal or quarternary ammonium salt) in dilute aqueous solution and in the presence of mineral and other low-molecular-weight (C_2–C_8) carboxylic acids without prior separation, by the formation and gas chromatography of the formyl derivative of any one of the following compounds: aniline and *o*-, *m*- and *p*-toluidine.

(b) The quantitative determination of aniline, *o*-, *m*- and *p*-toluidine, N-methyl aniline and N-methyl *p*-toluidine in aqueous and non-aqueous media by the gas chromatography of their formyl and acetyl derivatives.

The determination of formic acid by gas chromatography has largely involved the direct injection of the free acid, often in a mixture with other low-molecular-weight aliphatic carboxylic acids, into the instrument. Special stationary phase

combinations are formulated in order to avoid among other things the decomposition of formic acid[1-3]. Formic acid in cigarette smoke has been analysed by the gas chromatography of its methyl ester on 20% Carbowax 20 M coated on 60/80 mesh Chromosorb P[4]. The formyl derivatives of each of the amines mentioned above are stable, non-volatile and easy to prepare in all the possible aqueous conditions where formic acid or formate may be present.

Toluidines and certain other aromatic amines have been directly chromatographed on stationary phases coated on base-loaded supports[5-7]. However, the use of the derivatives rather than the free compounds often improves the quantitative gas chromatographic separations of most of these amines. The behaviour of the common derivatives on some selected stationary phases has been studied[8]. DOVE[9] described a method for the separation and determination of aniline and toluidine and other related amines via the gas chromatography of their trifluoroacetyl derivatives on a stationary phase mixture of 9.5% (w/w) Apiezon L and 3.5% (w/w) Carbowax 20 M coated on 80/100 mesh Aeropak 30. In the present work the formyl and unsubstituted acetyl derivatives are shown to facilitate the separation and determination of aniline and toluidine isomers on a single stationary phase coated on non-base-loaded Chromosorb G. The acetyl derivatives are also shown to be separable to a certain extent on the same stationary phase coated on the same support 1% (w/w) base-loaded with potassium hydroxide.

EXPERIMENTAL

A Perkin Elmer Model F 11 gas chromatograph equipped with dual flame ionisation detector was used for the analysis, and nitrogen served as the carrier gas.

Column preparation

2.5 g of sodium dodecylbenzenesulphonate (DBS) were dissolved in water–methanol (1:1) and the solution was added to 97.5 g of NAW 60/80 mesh Chromosorb G. After mixing by stirring the solvent was evaporated in the oven at 100° with occasional stirring until the solid appeared dry. It was cooled and packed by suction into 6 ft. (A) and 12 ft. (B) by 3 mm I.D. glass columns.

Column C. 1 g of potassium hydroxide was dissolved in 50 ml of methanol and added with more methanol to 99 g of NAW Chromosorb G. After mixing by stirring the solvent was removed. Then 97.5 g of the dry solid were added to 2.5 g of DBS dissolved in water–methanol, mixed thoroughly and the solvent removed as above in the oven. The solid was cooled and packed in a 6 ft. × 3 mm I.D. glass column (C).

The three packed columns A, B and C were conditioned at 220° for 18 h.

Instrument analytical conditions

The instrument conditions and the columns employed for the various separations and determinations are listed in Table I.

Materials

The chemicals were used as obtained from BDH without further purification. The formyl and acetyl derivatives of the anilines and toluidines were prepared as described by OPENSHAW[10] for retention time determinations. Dilute aqueous solutions

TABLE I

INSTRUMENTAL CONDITIONS FOR THE ANALYTICAL SEPARATIONS

Columns: (A) 6 ft. × 3 mm I.D. glass column packed with 2.5% (w/w) sodium dodecylbenzene-sulphonate (DBS) on NAW 60/80 mesh Chromosorb G; (B) the 12 ft. × 3 mm I.D. version of column A; (C) 6 ft. × 3 mm I.D. glass column packed with 2.5% (w/w) DBS on NAW 60/80 mesh 1% (w/w) base-loaded Chromosorb G.
The oxygen and hydrogen inlet pressures and the injection port temperature were the same for all the separations, *viz.* 26/18 lb./in.^{2} and 250°, respectively.

Chromatography of	*Instrumental conditions*
Formanilide (or any formyl derivative of any of the toluidines) for formic acid determination using methyl stearate as internal standard	Columm A; oven temperature, 200°; nitrogen inlet pressure, 10 lb./in.^{2}
Formyl derivatives of aniline, *o*-, *m*- and *p*-toluidine, N-methyl aniline, and N-methyl *p*-toluidine using methyl stearate as internal standard	Column B; oven temperature, 210°; nitrogen inlet pressure, 15 lb./in.^{2}
Acetyl derivatives of *o*-, *m*- and *p*-toluidine, N-methyl aniline, and N-methyl *p*-toluidine	Column B; oven temperature, 200°; nitrogen inlet pressure, 12 lb./in.^{2}

of formic acid were standardised by direct titration with standard sodium hydroxide solution using phenolphthalein as indicator. Standard formate solutions were made by treating measured aliquots of the standardised formic acid solution with little excess of sodium or tetramethyl- (or ethyl-) ammonium hydroxide in a volumetric flask and making up to the mark with distilled water, keeping the pH at 8–10.

PROCEDURE

Preparation of formic acid derivatives and chromatography

(a) Free formic acid only. 2 ml of 0.1 *M* aqueous solution of formic acid were pipetted into a 50-ml stoppered flask. 1 ml of 1 *M* hydrochloric acid, 0.2 g of amine and one small glass bead were added. The mixture was boiled for 10 min under reflux over a hot plate and cooled for 5 min in an ice bath. 1 ml of 2 *M* sodium bicarbonate solution was added and the mixture shaken. 5 ml of ethyl acetate containing 0.2% (w/w) methyl stearate were added and the flask stoppered and shaken vigorously for 1 min. After the two layers separated 2 µl of the ethyl acetate layer were taken with a hypodermic syringe and injected into the instrument (column A).

(b) Formic acid present as sodium/potassium or tetraalkylammonium formate. 2 ml of 0.5 *M* formate solution were placed into the stoppered flask. Drops of 1 *M* hydrochloric acid were added (*ca.* 1 ml) to bring the pH to 1. 1 g of amine and a glass bead was added and the mixture boiled under reflux for 10 min, cooled and treated with 1 ml of 2 *M* sodium bicarbonate solution and extracted with 5 ml ethyl acetate containing 0.2% (w/w) methyl stearate and injected into the instrument as in (a).

(c) Formic acid in the presence of mineral acids and/or other straight-chain C_2–C_8 carboxylic acids. 2 ml methanolic solution 1 *M* in each of formic and the carboxylic acids, and 1 ml of 2 *M* sulphuric acid were taken in the conical flask. Solid sodium bicarbonate was added in excess keeping the pH at 8–9. The pH of the solution was then brought down to 1 with drops of hydrochloric acid solution. 2 g of

amine were added and the mixture boiled under reflux, cooled and extracted as above (but using methyl myristate as internal standard) and injected into the instrument (column A or C).

Preparation of derivatives of aniline, o-, m- and p-toluidine, N-methyl aniline and N-methyl p-toluidine in a mixture and chromatography

(a) As formyl derivatives. 1 ml of the methanolic solution 0.5 *M* in each of the above amines was taken in the conical flask. 0.3 g of formic acid and 4 ml of water were added. The mixture was boiled under reflux for 10 min, cooled in an ice bath and shaken with 4 ml of 2 *M* sodium bicarbonate solution, and then extracted with 10 ml of ethyl acetate containing 0.2% (w/w) methyl stearate. The ethyl acetate layer was then injected into the instrument (column B) (Fig. 6).

(b) As acetyl derivatives. This method applies only to the toluidines and N-methyl aniline since *o*-toluidine and aniline derivatives were not separated on the available column. To a 1-ml aliquot of the methanolic solution 0.5 *M* in each of the amines 0.3 g of acetic anhydride was added in a 25-ml tube and the tube was placed in a hot water bath (75°) for 10 min and then cooled in an ice bath. 10 ml of ethyl acetate and 5 ml of 2 *M* sodium bicarbonate solution were added and the tube was shaken vigorously for 1 min. The ethyl acetate layer was injected into the instrument (columns B and C) (Figs. 7a and b).

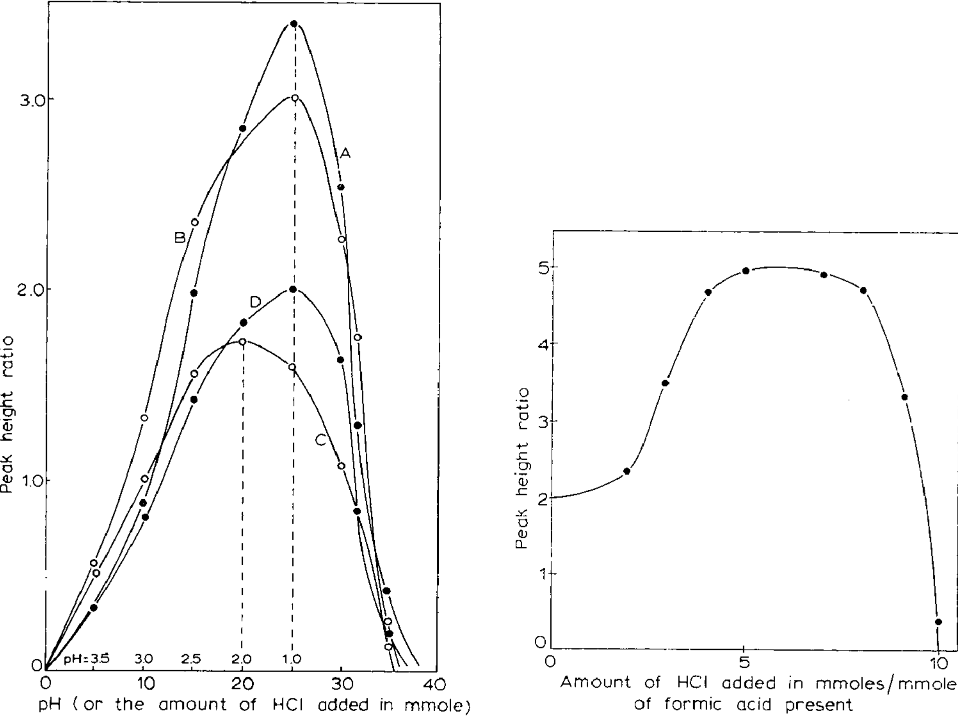

Fig. 1. Effect of the pH on the amount of the formyl derivatives of aniline and *o*-, *m*- and *p*-toluidine formed. (A) = *o*-toluidine; (B) = *m*-toluidine; (C) = *p*-toluidine; (D) = aniline.

Fig. 2. Effect of the amount of hydrochloric acid added to fixed amounts of formic acid and aniline on the amount of formanilide formed.

In the absence of N-methyl aniline, methyl myristate was used as the internal standard.

RESULTS AND DISCUSSION

Formic acid

The quantitative formation of the formyl derivative of each of the amines for formic acid determination was found to be dependent on three major factors, namely, the pH of the solution when dealing with a formate solution, the formic acid/hydrochloric acid molar ratio when dealing with free formic acid solution, and the amine/formic acid molar ratio.

The amount of derivative formed from a given quantity of a formate solution varied with the pH (Fig. 1), the maximum amount being formed at pH 1. A given quantity of free formic acid solution gave some derivative when reacted with any of the amines, but the amount of derivative formed with a fixed quantity of amine greatly increased with the addition of hydrochloric acid. Since free formic acid aqueous solution was found to exert a large buffer action when increasing amounts of hydrochloric acid were added to it, the effect of the presence of mineral acid on the amount of derivative formed from fixed quantities of formic acid and amine was investigated in terms of formic acid/hydrochloric acid molar ratio. The maximum amount of derivative was formed when the molar ratio of the two acids was 1:5 (Fig. 2). No significant loss in sensitivity and precision was observed when 4–8 mmoles hydrochloric acid per mmole of formic acid were used in derivative preparation.

At the correct pH or formic acid/hydrochloric acid molar ratio, the amount of derivative formed from a given amount of formate or formic acid also varied with amine/formic acid molar ratio (Fig. 3). The use of 6–10 mmoles of amine for each mmole of formic acid present proved quantitatively satisfactory.

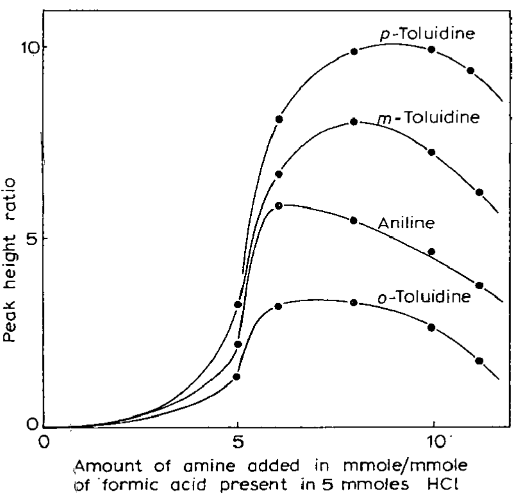

Fig. 3. Effect of the amine/formic acid molar ratio on the amount of derivative formed.

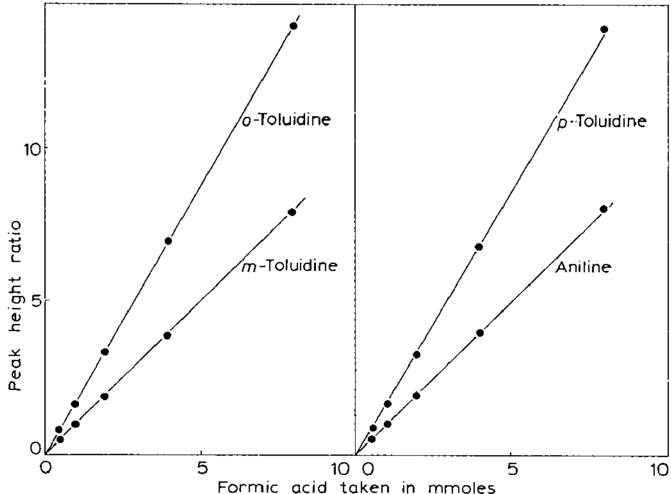

Fig. 4. Calibration curve for the formyl derivatives of aniline and *o*-, *m*-, and *p*-toluidine.

The calibration curves obtained (Fig. 4) by taking increasing aliquots of aqueous formic acid and proportionate amounts of hydrochloric acid and the amines indicated a linear relationship between the amounts of derivatives present and the formic acid taken. Up to 0.1 mg of formic acid may be determined by this method using any of the amines.

Amines

In the determination of the amines by formylation the formic acid/amine molar ratio has also been shown to affect the amount of derivative formed (Fig. 5). A reasonable excess of aqueous formic acid was necessary and the use of 2–6 mmoles of formic acid per mmole of amine present was found satisfactory.

The formylation method is useful when working in an aqueous medium and

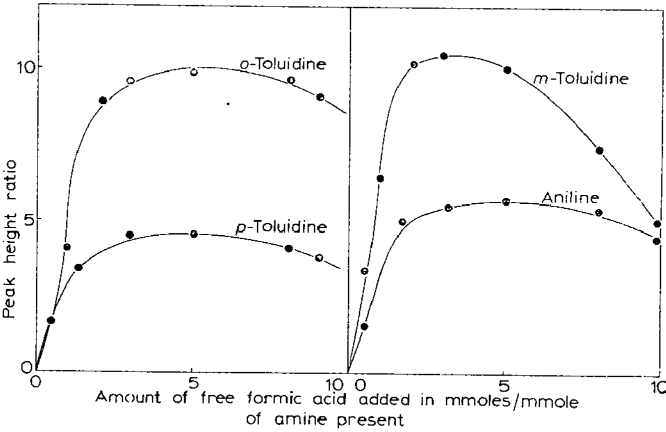

Fig. 5. Effect of the formic acid/amine molar ratio on the amount of derivative formed.

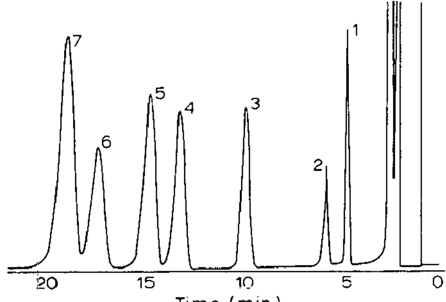

Fig. 6. Separation of formyl derivatives of N-methyl aniline (1), N-methyl *o*-toluidine (2), *o*-toluidine (4), aniline (5), *m*-toluidine (6) and *p*-toluidine (7) on a 12 ft. 2.5% DBS on Chromosorb G column, using methyl stearate (3) as internal standard.

with mixtures containing aniline and the toluidines. Acetylation on the other hand is useful in a non-aqueous medium and where only the toluidines and N-methyl aniline are present.

The formyl derivatives of the amines used here were quantitatively separated on column B but not on column C (Fig. 6). The acetyl derivatives on the other hand were separated on both columns though *m*- and *p*-toluidines were not quantitatively separated on column C (Figs. 7a and b).

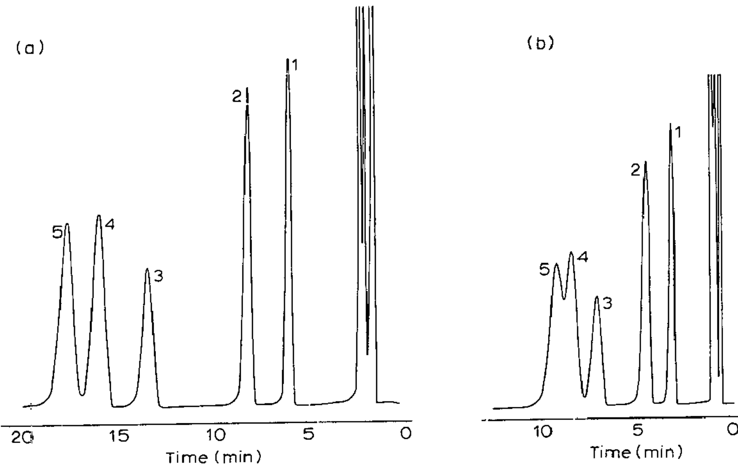

Fig. 7 (a) Separation of acetyl derivatives of *o*-, *m*- and *p*-toluidines, N-methyl aniline, and N-methyl *p*-toluidine on a 12-ft. 2.5% DBS on Chromosorb G column; 1 = N-methyl aniline, 2 = N-methyl *p*-toluidine, 3 = *o*-toluidine, 4 = *m*-toluidine, and 5 = *p*-toluidine. (b) Separation of acetyl derivatives of N-methyl aniline (1), N-methyl *p*-toluidine (2), *o*-toluidine (3), *m*-toluidine (4) and *p*-toluidine (5) on a 6-ft. 2.5% DBS on Chromosorb G column coated 1% with potassium hydroxide.

ACKNOWLEDGEMENT

I am indebted to Dr. F. GOODRIDGE of this department for his kind suggestions in the preparation of this manuscript.

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CHROM. 4855

SEPARATION AND DETERMINATION OF LOW-MOLECULAR-WEIGHT STRAIGHT-CHAIN (C_1–C_8) CARBOXYLIC ACIDS BY GAS CHROMATOGRAPHY OF THEIR ANILIDE DERIVATIVES

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(Received April 23rd, 1970)

SUMMARY

The analysis for formic, acetic, propionic, *n*-butyric, *n*-valeric, *n*-hexoic, *n*-heptoic and *n*-octoic acids in aqueous and non-aqueous mixtures by the gas chromatography of their anilide derivatives on sodium dodecylbenzenesulphonate coated on Chromosorb G is described. The derivatives of C_2–C_8 acids are formed in non-aqueous conditions via thionyl chloride treatment while the formic acid derivative is formed in aqueous conditions after pH adjustment. Optimum conditions for quantitative derivative formation are discussed. Variations of the pK_a values of the acids with derivative retention times and retention times and response factors with the number of carbon atoms are illustrated.

INTRODUCTION

Most of the methods described in the literature for the analysis of low-molecular-weight C_1–C_7 carboxylic acids by gas chromatography involve the direct injection of the acids into the column. This often necessitates the use of special combinations of stationary phases to obtain the optimum conditions for separation, but even then formic acid decomposition or lack of separation of it has been reported on some of these columns under the experimental conditions[1,2]. JANÁK AND KAPLANOVA[3] separated acetic and formic acids and water on a 1:3 mixture of bis(2-ethylhexyl) sebacate and citric acid coated on silanised Celite. MCKINNEY AND JORDAN[4] resolved formic, acetic, acrylic, propionic, *n*-butyric and isopentanoic acids and low-molecular-weight alcohols and aldehydes on 4% ethofat/2% isophthalic acid on Chromosorb T. A packing that will resolve a mixture of volatile fatty acids, C_1–C_5, has been reported by JACKSON[5], namely Ucon LB 550-X, sebacic acid and polypropylene glycol on 80/100 mesh AW Chromosorb W. Free formic and acetic acids in cigarette smoke have been determined by gas chromatography of their methyl esters on 20% Carbowax 20 M on 60/80 mesh Chromosorb P[6].

In the present work the anilide derivatives of formic, acetic, propionic, *n*-

butyric, *n*-valeric, *n*-hexoic, *n*-heptoic and *n*-octoic acids are chromatographed for quantitative determination on a single stationary phase coated on Chromosorb G. The derivatives are stable, non-volatile and easy to form. The method described lacks the disadvantages of the use of mixtures of stationary phases. The column packing remains stable and efficient over a long period of time because no free acids are passed through it.

EXPERIMENTAL

A Perkin Elmer Model F11 gas chromatograph equipped with dual flame ionisation detector was used for the analysis, and nitrogen served as the carrier gas.

Column preparation

2.5 g of sodium dodecylbenzenesulphonate (DBS) were dissolved in water–methanol (1:1) and the solution was added to 97.5 g of NAW 60/80 mesh Chromosorb G. After mixing by stirring the solvent was evaporated in the oven at 100° with occasional stirring until the solid appeared dry. It was cooled and packed by suction into 6 ft. (A) and 12 ft. (B) by 3 mm I.D. glass columns.

Column C. 1 g of potassium hydroxide was dissolved in 50 ml of methanol and added with more methanol to 99 g of NAW Chromosorb G. After mixing by stirring the solvent was removed. Then 97.5 g of the dry solid were added to 2.5 g of DBS dissolved in water–methanol, mixed thoroughly and the solvent removed as above in the oven. The solid was cooled and packed in a 6 ft. × 3 mm I.D. glass column (C).

The three packed columns A, B and C were conditioned at 220° for 18 h.

Intrument analytical conditions

The instrumental conditions and the columns employed for the various separations and determinations are listed in Table I below.

TABLE I

INSTRUMENTAL CONDITIONS FOR THE ANALYTICAL SEPARATIONS

Columns: (A) 6 ft. × 3 mm I.D. glass column packed with 2.5% (w/w) sodium dodecylbenzenesulphonate (DBS) on NAW 60/80 mesh Chromosorb G; (B) the 12 ft. × 3 mm I.D. version of column A; (C) 6 ft. × 3 mm I.D. glass column packed with 2.5% (w/w) DBS on NAW 60/80 mesh 1% (w/w) base-loaded Chromosorb G.
The oxygen and hydrogen inlet pressures and the injection port temperature are the same for all the separations, *viz.* 26/18 lb./in.^{2} and 250°, respectively.

Chromatography of the anilide derivatives of	*Instrumental conditions*
Formic and acetic or propionic or butyric acid using methyl stearate as internal standard	Columns A and C; oven temperature, 200°; nitrogen inlet pressure, 9 lb./in.^{2} (Fig. 2)
C_1–C_8 (formic, acetic, propionic, *n*-butyric, *n*-valeric, *n*-hexoic, *n*-heptoic and *n*-octoic) acids, using methyl myristate as internal standard	Column B; oven temperature, 200°; nitrogen inlet pressure, 15 lb./in.^{2} (Fig. 3)
	Columns A and C; oven temperature, 200°; nitrogen inlet pressure, 10 lb./in.^{2} (Fig. 5)
Acetic, propionic, *n*-butyric and *n*-valeric acids (for quantitative separation)	Column B; oven temperature, 200°; nitrogen inlet pressure, 10 lb./in.^{2} (Fig. 4)

Chemicals

The chemicals were used as obtained from BDH without further purification. The anilide derivatives of the carboxylic acids were prepared as described by OPENSHAW[7] for retention time determinations.

PROCEDURE

Preparation of the anilide derivatives of C_2–C_8 straight-chain carboxylic acids and chromatography

0.5 ml of the ethyl acetate solution of acetic, propionic, *n*-butyric, *n*-valeric, *n*-hexoic, *n*-heptoic, and *n*-octoic acids (0.5 *M* in each acid) were pipetted into a dry 50-ml stoppered tube containing 0.15 ml thionyl chloride. 0.5 ml aniline were then gently added. (The aniline was allowed to fall in drops directly onto the carboxylic acid–thionyl chloride mixture from a pipette without touching the walls of the tube.) The tube was placed unstoppered on a sand bath at 60–80° for 5 min and then cooled for 2 min in an ice bath. 10 ml of 0.1% (w/v) methyl myristate solution in ethyl acetate was added and the tube swirled round gently to mix. 10 ml of 1 *M* aqueous solution of sodium bicarbonate were added and the tube shaken unstoppered until the effervescence died down. It was then stoppered and shaken vigorously for 30 sec and left to stand for the layers to separate. 2 μl of the ethyl acetate layer were taken in a hypodermic syringe and injected into the instrument (columns B and C).

When the injection was not being done immediately after the derivative preparation the clear ethyl acetate layer was transferred with a clean dropping pipette to a 10–20 ml dry stoppered tube containing 2–5 g anhydrous sodium sulphate. The derivatives could stand in the solution for up to a week without deterioration.

RESULTS AND DISCUSSION

The anilide derivatives of a mixture of acetic to *n*-octoic acids may be prepared quantitatively by the method described only in non-aqueous conditions via thionyl chloride treatment. In aqueous medium and at the working concentrations (0.1–1.0 *M*) the anilides were hardly formed due to the ease of hydrolysis of the acid chlorides. On the other hand formanilide was hardly formed in non-aqueous medium especially after thionyl chloride treatment due to the reaction:

$$SOCl_2 + HCOOH \rightarrow CO + SO_2 + 2HCl$$

In aqueous medium, however, formanilide was obtained quantitatively in all working concentrations (0.05–1.0 *M*) even with thionyl chloride treatment.

When these acids are in an aqueous mixture they may be determined as described but must first be extracted into an organic (ethyl acetate or diethyl ether) layer. Though formic, acetic, propionic and *n*-butyric acids are miscible with water, they were extracted into the ethyl acetate layer in measurable amounts. However, the amount extracted individually into the organic layer, using equal volumes of distilled water and ethyl acetate and shaking vigorously for 1 min in a separatory funnel, varied with the amount of acid present, *e.g.* formic, acetic, propionic and *n*-butyric acids over a concentration range of 0.5 *M*–4.0 *M* in water were extracted 39.3–41.4%, 45.0–50.0%, 71.8–75.0% and 89.4–90.6%, respectively, into the organic layer. The rest of the acids gave 98–100% extraction when extracted individually.

Duplicate extractions of an equimolar (0.5 *M*) mixture of the C_2–C_8 acids gave a different extraction picture for each acid, *viz.* acetic acid, 15.5%; propionic acid, 30.3%; *n*-butyric acid, 36.8%; *n*-valeric acid, 39.2%; *n*-hexoic acid, 39.5%; *n*-heptoic acid, 39.5%; and *n*-octoic acid, 40.0%. The presence of mineral acids and salts also affected the carboxylic acid recoveries in the organic layer, so did the variation of the volume ratio of the organic and aqueous portions and the total acid concentration in the mixture. The investigation of these effects was not carried to the final conclusions as it might detract from the main object of this work. It must be pointed out that the recovery of each acid in a mixture would vary from situation to situation. It is therefore necessary to calibrate the extraction procedure for the acids in any given aqueous condition before proceeding with the derivative formation and gas chromatography.

The presence of water in the ethyl acetate solution of the acids reduces the amount of derivatives formed (hence lowers the sensitivity of the method) and distorts the chromatograms obtained from column B. Therefore after extraction of the acids from an aqueous medium the ethyl acetate layer must be dried with anhydrous sodium sulphate before being sampled for derivative formation.

Unknown acid mixtures may be determined by plotting calibration curves with standard mixtures, or by the use of the relative response factors (*i.e.*, weight of acid taken in mg/peak area *vs.* methyl myristate, Table III), using methyl myristate as the internal standard in both cases. The relative amount of each anilide formed (measured as the peak area ratio) in the mixture with any fixed amount of thionyl chloride present varies with the amount of aniline added (Fig. 1). Acetic, propionic and *n*-butyric acids on the one hand, and *n*-valeric, *n*-hexoic, *n*-heptoic and *n*-octoic acids on the other yield maximum amounts of derivatives at different concentrations of aniline. It was found reproducible and accurate enough in practice, however, to estimate first the number of mmoles of acid in an aliquot of the unknown mixture, and then to add thionyl chloride 1–2 mmoles in excess, followed by an amount of

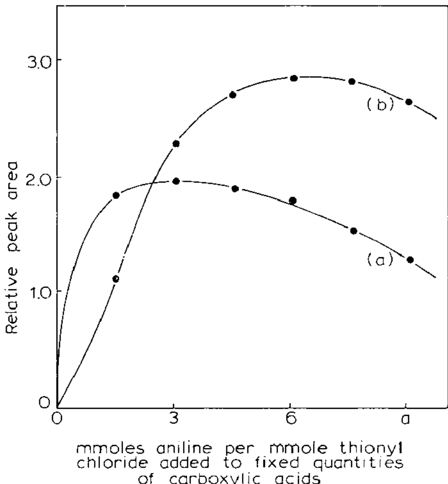

Fig. 1. Variation of quantity of derivative formed with increasing amounts of aniline. (a) *n*-Valeric–*n*-octoic acids; (b) acetic, propionic and *n*-butyric acids.

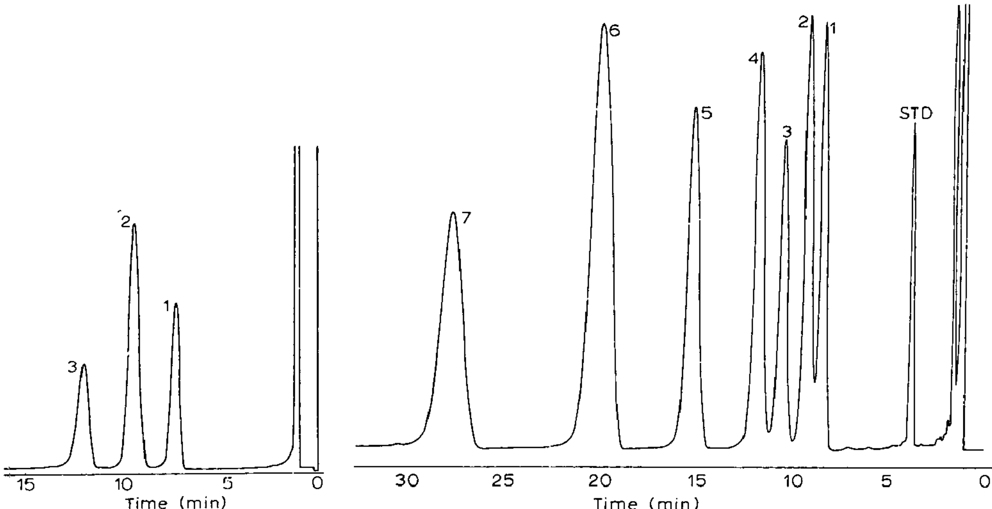

Fig. 2. Separation of acetanilide (1) and formanilide (3) with methyl stearate (2) as internal standard on a 6-ft. 2.5% DBS on Chromosorb G column.

Fig. 3. Separation of anilide derivatives of C_1–C_8 straight-chain carboxylic acids on a 12-ft. 2.5% DBS on Chromosorb G column. 1 = Propionic acid, 2 = *n*-butyric acid, 3 = acetic acid, 4 = *n*-valeric acid, 5 = *n*-hexoic acid, 6 = formic acid and *n*-heptoic acid, and 7 = *n*-octoic acid; STD = methyl myristate.

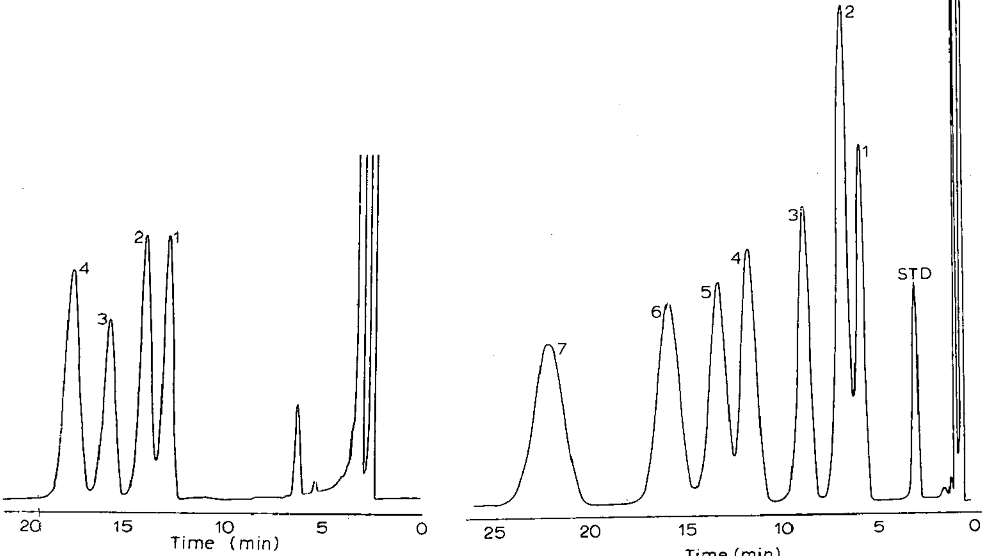

Fig. 4. Quantitative separation of anilide derivatives of (1) propionic acid, (2) *n*-butyric acid and (3) acetic acid on a 12-ft 2.5% DBS on Chromosorb G column. 4 = Valeric acid.

Fig. 5. Separation of anilide derivatives of C_1–C_8 straight-chain carboxylic acids on a 6-ft. 2.5% DBS on Chromosorb G column coated 1% w/w with potassium hydroxide. 1 = Propionic acid, 2 = acetic and *n*-butyric acid, 3 = *n*-valeric acid, 4 = *n*-hexoic acid, 5 = formic acid, 6 = *n*-heptoic acid, 7 = *n*-octoic acid; STD = methyl myristate.

TABLE II

RELATIVE RETENTION TIMES OF THE ANILIDES OF THE C_1–C_8 CARBOXYLIC ACIDS

Acid	*pKa (25°)*[8]	*Relative retention time*	
		Column B	*Column C*
Formic (C_1)	3.77	6.34	5.0
Acetic (C_2)	4.76	3.21	2.41
Propionic (C_3)	4.88	2.57	2.0
n-Butyric (C_4)	4.82	2.86	2.41
n-Valeric (C_5)	4.81	3.79	3.16
n-Hexoic (C_6)	4.85	4.92	4.33
n-Heptoic (C_7)	4.89	6.64	6.08
n-Octoic (C_8)	4.85	9.42	8.66

aniline three to six times the number of mmoles of thionyl chloride added. The relative amount of aniline added to sample and standard aliquots must be the same.

The anilides of the C_1–C_8 carboxylic acids were separated on columns A, B and C. Heptoic and formic acid anilides were not separated on column B but they were

TABLE III

Acid	*Relative response factor*	
	Weight (in mg) / *Peak area*	*Mmoles* / *Peak area*
Acetic	8.4	0.14
Propionic	8.14	0.11
n-Butyric	7.75	0.09
n-Valeric	7.14	0.07
n-Hexoic	6.96	0.06
n-Heptoic	7.02	0.05
n-Octoic	7.64	0.05

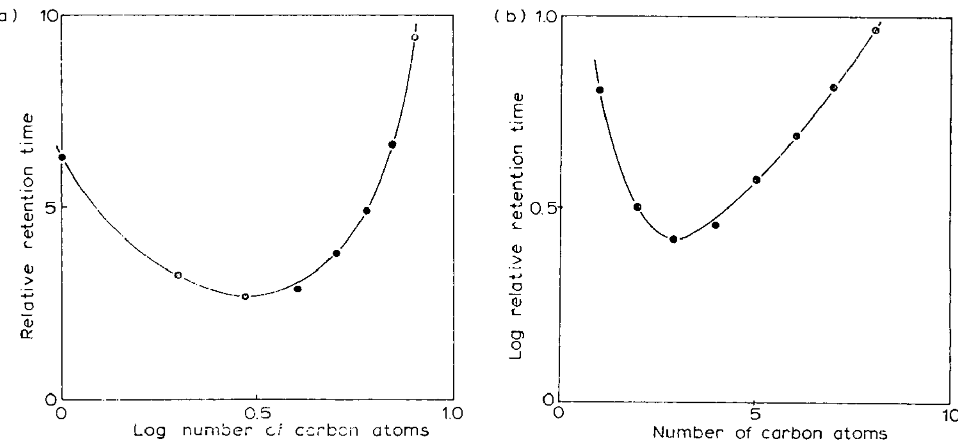

Fig. 6. (a and b) Variation of relative retention time with the number of carbon atoms on the carboxylic acids.

on columns A and C. Similarly, while *n*-butyric, acetic and propionic acid anilides were not resolved on columns A and C they were on column B. In the absence of *n*-butyric or acetic acid in the mixture the rest of the acids were quantitatively separated on columns A and C (Figs. 2–5).

The relative retention times (on columns B and C) and the response factors (on column B) of the acids are shown in Tables II and III, respectively. Their variations with the number of carbon atoms are illustrated in Figs. 6 and 7. The relative response factor decreases (in other words, the relative peak area per mmole of acid present increases) with increasing number of carbon atoms.

The effect of the pK_a of the acids on their relative retention times is not very clear. A plot of the pK_a (25°) *vs.* relative retention time produces the curves in Fig. 8. The pK_a values of formic, acetic, propionic and *n*-butyric acids lie on a straight line in relation to their retention times on column B.

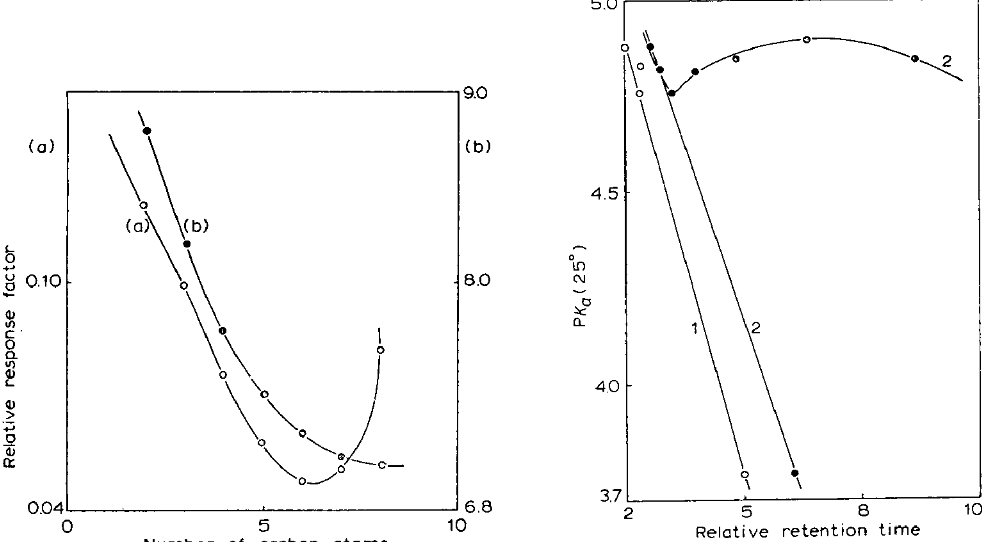

Fig. 7. Variation of relative response factors with the number of carbon atoms.
(a) (Weight of acid in mg/peak area) *vs.* number of carbon atoms
(b) (Mmoles acid taken/peak area) *vs.* number of carbon atoms

Fig. 8. pK_a (25°) *vs.* relative retention times of the anilides of the straight-chain C_1–C_8 carboxylic acids on columns B and C. (1) On column C; (2) on column B.

ACKNOWLEDGEMENT

I am indebted to Dr. F. GOODRIDGE of this department for his kind suggestions in the preparation of this manuscript.

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HEPTADECENOIC ACID AS AN INTERNAL STANDARD IN THE GAS CHROMATOGRAPHIC WEIGHT DETERMINATION OF FATTY ACIDS

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(Received May 6th, 1970)

SUMMARY

The method of calibration for the fatty acid methyl esters weight microdetermination (10–100 μg) by gas–liquid chromatography with an argon detector and dimethyldichlorosilane treated polyethyleneglycol adipate column is described. Methyl *n-cis*-9-heptadecenoate was used as an internal standard in the calibration. The acid was isolated from *Candida* sp. fat by liquid–liquid partition of the methyl esters, urea crystallization and preparative gas–liquid chromatography, and was identified by its melting point, refraction index, gas–liquid chromatography retention volume, oxidative degradation and infrared spectrum. Calibration was performed at different sample (soybean fatty acid methyl esters)/standard weight ratios. Good weight determination accuracy can be achieved in the 10.4–25.9 ratio range after multiplying the standard peak area by a correction coefficient. The corrected recovery value in the above range was 92.4–105.5%, with a relative standard deviation of $\pm$ 3.7%. Saponification of the sample–standard mixture, extraction of unsaponifiable and conversion of the fatty acids into their methyl esters again did not affect the accuracy and precision of the weight determination. Thus, methyl *cis*-9-heptadecenoate may be used as an internal standard in the microdetermination of the weight of unsaturated lipids in plant material.

INTRODUCTION

The colorimetric reaction resulting from hydroxamic acid formation has generally been used until recently to quantitate ester groups of lipids[1]. At present the determination of the weight of small amounts of lipid is generally carried out by gas–liquid chromatography (GLC) with an internal standard[2–6]. In addition to high sensitivity and speed of analysis, the internal standard technique has the advantage of compensating for fatty acid losses that inevitably occur during the working-up of the biological material and the GLC determination itself.

However, to make full use of this advantage the fatty acid chosen as an internal

standard should be similar in its characteristics to the fatty acids of the sample under investigation and should not be present in the sample. Several saturated acids have been used as standards up to now[2-4,6] but most are unsuitable for our work where it is necessary to estimate the non-extractable phospholipids in soybean seeds in which unsaturated acids predominate. Therefore, we decided to use unsaturated *n-cis*-9-heptadecenoic acid as an internal standard. Until recently it was believed that this acid occurs in natural sources of lipids in trace amounts only[7,8]. However, the investigations of DYATLOVITSKAYA *et al.*[9] have shown that its content in the lipids of the *Candida* sp. yeasts grown on "odd" hydrocarbons may be as high as 50% of the total fatty acids. Thus, in view of rapid growth of the microbiological industry the yeasts might become a convenient source for the preparative isolation of heptadecenoate.

Up to now the amount of the fatty acids has been determined by using only single ratios of the sample/standard concentrations[2-6]. However, it could be argued that the accuracy of the weight analysis at the various ratios may be different since the response of many chromatographic detectors is known to be non-linear.

The present paper describes the calibration of the procedure for the gas chromatographic weight determination of fatty acids. *n*-Heptadecenoic acid isolated from the lipids of *Candida* sp. yeasts was used as an internal standard. The results obtained at different sample/standard ratios made it possible to determine the range of these ratios in which fair accuracy of the weight determination may be achieved and to estimate the precision of the latter.

EXPERIMENTAL

Reagents

Hexane and methanol were purified and dried as described previously[10]. Dodecane, ethanol, isopropanol, chloroform and hydrochloric acid were distilled[11,12], toluene and benzene were treated with H_2SO_4 and distilled over metallic Na. Dimethyldichlorosilane (DMDCS, technical grade) was dried for several days with anhydrous Na_2SO_4 and distilled, the 70° b.p. fraction being collected. Reagent grade chemicals—anhydrous aluminium oxide, argon, urea, silver nitrate, $NaIO_4$, $KMnO_4$, $NaHCO_3$, $Na_2S_2O_3 \cdot 5H_2O$, $K_2S_2O_3$, KOH, H_2SO_4, HNO_3, acetyl chloride, *tert.*-butanol, as well as Sudan Black B (Feinchemie K.-H., Kallies KG, G.F.R.), methyl caprylate and methyl caprate (California Corporation for Biochemical Research, Los Angeles, U.S.A., purity > 99.5% and 99.8%, respectively) were used without further purification. Adipic, azelaic and sebacic acids were obtained from the Institute of Chemistry of Natural Products, U.S.S.R. Academy of Sciences, and technical fat isolated from *Candida* sp. yeasts was supplied by the Moscow Branch of the All-Union Research Institute of Fats. Lipids extracted by cold hexane from ground soybean seeds were used to prepare fatty acid methyl esters[10].

Isolation of the unsaturated acids of yeast as their methyl esters

A flow diagram of the methyl heptadecenoate preparation is shown in Fig. 1: Nos. I–XIV refer to separate fractions while Nos. 1–12 indicate single operations during isolation and purification. At every stage of the preparative separation the fatty acid composition of the fractions was checked by analytical GLC[10]. Yeast fat I (4 g) was dissolved in a minimal quantity of hexane and transferred to a 2.4 × 9 cm

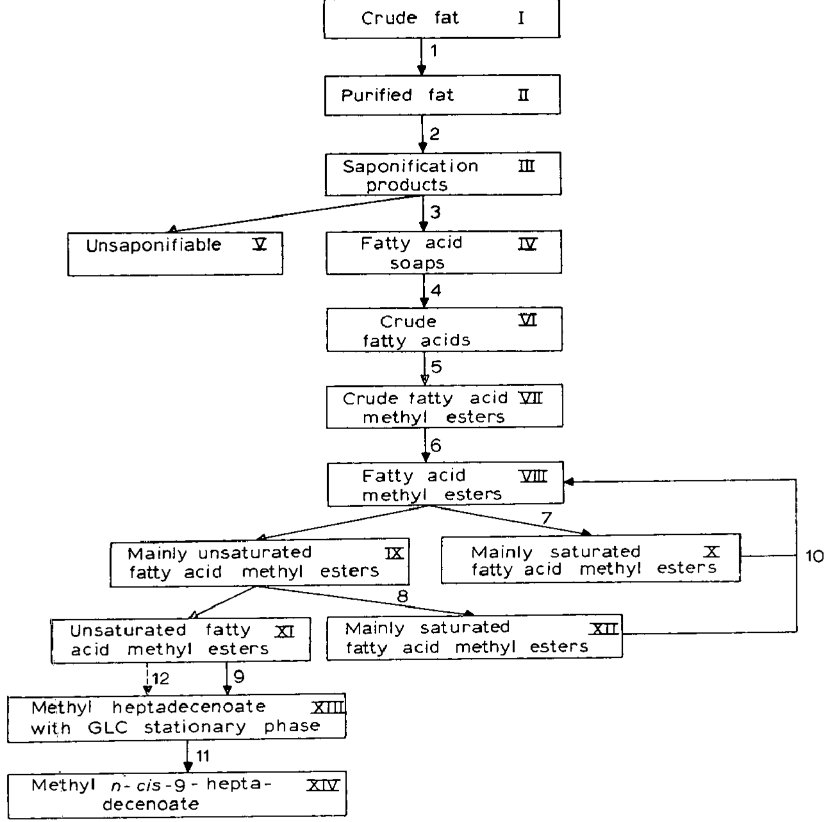

Fig. 1. Flow diagram of the preparative isolation of *n-cis*-9-heptadecenoic acid methyl ester. For designations of I to XIV and 1 to 12 see EXPERIMENTAL.

chromatographic column which was equipped with a No. 1 glass filter and contained 36 g of Al_2O_3. Triglycerides were eluted (1) from the column by 200 ml of hexane until the eluate became colorless. The partially purified fat II was saponified (2) in a solution of 500 mg of Na in 40 ml of ethanol for 1 h[10]. The ethanol was distilled off and the final traces were removed under vacuum at 50°. The saponification product III was transferred by 80 ml of H_2O to the continuous liquid–liquid extraction apparatus (total volume 100 ml) similar to that described previously[13] except that solvent vapors did not pass directly from the flask to the extraction chamber but to the upper end of the condenser of the apparatus. The aqueous phase was extracted with hexane for 13 h to remove most of color (3). Unsaponifiables V were discarded. Free fatty acids were regenerated from their soaps IV by H_2SO_4 acidification, and then extracted (4) with 10×7 ml of hexane and converted (5) to methyl esters VII[10,13] by 5 ml of methanol–acetyl chloride mixture (10:1). The esters VII were again purified (6) on Al_2O_3 as described above.

The purified esters VIII were dissolved in hexane to obtain a 10% (w/v) solution, an equal volume of saturated $AgNO_3$ solution in methanol was added and the mixture was shaken (7) in a separatory funnel. The upper phase containing mainly saturated esters X was separated and washed with water; hexane was distilled off, and the

residue was added (10) to fraction VIII during the next isolation. The mainly unsaturated esters of the lower phase IX were extracted with hexane after addition of excess water. The hexane was distilled off, and then a mixture of the esters IX (*A* g), urea (3.6 × *A* g), and methanol (16 × *A* ml) was heated until complete dissolution and allowed to stand overnight at room temperature. The supernatant fraction was filtered off on a Büchner funnel. The ester–urea complexes in both fractions obtained (XI and XII) were decomposed with an excess of hot aqueous HCl (20:1) and the esters were extracted with hexane (8). Mainly saturated esters XII as well as esters X were added (10) to fraction VIII.

Preparative GLC and storage of the preparation

The unsaturated esters XI were separated (9) on a preparative-scale gas chromatograph constructed in the Institute of Organic Chemistry, U.S.S.R. Academy of Sciences. The operating conditions were as follows: stationary phase, polyethylene glycol-2000 on Chromosorb P (20–30 mesh); column temperature, 200°; flash heater temperature, 220°; N_2 flow rate, 800 ml/min; column 20 m × 10–20 mm; HETP 1500; sample volume 1 ml. The isolated ester XIII was purified (11) by saponification (see above) from possible admixture of the stationary phase, unsaponifiables were repeatedly extracted with hexane in a flask with a side tube[14], and the heptadecenoic acid isolated after acidification was again converted into its methyl ester XIV.

The methyl heptadecenoate XIV so prepared was transferred to the storage ampul (Fig. 2) with a 5% (v/v) solution of methanol in benzene through the straight end 4. The joints were covered with a vacuum grease, and the stopcocks with female

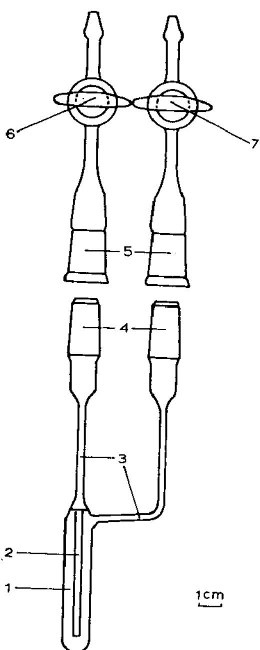

Fig. 2. Ampul for storage of the methyl heptadecenoate preparation. 1 = ampul; 2 = inert gas inlet tube; 3 = sealing points; 4 = male standard joint No. 14.5; 5 = female standard joint No. 14.5; 6 = gas inlet stopcock; 7 = outlet stopcock.

joints 5 were connected; the ampul was placed in an ice bath, and the argon flow was passed in through stopcock 6 at a rate of 10 ml/min and out through stopcock 7. 15 min later the ampul was immersed in solid carbon dioxide and the argon flow was maintained until the solution was frozen. Stopcocks 6 and 7 were then closed and the ampul was sealed, slightly opening the respective stopcocks immediately before sealing. The ampuled solution was stored in a frozen state at $-5°$.

Preparative partition chromatography

Preparation XIII can also be obtained (12) by partition chromatography of esters XI as their π-complexes with Ag ions[15]. The preparative isolation was performed using a 10% (v/v) dodecane solution and saturated $AgNO_3$ solution in 70% (v/v) aqueous methanol. Methyl heptadecenoate was eluted with hexane from unstained strips.

Determination of the physico-chemical characteristics of heptadecenoic acid and its methyl ester

The relative retention volume value was determined by GLC[10], the melting point was estimated according to TERENTYEV[16] and the refraction index n_D^{20} was measured at $20 \pm 2°$ (ref. 17) on an IRF-22 refractometer.

Investigation of the structure of heptadecenoate

Oxidative degradation of the acid was performed by the method described previously[18,19]. The oxidation products were extracted with ether and traces of iodine were destroyed with a diluted $Na_2S_2O_3$ solution. Mono- and dicarboxylic acids resulting from the oxidation and pure adipic, azelaic and sebacic acids were converted into their methyl esters with a CH_3OH–CH_3COCl mixture (see above). Esters of mono- and dicarboxylic acids were separated by GLC[10], the column temperature being 125° and 150° and the carrier gas flow rate being 14 and 60 ml/min, respectively.

The IR spectrum of methyl heptadecenoate was obtained on a UR-10 spectrometer (Carl Zeiss, Jena, G.D.R.) under the following conditions: chart speed 150 cm^{-1}/min; scale of registration 12 mm/100 cm^{-1}; slit program 4; amplification 4.5; crystal support, potassium bromide.

Preparation of sample–standard calibration mixtures

A sample of soybean fatty acid methyl esters (40.8 mg) containing (as weight per cent of esters) palmitic (16.7), stearic (5.7), oleic (30.7), linoleic (44.7), linolenic (1.8) and arachidic (0.5) acids and free of any impurities was weighed to the fourth decimal point and dissolved in toluene in a calibrated[17] volumetric flask (nominal volume 50 ml). The same procedure was employed to prepare a solution of the standard preparation (17.7 mg), both solutions being obtained at the same room temperature (20–22°). Using a calibrated pipette[17] (nominal volume 5 ml), 12 mixtures having different sample/standard ratios were obtained (see *Calibration for the weight determination*). Toluene was distilled off *in vacuo*, the mixtures were dissolved in benzene and stored in ampuls as described above.

Preparation of the GLC column for calibration

Celite-545, 100–120 mesh, acid-washed (W. G. Pye and Co., Ltd., Great Britain)

was further purified with hydrochloric acid and fine particles of the support were removed with water; pure dry Celite was treated with 5% (v/v) solution of DMDCS in toluene[20]. The inside surface of the glass column and the glass yarn plug to be placed in the column above the solid support stood for 1 h in the DMDCS solution, were washed 4 times with benzene and stood for 1 h in methanol. The glass yarn was dried at 120° and the column in a current of air without heating. Polyethyleneglycol adipate (PEGA, 1 g) in a minimal volume of benzene was treated with an equal volume of the DMDCS solution; 10 min later the solvents and excess DMDCS were distilled off *in vacuo* at 60° and the stationary phase was dissolved in 80 ml of chloroform. The Celite was coated with PEGA according to a previously described method[10,21], $CHCl_3$ being removed in a rotary film evaporator (PVO-64, Mikrotechna n.p., Modřany, Czechoslovakia). The column was packed and conditioned as usual[10].

Calibration

The gas chromatograph equipped with an argon ionization detector was used[10]. The analysis of the prepared series of calibration mixtures was performed without interrupting the carrier gas flow and column heating. Three chromatograms were usually obtained for each calibration mixture; the composition of some mixtures was determined 10 times in succession to estimate the precision of the analysis. Only chromatograms having a maximum peak height between 50% and 100% of the recorder scale were used for peak area calculation which was performed by a triangulation technique.

Calculation of the true sample weight of fatty acid methyl esters as determined by GLC using an internal standard

The GLC analysis of one of the standard preparations showed that it contained 89.4% of methyl heptadecenoate; it also contained 2.3% of an impurity whose retention time was equal to that of methyl stearate. Therefore, the actual sample and standard weights (p and p_s, μg) in a given calibration mixture were

$$p = f \cdot a \left(P + \frac{P_s \cdot y}{100} \right) \text{ and } p_s = (f \cdot a_s \cdot P_s \cdot t)/100,$$

where

f = $v/5V$, the correction factor for the volumetric glassware calibration;
V = true volume of the volumetric flask (ml);
v = true volume of the volumetric pipette (ml);
P = sample weight in V ml of the sample solution (μg) ($P = 4.08 \cdot 10^4$ μg);
P_s = weight of the standard preparation in V ml of the standard solution (μg) ($P_s = 1.77 \cdot 10^4$ μg);
a = the nominal volume of the sample solution used to prepare a given mixture (ml);
a_s = the nominal volume of the standard solution used to prepare a given mixture (ml);
t = methyl heptadecenoate content in the standard preparation (weight %) ($t = 89.4\%$);
y = content of impurities whose retention time is equal to that of any sample peak in the standard preparation ($y = 2.3\%$).

The GLC determination of the weight of the fatty acids by the internal standard method is based on the assumption that there must be a direct linear relationship between the weights of the sample and standard, on the one hand, and the areas of total sample peaks and standard peak (S and s, mm^2), on the other. On the basis of this assumption, the weight of the fatty acid methyl esters sample as determined by GLC using an internal standard (p', μg) is

$$p' = (p_s \cdot S)/s,$$

where

$S = h_1 \cdot b_1 + h_2 \cdot b_2 + \ldots + h_N \cdot b_N$;
$s = h_s \cdot b_s$;
h_i = height of the i-th peak of the sample (mm);
b_i = width of the i-th peak of the sample (mm);
N = number of peaks of the sample;
h_s = height of the peak of the standard (mm);
b_s = width of the peak of the standard (mm).

As mentioned above, the response of many gas chromatographic detectors is non-linear. Therefore, the accuracy of the weight of fatty acid esters p' found should be assessed by the recovery (k, %) of the sample weight during the GLC determination of the latter value; $k = (p' \cdot 100)/p$. If it is assumed that theoretical recovery (k = 100%) is confined to only one value of the actual sample/standard weight ratio $Q \doteq p/p_s$ or to a more or less narrow range of these values, then the true sample weight of fatty acid methyl esters as determined by GLC using an internal standard (p'_0, μg) in the region where k = 100% is $p'_{0,k=100\%} = p$, and beyond this region

$$p'_{0,k\neq 100\%} = p' \cdot \frac{100}{k} = \frac{f \cdot a_s \cdot P_s \cdot t\,(h_1 \cdot b_1 + h_2 \cdot b_2 + \ldots + h_N \cdot b_N)}{h_s \cdot b_s \cdot k}$$

In successive recovery determinations (see *Calibration*) yielding a number of k_i values for one or several calibration mixtures, the arithmetic mean of the recovery was calculated as $\bar{k} = \Sigma\, k_i/n$, where n is the number of measurements. In the $k \neq 100\%$ region $\bar{k}$ may be used for calculations of the corrected single recovery value $k'_i = 100\, k_i/\bar{k}$. The relative standard deviation S_{rel} for a number of $\bar{k}$ or k'_i values was determined by a previously described procedure (see Table II in ref. 17).

RESULTS AND DISCUSSION

Isolation and characteristics of heptadecenoic acid and its methyl ester

As mentioned above, the fatty acids previously used as internal standards in the weight determination of lipids[2-6] are sometimes inadequate for this work. Esters of normal acids with less than 16 carbon atoms in the chain (pentadecanoic, myristic) which have been used as standards owing to a low concentration of these acids in many living tissues[2] give very sharp peaks at isothermal column conditions. Measurement of the width of these peaks involves considerable error, and the appreciable volatility of the esters at room temperature may result in the loss of a standard during the working-up of the biological material before analysis. The disadvantages of margaric acid, which has frequently been used as a standard[3,4], are its high melting point (61°), difficulties involved in its purification and its occurrence in

TABLE I

COMPOSITION OF FATTY ACID METHYL ESTERS OF THE YEAST FAT VII AND THE FRACTION OF UNSATURATED ACID METHYL ESTERS XI (WEIGHT % OF ESTERS)

Fatty acid[a]	*Fraction VII*	*Fraction XI*
16:0	14.4	0.0
16:1	4.1	8.3
17:0	16.7	0.0
17:1	31.0	51.3
"18:0"	6.0	3.9
18:1	22.8	22.3
18:2	5.0	14.2

[a] 16:0 = palmitic; 16:1 = hexadecenoic; 17:0 = heptadecanoic; 17:1 = heptadecenoic; "18:0" = unidentified fatty acid: the relative retention volume of its methyl ester is close to that of methyl stearate, but according to the fractionation pattern this acid is similar to unsaturated acids; 18:1 = octadecenoic; 18:2 = octadecadienoic.

some natural fats. Branched-chain 2-methylalkanoic acids which have been employed as standards by NAPIER[5] can only be prepared by special synthesis, and so are not readily available.

The main requirements to be fulfilled by a fatty acid for use as an internal standard in the GLC weight determination of the residual lipids of soybean seeds are as follows: first of all it should not occur in this plant and moreover it should be as close as possible to the unsaturated acids predominantly occurring in soybean lipids in its physical characteristics—m.p., solubility, etc. Among the natural fatty acids *n-cis*-9-heptadecenoic acid meets these requirements best of all; during GLC on polar liquid phases the peak of its methyl ester is situated in the region between the peaks of methyl palmitate and methyl stearate usually not occupied by other esters.

The fat of *Candida* sp. yeasts used for the preparative isolation of heptadecenoic acid contains about 30% of this acid (Table I); the fractionation of Ag^{+} coordination complexes and the urea precipitation result in an increase of the 17:1 content up to 50% and in complete removal of the saturated esters. The unsaturated fraction contains no methyl heptadecanoate (17:0) which would be difficult to separate from methyl heptadecenoate by preparative chromatography on a polar liquid phase because of the incomplete separation of their peaks. About 400 mg of 100% pure methyl heptadecenoate were isolated by GLC. The content of the major component in the preparation obtained by partition chromatography is about 98%, but the method itself is laborious and time-consuming.

TABLE II

CHARACTERISTICS OF *n-cis*-9-HEPTADECENOIC ACID AND ITS METHYL ESTER[a]

	V_R^{rel}		*Melting point (°C)*		n_D^{20}	
	Found	*Ref. 22*	*Found*	*Ref. 23*	*Found*	*Ref. 23*
Acid	—	—	14.0–14.1	8.7–9	1.4611	1.4594
Ester	3.12	3.12	4.1– 4.2	(−30.8)–(−30.3)	1.4533	1.4516

[a] V_R^{rel} = relative retention volume (V_R^{rel} of methyl myristate = 1.00).

To establish the structure of the isolated heptadecenoate its relative retention volume, melting point and refractive index were compared with the corresponding known data for natural and synthetic methyl *cis*-9-heptadecenoate and the free acid. As shown in Table II, the relative retention volume of methyl heptadecenoate is equal to that obtained for 17:1 from *Candida* lipids (see Fig. 1 in ref. 22). At the same time the melting point of synthetic *n-cis*-9-heptadecenoic acid and methyl heptadecenoate[23] are below those reported here. On the assumption that the ester melting point is −30.3° (Table II) we attempted to separate methyl heptadecenoate from other esters of fraction XI by crystallization in acetone at −78°; however, the 17:1 ester was always precipitated with methyl oleate. Found n_D^{20} values of both acid and ester were by 0.0017 higher than the data reported for the respective synthetic products.

Because of the above discrepancies, the position of the double bond in the chain of isolated methyl heptadecenoate was determined by oxidative degradation[18,19]. The retention volumes (V_R) of the methyl esters of standard mono- and dicarboxylic acids and heptadecenoate oxidation products are shown in Table III.

TABLE III

Methyl esters of	V_R, *ml Ar*
Caprylic acid	290
Capric acid	780
Adipic acid	265
Azelaic acid	969
Sebacic acid	1464
Oxidation products	294
	966

These data confirm the results of a previous investigation[9] according to which the double bond in the chain of *n*-heptadecenoic acid from *Candida* yeast is situated between the 9th and 10th carbon atoms. Higher acid and ester melting points found here might be supposed to be brought about by partial *cis, trans*-isomerization of the double bond during isolation and purification[23]. However, the absence of the *trans*-bond absorption band at 965 cm^{-1} in the IR spectrum of methyl heptadecenoate (Fig. 3) confirms the *cis*-configuration of the double bond of this ester reported previously[9].

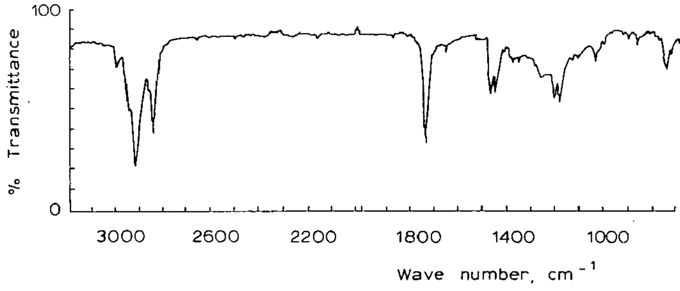

Fig. 3. IR spectrum of methyl *n-cis*-9-heptadecenoate.

Thus, the preparation isolated is the methyl ester of *n-cis*-9-heptadecenoic acid. The data available so far are insufficient to account for the differences in n_D^{20} and melting point between our preparation and the respective synthetic sample. It cannot be ruled out that this discrepancy might be associated with polymorphic differences in the crystals used in the m.p. measurements.

Calibration for the weight determination

The methyl heptadecenoate preparations obtained were used as an internal standard in the determination of the weight of total fatty acids. In practice it may become necessary to use the preparation of standard containing foreign impurities. Therefore, the calibration of the method was performed using one of the preparations containing 89.4% heptadecenoate, and corresponding correction factors t and y were introduced into the equations used to calculate the true sample weight (see EXPERIMENTAL).

The calibration was performed on a chromatographic column all components of which—inside surface, solid support and liquid phase—had been pretreated with dimethyldichlorosilane[20] to prevent selective adsorption of fatty acid methyl esters which could be affected by free silanol and hydroxyl groups of these components[24].

As shown in Fig. 4, the theoretical recovery of the sample weight ($\bar{k} \approx 100\%$) can be achieved only at $Q = 5.2$. Practical determination of the lipid weight requires, however, a more or less extensive range of Q ratios which should be characterized by equal or similar recovery values k. If this range were found, then by changing the weight of the added standard p_s it should be possible to obtain Q values for the lipid sample under investigation which will be within the limits of this range. There are horizontal sections of the curve (Fig. 4) at both $k < 100\%$ and $k > 100\%$. However, the $Q \leqslant 2.1$ region, owing to the small peak heights of the sample, does not provide sufficient precision for the determination of the weight and there is considerable scattering of the experimental points (Fig. 4). It was found that the range from

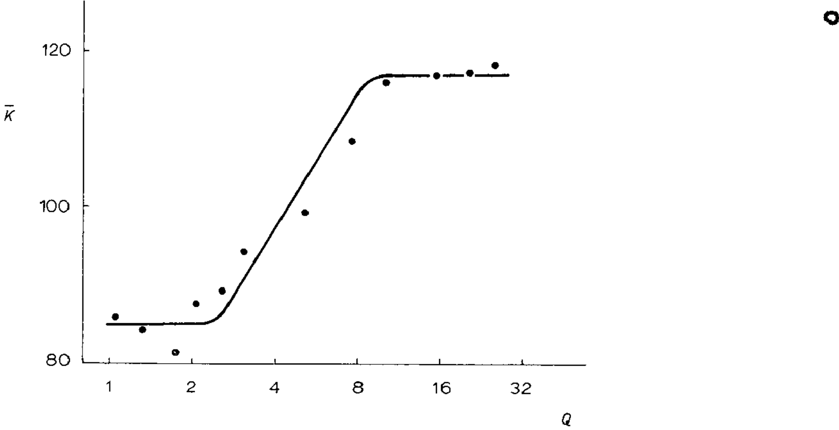

Fig. 4. Dependence of the arithmetic mean $\bar{k}$ ($n = 3$) of sample weight recovery as determined by GLC using an internal standard on the actual sample/standard weight ratio Q (Q values are plotted on the logarithmic scale).

$Q = 10.4$ to $Q = 25.9$ is most suitable for practical purposes since in this range the scattering does not exceed the error of replicate determinations of the same mixture (see below).

In the above range $\bar{k} = 115.8\%$ ($n = 12$). It should be pointed out that our preliminary data suggest that the absolute value of the correction coefficient $\bar{k}$ varies somewhat depending on the condition of the argon detector, even if basic process parameters remain unaltered[10]. Therefore, after a long time interval between experiments it is necessary to stabilize the detector by heating and to reascertain the $\bar{k}$ value using ready-made calibration mixtures. Over the whole working range $S_{rel} = \pm$ 3.7% ($k'_i = 92.3$–105.5%); an increase in the $\bar{k}$ value by several unit per cent does not change the S_{rel} value.

In order to assess the comparative precision of the determination of the weights in the selected range, the S_{rel} values for the mixtures with $Q = 15.5$ and 25.9 were compared with the S_{rel} value at $Q = 5.2$ ($\bar{k} \approx 100\%$). The S_{rel} values found were $\pm$ 1.5%, $\pm$ 2.8% and $\pm$ 3.1%, respectively ($n = 10$ in all cases). It can be seen that there is not much difference between the errors in the determination of the weight at arbitrary points of the range, at the point where $\bar{k} \approx 100\%$ and in the whole working range ($S_{rel} = \pm$ 3.7%). It can be concluded that this range provides not only fair accuracy but also sufficient precision for the weight analysis.

In a determination of the weight of the lipids in living tissues, the standard is usually added to the untreated plant material which is subsequently subjected to saponification. To assess the effect of this treatment on the accuracy and precision of the weight determination of the fatty acid methyl esters, the calibration mixture of the esters with $Q = 25.9$ was saponified, unsaponifiables were repeatedly extracted and fatty acids were again converted to their methyl esters. Before and after saponification mean arithmetic values of k'_i ($n = 10$) were 101.6 and 101.5%, respectively; the error of analysis did not change due to the saponification ($S_{rel} = \pm$ 2.8%).

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CHROM. 4835

A QUANTITATIVE GAS–LIQUID CHROMATOGRAPHIC DETERMINATION OF AROMATIC ALDEHYDES AND ACIDS FROM NITROBENZENE OXIDATION OF LIGNIN

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(Received April 22nd, 1970)

SUMMARY

A simplified gas–liquid chromatographic technique for the separation and quantitation of the major aromatic aldehydes and acids produced by the alkaline nitrobenzene oxidation of lignin has been described. Separation of *p*-hydroxybenzaldehyde, vanillin, syringealdehyde, *p*-coumaric acid, ferulic acid and four aromatic compounds appearing in low concentration has been achieved by a temperature programmed analysis using 5% OV-25 on Gas-Chrom Q as column packing. It was shown that the method can be applied to monitoring changes in the yield of phenolic compounds from the oxidation of isolated (Kraft) and *in situ* (corn plant) lignin which had been subjected to heat and digestive treatments.

INTRODUCTION

Alkaline nitrobenzene oxidation has been used in the elucidation of chemical composition of plant lignin[1]. Paper chromatography of the aromatic aldehydes and acids produced by the oxidation of plant lignin facilitated their quantitative determination[2]. REALE[3] introduced thin-layer chromatography to expediate this analysis. In our experience, however, both paper chromatography and thin-layer chromatography proved lengthy and were inconvenient for application to the routine analyses of the chemical composition of lignin oxidation products. Instead, it was decided to adapt the gas chromatographic methods of phenols and phenolic compounds[4–6] to the analysis of the alkaline nitrobenzene oxidation products of lignin. The use of the method has been extended to monitoring the changes which occur in lignin as a result of heat treatment and biodegradation of plant material.

EXPERIMENTAL

All compounds used were obtained commercially. Reference material included *p*-hydroxybenzaldehyde, vanillin, syringealdehyde, vanillic acid, *p*-hydroxybenzoic

acid, ferulic acid, *p*-coumaric acid, syringic acid and caffeic acid. Octyl ether was used as an internal standard since it was stable and had a retention time which did not interfere with the phenolic compounds. Tri-Sil (TMS) was purchased from Chromatographic Specialties Ltd. All solvents were redistilled and particular care was exercised in obtaining pure nitrobenzene.

Trimethylsilylation

The method of PELLIZZARI *et al.*[5] was used. Samples once prepared were immediately chromatographed.

Gas–liquid chromatography

Analyses were performed on a Hewlett-Packard Instrument (F & M 402) equipped with dual glass columns and a flame ionization detector. Glass columns were 4.0 ft. (3 mm I.D., 5 mm O.D.), packed with Gas-Chrom Q (80–100 mesh), coated with 5% OV-25 (Applied Science Co.). Various other packings were investigated in an attempt to obtain complete resolution of the aromatic compounds from lignin oxidation which were to be examined. 1–10% Carbowax 20 M/Chromosorb W and 2.5% OV-1/Chromosorb W packings gave serious peak tailing. 3% OV-1/Gas-Chrom Q and 3–5% OV-17/Gas-Chrom Q did not give complete resolution of peaks.

The flow rate of the carrier gas (nitrogen) was 80 ml/min. The injector port and detector temperatures were 240° and 225°. The column temperature was programmed between 120–210° at 2°/min. Peak areas were calculated by use of an integrator (Infotronics, Digital Readout System).

In order to facilitate the identification of the individual phenolic compounds in samples of unknown composition, the relative retention times of reference standards were used. Quantitative analysis of samples was performed by measuring peak areas of accurately weighed samples (2–6 mg) of reference standards using octyl ether as an internal standard.

In our experience the liquid phase maintained its efficiency for seventy-five injections. For this number of analyses the relationship of peak area to weight was not altered, and only a minimal decrease in retention times was observed.

Alkaline nitrobenzene oxidation of lignins

The procedure of GEE *et al.*[7] was used except the oxidation was carried out in sealed glass tubes instead of the stainless steel oxidation bombs because oxidation in the bombs led to the formation of extraneous compounds. Lignin (25–50 mg), nitrobenzene and 2.0 *N* NaOH were added to a 15 ml pyrex test tube; the test tube was sealed and placed in an oven at 160° for 3 h.

The oxidation products were purified by extracting nitrobenzene and its derivatives at a high pH (~13) with one washing of CH_2Cl_2. The solvent fraction was washed with 50 ml of 1 *N* NaOH. The combined sodium hydroxide layer and the original aqueous layer were acidified to pH 1 with 6 *N* HCl followed by extraction with 3 × 50 ml washings of CH_2Cl_2 and 2 × 50 ml washings of diethyl ether. The combined solvent fractions were dried over anhydrous sodium sulfate, reduced to a small volume on a rotary evaporator, then transferred to a 1 dr. vial and taken to near dryness (0.1–0.3 ml) on a sand bath (95°) under nitrogen. Care was taken to maintain an anhydrous sample. 1 ml of TMS was added. The sample was allowed to stand for

5 min, then immediately chromatographed. Recovery of reference standards was monitored for all extraction steps.

In vitro incubation

Ground corn stalk and leaf were extracted for 6 h each in ether and acetone; 10 g of the mixture were sterilized at 120° at 15 p.s.i. for 30 min in a 150 ml fermentation flask (construction of flasks is described elsewhere[8]) in the presence of 40 ml of culture medium[9]. To ascertain differences, if any, brought about in the composition of lignin oxidation products due to sterilization, another sample of the same corn material plus 40 ml of culture medium was gas sterilized at 40 ± 5° for 18 h. The sterile medium was inoculated with 80 ml of rumen fluid.

The standard incubation procedure included the bubbling of sterile carbon dioxide into the fermentation medium for 10 min. Subsequently, the head space was flushed continuously with sterile oxygen-free nitrogen. The gaseous effluent was passed through 0.1 *N* NaOH solution. The viability of microbial activity was monitored by recording electrical conductivity produced in an 0.1% NaOH solution. The pH of the incubation was maintained at 6.8 ± 0.5 during the entire 36 h incubation with appropriate base or acid addition. At the conclusion of the incubation, the medium was heated to 100° and the residue recovered by centrifugation and freeze-dried. The dry residue was subsequently extracted with DMF to obtain DMF lignin.

The Kraft lignin incubation medium consisted of 1 g of Kraft lignin, 20 ml of culture medium, and 40 ml of rumen fluid. To ascertain the effect of the sterilizations on the lignin, control incubations were carried out using heat deactivated rumen fluid. The rumen fluid was obtained from a fistulated steer, fed a maintenance diet of concentrate and forage.

In vivo incubation

The corn material was treated as above and 15 g were placed in each of 2 nylon bags made of 20 × 20 cm nylon. The bags were suspended for 36 h in the rumen of the above described animal. The residue was freeze-dried and extracted as before. Statistical analyses were performed according to STEEL AND TORRIE[10].

RESULTS

It has been shown that the major phenolic products of lignin oxidation with nitrobenzene in an alkali medium are *p*-hydroxybenzaldehyde, vanillin, syringealdehyde, *p*-coumaric acid, ferulic acid, and syringic acid[2]. Caffeic, *p*-hydroxybenzoic, and vanillic acid appear as minor products[2]. In addition some phenolic compounds accumulate in trace amounts[11].

The gas chromatographic method described has facilitated the resolution of all major and minor phenolic components of lignin oxidation by a single analysis (Fig. 1A). The recovery of pure standards was complete (Table I). Although results are only shown for one phenolic aldehyde and one phenolic acid, the observations were typical of all major compounds. The partial overlap of *p*-coumaric acid and syringic acid, and caffeic acid and ferulic acid did not appear to disturb the quantitative aspect of the analysis.

Changes in yield of aromatic compounds of 100 μg per g of lignin were detect-

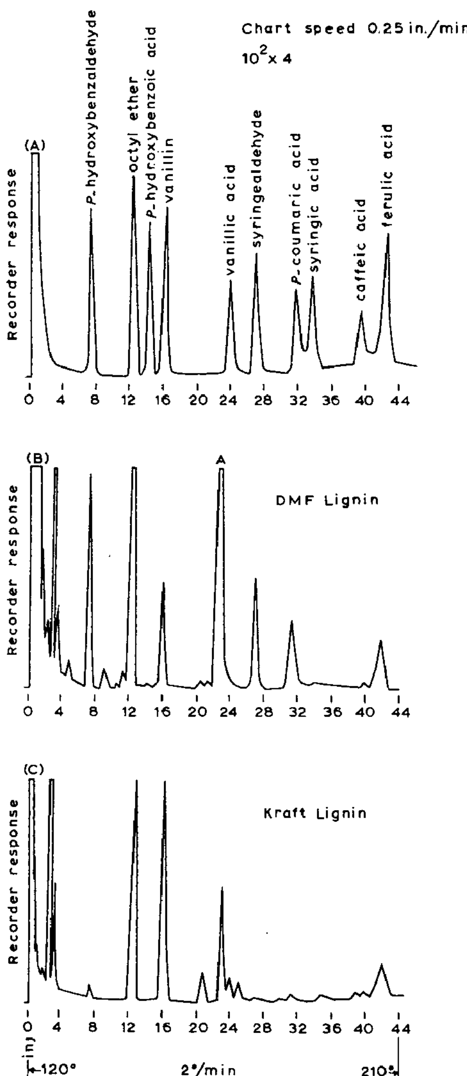

Fig. 1. (A) Chromatograms of an equimolar mixture of standard aromatic aldehydes and acids. (B) Typical chromatogram of alkaline nitrobenzene oxidation products of DMF lignin from a composite mixture of ground corn plant material. (C) Typical chromatograms of alkaline nitrobenzene oxidation products of Kraft lignin.

able by this method. The reproducibility of values between samples from the same lignin for individual components varied between 1–12.5%. Maximum accuracy was achieved when yield of components from the starting material was greater than 5 mg/g of lignin.

The chromatograms of the oxidation products of DMF and Kraft lignins are shown in Fig. 1B and C. Two unidentified peaks, one major (A) and one minor, were observed. The minor peak eluted between syringic acid and caffeic acid. Its concentration was decreased to trace levels by careful purification of the nitrobenzene. How-

TABLE I

RECOVERY OF VANILLIN AND *p*-COUMARIC ACID

Compound	*Amount added (mg)*	*Amount recovered (mg)*	*% recovery*
Vanillin	2.40	2.37	98.9
	3.54	3.59	101.4
	2.75	2.69	97.8
			Av. 99.3
p-Coumaric acid	4.22	4.19	99.3
	4.41	4.38	99.3
	3.55	3.555	100.1
	3.18	3.13	98.4
			Av. 99.3

ever, peak A was always present and probably is a reaction product of nitrobenzene. Neither compound interferes with the analysis of the major phenolic compounds from the oxidation of lignin. Only at high concentrations of compound A was interference in resolution of the vanillic acid observed.

Preliminary observations showed that the recovery of Kraft lignin was reduced by sterilization treatment. However, little if any additional lignin solution occurred during *in vitro* incubation in rumen fluid. The yield of DMF lignin from high temperature sterilized corn was marginally increased. Low temperature sterilization did not appear to affect the yield of DMF lignin. Marginal decreases in DMF lignin are associated with *in vitro* digestion. The recovery of DMF lignin from the *in vivo* digested corn material was reduced. The complete results will be presented in a subsequent paper.

Concentrations of the major phenolic products from the oxidation of the DMF lignins are shown in Table II. Quantitative comparison was not valid with results obtained by others due to the variability of the lignin in the starting material[12–14]. However the orders of magnitude and concentration for the phenolic aldehydes of DMF lignin from the composite mixture of corn leaf and stalk were similar to those reported by GEE *et al.*[7] for corn stalk. The higher absolute amounts of phenolic aldehydes in corn stalk probably reflect the greater concentration of lignin in this material.

The composition of the oxidation products from Kraft lignin was altered by treatment (Fig. 2A). The sensitivity of the method renders the changes in vanillin, syringealdehyde, *p*-coumaric acid and ferulic acid yield between original and treated

TABLE II

YIELDS OF MAJOR PHENOLIC OXIDATION PRODUCTS OF UNTREATED KRAFT AND DMF LIGNIN

Lignin	*Aromatic reaction products*				
	p-Hydroxy-benzaldehyde	*Vanillin*	*Syringe-aldehyde*	*p-Coumaric acid*	*Ferulic acid*
Kraft	1.0 ± 0.12	69.8 ± 2.34	5.8 ± 0.69	1.6 ± 0.20	3.0 ± 0.31
DMF	22.4 ± 2.68	27.8 ± 2.57	33.7 ± 2.11	21.6 ± 2.83	10.6 ± 0.46

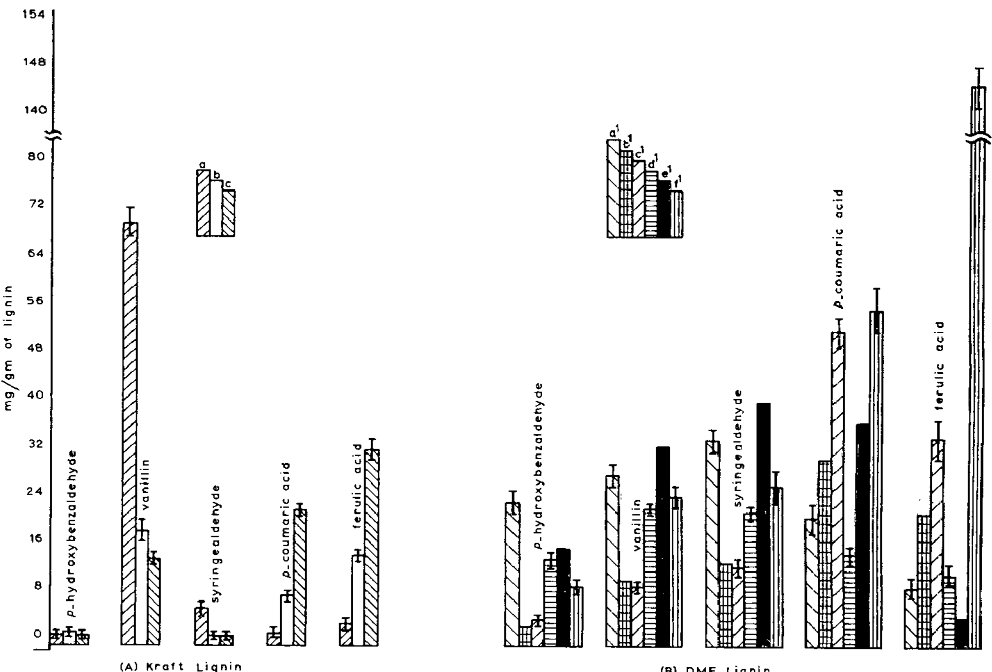

Fig. 2. (A) Effect of treatment on the yield of the five major aromatic oxidation products from Kraft lignin. Explanation of key: a, untreated; b, sterilized at 120°, 15 p.s.i.; c, *in vitro* digestion of high temperature sterilized Kraft lignin in rumen fluid for 36 h. (B) Effect of treatment on the yield of five major aromatic oxidation products from DMF lignin from corn plant. Explanation of key: a^1, untreated; b^1, sterilized at 120°, 15 p.s.i.; c^1, *in vitro* digestion of b^1 in rumen fluid for 36 h; d^1, gas sterilized at 40 ± 5° for 18 h; e^1, *in vitro* digestion of d^1 in rumen fluid for 36 h; f^1, *in vivo* suspension in the rumen for 36 h. I = standard deviation; where not shown insufficient sample was obtained to ascertain significance.

material highly significant. The differences in yield between treatments (b) and (c) are significant only for *p*-coumaric acid and ferulic acid. The most prominent change was a loss in yield of vanillin of 73.4% as a result of sterilization and 79.2% in consequence of sterilization and digestion. Both phenolic acids increased in concentration.

The analysis of the DMF lignin from corn plant which had been subjected to high and low heat sterilization, *in vitro* incubation in rumen fluid, and *in vivo* suspension in the rumen are given in Fig. 2B. Insufficient material was available to establish the significance of the changes in yield of phenolic compounds due to *in vitro* treatments. High temperature sterilization resulted in the reduction of phenolic aldehydes and an increase in phenolic acids. Low temperature sterilization brought about the same pattern of changes in phenolic products but the magnitude of change was smaller. *In vitro* digestion of high temperature sterilized corn material resulted in only minor, insignificant changes in yield of phenolic compounds. *In vitro* digestion of low temperature sterilized corn material was associated with an increase in all phenolic aldehydes and *p*-coumaric acid relative to sterilized material. The yield of vanillin, syringealdehyde and *p*-coumaric acid was also increased relative to the starting material. Comparison of the changes in the yield of phenolic products induced by indi-

vidual treatments suggests that the more severe the pre-digestive treatment, the lower the effects of digestion in an *in vitro* system.

Large differences in yield of phenolic compounds were observed as a result of the *in vivo* treatment of corn material. The sensitivity of the method has rendered the changes in *p*-hydroxybenzaldehyde, syringealdehyde, *p*-coumaric acid and ferulic acid yield between starting material and treated material highly significant. The changes were manifested by a decrease in phenolic aldehydes and increases in both phenolic acids. The ferulic acid yield increased by an order of magnitude.

DISCUSSION

Although lignin is believed to be resistant to the digestive process of the ruminant animal there is evidence that changes occur in the lignin during digestion of forages[15] and under some circumstances lignin itself is digested[16–17]. These studies on the effects of digestion on lignin have largely been based on data obtained by proximate analyses[18,19]. These methods define lignin as a residue resistant to certain chemical treatments and furthermore these methods do not permit the examination of the polymer itself. Therefore, although it is well established that the lignin polymer from different sources varies in composition[2,12], no systematic study of the relationship between lignin structure and digestive alteration has been possible. Most attempts to correlate lignin structure with breakdown have been conducted using model lignin compounds[20,21]. This method, however, has obvious limitations.

The method described here lends itself to an evaluation of chemical composition of lignin possibly irrespective of its method of preparation and permits the evaluation of chemical changes within the lignin polymer brought about by treatments. It is applicable to the characterization of treatment effects on the chemical composition of either isolated lignin or *in situ* lignin.

The application of the method in evaluating the digestion of forages with lignin of varying chemical composition will be reported in a subsequent paper.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. B. L. WALKER of the Nutrition Department for his interest and valuable assistance in developing the GC technique.

Financial support by the Canadian Department of Agriculture and the Ontario Department of Agriculture and Food is gratefully acknowledged.

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CHROM. 4814

A MINIATURIZED ULTRAVIOLET FLOW PHOTOMETER FOR USE IN LIQUID CHROMATOGRAPHIC SYSTEMS*

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(Received May 4th, 1970)

SUMMARY

A miniaturized ultraviolet photometer for continuously monitoring chromatographic column effluents at 254 nm and 280 nm has been built and tested at the Oak Ridge National Laboratory. The instrument is of double-beam design and employs dual solid-state electronic circuits for operation without wavelength switching and multipoint recorder synchronization. Performance tests have shown that the ultraviolet photometer can provide greater sensitivity and less band spreading of chromatographic peaks than the modified spectrophotometer that was used previously.

INTRODUCTION

Several analytical instruments now in use or under development use high-resolution liquid chromatography for the separation step. One instrument of this type, called the UV analyzer, is being developed in the Body Fluids Analyses Program at the Oak Ridge National Laboratory (ORNL). It utilizes high-pressure ion-exchange column chromatography for separation and UV photometry for detection of the UV-absorbing constituents in body fluid samples[1,2].

Prototype systems of the UV analyzer were built at ORNL and are now being tested at several clinical laboratories[3]. Examination of the design of the UV analyzer and of the service records for the prototypes showed that the UV detector (a commercial spectrophotometer modified for automatic wavelength shifting) was a major contributor to the cost, size, and maintenance requirements of the systems. Therefore, it was desirable to replace the large, general-purpose spectrophotometer with a small UV photometer designed specifically for the application at hand.

The criteria established for an acceptable UV photometer include the following:

(1) The detector head must be of the smallest size practical to permit mounting as close to the column discharge as possible.

* Research supported by the National Institute of General Medical Sciences, National Institutes of Health, Bethesda, Md., U.S.A.

** Operated for the U.S. Atomic Energy Commission by Union Carbide Corporation.

(2) The flow cells for the photometer must have a minimal volume, and their bubble-clearing characteristics should be optimized.

(3) Two wavelengths, 280 nm and either 254 or 260 nm, should be monitored continuously.

(4) The photometer must operate as a double-beam instrument at both of the designated wavelengths so that it will be useful with chromatographic systems in which gradient elution is used.

(5) The sensitivity of the photometer must be as high as that of a moderately-priced spectrophotometer equipped with flow cells having a path length of 1 cm.

(6) The instrument should be simple in design, require little maintenance, and have a low fabrication cost.

A number of commercially available UV photometers were examined or tested; however, while each had some acceptable feature, no single model contained all of the required characteristics. Thus the two-wavelength, dual-beam photometer (designated as Mark I) described below was developed to meet the established criteria.

DESCRIPTION

The Mark I photometer system (Fig. 1) consists of a photometer head, a small electronic chassis, and an interconnecting cable.

The photometer head (Figs. 1 and 2) is 1½ in. in diameter and 5 in. long. Reference and sample streams flow upward through 3-mm-I.D. flow cells made of drawn quartz. A low-pressure mercury pencil lamp supplies the 254 nm radiation. Excess 254 nm radiation excites a pair of phosphor rods, which emit 280 nm radiation normally absent from the mercury spectrum. The 254 nm channel consists of reference and measuring beams that cross the respective flow cells near their lower end, a com-

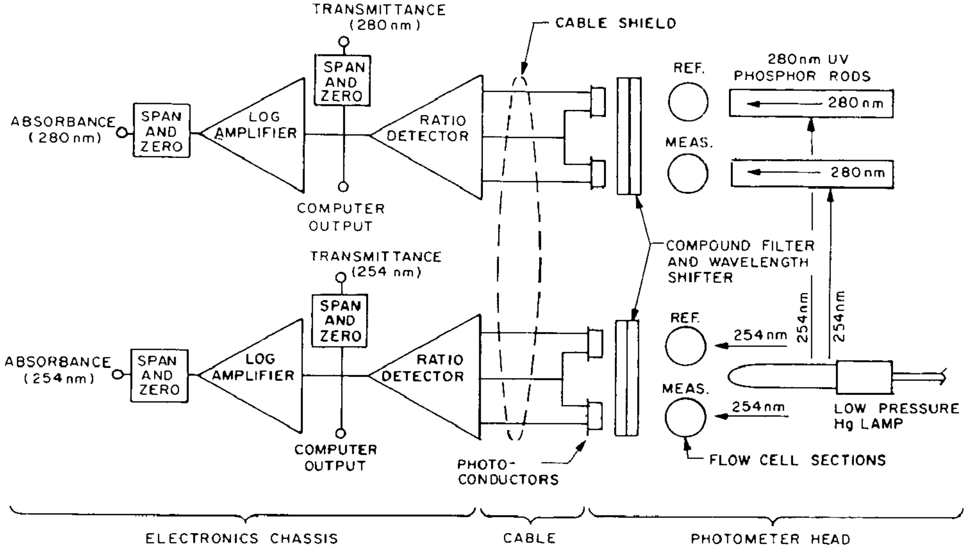

Fig. 1. Schematic diagram of the photometer system.

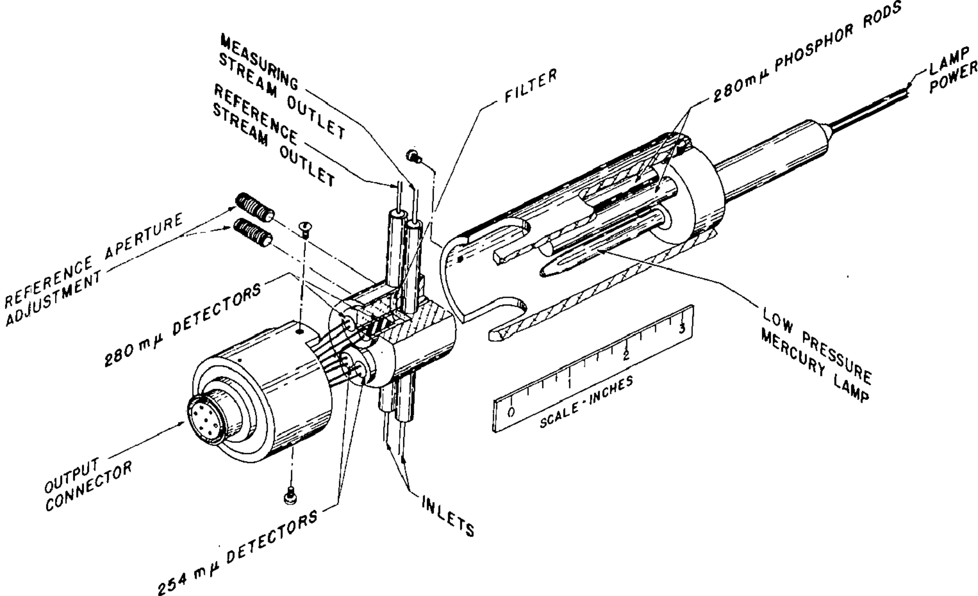

Fig. 2. Two-wavelength UV photometer detector head.

pound optical filter and wavelength shifter, and a pair of photoconductive detectors to sense the intensity of the two transmitted beams. The parallel 280 nm channel crosses the two flow cells near their upper end. The optical apertures of the reference channels can be adjusted to provide reference cell illumination at the same level as that of the measuring cell. The outer cylindrical case with its two end caps supports the photometer capsule, the UV lamp, the phosphor rods, and the signal connector; it also aligns the optical components. The photometer head is connected to the electronics chassis by a six-conductor shielded cable.

The reference and measuring photoconductors* in each channel have the relationship:

$$R = CI^{-k},$$

where R = resistance of photoconductor,
I = illumination level
C = resistance at unit illumination,
k = slope constant for photoconductor (approximately 1 at low light levels).

The signals from the two photoconductors provide the input to a ratio detector (Fig. 1), which, in turn, provides an output voltage E_o:

$$E_o = E_i \left(\frac{R_{\mathrm{REF}}}{R_{\mathrm{MEAS}}}\right) = E_i \left(\frac{I_{\mathrm{MEAS}}}{I_{\mathrm{REF}}}\right)^k = E_i T^k,$$

where E_i = 10 V, T = transmittance, and $k \approx 1$. Accomodations are available for setting the span and the zero point of the ratio detector output so that the trans-

* Clairex type 905 HLL-T, manufactured by Clairex Corp., N.Y., U.S.A.

mittance of the test liquids can be displayed on a 10-mV recorder at any sensitivity from 0–100% to 90–100%.

The output of the ratio detector can also be fed to a logarithmic amplifier, which provides an output E_L:

$$E_L = g \log E_i T^k + gz = g(\log E_i + z) + gk \log T,$$

where g is an adjustable gain and z is an adjustable voltage that can be set as $z = -\log E_i$.
Thus

$$E_L = gk \log T = GA_z,$$

where A_z is the absorbance for an optical path length, z (in cm). However, since $A_{1.0\ \text{cm}} = A_z(1/z)$, the scale factor G can be set to display $A_{1.0\ \text{cm}}$.

The transmittance and absorbance output signals are displayed on a 10-mV multipoint recorder. In addition, transmittance output of 0 to -10 V is available for interfacing with a digital computer.

EXPERIMENTAL RESULTS

The Mark I UV photometer was tested for linearity, sensitivity, and general

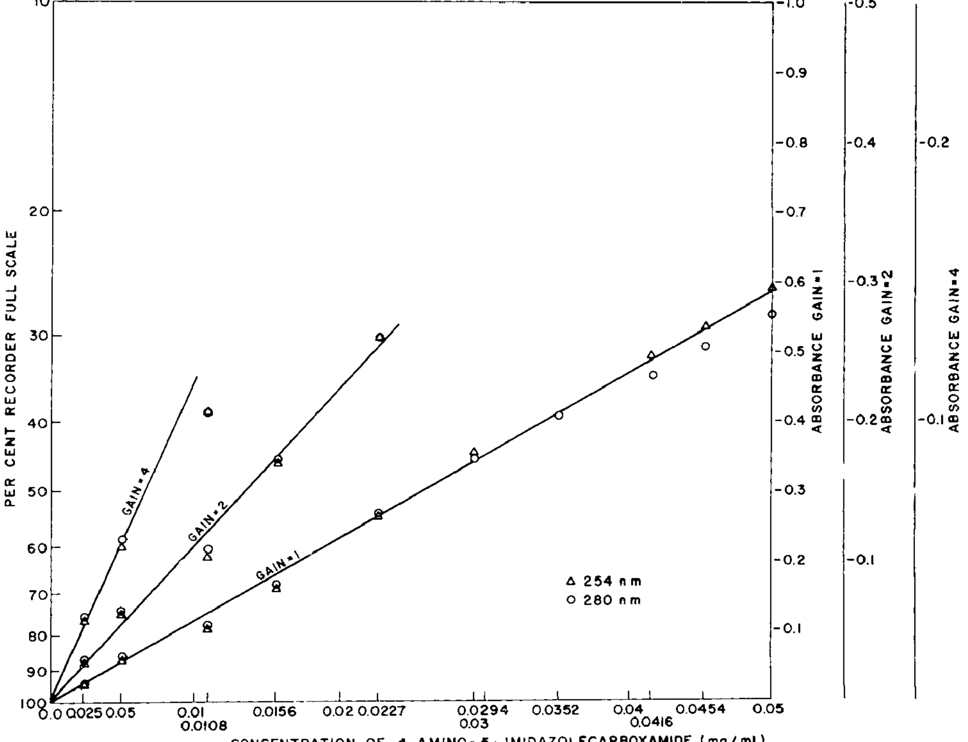

Fig. 3. Calibration curves for the Model Mark I UV photometer.

utility and was found to be suitable for use as a detector in liquid chromatographic systems.

Calibration

The response of the Mark I photometer to aqueous solutions of known compounds was determined and compared with that of a modified Beckman DB spectrophotometer. The photometer was calibrated at absolute electronic gains of 1X, 2X, and 4X, using solutions of 4-amino-5-imidazolecarboxamide. Calibration curves obtained from these tests are shown in Fig. 3. The data show that the dual-beam photometer has essentially a linear response to changes in concentration. Results of similar calibration tests made using the modified DB spectrophotometer indicate that the linearity of the Mark I UV photometer is about the same as that of the DB spectrophotometer.

The effective path length of the photometer, using 3-mm-I.D. quartz tubes as flow cells, was determined by comparing the absorbance values of the reference solution in the photometer with those obtained from the spectrophotometer with cells of 10-mm path length (see Fig. 4). From the slope of the straight line of this plot, the effective path length of the photometer flow cells was found to be 2.6 mm.

Peak broadening

The dispersion number of the Mark I photometer was measured by the pulse injection technique in order to determine the extent to which the photometer contributes to extra-column peak broadening[4]. In this technique, a UV-absorbing substance is injected, as a pulse, into a stream flowing through the flow cells, and the absorbance of the stream is measured as a function of time and recorded on a strip-

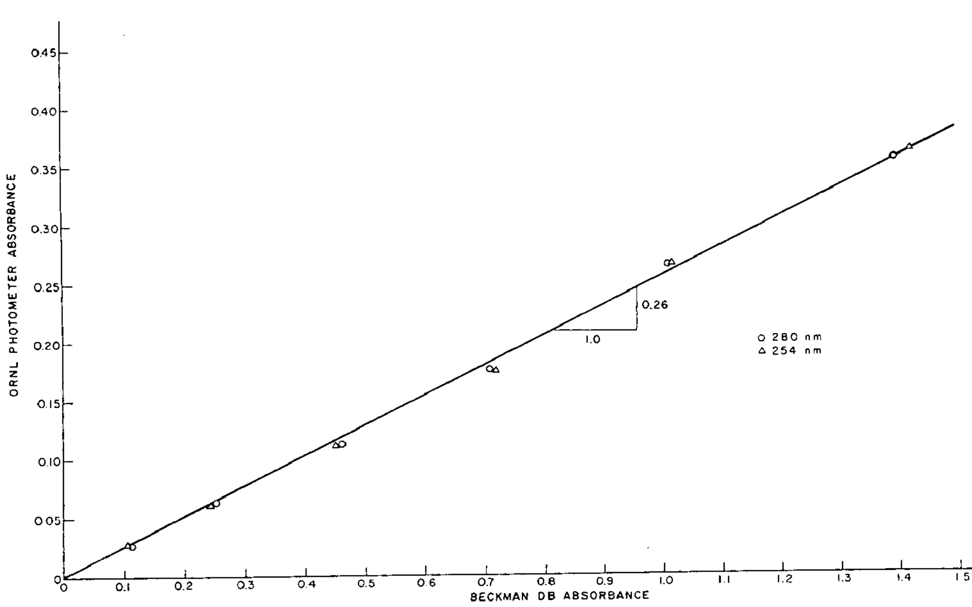

Fig. 4. Comparison of ORNL photometer with Beckman DB.

chart. The resulting increase in peak width is a result of flow through the flow cell and is thus the measure of the peak broadening due to the cell.

The dispersion number is a dimensionless group that uniquely characterizes the degree of longitudinal mixing that occurs during flow. Depending on the experimental setup, this parameter can be determined from experimental concentration *vs.* time curves or by measuring the standard deviation of a chromatographic peak. For the experimental setup used, the dispersion number was determined by:

$$8\left(\frac{D}{\mu L}\right)^2 + 2\left(\frac{D}{\mu L}\right) = \frac{J^2}{E^2}$$

where $D/\mu L$ is the dispersion number; J is the standard deviation of the peak in time units; and E is the mean resident time. A small value for the dispersion number

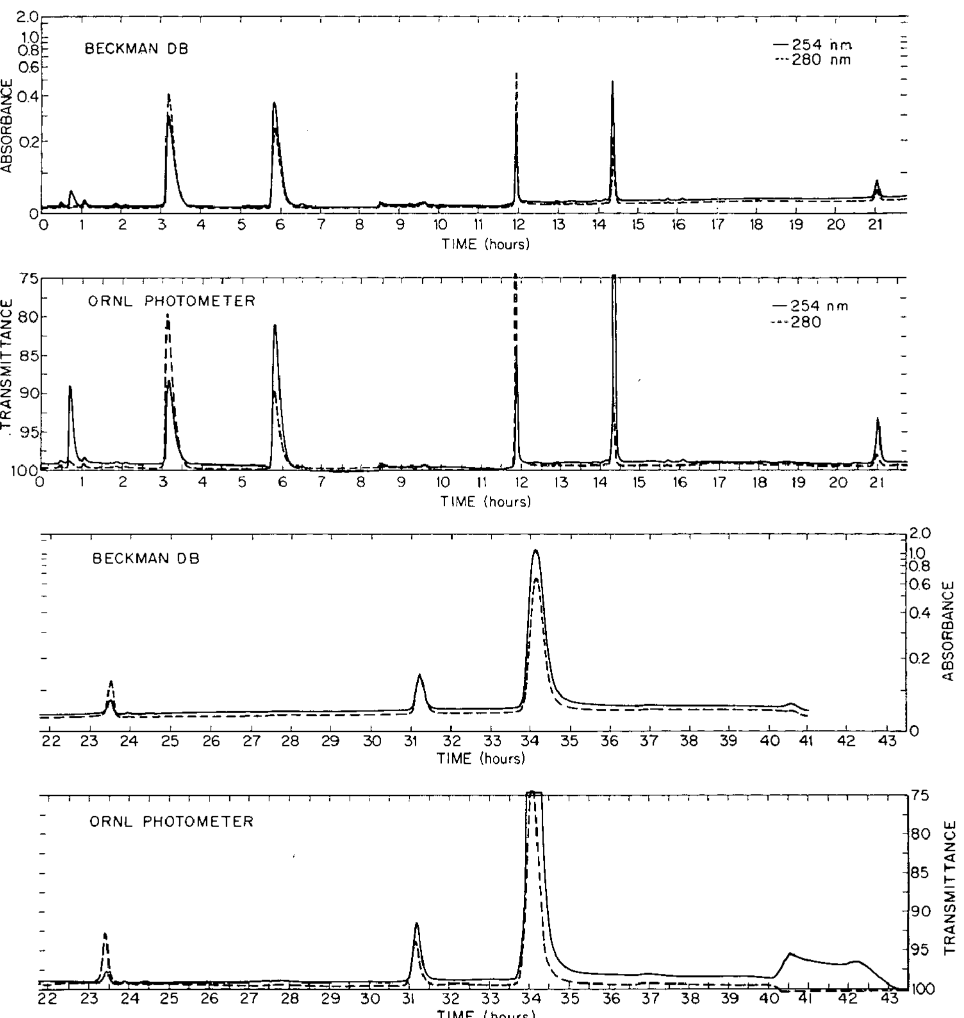

Fig. 5. Comparison of the UV photometer with the Beckman DB spectrophotometer as column monitors.

indicates that there is only a small amount of backmixing or peak broadening and suggests that the cell is well designed.

The dispersion numbers determined for a flow rate of 5.0 ml/h (*i.e.*, the flow rate used with a 0.22-cm-diam. column in the UV analyzer) were 0.032 and 0.045 for the 254-nm portion and the 280-nm portion, respectively, of the photometer flow cell. For comparison, a typical dispersion number for the DB spectrophotometer with a Pyro-Cell* was 0.065. This indicates that the contribution to peak broadening of the Mark I photometer flow cell is significantly less than that of the flow cell used in the DB spectrophotometer.

Chromatographic detector

In order to determine the comparative utility of the Mark I UV photometer and the modified Beckman DB spectrophotometer as chromatographic column monitors, the two instruments were placed in series on a standard Mark II UV analyzer prototype**. Fig. 5 shows the detector signals, as recorded on strip-chart recorders, of the 280 and 254 nm wavelength channels of each instrument. The UV photometer was operated at an electronic gain of 4 × (transmittance range of 75–100%) during these tests, and its response to the separated urinary constituents was somewhat greater than that of the DB spectrophotometer. Also, the chromatographic peaks showed less band spreading. The gain of the photometer can be increased additionally by about a factor of 2 without significantly increasing the noise level.

After this initial test, the Mark I UV photometer was used successfully as the primary detection system in more than 50 UV analyzer runs.

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* Flow cell No. 5011, with a 1-cm path length, from Pyro-Cell Manufacturing Co., Westwood, N.J., U.S.A.

** Available as CAPE-1753 from the Clearing House for Federal Scientific and Technical Information, U.S. Department of Commerce, 5285 Port Royal Road, Springfield, Va. 22151, U.S.A.

CHROM. 4806

THE DETERMINATION OF THE BASE COMPOSITION OF RNA BY HIGH-PRESSURE CATION-EXCHANGE CHROMATOGRAPHY

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(Received April 27th, 1970)

SUMMARY

A method has been developed to determine the base composition of micro- and nanogram quantities of RNA in 4 min. The RNA's were hydrolyzed (either chemically or enzymatically) to their nucleoside level and separated by cation-exchange chromatography. The nanogram quantities of ribonucleosides were separated in less than 4 min by eluting with formate buffer at a flow rate and pressure of 50 ml/h and 3000 p.s.i., respectively. The separation time is reduced to 2 min by eluting at 80 ml/h and 4600 p.s.i. The column was monitored with a sensitive UV detector which permitted absorbance to be recorded linearly and allowed analysis of nanomole to picomole quantities of nucleosides with a relative standard error of 2–3%. Data from four RNA samples are presented. The method is also applicable to the determination of the base composition of DNA since the deoxynucleosides are also separated by the procedure.

INTRODUCTION

The determination of the base sequence of a nucleic acid or oligonucleotide requires hydrolysis (either chemically or enzymatically) followed by separation and quantitation of the resultant products. With the increased interest in the sequencing of nucleic acids has come the need for a rapid and sensitive method for base composition analysis.

Previously, the most widely employed method for the determination of base composition required hydrolysis of the nucleic acid or oligonucleotide to the nucleotide level, followed by separation and quantitation of the resultant mixture by either anion-exchange[1–8] or cation-exchange chromatography[9–13]. An alternative method has been hydrolysis to the nucleoside or N-base level followed by separation and quantitation by cation-exchange chromatography[8,13,14–17] although anion-exchange chromatography[5–6] has been used in some instances.

Recently, UZIEL *et al.*[16] have demonstrated the advantages of enzymatically hydrolyzing nucleic acid to the nucleoside level followed by separation and quantitation by cation-exchange chromatography. Using an 0.6 × 20 cm column filled

with an efficient cation-exchange resin, and by operating at a low flow rate and pressure, they were able to separate and quantitate nanomole quantities of nucleosides in less than 1 h. With this technique they were able to avoid both the isomeric peaks that arise from alkaline hydrolysis of nucleic acids and the more drastic hydrolytic procedures required to realize bases.

To decrease the time required for the nucleoside separation, we have employed a high-resolution liquid chromatograph that utilizes a column filled with a cation-exchange resin of smaller particle size than that used in the method of UZIEL *et al.*[16] By monitoring the system with a sensitive UV photometer and by operating at high linear velocities (1 cm/sec) and pressures (3000–5000 p.s.i.), we demonstrate the separation and quantitation of nano- and picomole quantities of nucleosides in less than 4 min. Using this separation a method is described for the determination of the base composition of transfer and ribosomal RNA hydrolysates.

EXPERIMENTAL

Materials

Chemicals. The ribonucleosides were purchased from either Calbiochem or Sigma Chemicals and the deoxyribonucleosides from Schwarz Bioresearch, Inc. Isopentenyladenosine was kindly supplied by M. SCHWEIZER, Nucleic Acid Institute, International Chemical & Nuclear. Alkaline phosphatase from *Escherichia coli* and venom phosphodiesterase *(Crotalus adamanteus)* were purchased from Schwarz Bioresearch Inc. Yeast tRNA and *E. coli* B tRNA were purchased from General Biochemicals, and *E. coli* K-12 tRNA and *E. coli* Q-13 ribosomal RNA were kindly supplied by Z. B. EGAN and A. D. KELMERS, Oak Ridge National Laboratory.

Resins. The cation-exchange resin VC-10, having a particle size range of 7–14 μ, was kindly supplied by Sondell Scientific Instruments, Inc. The cross-linking in this resin (*i.e.*, % of divinylbenzene in the polymer bead) was 10%. The resin, as received from the manufacturer, was swollen for 24 h in 0.4 *M* ammonium formate, pH 4.50, before column packing.

Methods

Column preparation. A 0.24 × 25 cm stainless steel column was "dynamically packed" with the VC-10 resin as described by SCOTT AND LEE[18]. A short extension (0.54 × 10 cm) was added to the column and both filled with a 50 : 50 slurry of the resin and 0.4 *M* ammonium formate, pH 4.50. The column was packed under a pressure of 1000 p.s.i. using a Milton Roy Minipump, and equilibrated for several hours with buffer. The extension was then removed and the column placed in the chromatographic system.

Chromatographic system. The system used was the Varian Aerograph Model 4100 liquid chromatograph. A schematic of the system is seen in Fig. 1. The chromatograph utilizes a 5000 p.s.i., pulseless, constant-displacement pump capable of delivering solvents at flow rates from 1 to 200 ml/h. It has a reservoir capacity of 250 ml. The pressure generated by the pump is monitored by means of a pressure-sensitive transducer and displayed on an optical meter. A safety circuit is incorporated into the system which allows the operator to select any pressure limit up to 5000 p.s.i. Additional protective devices automatically shut down the pump if the pressure

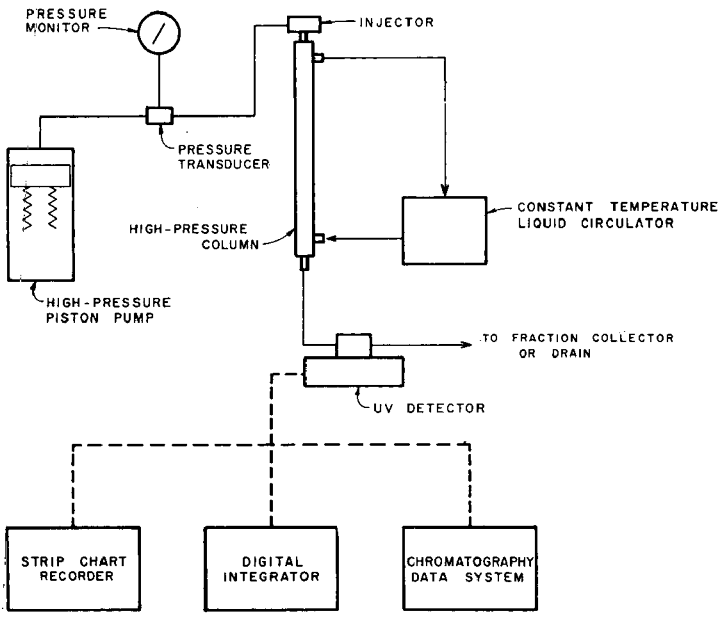

Fig. 1. Schematic of the Varian Aerograph Model 4100 liquid chromatograph.

exceeds 5050 p.s.i. and 6000 p.s.i., respectively. The sample is introduced into the column by a Hamilton syringe through a septumless injector which is attached to the chromatographic column. Water-jacketed columns are constructed of 0.24 cm I.D. 316 stainless steel tubing. A 0.5 μ stainless steel frit supports the column bed and prevents resin from contaminating the column detector. The temperature of the column was maintained within $\pm$ 0.01° of the desired temperature by means of a liquid bath. The column effluent is monitored by a sensitive UV photometer, operating at 254 nm, which is equipped with a cylindrical flow cell having a 1-mm diameter and 10-mm path length (*i.e.*, 8-μl cell volume). The detector output is linear in absorbance units, therefore, linear with respect to solute concentration in accordance with Beer's Law. Full-scale absorbance ranges from 0.005 up to 0.64 are provided in binary steps. In addition, a non-linear high absorbance range is provided for qualitative monitoring of highly absorbing samples. The photometer output (10 mV) is displayed on a strip chart recorder or fed into a digital integrator or a chromatography data handling system for data acquisition and processing. The column effluent, after passing through the detector, is routed either to a drain or to a fraction collector.

Sample preparation. The RNA samples were hydrolyzed to their nucleoside level by either (I) alkaline hydrolysis with 1 *N* sodium hydroxide followed by enzymatic hydrolysis with alkaline phosphatase, or (II) a dual enzymatic hydrolysis using a combination of venom phosphodiesterase and alkaline phosphatase. *Procedure I.* A 20-μl aliquot containing 6 μg of RNA was placed in a small glass test tube and diluted with 10 μl of 1 *N* sodium hydroxide and incubated for 40 min at 80°. UZIEL *et al.*[16] have reported this time sufficient for quantitative hydrolysis with minimal loss of major components. The sample was partially neutralized with

9 μl of 1 N formic acid and adjusted to pH 8.8 with 20 μl 0.2 M ammonium acetate. Approximately 0.15 units of alkaline phosphatase in 4 μl of H_2O were added and the solution incubated for 4 h at 37°. A 5-μl aliquot representing 0.5 μg of hydrolyzed RNA was analyzed. *Procedure II.* Three micrograms of RNA were incubated for 4 h at 45° in 20 μl of 0.2 N sodium acetate, pH 8.8, containing 0.023 M magnesium acetate, 0.03 unit/ml of venom phosphodiesterase, and 7.4 units/ml of alkaline phosphatase. Two to five microliters of the hydrolysate representing 0.3 to 0.7 μg of RNA were then analyzed.

Calibration. Stock solutions of the four common ribonucleosides were prepared by dissolving 6 mg of each nucleoside in 10 ml of distilled water. Each stock solution was diluted 1 : 100 with distilled water and precisely calibrated by UV spectrophotometry. A working nucleoside mixture, containing 0.06 mg/ml of each nucleoside, was prepared by pipetting 1 ml of each nucleoside stock solution into a common vial and diluting the resultant mixture to 10 ml with water. Aliquots of this mixture were then chromatographed and the nucleoside peak areas determined from the resultant chromatograms by peak area integration. Response factors for each nucleoside were obtained by dividing the quantity of each nucleoside by its respective peak area.

Cation-exchange chromatography. To inject a sample the flow was stopped, pressure reduced to atmospheric by means of a valve, and the sample injected through the septumless injector directly into the column bed with a 10-μl Hamilton syringe. The syringe was withdrawn, the injector closed, and the flow rate and pressure quickly (<30 sec) brought to the desired level by means of a fast pump switch on the pump control module. Since the separation requires only a single eluent, no regeneration of the column was necessary between chromatographic runs.

Calculation of base composition. Response factors for each nucleoside were obtained as described under "calibration". The nucleoside peak areas from the RNA hydrolysates were determined by peak area integration. The quantity of nucleoside that each peak represented was obtained by multiplying each peak area by its respective response factor. Within each sample the nucleoside quantities may be normalized to obtain a base composition on a weight % basis or converted to moles and normalized to a mole % basis. The data presented here have been calculated on the mole % basis.

RESULTS

Fig. 2 illustrates a typical chromatogram obtained from the analysis of an aliquot of the standard nucleoside mixture. An aliquot of the standard mixture containing 0.1 μg of each nucleoside was separated in less than 4 min by eluting with 0.4 M ammonium formate at a flow rate and pressure of 50 ml/h and 3000 p.s.i., respectively. The separation time was reduced to less than 2 min by increasing the flow rate and pressure to 80 ml/h and 4600 p.s.i., respectively (Fig. 3). The 4-min, rather than the 2-min separation, was used for the quantitation of the RNA hydrolysates since the increased resolution, obtained at the lower flow rate, facilitated the integration of the individual peak areas.

Fig. 4 illustrates the four chromatograms obtained from the analysis of the alkaline hydrolysates of RNA. Separation of the four ribonucleosides was complete

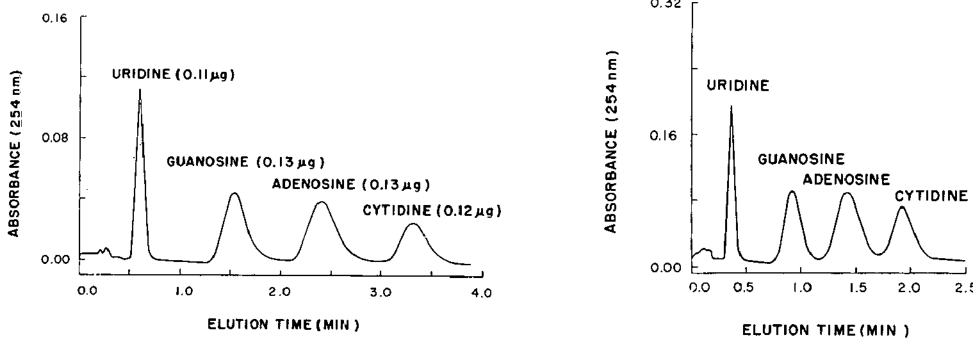

Fig. 2. Separation of the ribonucleosides by high-pressure, cation-exchange chromatography. Conditions: column, 0.24 × 25 cm; resin, 7–14 μ VC-10 (10X); eluent, 0.4 *M* ammonium formate, pH 4.50; flow rate, 50 ml/h; pressure, 3000 p.s.i.; column temperature, 75°; sample, mixture containing 0.1 μg of each nucleoside.

Fig. 3. Rapid separation of the ribonucleosides by high-pressure, cation-exchange chromatography. Conditions: column, 0.24 × 25 cm; resin, 7–14 μ VC-10 (10X); eluent, 0.4 *M* ammonium formate, pH 4.50; flow rate, 80 ml/h; pressure 4600 p.s.i.; column temperature 75°; sample, mixture containing 0.2 μg of each nucleoside.

in less than 4 min and the nucleoside peak areas were used to determine the base composition of the RNA's as described in METHODS. Since unhydrolyzed RNA and nucleotides were found to elute at the same time as the large peak that preceded the uridine peak, the column eluent containing this peak was collected and tested for its RNA and nucleotide content. Complete hydrolysis to the nucleoside level was indicated in both procedures as this fraction contained neither unhydrolyzed RNA, as determined by the procedure of MILLER *et al.*[19], nor nucleotides, as determined by the procedure of HORVATH *et al.*[7]. Chromatography of a sample blank indicated that this peak was due to the enzyme and neutralization salt of the hydrolytic procedure. Since this peak was resolved from those of the nucleosides and did not contain material of nucleic acid origin, it did not interfere with the quantitation.

The base compositions, as determined from their alkaline hydrolysates, of the four RNA samples are found in Table I. The base composition of both the B and K-12 strains of *E. coli* transfer RNA were similar. The yeast tRNA contained similar quantities of guanosine and adenosine when compared with the *E. coli* strains, but was characterized by a decreased cytidine and increased uridine content. As expected, the ribosomal RNA had a different base composition than did the tRNA's as it had a lower guanosine and cytidine content and a higher uridine and adenosine content. In all of the samples the guanosine content was highest, representing 30–34 mole % of the individual RNA samples.

To minimize decomposition of alkaline labile components or to hydrolyze alkali-resistant linkages, a dual enzymatic hydrolysis may be performed with the RNA first being hydrolyzed to the nucleotide level by venom phosphodiesterase and then to the nucleoside level by alkaline phosphatase. The four RNA samples were therefore enzymatically hydrolyzed (Procedure II) and the chromatograms obtained from analysis of the hydrolysates are seen in Fig. 5. In general, the chromatograms are similar to those obtained from the alkaline hydrolysates with the four nucleosides

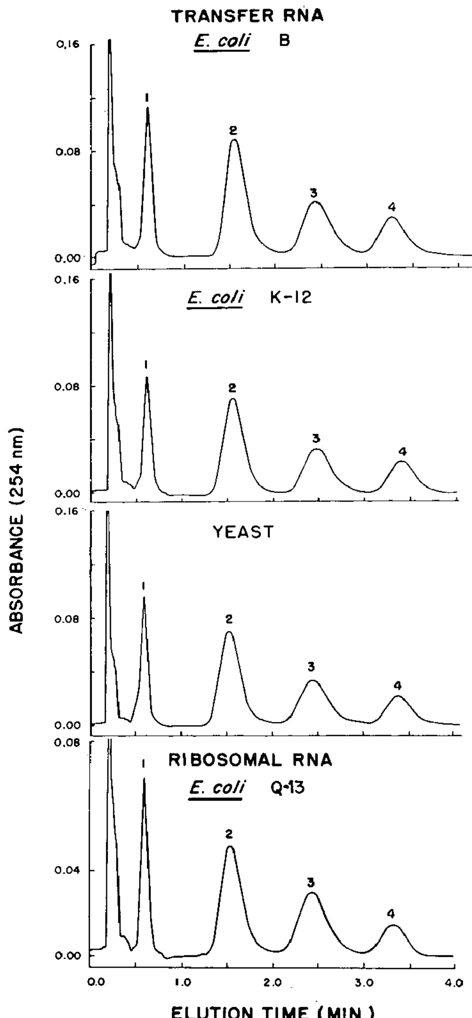

Fig. 4. Separation of the ribonucleosides obtained from the alkaline hydrolysis of various RNA samples. Conditions: column, 0.24 × 25 cm; resin, 7–14 µ VC-10 (10X); eluent, 0.4 *M* ammonium formate, pH 4.50; flow rate, 50 ml/h; pressure, 3000 p.s.i.; column temperature, 75°; sample, aliquots representing 0.5 µg of RNA that had been hydrolyzed by a combination of NaOH and alkaline phosphatase hydrolysis (see text for hydrolysis conditions). 1 = Uridine; 2 = guanosine; 3 = adenosine; 4 = cytidine.

being separated in less than 4 min. A noticeable difference is the reduction in the large peak eluting before uridine. Analysis of this peak for its unhydrolyzed RNA and nucleotide content proved negative. The subsequent chromatography of an aliquot of a sample blank indicated that this peak was due to the enzymes used in the hydrolysis. The smallness of this peak, when compared with the large peak in the alkaline hydrolysate chromatograms, indicates that the size of the latter was due to the sodium formate salt arising from the neutralization of the NaOH with the formic acid. It is probable that the detector response to this salt peak is not due to

TABLE I

MOLAR BASE COMPOSITION OF ALKALINE HYDROLYZED[a] RNA

Chromatographic conditions: column, 0.24 × 25 cm; resin VC-10 (7–14 μ); flow rate, 50 ml/h; pressure, 3000 p.s.i.; monitored at 254 nm, full scale either 0.08 or 0.16 absorbance units; eluent, 0.4 *M* ammonium formate, pH 4.50.

RNA type	*Mole %*			
	Uridine	*Guanosine*	*Adenosine*	*Cytidine*
Transfer				
E. coli B	19.6[b]	33.5	20.9	26.1
E. coli K-12	19.9	33.6	21.0	25.7
Yeast	22.1	33.9	21.1	23.0
Ribosomal				
E. coli Q-13	23.5	30.7	23.9	21.9

[a] Samples hydrolyzed to their nucleoside level by a combination alkaline (NaOH) and alkaline phosphatase hydrolysis.

[b] Numbers represent the mean ± relative standard error of 2–3% for nine determinations.

the UV absorption of the salt, since sodium formate has a low molar absorptivity at 254 nm. Instead, it is probably dependent upon a change in refractive index since it has been previously demonstrated that the small dead volume UV detectors are sensitive to changes in the refractive index[17] of the solvent.

The base composition was determined for the enzyme hydrolysates of the four RNA samples (Table II). Again, a close similarity was observed for the base composition of the two strains of *E. coli* tRNA. Yeast tRNA had similar adenosine and guanosine content, but had a decreased cytidine and increased uridine content when compared to the *E. coli* samples. The ribosomal RNA had lower guanosine and cytidine and higher adenosine and uridine content than did the transfer RNA's.

In general, the base compositions of the tRNA's were similar using either hydrolysis procedure. The slight increase in the uridine content of the alkaline

TABLE II

MOLAR BASE COMPOSITION OF ENZYMATIC HYDROLYZED[a] RNA

Chromatographic conditions: the same as in Table I.

RNA type	*Mole %*			
	Uridine	*Guanosine*	*Adenosine*	*Cytidine*
Transfer				
E. coli B	18.3[b]	34.0	21.7	26.0
E. coli K-12	17.9	34.6	21.7	25.8
Yeast	19.3	34.9	21.9	23.9
Ribosomal				
E. coli Q-13	20.4	32.9	22.9	23.8

[a] Samples hydrolyzed to their nucleoside level by enzymatic hydrolysis using venom phosphodiesterase and alkaline phosphatase.

[b] Numbers represent the mean ± relative standard error of 2–3% for nine determinations.

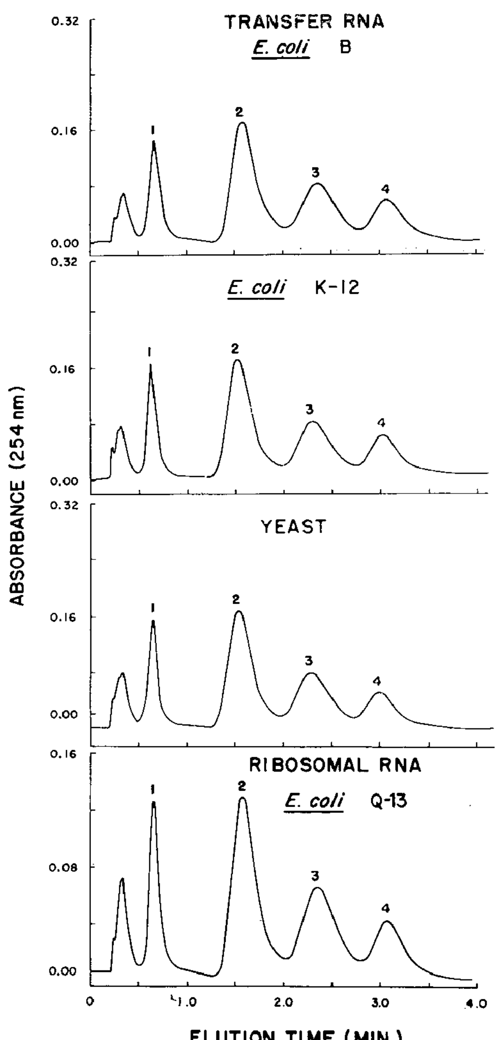

Fig. 5. Separation of the ribonucleosides obtained from the enzymatic hydrolysis of various RNA samples. Conditions: column, 0.24 × 25 cm; resin, 7–14 μ VC-10 (10X); eluent, 0.4 M ammonium formate, pH 4.50; flow rate, 50 ml/h; pressure, 3000 p.s.i.; column temperature, 75°; sample, aliquots representing 0.3–0.7 μg of RNA that had been hydrolyzed by a combination venom phosphodiesterase and alkaline phosphatase (see text for hydrolysis conditions). 1 = Uridine; 2 = guanosine; 3 = adenosine; 4 = cytidine.

hydrolysates, when compared to the enzymatic hydrolysates, is probably due to deamination from the alkaline conditions of Procedure I. The resultant deaminated products (*i.e.*, xanthosine, inosine and uridine) would elute with the uridine peak which, upon quantitation, would result in an increase in the uridine content of the sample. However, the observed increase was small, which confirms the work of UZIEL *et al.*[16], who found minimal deamination under the conditions of Procedure I.

The largest difference in the base composition as determined by the two

TABLE III

ELUTION PARAMETERS OF THE NUCLEOSIDES OF THE UNUSUAL BASES

Chromatographic conditions: column, 0.24 × 25 cm; resin, Sondell VC-10; flow rate, 25 ml/h; pressure, 1400 p.s.i.; temperature, 75°; eluent, 0.4 *M* ammonium formate, pH 4.5.

Nucleoside	*Elution time (min)*	*Elution volume (ml)*
Dihydrouridine	1.2	0.50
Pseudouridine	1.4	0.58
Uridine	1.5	0.62
5-Hydroxyuridine	1.6	0.67
Thymine riboside	1.6	0.67
Deoxyuridine	1.9	0.79
Thymidine	1.9	0.79
Xanthosine	2.4	1.00
Inosine	2.4	1.00
7-Methylxanthosine	2.4	1.00
Guanosine	3.3	1.37
Deoxyguanosine	4.5	1.87
Adenosine	4.7	1.96
Cytosine arabinoside	5.7	2.37
Cytidine	6.1	2.54
6-Methyladenosine	6.3	2.62
Deoxyadenosine	6.5	2.71
Deoxycytidine	6.8	2.83
6-Dimethyladenosine	11.8	4.92
Isopentenyladenosine	15.1	6.29
3-Methylcytidine	26.0	10.83
7-Methylinosine	30.0	12.49
1-Methyladenosine	30.5	12.70
7-Methylguanosine	52.8	22.0

hydrolysis procedures was observed with ribosomal RNA. A 2% increase in both guanosine and cytidine and a 4% decrease in uridine content was observed when the enzymatic hydrolysis is compared to the alkaline hydrolysis. Again, this is probably due to deamination of the guanosine and cytidine to xanthosine and uridine under the alkaline conditions of Procedure I. Since the possibility of deamination does exist with alkaline hydrolysis, it is probable that the milder enzyme hydrolysis gives a more accurate measurement of the actual base composition of an RNA sample.

Since tRNA is known to contain "unusual bases", samples of the nucleosides of several of these bases were obtained and aliquots of solutions of them were chromatographed. Table III indicates the elution times and volumes of these nucleosides. The elution times and volumes of the eight common ribo- and deoxynucleosides are also included in this table for comparative purposes. To obtain increased resolution, the flow rate and pressure for the separation of the nucleosides of the unusual bases to the separation was decreased to 25 ml/h and 1400 p.s.i., respectively. Under these conditions, nucleosides of the unusual bases were found in the three tRNA hydrolysates with the yeast tRNA hydrolysate containing the highest number of these compounds. The enzymatic hydrolysis (Procedure II) was preferred, when analyzing for the nucleosides of the unusual bases, since many of these compounds are labile to the alkaline condition of Procedure I. The nucleosides of the unusual bases were not found in the ribosomal RNA hydrolysates.

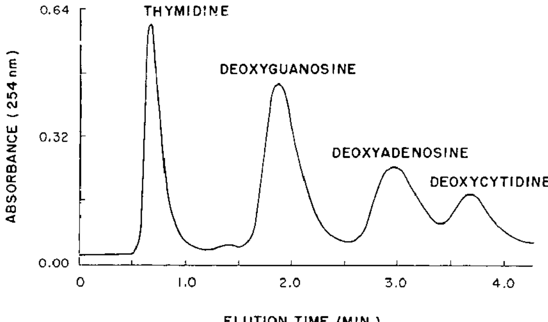

Fig. 6. Separation of deoxyribonucleosides by high-pressure, cation-exchange chromatography. Conditions: column 0.24 × 25 cm; resin, 7–14 μ VC-10 (10X); eluent, 0.4 *M* ammonium formate, pH 4.50; flow rate, 50 ml/h; pressure, 3000 p.s.i.; column temperature, 75°; sample, mixture containing 0.4 μg of each deoxynucleoside.

Although the base composition of DNA was not determined in this study, the method should be applicable for such an analysis since a mixture of the four deoxynucleosides commonly found in DNA was found to separate in less than 4 min (Fig. 6). Therefore, a DNA sample, hydrolyzed to its nucleoside level, could be separated and quantitated as previously described. It should be noted, however, that the enzymatic hydrolysis (Procedure II) would have to be used due to the resistance of DNA to alkaline hydrolysis.

DISCUSSION

The advantage of operating columns, filled with an efficient cation-exchange resin of small particle size, at high flow rates and pressures is obvious. The time required to separate a mixture of the four common ribonucleosides has been reduced to less than 4 min. Previously, one was only able to attain these speeds of analysis with gas chromatography[20–22]. However, the gas chromatography analysis of the nucleosides requires derivatization of the nucleosides, which consists of several reaction steps. Using liquid chromatography, we have shown rapid separations of the nucleosides without the need for a time-consuming derivatization procedure.

The RNA base compositions determined in this study, using either hydrolysis procedure, agree with published data[11,23–34]. For example, the base composition of *E. coli* B tRNA, expressed as nucleoside mole %, was reported[11,24] to be uridine 20.0–21.4%, guanosine 31.0–33.7%, adenosine 18.2–19.6%, and cytidine 28.0–28.1%. For *E. coli* ribosomal RNA nucleoside mole % values of 21.1–21.4% uridine, 31.5–33.8% guanosine, 23.6–25.0% adenosine, and 21.4–22.1% cytidine have been reported[11,23]. These values are similar to those reported here (Tables I and II) but the techniques used to obtain them are characterized as being time consuming and lacking in sensitivity. In addition, the precision of these techniques ranged from 2–8% as compared to the 2–3% obtained with the method presented here.

Using a sensitive UV photometer as a column monitor, the method was found

to be very sensitive. The results presented here were obtained by hydrolyzing a 3–6 μg sample of RNA, of which a 0.5-μg aliquot was chromatographed and quantitated with a relative error of 2–3%. For this size of sample, the detector was operated at either 0.16 or 0.32 absorbance units full scale. Since the detector can be further attenuated, the method could be used for base composition determination on nanogram samples of RNA. However, by using smaller sample sizes and operating at the higher sensitivities, the accuracy and precision of the method would be expected to decrease due to the difficulties arising in the handling and analysis of these small quantities.

A note as to the operation and stability of conventional cation-exchange resins at high pressures: When a new column is operated at high pressures, we have observed an initial settling of the column bed. However, after the initial settling, the column will operate routinely at pressures up to 5000 p.s.i. with no further compression of its bed. For example, we have operated the column used in this study for 6 months, without encountering difficulty. We attribute the stability of this resin to high pressure to the fact that we have used resin of 10% cross-linkage* which contributes to the mechanical stability of the resin. The compression of columns filled with exchange resin was previously reported[25] but 4% cross-linked resins were used in these studies. In addition, the VC-10 resin was a narrow cut of small particle size which, when "dynamically packed", has been shown by SCOTT AND LEE[18] to yield a uniformly packed column bed that is operable at high pressures. In addition to the 10% cross-linked cation resin used in this study, we have used 8% cross-linked cation resin from two manufacturers and have observed no difficulty in operation and stability of these resins at pressures up to 5000 p.s.i.[26]. The feasibility of using conventional anion-exchange resin at high pressures has been previously demonstrated by SCOTT and co-workers[27–29].

In conclusion, this study has shown that base composition analysis of RNA may be determined rapidly, precisely and accurately by liquid chromatography. Using conventional cation-exchange resin as the chromatographic support and by operating at high flow rates and pressures, the four ribonucleosides are separated in less than 4 min. Since a single solvent is used, no equilibration between analyses is required. A sensitive UV detector used as a column monitor allows analysis of nanomole to picomole quantities of nucleosides with a precision of 2–3%. The method is also applicable to DNA base composition analysis since the four deoxynucleosides are also separated in less than 4 min.

ACKNOWLEDGEMENTS

The contributions of the following people are gratefully acknowledged: JIM PATTERSON of Sondell Scientific Instruments, Inc., who provided as a gift the cation-exchange resin used in these studies; ZANE EGAN and A. D. KELMERS of the Oak Ridge National Laboratory, who provided the *E. coli* K-12 tRNA and Q-13 ribosomal RNA samples; and to MARTIN SCHWEIZER of the Nucleic Acid Institute, International Chemical & Nuclear, who provided the isopentenyladenosine sample.

* %cross-linking indicates the % of divinylbenzene that is incorporated into the polymer beads prior to attaching ionic groups.

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CHROM. 4813

AN AUTOMATED SYSTEM FOR ION-EXCHANGE CHROMATOGRAPHY OF ACID-SOLUBLE NUCLEOTIDES AT THE NANOMOLE LEVEL

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(Received May 1st, 1970)

SUMMARY

An automated ion-exchange column chromatography system for the separation of acid-soluble nucleotides from various biological sources is presented. This system, which is a modification of earlier methods used, gave satisfactory recoveries of the nucleotides at the nanomole level.

INTRODUCTION

The study of the free nucleotides of a micro-organism is often laborious because of the complex nature of the nucleotide pool. Furthermore, the rapid growth of the organism and the technical difficulties in obtaining the extract may complicate the analysis[1-3]. In order to study the qualitative and quantitative variation of a nucleotide pool of a micro-organism under *e.g.* different growth conditions, an analytical method with good resolution and sensitivity is necessary.

Separation of nucleotides by means of ion-exchange chromatography was introduced by COHN[4,5], gradient elution was added to the procedure by HURLBERT *et al.*[6] and SCHMITZ *et al.*[7], and since then several other modifications have been published[8-13]. In addition to ion-exchange resins, cellulose based exchangers have been used[1,2,14-19]. Two-dimensional paper chromatography, paper electrophoresis combined with paper chromatography, ion-exchange paper chromatography, and recently thin-layer chromatography have also been used for nucleotide separations[1,2,20-35].

The optimum amount of nucleotides suitable for paper chromatography is 10–200 μg, for paper electrophoresis 100–500 μg, for ordinary thin-layer chromatography 0.2–30 μg per spot, and for column chromatography from 50 μg up to several hundred mg per column.

The best resolutions in the separation of complex nucleotide mixtures are obtained by thin-layer and column chromatography[34]. Column chromatography seems to be most suitable for primary separations of complex nucleotide mixtures because it can be used both as an analytical and a preparative tool simultaneously.

In our studies on the nucleotide metabolism of mycoplasma, ion-exchange column chromatography was used for the separation and partial identification of the

nucleotides. In this connection we have further modified the separation methods previously used in this laboratory[2,3]. As a result an automated, sensitive and flexible system, useful for the analysis of nucleotides of biological material, is presented.

MATERIALS AND METHODS

Chemicals

All chemicals were of analytical grade. The nucleotide standards were purchased from Calbiochem, Luzern, Switzerland, and checked for purity by means of paper chromatography.

Biological test sample

The biological test sample was a cold perchloric acid extract, 2–40 ml[2,3] of cells of *Mycoplasma laidlawii* A, grown in a liquid medium[36].

Anion-exchange columns

The resin was Dowex-2, 200–400 mesh, 8% cross linked, converted to the HCOO-form, and packed into conventional glass columns. The regeneration of the columns was performed as described previously[2,3].

All connections were made by means of small-bore teflon or acid-resistant rubber tubing (I.D. 0.045 and 0.081, "Acidflex", Technicon Corp., Tarrytown, New York, U.S.A.) and by using teflon connectors from the same manufacturer.

Components of the automatic system

A constant flow of the solutions from a gradient mixer (Varigrad, rectangular model, nine compartments, 500 ml each, Buchler Instruments Inc., Fort Lee, N.J., U.S.A.) was achieved by using a reciprocal pump (Accu-Flo, flow rate 3–700 ml/h, Beckman Instruments Inc.). The elution was followed at two wavelenghts (260 and 275 nm) with a multiple sample recorder (Gilford 2000, Gilford Instruments Laboratories Inc., Oberlin, Ohio, U.S.A.), which was used in combination with a Beckman DU Monochromator (Beckman Instruments Inc., Palo Alto, Calif., U.S.A.). The effluent was collected in 5 ml fractions (Radi Rac Universal fraction collector, with distributor and controller, LKB, Stockholm, Sweden), and these were marked on the chart paper by an event marker connected to the system.

Recovery of nucleotides

The primary identification of the components was made from the elution pattern and the ratio of $E_{275} : E_{260}$, which is characteristic for each individual nucleotide base. The final identification was made after the fractions were combined, lyophilized and purified as reported earlier[2,3].

EXPERIMENTAL

The improvements in the resolution and sensitivity of the nucleotide column chromatography techniques were achieved in the following manner.

Gradient

Several different formic acid–ammonium formate gradients were tested. The best resolution of the nucleotides from our biological extracts was obtained with increasing concentrations of formic acid and ammonium formate, in seven successive chambers of the gradient mixer, as follows: chamber 1: distilled water; chamber 2: 0.5 *M* formic acid; chambers 3–4: 4.0 *M* formic acid; chambers 5–7: 4.0 *M* formic acid–1.0 *M* ammonium formate. The volume of liquid in the chambers was 150–300 ml depending on the desired slope of the gradient. The best flow rate of the eluant proved to be 25–36 ml/h.

Column

Preliminary separations of large samples of biological material were performed with ordinary 1.0 × 22.0 cm glass columns. 0.8 × 12.0 cm columns proved to be practical and gave even better resolution in the separation of small amounts of nucleotides than columns with larger volumes of ion-exchange resin.

Flow cells

The detection sensitivity of the system was satisfactory with the original microcuvettes with a 10 mm light path. In practice, however, the elution of the column with a formic acid–ammonium formate gradient caused the formation of gas bubbles which were trapped in these flow cells, thus making automatic UV-monitoring impossible.

This detrimental interference was avoided by our modification of the flow cell (Fig. 1). The roof of the cell was made to slope in order to allow the bubbles to flow through the cell without entering the light path. The angle of inclination of the roof was kept as small as possible (30°, Fig. 1) in order to avoid loss of sensitivity due to any unnecessary increase of the cell volume. The final volume of the cuvette was

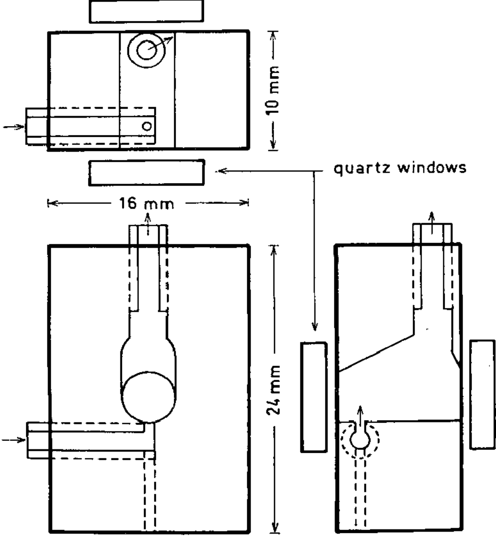

Fig. 1. A flow cell, drilled out of teflon, 0.25 ml capacity, 10 mm optical path length.

0.25 ml, the light path 10 mm, and the diameter of the cuvette window 4 mm. The flow cell was simply drilled out of a teflon block size 10 × 16 × 24 mm. Short pieces of teflon tubing were cemented to the outlet and inlet holes for attachment to the flexible tubing. The holes which were not used (dotted line in Fig. 1) were filled with cement.

UV-recording

The recorder was calibrated to the desired level of sensitivity with standard solutions of adenine of known absorbance measured with a Beckman DK-1 spectrophotometer. With maximum sensitivity a full-scale deflection on the Gilford-recorder corresponded to 0.05 absorbance units measured at 260 nm with a 10 mm light path.

The ratio between the readings at 260 and 275 nm is characteristic for each purine and pyrimidine base and therefore a valuable aid in the identification of the different peaks. For this purpose a dual-wavelength selector was connected to the UV-monitoring system, which made it possible to make recordings alternatively at 260 and 275 nm.

Background neutralization

In nucleotide separations with a formic acid–formate gradient, the increasing ammonium formate concentration of the effluent causes the base-line to drift, especially during the latter part of the run, thus disturbing the detection and quantitative evaluation of small fractions. We were able to avoid this by using two modified flow cells together with an automatic blank compensator. This assembly maintained the base-line constant when the eluant was run through the reference cell, and the effluent through the measuring cell.

Procedure in practice

The assembly of apparatus is represented schematically in Fig. 2. The recording was started and the sample was run through the column at a rate of 0.1–0.5

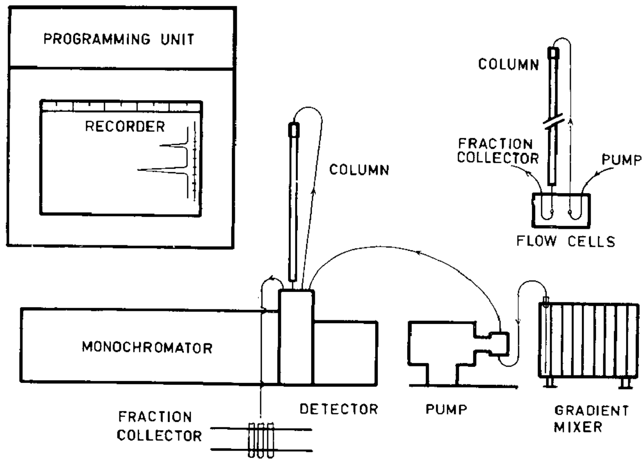

Fig. 2. Scheme for the automatic column chromatography apparatus.

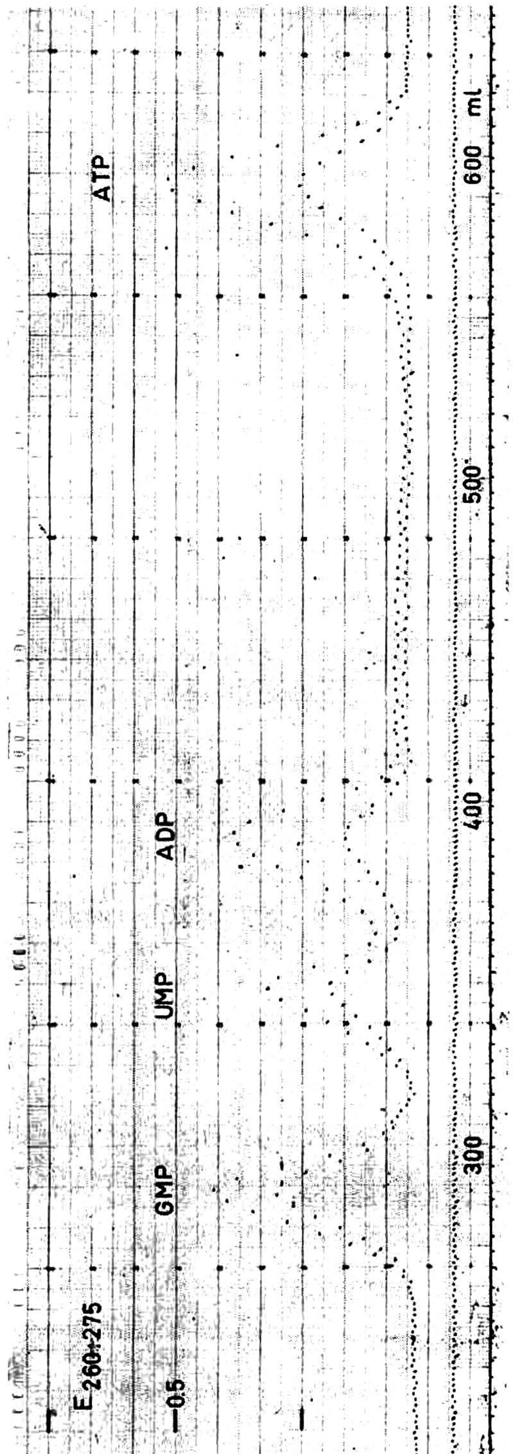

Fig. 3. Separation of 100 μg GMP, UMP, ADP and 150 μg ATP. Resin: Dowex-2, $HCOO^-$; column size: 0.8 × 12.0 cm, gradient: 7 × 150 ml (see text for further details), temperature 22°. The eluant was recorded at 2 min intervals, alternatively at 260 nm (upper curve) and at 275 nm (lower curve). The event marker line (5 ml fractions) is shown at the bottom of the chart paper.

ml/min. Occasionally, positive pressure from a nitrogen flask was used. The material which was not retained by the resin at neutral pH was washed out with distilled water. The gradient mixer was then connected to the pump. Thereafter the assembled apparatus completed the nucleotide separation unattended. The results were printed out on the chart paper, from which the relative and quantitative amounts of the nucleotides could be calculated.

Good separation and resolution of nucleotides were achieved when standard mixtures were separated with this system (Fig. 3).

Recovery of nucleotides

The recovery obtained with 100 nmoles of UMP, ADP, UDPAG and UDP added to a mycoplasma extract was satisfactory. The quantitative calculations made from the peak areas of three different separations gave the following percentages of recovery: UMP 94.0 ± 14.0%, ADP 104.0 ± 16.7%, UDPAG 104.5 ± 2.0% and

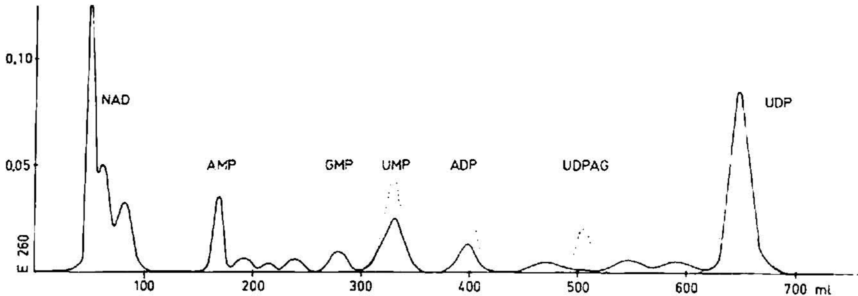

Fig. 4. Recovery of 100 nmoles UMP, ADP, UDPAG and UDP (dotted lines) added to a mycoplasma extract. Column: 0.8 × 12.0 cm, gradient: 7 × 200 ml, 22°, 24 h.

UDP 120.5 ± 8.6% (Fig. 4). The volume of the fractions was 40–70 ml. The concentration of the standard nucleotides and the mycoplasma extract could be varied between 100–1000 nmoles without significant effect on the elution pattern or the resolution of the individual nucleotides.

DISCUSSION

In several cases where good resolution has been achieved[8–10,16], long (150 cm) columns or small bore spiral plastic tubing[37] have been used. In practice, the use and servicing of these columns are laborious and difficult for multiple analysis. In our system the use of either long or short columns is possible, but because of the possible leakage of UV-absorbing material from the resin during a run[9], smaller columns are to be preferred.

A formic acid–ammonium formate gradient was chosen because of the known nucleotide elution pattern and certain other advantages[2]. The gradient mixer, baseline neutralization and modified flow cells made automation possible and eliminated the practical problems of this gradient system.

The detection sensitivity level depends on the resolution, the slope of the gradient,

the length of the light path, and on the volume of the measuring cell. With the use of capillary or cylindrical cells (Beckman), with up to a 50–100 mm light path, it would be possible to improve the sensitivity still further; practical difficulties would then, however, arise in proportion to the cell diameter and volume. The present method can be adapted for preparative work by using flat cuvettes, adjusting the recording sensitivity and splitting the effluent stream.

The application of an automatic UV-monitoring system to nucleotide column chromatography was first introduced by ÅGREN[39]. He used a monochromator connected to a photomultiplier and a recorder. ANDERSON[8] has described two useful UV-monitoring systems assembled partly on commercially available components. He used a single or double-beam system with two wavelengths and constructed a base-line compensation and flow cells. He was able to separate standard nucleotide mixtures (bases, nucleosides and nucleotides) in a single run with very good resolution[9]. The nucleotides of yeast were also separated with this nucleotide analyzer[10]. Recently, this instrumentation has been further modified and sensitized down to the 2 nmolar level in the separation of bases and nucleosides[40]. At the present time, there are also several commercial apparatus, which have been used for the partial automation of nucleotide analysis[13,41–43].

The present system was intended primarily for work with biological samples containing only small amounts of free nucleotides. The UV spectra of the nucleotides depend upon the pH, and therefore the automatic scanning cannot produce quantitative records of chromatograms with pH gradients[1]. With our modification, however, the reproducibility of chromatograms of test samples was good and the recovery of individual nucleotides satisfactory. The method has been proven useful in the quantitative determination of the changes in the concentration of different nucleotides, *e.g.* during the growth of mycoplasma[36].

ACKNOWLEDGEMENTS

The author wishes to thank Prof. J. J. SAUKKONEN AND V. KAUPPINEN Ph. D. for their valuable help and advice, and the Finnish Foundation for Medical Research for the necessary financial support.

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CHROM. 4822

ANALYSIS OF RIBONUCLEOTIDES AND DEOXYRIBONUCLEOTIDES USING HIGH-PRESSURE LIQUID COLUMN CHROMATOGRAPHY

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(Received April 16th, 1970; revised manuscript received May 12th, 1970)

SUMMARY

Conditions for the separation of some mononucleotides, dinucleotides, nucleoside diphosphates and deoxymononucleotides on a pellicular anion-exchange column are described. These chromatographic systems allow quantification and identification of nucleotide materials in nanomole quantities and can be achieved within 100 min. The applicability of the present systems to the analysis of base composition of polynucleotides has been demonstrated by analyzing some oligonucleotides of known structure.

INTRODUCTION

In the sequence analysis of RNA molecules, characterization and quantification of base components is an important operation. Paper and thin-layer chromatography and electrophoresis have been used commonly as well as various column chromatographic methods[1,2]. Recently, SHMUKLER[3] reported the separation of ^{32}P-labeled AMP, ADP and ATP on a commercial nucleic acid analyzer. Analysis of some ribonucleosides and bases using a similar column has been reported also by HORVATH AND LIPSKY[4].

This communication describes the separation of some mono- and dinucleotides, nucleoside diphosphates and deoxymononucleotides on a pellicular anion-exchange column. In essence the present method involves a high-pressure liquid chromatography ion-exchange system. A mixture of mononucleotides can be cleanly separated within 1 h and is quantifiable for as little as 0.3 nmole of each mononucleotide. In addition, some dinucleotides, nucleoside diphosphates and deoxymononucleotides can be separated on the column.

MATERIALS AND METHODS

Mononucleotide samples, Cp (2′ + 3′), Up (2′ + 3′), Ap (3′) and Gp (2′ + 3′)

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obtained from P-L Biochemicals were used as standards. They were prepared as aqueous solutions whose concentrations were determined by measurement of the $O.D._{254m\mu}$ of an aliquot in 0.2 *M* KH_2PO_4, pH 8.5. Nucleoside diphosphates (Li salts) were obtained from Mann Research Laboratories.

Four dinucleotides, ApCp, GpCp, ApUp, and GpUp were prepared by digestion of a sample of *Escherichia coli* sRNA (General Biochemicals) with pancreatic RNase. The digest was chromatographed on EDTA-washed Whatman No. 3 MM paper, in *n*-propanol–ammonia–water (55:10:35). The band containing dinucleotides was eluted with 0.05 *M* NH_4OH, and re-spotted on washed Whatman No. 3 MM paper for electrophoresis (0.05 *M* NH_4COOH, pH 3.0; 2000 V for 3 h). The dinucleotides were eluted with 0.05 *M* NH_4OH, evaporated to dryness, and redissolved in distilled water. A sample of each dinucleotide was hydrolyzed in 0.3 *N* KOH at 37° for 18 h. Each dinucleotide was identified by measurement of its mononucleotide components obtained from the KOH hydrolysate.

Apparatus

A Picker Nuclear LCS-1000 analyzer equipped with an anion-exchange column was used for this study.

Preparation of buffers

In all buffers prepared for the analyzer, the precise adjustment of pH was found to be of the utmost importance. Concentrated and dilute buffers described here contained Fisher "ACS Certified" KH_2PO_4. The salt used for buffers in linear gradient systems was not purified further, but that for exponential gradient systems (see nucleoside diphosphates, dinucleotides below) was purified as follows: Distilled water was added to 800 g KH_2PO_4 to a volume of 1200 ml. The mixture was boiled gently until all salt had dissolved, then cooled slowly and allowed to stand overnight at room temperature. The crystals which had formed were discarded; the supernatant was filtered through cotton to remove gross impurities. The concentration of KH_2PO_4 in the filtered supernatant was determined. Appropriate dilutions were then made to make the desired buffer concentrations; the pH was adjusted with concentrated H_3PO_4, using a Leeds and Northrup pH meter.

Operational conditions

While not in use, the column was maintained at 40°. Prior to each chromatographic run, the column was conditioned at high flow rates for 10 min with dilute degassed buffer at 40°, then at 70° (or the temperature to be used during the analysis) for about 30 min at which time the baseline usually had leveled off. Samples were injected with a Hamilton syringe. Elution was effected with a linear or exponential gradient at a flow rate of 0.2 ml/min. Chart paper speed was 1 in./5 min.

Sample preparations and elution conditions

System I. This buffer system was used for the separation of mononucleotides. Samples containing between 0.25 and 2.7 nmole of each mononucleotide in a solution containing 0.49 μmole $KClO_4$, 0.26 μmole $HClO_4$ and 0.80 μmole KH_2PO_4, pH 8.5, were injected onto the column. The column was maintained at 70°. Initially, the mixing chamber contained 40 ml of dilute buffer (0.005 *M* KH_2PO_4, pH 2.4). Gradient-

forming concentrated buffer (1.0 M KH_2PO_4, pH 3.6) was introduced into the mixing chamber 10 min after the beginning of the run, at a rate of 0.1 ml/min.

System II. This system was used for the separation of ribo- and deoxyribo-mononucleotides. Samples containing between 0.3 and 2.0 nmole of each mononucleotide in a solution containing 0.12 μmole $KClO_4$, 0.12 μmole $HClO_4$ and 1 μmole KH_2PO_4 were injected onto the column. The column was maintained at 70°. The dilute buffer in System II was 0.001 M KH_2PO_4, pH 3.5, filled to a level of 50 ml in the mixing chamber. Without any gradient delay, concentrated buffer, KH_2PO_4, 1.0 M, pH 4.3 flowed into the mixing chamber at a rate of 0.1 ml/min.

System III. For the separation of nucleoside diphosphates and dinucleotides, samples were made up as for System II for mononucleotides; the volume injected contained about 0.5 μg of each diphosphate. Dilute buffer was 0.01 M KH_2PO_4, pH 2.4, and filled the mixing chamber to 40 ml. Concentrated buffer (1.0 M KH_2PO_4, pH 3.6) was added, without any gradient delay, at a rate of 0.2 ml/min, *i.e.*, at a rate equal to the flow of buffer through the column. This established a system with an exponential gradient.

RESULTS AND DISCUSSION

Separation of mononucleotides

R values of the mononucleotides separated by System I are shown in Table I. Fig. 1 illustrates a typical run. The system separates 2′ and 3′ isomers with the ex-

TABLE I

R VALUES FOR SOME NUCLEOTIDES IN THREE BUFFER SYSTEMS

Nucleotide	*R values (cm)*		
	System I	*System II*	*System III*
Cp (2′)	1.3	9.9	
Cp (3′)	1.3	11.5	
Up (2′)	13.0	14.6	
Up (3′)	15.3	16.4	
Ap (2′)	4.3	19.8	
Ap (3′)	8.6	24.5	
Gp (2′)	23.2	25.8	
Gp (3′)	28.2	31.2	
CDP			14.4
UDP			24.8
ADP			27.3
GDP			44.6
ApCp			18.2
GpCp			32.3
ApUp			33.7
GpUp			51.6
dCMP		10.4	
dAMP		18.2	
dGMP		26.3	

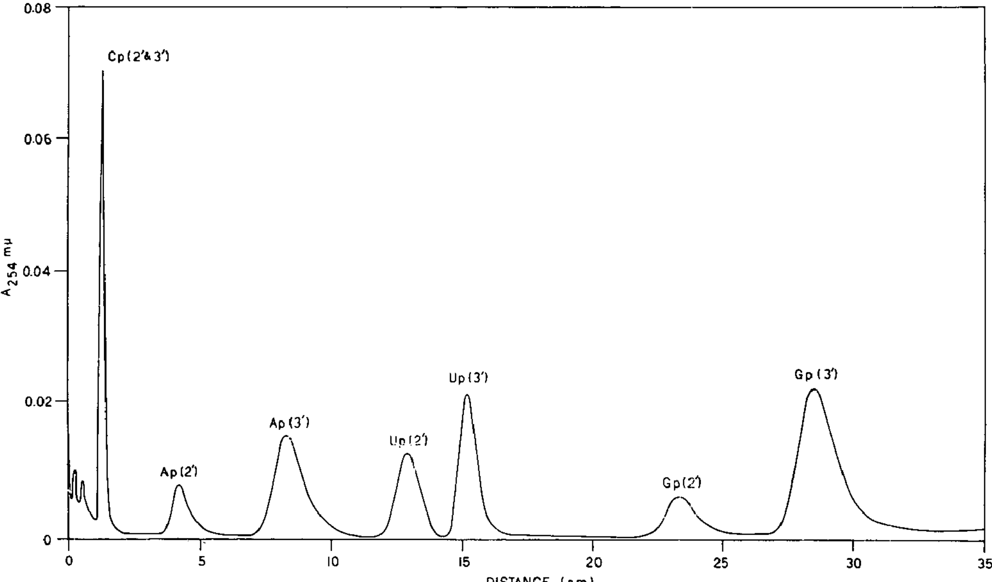

Fig. 1. Chromatogram of mononucleotides. Chromatographic conditions are described in the text.

ception of those of Cp which are eluted as a single peak. The extreme sharpness of Cp (2' + 3') as well as its proximity to the "noise" peak (marking beginning of run) would seem to increase the error in the quantitation of Cp. In spite of this apparent disadvantage, standard curves for this system show limited variation in the points depicting Cp values (Fig. 2). The linearity of all four curves indicates that the measure-

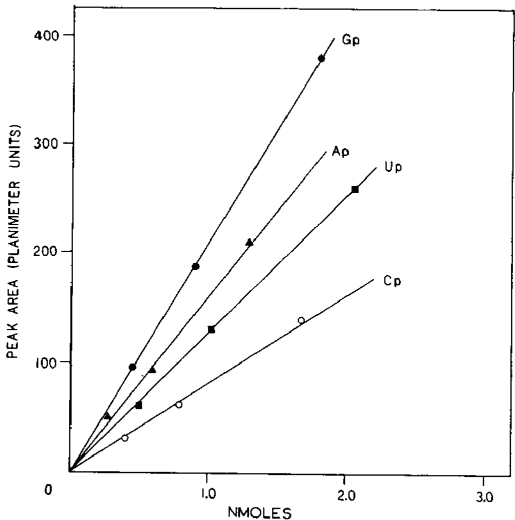

Fig. 2. Standard curves for mononucleotides showing the linear relationship between quantities of each mononucleotide and peak area of chromatogram. Chromatographic system I was used.

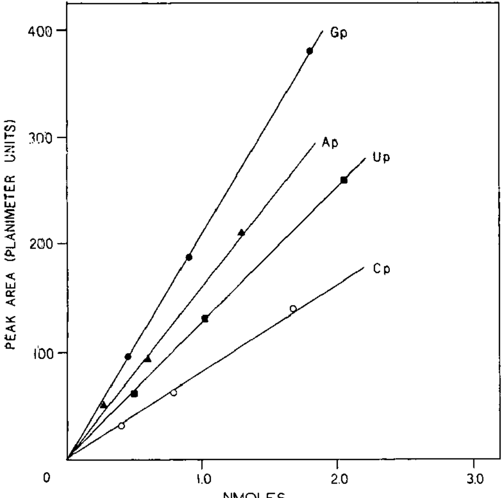

Fig. 3. Standard curves for mononucleotides. Chromatographic system II was used.

ment of peak areas from System I can be used for quantitation of the mononucleotides.

The additional possibility in System I of contamination of Cp peaks with nucleosides (which would be eluted from the column near the beginning of the run) suggested that an improved separation system should be developed in which Cp peaks were retained on the column for a longer time. It was found that System II had such an effect.

R values of some mononucleotides separated by System II are shown in Table I. Standard curves for these mononucleotides using System II are shown in Fig. 3. The linearity of these curves indicates that quantitative measurement of each nucleotide may be obtained through measurement of peak areas.

TABLE II

CHARACTERIZATION OF PRODUCTS FROM ALKALINE HYDROLYSIS OF DINUCLEOTIDES

Dinucleotide	*Mono-nucleotide products*	*R*	*Area*	*nmoles*	*Ratio*
ApCp	Cp (3′)	12.2	510	6.63 }	
	Ap (2′)	20.3	537 }	7.44 }	1.22
	Ap (3′)	25.0	722 }		
ApUp	Up (3′)	17.1	366	2.93 }	
	Ap (2′)	20.3	256 }	3.48 }	1.19
	Ap (3′)	25.2	333 }		
GpUp	Up (3′)	16.7	38	0.31 }	
	Gp (2′)	26.0	27 }	0.29 }	1.07
	Gp (3′)	31.6	34 }		
GpCp	Cp (3′)	11.2	40	0.52 }	
	Gp (2′)	26.5	56 }	0.56 }	1.08
	Gp (3′)	32.1	61 }		

It was of particular interest to test the applicability of the present systems to the analysis of base composition of polynucleotides. Table II shows the data of such analysis on dinucleotides of known structure.

Hence, the two systems used for the separation of mononucleotides each have their own merits. System I is valuable because it separates the four mononucleotides completely from each other although the 2′ and 3′ isomers of Cp cannot be measured individually. However, the system remains useful for measurement of total Cp, Ap, Up and Gp, respectively, as illustrated by the analysis of dinucleotides (Table II).

System II for mononucleotides gives excellent separation of 2′ and 3′ isomers of all four mononucleotides with the exception of Ap (3′) and Gp (2′), which overlap. This system is therefore ideal for analysis of KOH hydrolysates of oligonucleotides derived from T_1 digests of RNA, since these oligonucleotides should not contain Gp (2′). Several such analyses have been done and have been reported previously[5].

Separation of nucleoside diphosphates

Table I also shows the *R* values obtained for some nucleoside diphosphates. The increase in salt concentration of the buffer to a much higher level during the exponential gradient run led to the problem of a "significant" rise in the baseline after a period of about 50 min. Since the run described above took 105 min, purification of the buffers was necessary (see MATERIALS AND METHODS) in order to eliminate this baseline rise during as much of the run as possible. With the use of "purified" KH_2PO_4, the baseline increased less than 0.002 O.D. units during the first 100 min of a run.

Separation of dinucleotides

In addition to being a method for base composition analysis, it would be useful if the present method can also be suited as a finger printing method for studies of the primary structure of RNA's. Thus, we examined the elution pattern of the dinucleotides produced from a pancreatic ribonuclease digest of RNA. The *R* values of the four dinucleotides are shown in Table I.

Separation of deoxynucleotides

The results shown above represent only a beginning in the development of buffer systems for the purpose of separating nucleic acid components. Although the present data deal with ribonucleotides, preliminary data show that a mixture of dCMP, dAMP, and dGMP may also be separated on this column using System II. *R* values for such compounds are shown in Table I.

ACKNOWLEDGEMENTS

The authors are indebted to Professor V. M. INGRAM for his interest and support and the use of his laboratory during the course of this investigation. We also wish to thank Picker Nuclear for the loan of an LCS-1000 analyzer.

This investigation was supported by a grant No. AM 08390 from the U. S. Public Health Service to Professor INGRAM.

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CHROM. 4810

SEPARATION AND DETERMINATION OF LINEAR AND BRANCHED CHAIN ALKYLBENZENE SULFONATES BY SALTING-OUT CHROMATOGRAPHY

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(Received May 1st, 1970)

SUMMARY

The separation and determination of biologically "soft" (LAS) and "hard" (ABS) alkylbenzene sulfonates in blends of the two was investigated by salting-out chromatography. The analytical conditions were as follows: column, 26 mm I.D. and 500 mm long; resin, Amberlite CG-50 (100–200 mesh); developing solution, 0.5 *M* ammonium sulfate–43% methanol solution; and column temperature, 50°.

Quantitative separation of LAS and ABS was satisfactory and the peak resolution between them more than unity.

INTRODUCTION

There are two types of biologically "soft" (LAS) and "hard" type (ABS) alkylbenzene sulfonates used as raw materials for household synthetic detergents. Actually a mixture of the two types is in everyday use in our country although the conversion from the hard to the soft type can be carried out in the same way as in other countries.

Thus, it would be worth while establishing a rapid and precise analytical method for both types of sulfonate mixture. Many methods have been studied for the analysis of mixtures of LAS and ABS.

The JIS (Japanese Industrial Standard)[1] method is based on the measurement of biological degradation and takes a long analysis time. In IR methods[2–6], the extent to which the LAS to ABS ratio can be determined is limited, because the overlap of the absorption LAS and ABS bands makes the quantitative analysis of a mixture difficult when one sulfonate is present in a much higher concentration than the other. NMR[7–9] and GC[10–13] methods necessitate desulfonation of the sulfonate mixture.

ROSEN AND GOLDSMITH[14] have investigated extensively the separation and determination of mixtures of ionic and nonionic surface active agents by ion-exchange chromatography. However, there have been no reports on the separation and determination of mixtures of several ionic surface active agents.

On the other hand, SARGENT AND RIEMAN[15] succeeded in separating for the first time a mixture of nonelectrolytic organic compounds by salting-out chromato-

graphy using an ion-exchange resin. FUNASAKA and co-workers[16,17] satisfactorily applied this technique to the analysis of organic sulfonic isomers (*e.g.* dyestuff intermediates).

The present paper describes the separation and the determination of LAS and ABS mixtures by a salting-out chromatography technique.

EXPERIMENTAL

Apparatus

The distribution coefficient was measured by a batch method using a 50 ml conical flask with a stopper. A shaker (Model KM, Iwaki Co., Ltd.) was employed to obtain the adsorption equilibrium rapidly. Absorbance of the material separated was measured by a spectrophotometer (Model QV-50, Shimadzu Co., Ltd.) with a quartz cell (cell thickness 2 cm). The glass column (26 mm I.D. and 500 mm long) was equipped with a jacket in order to control the column temperature.

The temperature of the circulating water was controlled by a constant temperature water circulation unit (Model 1, Shimadzu Co., Ltd.). A Uvicord II detector (LKB-Produkter AB Co., Ltd., Sweden; wavelength 254 mμ) was employed for automatically recording the elution chromatogram. The polyethylene tube (1.8 mm outside diameter) was used to connect the column to the Uvicord II detector. After passing through the Uvicord II detector, the eluate was directed to the fraction collector (model SF-160K, Toyo-Kagaku Co., Ltd.) where it was collected in a volumetric flask.

Reagents and samples

Amberlite CG-50 (100–200 mesh), a weakly acidic cation-exchange resin, was used as a column substrate. The methanol in the eluent was weighed in order to make the eluent composition precise.

The LAS and ABS were manufactured by our company and purified by the following procedure. 3 g of sample was dissolved in 250 ml of 50% of aqueous ethanol solution and extracted three times with 100 ml of petroleum ether to remove unreacted substances. The aqueous ethanol solution was evaporated to dryness and then the sample was redissolved in ethanol. The ethanol solution was filtered to remove sodium chloride and the filtrate was dried on the waterbath.

A standard sample solution was prepared by dissolving a purified sulfonate in water. It was titrated with 0.01 *M* cetylpyridinium chloride solution using Methylene Blue as indicator.

Procedure

Measurement of distribution coefficient. In order to find suitable column conditions, the distribution coefficients of LAS and ABS with the resin were first measured by a batch method. The ion-exchange resin was washed with ethanol using a Soxhlet extractor, treated with 3 *N* sodium hydroxide and 3 *N* aqueous hydrogen chloride solution alternately, then washed thoroughly with water and finally dried at 105° for 5 h. About 1 g of this dried resin was weighed out accurately and put into a 50 ml stoppered conical flask. 25 ml of each salting-out reagent solution, of various concentrations, was added to each flask containing 1 g of resin. The flasks were allowed to

stand for 1 h in order to swell the resin. 1 ml of sample solution containing LAS and ABS (1 ml of solution contains 10 mg of sample) was added to each respective flask, which was shaken vigorously for 20 min at intervals of 1 h during first 3 h, then allowed to stand for 15 h in order to obtain adsorption equilibrium. The resin was filtered off and absorbances of the filtrates were measured against a blank solution.

The distribution coefficient K_d was calculated from the following equation:

$$K_d = \frac{\text{amount of sample adsorbed on 1 g of resin (mg/g)}}{\text{amount of sample contained in 1 ml of solution (mg/ml)}}$$

The maximum adsorption wavelength of both LAS and ABS in the UV region was 261 mμ.

Salting-out chromatography. The salting-out chromatographic procedure was performed as follows. The resin was poured into the column by the wet method, after which the resin bed in the column was settled by passing the eluent (0.5 M ammonium sulfate–43% methanol solution) through it for 1 h. For column temperatures higher than room temperature, the eluent solution with the resin suspended in it was heated a little higher than the column temperature before being poured into the column.

1 ml of sample solution was adsorbed carefully on the top of the resin bed, then the inside wall of the column was washed several times with the same eluent.

The flow rate was controlled by a stopcock, the eluate was passed through the Uvicord II detector and the elution chromatogram recorded.

Quantitative analysis, *i.e.* an operation to calculate the recovery of a sample, was carried out according to the following method. The chromatogram recorded by the Uvicord II detector was used as a monitor, the eluate of a section showing an elution peak was collected in a volumetric flask, and diluted with eluent to volume. A reference solution was prepared by diluting 1 ml of sample solution to the same volume. The absorbance of both solutions was measured at 261 mμ using eluent as the blank solution; recovery was calculated from the absorbance ratio of the sample solution against the reference solution.

Substances which were not eluted with this eluent were eluted with 43% aqueous methanol solution containing no salting-out reagent. The column could be used again to analyze the next sample.

RESULTS AND DISCUSSION

Salting-out reagent and distribution coefficient

Four kinds of salting-out reagents (sodium chloride, ammonium sulfate, am-

TABLE I

EFFECT OF SALTING-OUT REAGENTS ON DISTRIBUTION COEFFICIENTS OF LAS AND ABS

Reagent	*Distribution coefficient (ml/g)*		*Ratio* K_{d1}/K_{d2}
	K_{d1}*(LAS)*	K_{d2}*(ABS)*	
NaCl	152	104	1.46
NH_4NO_3	214	101	2.12
$(NH_4)_2SO_4$	256	104	2.46
$CaCl_2$	—	—	

monium nitrate and calcium chloride) were tested for their effect on the distribution coefficients of LAS and ABS, by a batch method.

Table I shows the distribution coefficients where the concentration of each salting-out reagent was 0.05 *M*.

With calcium chloride, a measurement was impossible on account of the formation of white turbidity due to the strong salting-out action.

Eluent

In the separation of both sulfonates by the column method, it is desirable that the distribution coefficient ratio is greater than 1.5, and, furthermore, that the distribution coefficients are less than 100.

FUNASAKA and his co-workers[17] reported that the amount of adsorption decreased when alcohol was present in the salting-out reagent solution.

Thus, a salt–methanol solution system was investigated. At first, the effect of the concentration of the salting-out reagent and the methanol on the distribution coefficient was investigated for a sodium chloride–methanol system. In the experimental range in which the elution is possible, the distribution coefficient ratio of LAS and ABS was small. As a result of this the system was unsatisfactory as a developing solvent. Ammonium sulfate–methanol mixtures were next investigated, because the distribution coefficient ratio was larger than in the case of sodium chloride. The results

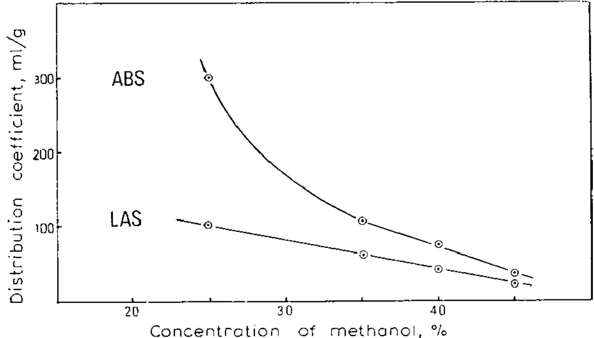

Fig. 1. Effect of the concentration of methanol on the distribution coefficients of LAS and ABS. Concentration of ammonium sulfate solution was 0.5 *M*.

are presented in Fig. 1, which shows that the larger the concentration of methanol, the lower the distribution coefficient. It seems that the molecules of alcohol tend to accumulate on the surface of the resin, thus preventing the sample being adsorbed on the resin and that the solubility of the surface active agent is greater in alcohol solution than in water. It is thought that the distribution coefficient is lowered by these two factors. A mixture of 0.5 *M* ammonium sulfate and 43% of methanol solution was selected as the eluent from the results presented in Fig. 1.

Column temperature

The effects of the column temperature on the separation and recovery of LAS and ABS mixtures were investigated. The preparation was carried out at 40°, 45° and 50° under constant conditions except for the column temperature.

TABLE II

EFFECT OF COLUMN TEMPERATURE ON RECOVERIES OF LAS AND ABS
Sample taken: LAS 8.70 mg; ABS 9.20 mg.

Column temp. (°C)	*Recovery (%)*	
	LAS	*ABS*
50	98.9	100.1
45	84.5	97.1
40	76.0	93.0

When the column temperature was 40° or 45°, the recoveries of both sulfonates were lower than 100% as shown in Table II. Table II shows that the higher the column temperature the more suitable were the column conditions. The column temperature was fixed at 50°, taking into account the use of methanol in the eluent and the difficulty of the operation.

Separation by the column method

The separation of LAS and ABS was carried out under the following conditions: eluent, 0.5 *M* ammonium sulfonate–43% methanol solution; column size, 500 × 26 mm I.D.; column temperature, 50°.

The chromatogram is shown in Fig. 2. Two small peaks overlapping in front of

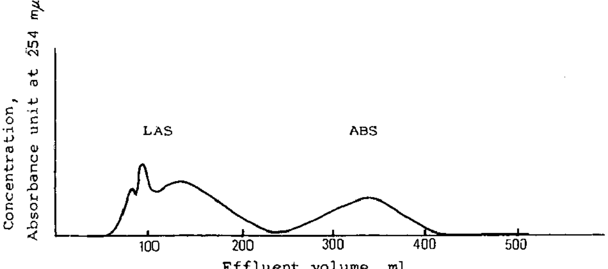

Fig. 2. Elution curve of a mixture of LAS and ABS. Column: 500 × 26 mm I.D.; resin: Amberlite CG-50 (100–200 mesh); eluent: 0.5 *M* $(NH_4)_2SO_4$–43% CH_3OH; flow rate: 0.45 ml/min; column temperature: 50°; weight of sample: 8.70 mg LAS, 9.20 mg ABS.

the LAS peak are assumed to be either due to the elution of by-products, such as disulfonate, or peaks based on the molecular weight distribution of LAS. Further investigation of these peaks was not carried out.

Peak resolution[18], which is the difference between the retention volumes of the two sulfonate peaks divided by the average value of the width of the two peaks, was more than unity.

Reproducibility and recovery

Recoveries of LAS and ABS from the column were each investigated independently in triplicate. Average recoveries of LAS and ABS were 93.4% and 99.2% respectively, and the reproducibility was satisfactory. The recoveries of LAS are

TABLE III

DETERMINATION OF EACH STANDARD SOLUTION AND KNOWN MIXTURES

Sample	*Taken (mg)*	*Found (mg)*	*Recovery (%)*	
LAS	7.90	7.35	93.1	
LAS	7.90	7.38	93.5	93.4
LAS	7.90	7.39	93.6	
ABS	8.80	8.73	99.2	
ABS	8.80	8.73	99.2	99.2
ABS	8.80	8.73	99.2	
LAS	8.70	8.74	100.5	
ABS	17.85	18.12	101.5	
LAS	17.40	17.41	100.1	
ABS	18.35	18.46	100.6	
LAS	17.40	16.96	97.5	
ABS	8.80	9.08	103.2	
LAS	26.10	25.38	97.3	
ABS	9.18	9.60	104.5	
LAS	25.50	24.60	96.3	
ABS	4.40	4.66	105.9	

somewhat lower and this may be due to by-products such as dialkylbenzene sulfonate contained in the sample. Accordingly, in case of the determination of LAS, a correction factor, 1.07, was used.

Known mixtures, prepared by mixing LAS and ABS in certain proportions, were analyzed and the results were shown in Table III.

From Table III, it appears that the agreement between the calculated and found values is satisfactory.

MECHANISM OF SEPARATION

It is thought that these surface active agents form micelles in aqueous solution, because the concentrations of both LAS and ABS used are only 10–20 mg/ml. In the case of a mixed sample, it is assumed that mixed micelles are formed at the first stage of

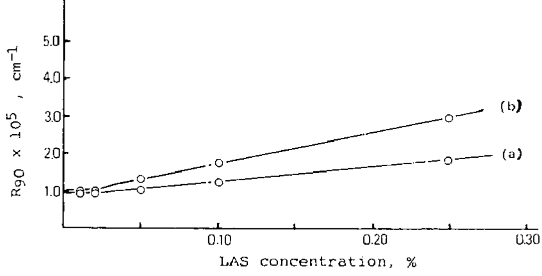

Fig. 3. Experimental Rayleigh ratios for solutions of LAS in 0.5 *M* ammonium sulphate–methanol. (a) 43% v/v methanol; (b) 35% v/v methanol.

sample addition. However, separation of LAS and ABS will not occur if the sample passes through the column in the form of mixed micelles. Therefore, it is presumed that the mixed micelles are each resolved to uni-molecules on addition of the eluent, and that LAS and ABS pass through the column in the state of uni-molecules.

PARFITT AND WOOD[19] examined the effect of methanol on micelle formation with respect to sodium dodecylsulfate in a methanol–water system. According to that paper, the micelles were not formed even though the sample concentration was increased and the molar fraction of methanol was more than 0.27 (about 40%).

In the present study, micelle formation in the eluent was investigated by a light scattering method. From Fig. 3, it appears that in an 0.5 *M* ammonium sulfate–43% methanol solution system micelles are not formed or only just begin to form. The inflexion point in Fig. 3 indicates micelle formation in 0.5 *M* ammonium sulfate–35% methanol solution system.

Accordingly, it was considered that the mixed sample was separated in the column in a uni-molecular state. When the concentration of alcohol in the eluent

TABLE IV

EFFECT OF CONCENTRATION OF METHANOL ON RECOVERIES OF LAS AND ABS

Sample taken: LAS 8.70 mg, ABS 9.20 mg. Concentration of ammonium sulfate: 0.5 *M*.

Concn. of methanol (%)	*Recovery (%)*	
	LAS	*ABS*
43	98.9	100.1
40	83.6	95.0
35	73.5	89.0

decreased, the recoveries of LAS and ABS also decreased as shown in Table IV. From these facts, it is apparent that the alcohol in the eluent plays an important role in the separation and determination of both sulfonates.

CONCLUSION

The mixture of LAS and ABS could be separated quantitatively by salting-out chromatography. From the point of view of molecular structure, LAS and ABS have only small differences in the alkyl chains which contribute to separate both sulfonates. This shows that the separation by salting-out chromatography is based on a delicate mechanism.

Further applications to the analysis of ionic surface active agent mixtures are being studied.

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CHROM. 4808

THIN-LAYER AND GAS–LIQUID CHROMATOGRAPHY OF DERIVATIVES OF ISOMERIC CHLOROPHENOLS

CHLOROPHENYL FLUOROSULFONYL-BENZENESULFONATES AND RELATED COMPOUNDS

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(Received May 8th, 1970)

SUMMARY

The thin-layer and gas–liquid chromatography of derivatives of the isomeric chlorophenols, *e.g.* the isomeric chlorophenyl fluorosulfonyl-benzenesulfonates, were compared with the respective precursor isomeric fluorosulfonyl-benzenesulfonyl chlorides and acaricidal chlorophenyl benzenesulfonates. The thin-layer chromatographic investigation utilized four solvent systems and five electron-acceptor reagents to separate and distinguish the various isomeric derivatives. Gas–liquid chromatography was best accomplished using 5% OV-17 and SE-30 with flame ionization detection.

INTRODUCTION

Isomeric chlorophenols and their esters are important environmental agents that are extensively used as insecticides[1], herbicides[2,3], growth regulators[4,5], nematocides[6,7], germicides[8] and as fungicides in many industries including the textile, leather, paint and adhesive manufacturing processes.

Chlorophenols such as *p*-chlorophenol and 2,4-dichlorophenol are also hydrolysis products of the acaricides ovex (*p*-chlorophenyl *p*-chlorobenzenesulfonate) (I), fenson (*p*-chlorophenyl benzenesulfonate) (II) and genite (2,4-dichlorophenyl benzenesulfonate) (III), respectively.

(I) (II) (III)

Earlier papers have described the detection and thin-layer (TLC) and gas–liquid chromatography (GLC) of a number of derivatives of isomeric chlorophenols, *e.g.*, N-(trichloroacetyl) carbamates[9,10] and *m*-fluorosulfonyl-benzoate esters[11].

The purpose of this investigation was to elaborate further the effect of structure on TLC and GLC behavior of additional derivatives of the isomeric monochlorophenols, *e.g.*, the isomeric chlorophenyl fluorosulfonyl-benzenesulfonates as well as to compare their chromatographic behavior with precursor isomeric fluorosulfonyl-benzenesulfonyl chlorides and related derivatives such as the acaricides ovex and genite.

EXPERIMENTAL

Materials

Detecting reagents were: (1) DDQ reagent: 2% 2,3-dichloro-5,6-dicyano-1,4-benzoquinone in benzene[12]; (2) TCNE reagent: 2% tetracyanoethylene in benzene[12]; (3) Gibbs' reagent: 2% 2,6-dibromo-N-chloro-*p*-benzoquinoneimine in benzene; (4) TNF reagent: 2% 2,4,7-trinitrofluorenone in benzene; (5) Chloranil: 1% tetrachloro-*p*-benzoquinone in benzene.

The developing solvents utilized were: (a) 2.5% acetone in benzene; (b) chloroform–acetic acid (5:1); (c) toluene–ethyl acetate (1:1); and (d) 5% ethylene dichloride in benzene.

The chlorophenyl *o*-, *m*- and *p*-fluorosulfonyl-benzenesulfonates were prepared via the reaction of the respective fluorosulfonyl-benzenesulfonyl chloride with the isomeric monochlorophenols in benzene and triethylamine for 1 h at 85° and the product recrystallized from benzene–acetone (3:1). The isomeric fluorosulfonyl-benzenesulfonyl chlorides and chlorophenols were obtained from Aldrich Chemical Co., Milwaukee, Wisc., U.S.A.; the pesticidal acaricides ovex and genite from the Pesticides Research Laboratory, Perrine, Fla., U.S.A.; the detecting reagents 2,3-dichloro-5,6-dicyano-1,4-benzoquinone, 2,6-dibromo-N-chloro-*p*-benzoquinoneimine, and tetrachloro-*p*-benzoquinone from J. T. Baker Corp., Phillipsburg, N.J., U.S.A.; and tetracyanoethylene and 2,4,7-trinitrofluorenone from Distillation Industries, Rochester, N.Y., U.S.A.

Thin-layer chromatography

Chromatoplates prepared according to MORLEY AND CHIBA[13]. Silica Gel DF-5* was applied on 8 × 8 in. plates to a thickness of 280 µ. After air-drying, the plates were activated in an oven for 30 min. Acetone solutions (1–2 µl containing 1–10 µl of test substance) were applied along a line 2.5 cm from the lower end of the plate and developed by the ascending method. After evaporation of the solvent, the spots were located on the plate by UV detection, then sprayed with one of the chromogenic reagents and the initial color development as well as subsequent color changes noted.

Pre-coated sheets. MN-Polygram SILN-HR/UV_{254} (Brinkmann) precoated silica gel 20 × 20 cm sheets were used without further activation.

Gas–liquid chromatography

The following gas chromatographs were employed: (1) Varian Model 1200 equipped with a flame ionization detector and a 5 ft. × $\frac{1}{8}$ in. O.D. stainless steel column

* Obtained from Camag, Muttenz, Switzerland.

coated with 3% SE-30 on 100/120 mesh Varoport 30 and a 10 ft. × $\frac{1}{8}$ in. O.D. stainless steel column coated with 10% OV-1 on 80/100 mesh Supelcoport; (2) Hewlett-Packard Model 700-1099F equipped with a flame ionization detector and a 6 ft. × $\frac{1}{8}$ in. I.D. stainless steel column coated with 5% OV-17 on 80/100 Supelcoport; and (3) Hewlett-Packard Model 5750 equipped with both flame ionization and ^{63}Ni electron-capture detectors and (a) 6 ft. × $\frac{1}{4}$ in. O.D. spiral glass containing 4.9% OV-17 on 100/120 mesh Gas-Chrom Q; (b) 6 ft. × $\frac{1}{8}$ in. O.D. stainless steel coated with 3% OV-1 on 100/120 mesh Gas-Chrom Q columns. Standard derivative solutions were prepared in benzene in concentrations of 1 mg/ml. Specific analytical operating conditions are given in Table IV.

TABLE I

R_F × 100 VALUES OF ISOMERIC CHLOROPHENYL FLUOROSULFONYL-BENZENESULFONATES ON SILICA GEL DF-5 CHROMATOPLATES AND MN-POLYGRAM SILN-HR/UV_{254} PRE-COATED SHEETS

Solvents: (A) 2.5% acetone in benzene, (B) chloroform–acetic acid (5 : 1), (C) toluene–ethyl acetate (1 : 1), and (D) 5% ethylene dichloride in benzene.

Compound No.	*Derivatives*	*M.p. (°C)*	*Silica Gel DF-5*				*MN-Polygram SILN-HR/UV₂₅₄*			
			A	*B*	*C*	*D*	*A*	*B*	*C*	*D*
Chlorophenyl o-fluorosulfonyl-benzenesulfonates										
1	*o*-Chlorophenyl	145–147	38	46	12	31	34	48	15	28
2	*m*-Chlorophenyl	87– 89	44	51	18	39	41	54	20	34
3	*p*-Chlorophenyl	140–142	56	57	25	45	51	59	27	41
Chlorophenyl m-fluorosulfonyl-benzenesulfonates										
4	*o*-Chlorophenyl	157–159	45	53	10	37	40	57	16	30
5	*m*-Chlorophenyl	149–151	53	58	15	44	46	61	20	36
6	*p*-Chlorophenyl	161–162	64	62	21	52	54	66	26	45
Chlorophenyl p-fluorosulfonyl-benzenesulfonates										
7	*o*-Chlorophenyl	186.5–187.5	60	57	31	60	57	60	35	55
8	*m*-Chlorophenyl	192–194	67	61	38	66	65	65	41	61
9	*p*-Chlorophenyl	226–228	77	66	46	74	74	70	48	69

RESULTS AND DISCUSSION

Thin-layer chromatography

Tables I and II depict the R_F × 100 values of the isomeric chlorophenyl fluorosulfonyl-benzenesulfonates, fluorosulfonyl-benzenesulfonyl chlorides and acaricidal chlorophenyl benzenesulfonates, respectively, on Silica Gel DF-5 plates and pre-coated Polygram sheets developed in four solvent systems, *e.g.*, (a) 2.5% acetone in benzene, (b) chloroform–acetic acid (5:1), (c) toluene–ethyl acetate (1:1), and (d) 5% ethylene dichloride in benzene.

The isomeric chlorophenyl sulfonate derivatives as well as the precursor fluorosulfonyl-benzenesulfonyl chlorides were separated without decomposition in all of the solvent systems employed. The 2.5% acetone in benzene system was the solvent of

TABLE II

$R_F \times 100$ VALUES OF ISOMERIC FLUOROSULFONYL-BENZENESULFONYL CHLORIDES (I) AND ACARICIDAL CHLOROPHENYL BENZENESULFONATES (II) ON SILICA GEL DF-5 CHROMATOPLATES AND MN-POLYGRAM SILN-HR/UV_{254} PRE-COATED SHEETS

(I) (II)

Solvents: (A) 2.5% acetone in benzene, (B) chloroform–acetic acid (5:1), (C) toluene–ethyl acetate (1 : 1), and (D) 5% ethylene dichloride in benzene.

Compound No.	Derivatives	M.p. (°C)	Silica Gel DF-5				MN-Polygram SILN-HR/UV_{254}			
			A	B	C	D	A	B	C	D
Fluorosulfonyl-benzenesulfonyl chlorides										
10	*o*-Fluorosulfonyl	88–89	48	80	39	45	42	85	43	40
11	*m*-Fluorosulfonyl	110–112	60	85	44	61	51	91	48	49
12	*p*-Fluorosulfonyl	156–158 5 mm	71	91	52	74	63	97	56	62
Acaricidal chlorophenyl benzenesulfonates										
13	Ovex (R_1 = Cl; R_2 = H)	86.5–87	70	86	53	61	66	80	60	52
14	Genite (R_1 = H; R_2 = Cl)	42–43	64	81	47	50	58	74	52	40

TABLE III

COLOR COMPLEXES FORMED ON SILICA GEL DF-5 WITH ELECTRON-ACCEPTOR REAGENTS

Designation of colors: Bg = beige, Bn = brown, Gr = grey, M = maroon, O = orange, P = purple, R = rose, T = tan, V = violet, and Y = yellow.

Compound No.	Derivatives	Detecting reagents				
		DDQ	TCNE[a]	Gibbs'	TNF	Chloranil
Chlorophenyl o-fluorosulfonyl-benzenesulfonates						
1	*o*-Chlorophenyl	R→P	O-Y	Bg→T	T→Bn	R→V
2	*m*-Chlorophenyl	R→P	O-Y	Bg→T	T→Bn	R→V
3	*p*-Chlorophenyl	P	O	O-Bn	Bn	V
Chlorophenyl m-fluorosulfonyl-benzenesulfonates						
4	*o*-Chlorophenyl	R→P	O-Y	T	Bn	Bn→V
5	*m*-Chlorophenyl	R→P	O	T	Bn	Bn→V
6	*p*-Chlorophenyl	P	O	O-Bn	Bn	V
Chlorophenyl p-fluorosulfonyl-benzenesulfonates						
7	*o*-Chlorophenyl	Bn→P	O-Y	T	T→Bn	Bn-V
8	*m*-Chlorophenyl	Bn→P	O-Y	T	Bn	Bn-V
9	*p*-Chlorophenyl	P	O	O-Bn	Bn	V
Fluorosulfonyl-benzenesulfonyl chlorides						
10	*o*-Fluorosulfonyl	Bn→M	O	Bg→T	Y	T
11	*m*-Fluorosulfonyl	Bn→M	O	Bg→T	Y	T
12	*p*-Fluorosulfonyl	M	O	T	Y	Bn
Acaridical chlorophenyl benzenesulfonates						
13	Ovex	Y	O	Gr	Bn-V	Y-Gr
14	Genite	Y	O	Gr	Bn-V	Y-Gr

[a] Colors after exposure to ammonia vapors (2 min).

choice in the former case while 5% ethylene dichloride in benzene resolved the latter derivatives as well as the acaricides ovex and genite most efficiently. The order of R_F values for both series of isomeric derivatives was $p > m > o$ in all of the solvent systems.

Table III depicts the color complexes formed on Silica Gel DF-5 with electron-acceptor reagents. A number of general observations can be made as to the reactivity of the various classes of derivatives. Both the isomeric chlorophenyl fluorosulfonyl-benzenesulfonates and the acaricidal chlorophenyl benzenesulfonates are more reactive than the fluorosulfonyl-benzenesulfonyl chlorides toward all of the reagents screened, *e.g.*, the above two classes yield colors (from instant to 5 min), whereas the fluorosulfonyl-benzenesulfonyl chlorides are generally detected after 15 min or require development at 80°. It is of interest to note that in earlier work[14] sulfoxides were found to be more reactive than sulfones. The latter are weaker bases (poorer donors) due to the net decrease in the electron density on the sulfur and oxygens. A comparison of the class formulas (Tables I and II) suggests that analogously, the fluorosulfonyl-benzenesulfonyl chlorides are poorer donors than the oxygen-enriched chlorophenyl fluorosulfonyl-benzenesulfonates.

Table III also indicates that it is possible to distinguish the various classes of derivatives studied using a number of detecting reagents. For example, utilizing the DDQ reagent, the isomeric chlorophenyl fluorosulfonyl-benzenesulfonates, fluorosulfonyl-benzenesulfonyl chlorides and the acaricidal chlorophenyl benzenesulfonates formed purple, maroon and yellow spots, respectively, on a light tan background. The lower limits of detection for the above categories were 2, 4 and 1 μg per spot with the DDQ reagent. Similarly, chloranil and Gibbs' reagent permitted facile differentiation of the same three tested categories of derivatives with limits of detection in the order of 2, 4, 2 for chloranil and 3, 5 and 3 μg per spot for Gibbs' reagent, respectively. The use of TCNE reagent followed by exposure of the chromatoplates to ammonia yielded orange or orange-yellow spots for all the derivatives, with detection in the order of 5 μg per spot. The TNF reagent was least sensitive, with detection of all derivatives in the order of approx. 6–10 μg per spot. The substituted *p*-benzoquinone-type detectors, *e.g.*, DDQ and chloranil (detectors 1 and 5) were more sensitive than the halogenated quinoneimine detector (Gibbs' reagent, detector 3). The overall utility of the electron-acceptor reagents toward the classes of derivatives studied was: DDQ, chloranil > Gibbs' > TCNE > TNF.

With regard to steric effects in the series of isomeric chlorophenyl fluorosulfonyl-benzenesulfonates and fluorosulfonyl-benzenesulfonyl chlorides, the reactivity of these derivatives with the electron-acceptor reagents was generally in the order of $p > m > o$; *e.g.*, the respective *para* isomers (compounds 3, 6, 9 and 12) yielded instant colors, whereas the *meta* isomers (compounds 2, 5, 8 and 11) after 1–2 min and the *ortho* isomers (compounds 1, 4, 7 and 10) yielded colors only after approx. 5 min with somewhat decreased sensitivity.

The use of pre-coated silica gel sheets (MN-Polygram SILN-HR/UV_{254}, Brinkmann) permitted the UV detection of the above classes of compounds in amounts of approximately 5 μg per spot.

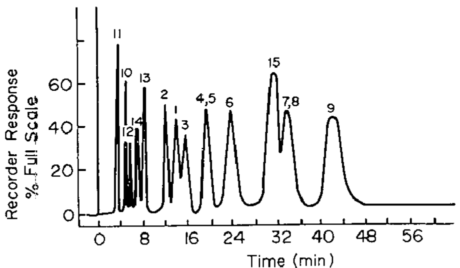
Gas chromatography

Five different procedures were surveyed in performing the analysis. For each

TABLE IV

GAS CHROMATOGRAPHIC PARAMETERS

	Determination				
	A	*B*	*C*	*D*	*E*
Instrument	Hewlett-Packard Model 700-1099F	Varian Model 1200	Varian Model 1200	Hewlett-Packard Model 5750	Hewlett-Packard Model 5750
Detector and temperature	Flame ionization; 290°	Flame ionization; 230°	Flame ionization; 230°	Electron-capture (^{63}Ni); 255°	Flame ionization; 260°
Column	Spiral of stainless steel; 6 ft. × 1/8 in. I.D.; packing: 5% OV-17 on 80/100 mesh Supelcoport	Spiral of stainless steel; 5 ft. × 1/8 in. O.D.; packing: 3% SE-30 on 100/120 mesh Varoport 30	Spiral of stainless steel; 10 ft. × 1/8 in. O.D.; packing: 10% OV-1 on 80/100 mesh Supelcoport	Spiral glass 6 ft. × 1/4 in. O.D.; packing: 4.9% OV-17 on 100/120 mesh Gas-Chrom Q	Spiral of stainless steel 6 ft. × 1/8 in. O.D.; packing: 3% OV-1 on 100/120 mesh Gas-Chrom Q
Column temperature	225°	200°	200°	240°	Programmed to 160° for 3 min; then 15°/min to 240°
Injector temperature	260°	250°	250°	255°	280°
Carrier gas	Helium, 50 ml/min	Helium, 20 ml/min	Helium, 20 ml/min	Helium, 70 ml/min; 5% methane in argon purge	Helium, 50 ml/min
Recorder and paper speed	Model H10-7127A strip chart; 0.25 in./min	Sargeant-Welch Model SRLG; 0.25 in./min	Sargeant-Welch Model SRLG; 0.25 in./min	Model 7128A strip chart; 0.25 in./min	Model 7128A strip chart; 0.25 in./min
Attenuation	5×10^2	8×10^2	8×10^2	8×10^2	8×10^2



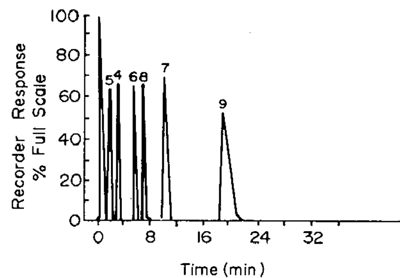


Fig. 1. Separation of the components of the isomeric chlorophenyl fluorosulfonyl-benzenesulfonates, fluorosulfonyl-benzenesulfonyl chlorides and acaricides on 5% OV-17 at 225°. The numbering of the peaks corresponds to that in Table V.

Fig. 2. Separation of the isomeric chlorophenyl *m*- and *p*-fluorosulfonyl-benzenesulfonates on 3% SE-30 at 200°. The numbering of the peaks corresponds to that in Table V.

TABLE V

GAS CHROMATOGRAPHY OF ISOMERIC CHLOROPHENYL FLUOROSULFONYL-BENZENESULFONATES, FLUOROSULFONYL-BENZENESULFONYL CHLORIDES AND ACARICIDES

Compound No.	*Derivatives*	*Retention times (min)*[a]				
		A[b]	*B*	*C*	*D*	*E*
Chlorophenyl o-fluorosulfonyl-benzenesulfonates						
1	*o*-Chlorophenyl	15.01	3.36	8.6	11.05 [6.45, 13.6]	11.24
2	*m*-Chlorophenyl	13.96	2.94	8.0	10.19	11.20
3	*p*-Chlorophenyl	15.87	4.60	10.5	12.01 [2.04, 5.13]	11.53
Chlorophenyl m-fluorosulfonyl-benzenesulfonates						
4	*o*-Chlorophenyl	19.78	3.65	10.0	10.02	11.21
5	*m*-Chlorophenyl	19.70	2.35	8.3	9.38	11.00
6	*p*-Chlorophenyl	24.00	6.34	10.2	5.05 [2.61, 11.19]	11.74
Chlorophenyl p-fluorosulfonyl-benzenesulfonates						
7	*o*-Chlorophenyl	35.10	10.67	12.65	11.57	—
8	*m*-Chlorophenyl	35.00	7.17	12.01	5.32 [2.61, 11.9]	—
9	*p*-Chlorophenyl	42.00	19.43	22.15	5.41 [2.22, 11.80]	—
Fluorosulfonyl-benzenesulfonyl chlorides						
10	*o*-Fluorosulfonyl-	5.15	5.07	10.1	—	2.92
11	*m*-Fluorosulfonyl-	3.80	3.51	8.5	—	2.64
12	*p*-Fluorosulfonyl-	6.05	6.34	11.5	—	4.54
Acaricides						
13	Ovex	8.04	4.18	10.4	—	9.00
14	Genite	6.90	3.60	10.1	—	8.94
15	Tedion (*p*-Chlorophenyl 2,4,5-trichlorophenylsulfone)	31.70	10.22	16.1	—	13.62

[a] Underlined values indicate major peak. Bracket values indicate trace quantities.
[b] Gas chromatographic parameters described in Table IV.

of these, experimental conditions and retention times are presented in Tables IV and V, respectively.

Fig. 1 illustrates the separation of the components of the isomeric chlorophenyl fluorosulfonyl-benzenesulfonates, fluorosulfonyl-benzenesulfonyl chlorides and acaricides on 5% OV-17 at 225°. Fig. 2 illustrates the separation of the components of the isomeric chlorophenyl *m*- and *p*-fluorosulfonyl-benzenesulfonates on 3% SE-30 at 200°. The numbering of the peaks corresponds to that in Table V. The use of a 5% OV-17 column at 225° (determination A, Tables IV and V) permitted the largest separation of isomeric constituents as well as the acaricides. However, this column does not separate the isomeric pairs 4 and 5 as well as 7 and 8 (*e.g.*, *o*- and *m*-chlorophenyl sulfonate isomers of the chlorophenyl *m*- and *p*-fluorosulfonyl-benzenesulfonates, respectively). However, the above classes of isomers were separated on 3% SE-30 at 200°. A 10 ft. × $\frac{1}{8}$ in. O.D. 10% OV-1 column at 200° (determination C, Tables IV and V) was less effective in resolving the various isomeric components while the use of a spiral glass 6 ft. × $\frac{1}{4}$ in. O.D. column coated with 4.9% OV-17 at 240° (determination D) effected poor separation (with some concomitant degradation) of the various isomers of the chlorophenyl fluorosulfonyl-benzenesulfonates.

The order of retention of both the isomeric chlorophenyl fluorosulfonyl-benzenesulfonates and fluorosulfonyl-benzenesulfonyl chlorides on the non-polar SE-30, OV-1 and OV-17 liquid phases was $p > o > m$.

The use of an electron-capture detector (^{63}Ni) permitted an enhanced sensitivity compared to flame ionization for the detection of the isomeric chlorophenyl fluorosulfonyl-benzenesulfonates (*e.g.*, 0.2–0.5 ng *versus* 1–3 ng for the latter). The response of these isomers compared to *p,p'*-DDT was 0.5 on a weight basis.

Attempts to achieve adequate separation of the variety of derivatives utilizing 3% OV-1 with temperature programming were generally unrewarding (Tables IV and V). It is also of interest to note that the retention times of the isomeric fluorosulfonyl-benzenesulfonyl chlorides were 2.9, 2.6 and 4.5 min for the *ortho*, *meta* and *para* isomers, respectively, *versus* 5.3, 5.3 and 7.7 min for their hydrolysis products (the respective isomeric fluorosulfonyl-benzenesulfonic acids, as determined by temperature programming determination E, Table IV).

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CHROM. 4849

APPLICATION OF THIN-LAYER CHROMATOGRAPHY TO HYDROCARBON-TYPE ANALYSIS

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(First received April 1st 1970; revised manuscript received May 22nd, 1970)

SUMMARY

Thin-layer chromatography has been applied to the hydrocarbon-type separation of heavy oils, with emphasis on separation of petroleum resins. A three-stage discontinuous layer-gradient plate was employed and found to give better separation of hydrocarbon types than a single adsorbent plate. Various solvent combinations were utilized and placed in an eluotropic series as applied to petroleum resin separation. Semi-quantitative information was obtained from a completed chromatogram plate by measuring the size of the bands or spots with a ruler or photoelectric scanner.

INTRODUCTION

Many petroleum processing studies require analyses of heavy oil samples in terms of hydrocarbon types. Hydrocarbon-type separations can be carried out by conventional liquid–solid elution chromatography. Resins can be removed by adsorption on an activated clay, and components in the deresined oil can be separated with a column containing both alumina and silica gel. In general terms, the goal is to separate the oil sample into saturates, monoaromatics, polyaromatics, and resins fractions. Conventional chromatographic separation is quite time consuming, and a simpler and faster method is needed to make the technique more widely applicable. Thin-layer chromatographic (TLC) separations can generally be carried out in 20–30 min, and if the developed zones could then be measured rapidly, a considerable advantage would be realized. Several techniques for separating heavy oils by TLC and the measurement of the developed plates have been investigated and are described below.

EXPERIMENTAL

Single adsorbent plates

Single adsorbent plates were prepared using TLC adsorbents with binder and spread in 250-μ layers with a Research Specialties Company variable thickness spreader. The plates were activated in an oven at 105° for 40 min. A rectangular glass

jar was used as a developing chamber. In addition, Eastman Chromagram Sheets, Type K 301-R2 (silica gel without fluorescent indicator) were used. These plates were heated in an oven just before use and developed in a sandwich-type apparatus. The ascending elution technique was used in all cases.

Three-stage plates

CRUMP[1] has reported that the calcium sulfate binder used in conventional silica or alumina thin-layer plates interferes with aromatic hydrocarbon separation and, for this reason, employed a "loose-layer" technique (thin adsorbent layer spread loosely on a glass plate without binder). However, the loose-layer technique is rather difficult to carry out experimentally since any slight jar will disturb the adsorbent bed. For greater handling ease, an investigation of a multi-stage TLC technique with binders was begun in this laboratory. A multi-stage TLC plate is composed of several different adsorbents (three in this case) placed side by side on a single glass plate. This three-stage technique permits the simulation of the larger scale multi-adsorbent separation.

Multi-stage or gradient-layer TLC was suggested by STAHL in 1964[2,3]. He designed special spreaders to prepare gradients of various configurations or several side-by-side layers. BERGER *et al.*[4] separated mixtures of iodide and iodinated derivatives of tetrachlorofluorescein on plates composed of side-by-side layers of silver chloride and a Dowex ion-exchange resin. A simple gradient spreading device that can be applied to the Shandon spreader was reported by WARREN[5]. OULLETTE AND BALCIUS[6] used mixtures of cellulose and Silica Gel G to separate iodine-containing compounds. For our work, a Research Specialties Company Model 200-11 spreader was adapted for three-stage plates by a very simple device, namely two aluminum dividers cut to fit snugly inside the spreader. A bevel or knife edge was placed on the bottom of the dividers. The three-stage plates were prepared using 250-μ layers of Silica Gel G, Aluminum Oxide G, and Florisil G with phosphor.

Spot measurement techniques

The simplest technique for measuring the separated spots or zones is to use a ruler. Measurement with a ruler is facilitated if the plate is marked off in narrow columns (0.3–0.4 cm wide) to confine the developed sample. The separated zones were also measured with two different photoelectric scanner arrangements. The photometer used in both cases was a photomultiplier type that can accommodate several different tube types interchangeably. A long wavelength (3660 Å) UV and a tungsten lamp (15 W, frosted) were used in one arrangement. In addition to these, a short wavelength (2537 Å) UV lamp and 6 W clear tungsten lamp were used in another arrangement.

In the first arrangement, the light source and plate were held fixed and the phototube was moved manually behind the plate. The second scanner system was used principally with strips of Eastman Chromagram Sheet that were pulled across the face of the phototube. The photometer used was an Eldorado Electronics Universal Photomultiplier Photometer Model PH-200, and the photometer signal was fed to a 10 mV Brown recorder. A 1P21 phototube was employed for most of the work. The slit positioned between the TLC strip and the phototube had a width of 0.1 cm and a length of 1.1 cm and was found to give better chromatograms than a

smaller slit that was used initially. The drive motor for the TLC strips was a Gra-Lab timer with the hands removed (speed: 0.5 in./min). A set of cardboard guides and a table were placed over the lower slit so that a strip could be pulled smoothly across the slit. A small hole was placed in the middle of each end of the strips to fasten a small hook for pulling in the forward direction and a counter weight to be fastened to the rear. AMOS[7] has used several photoelectric densitometers to measure additive depletion in used lubricating oils and found that precision of $\pm$ 5% could be obtained with the Joyce-Loebl Chromoscan.

RESULTS AND DISCUSSION

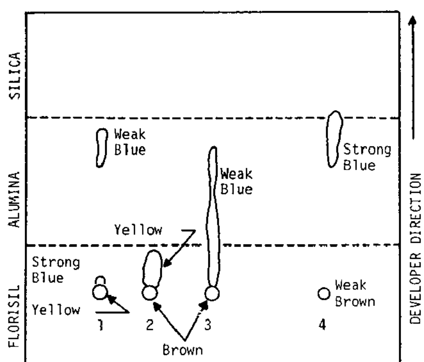
Three-stage plates with adjacent layers of silica gel, alumina, and Florisil were used to separate monoaromatic, polyaromatic, and resin cuts from a middle distillate (average molecular weight = 373). The development direction was from the Florisil to the silica gel layer. By the use of mixed developers such as cyclohexane–benzene–ethyl acetate (105 : 1 : 1), it was possible to obtain a developed plate containing one of the separated cuts in each stage. With a mixed developer composed of petroleum ether–ethyl acetate–acetone (380 : 1 : 1), separation was obtained between *n*-hexadecane and an alkylbenzene in the silica gel stage.

For qualitative comparison, several resin fractions removed from different oil samples were chromatographed on three-stage plates. These fractions were obtained from molecular distillation of West Texas straight run residues having average molecular weights 249, 373, and 959. From the developed chromatograms, it was observed, as expected, that resins from the 249 molecular weight fraction contain less heavy material than the other resins from this series. Resins from the 959 molecular weight fraction cover the largest area on the plate. Two other resin cuts, raw catalytically cracked heavy gas oil (CCHGO) and hydrotreated flashed distillate, formed long streaks from the Florisil up into the alumina, whereas most of the other chromatographed resins formed separated spots in the Florisil and alumina layers. Fig. 1 shows a sketch of the separation obtained with some of these resins.

To determine if three-stage TLC plates offer a definite advantage over plates with one adsorbent alone, a comparison was made of the separated cuts from the 373 molecular weight fraction (1, monoaromatics; 2, polyaromatics; 3, resins) on a three-stage plate and on plates of Alumina G, Silica Gel G, and Florisil G alone. The developer used in all three cases was cyclohexane–benzene–ethyl acetate (42 : 1 : 1). For this investigation, the plates were not sectioned off in columns.

The three-stage plate gave reasonable separation of all three cuts from the 373 molecular weight fraction. Alumina G did not give separation of the mono- and polyaromatics, but the resins remained near the starting point, except for the usual portion that moves with the polyaromatics. Silica Gel G very nearly gave separation of the mono- and polyaromatics, but considerable overlap occurred with the polyaromatics and resins. A Florisil plate gave a fair separation of the three types, but long tails were observed, both with the polyaromatics and resins. The separations obtained in these cases are illustrated in Fig. 2, 3, 4, and 5.

Somewhat different results were obtained when cyclohexane alone was used as developer. On Alumina G, the separation with cyclohexane was slightly better than with the mixed solvent above, but still not equal to that of the three-stage plate. In



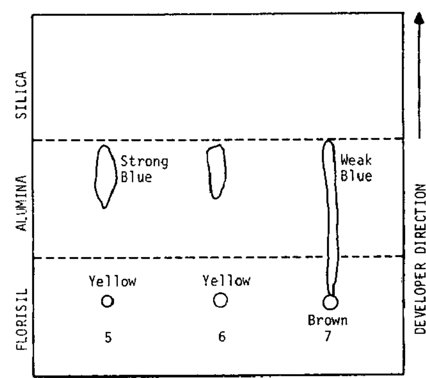
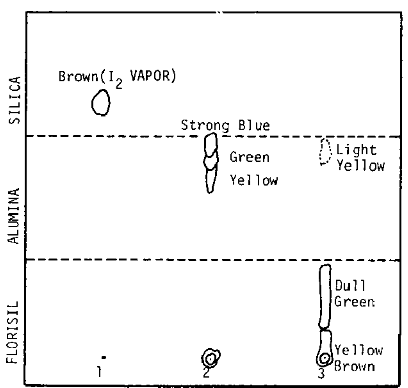


Fig. 1. Three-stage TLC of resin cuts. Developer: cyclohexane–benzene–ethyl acetate (105 : 1 : 1, v/v/v). (1) 249 molecular weight fraction; (2) 373 molecular weight fraction; (3) 959 molecular weight fraction; (4) hydrotreated flashed distillate; (5) hydrotreated CCHGO; (6) hydrotreated CCHGO; (7) hydrotreated flashed distillate.



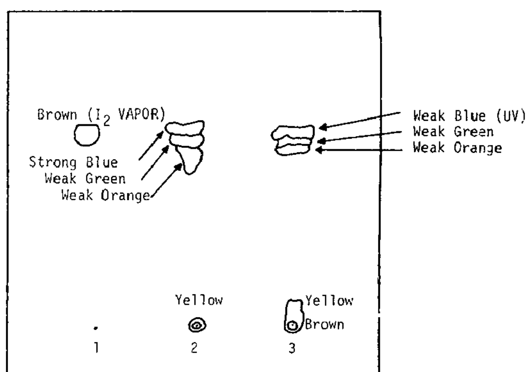


Fig. 2. Comparison of TLC adsorbents—three stage. Developer: cyclohexane–benzene–ethyl acetate (42 : 1 : 1, v/v/v). (1) monoaromatics; (2) polyaromatics, (3) resins.

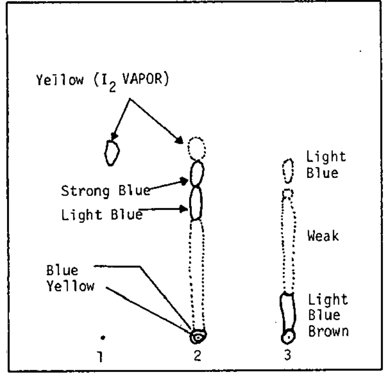
Fig. 3. Comparison of TLC adsorbents—Alumina G. Developer: cyclohexane–benzene–ethyl acetate (42 : 1 : 1, v/v/v). (1) monoaromatics; (2) polyaromatics; (3) resins.

the silica gel case, cyclohexane development was worse than with the mixed solvent. Development of the Florisil plate with cyclohexane alone gave good separation of the monoaromatics, but the polyaromatics and resins remained near the starting point.

It is apparent from the separations illustrated in Fig. 1 through 5 that not only are the polyaromatic fractions at least partially resolved on the TLC plates (presumably according to molecular polarity) but also the resin fractions can be separated into sub-classes in rather striking fashion in some cases.

Correlation of sample weight and zone length

If different amounts of the same resin sample are developed with cyclohexane on a plate of Florisil alone, a relatively linear calibration curve can be obtained that



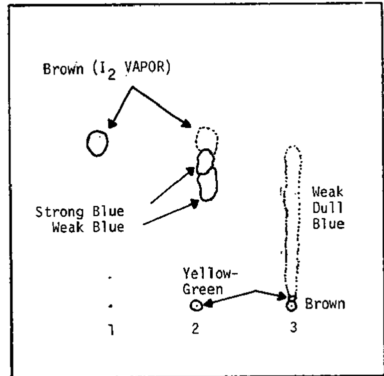


Fig. 4. Comparison of TLC adsorbents—Florisil G. Developer: cyclohexane–benzene–ethyl acetate (42 : 1 : 1, v/v/v). (1) monoaromatics; (2) polyaromatics; (3) resins.

Fig. 5. Comparison of TLC adsorbents—Silica Gel G. Developer: cyclohexane–benzene–ethyl acetate (42 : 1 : 1, v/v/v). (1) monoaromatics; (2) polyaromatics; (3) resins.

TABLE I

EFFECT OF SAMPLE WEIGHT ON ZONE LENGTH

Sample wt. (μg)	*Developed distance (cm)*	
	Run 1	*Run 2*
50	3.30	3.10
186	4.65	4.75
304	6.20	6.40
444	7.85	7.70

relates weight of applied sample to length of fluorescent zone. Shown in Table I is a comparison of the zone lengths obtained from duplicate chromatograms of a given resin fraction previously separated from a deasphalted oil with a molecular weight of 772. These samples were developed with cyclohexane for a distance of 15 cm on 500-μ thick plates of Florisil.

The resin content of a sample of heavy flasher tops was determined several times by large-scale liquid–solid chromatography, giving an average value of 6.6 wt. %. This sample was separated with a petroleum ether–ethyl acetate (280 : 1) developer on a three-stage TLC plate marked off in 0.4-cm columns. Based on the length of the fluorescent resin spot in the Florisil layer compared to the total sample length, the results of several analyses are given in Table II. It is obvious from the data in Table II that some uncertainty exists in the result; however, some scatter also occurs in the large-scale liquid–solid chromatographic technique. In one series of large-scale separations, the resin content of repeat runs on a Kuwait flashed distillate varied from 5 to 9%.

Further tests were made with a series of synthetic gas oils prepared from raw CCHGO components. A resin fraction and a deresined oil fraction, separated previously from a CCHGO sample, were recombined to give gas oil samples of about 2, 4, 6, and 10 wt. % resins. The results for resin determinations on these samples are

TABLE II

RESINS IN HEAVY FLASHER TOPS

Run	*% resins*
1 through 10	4.6, 7.4, 7.7, 7.4 7.1, 7.2, 6.5, 5.4, 5.9, 6.3
Average	6.6

given in Table III. Runs 2 and 3 were duplicates developed on the same plate. Run 5 was different from the others in that samples were diluted so that about the same weight of resin was applied to all spots. After development, the measured lengths were multiplied by the proper factors to account for dilution. This observation suggests that sample size is important and that too large a sample size leads to low results.

TABLE III

RESINS OBSERVED IN SYNTHETIC GAS OIL

Run	*Developer*	*Nominal wt. % resin*			
		2	*4*	*6*	*10*
1	Cyclohexane–benzene–ethyl acetate (105 : 1 : 1)	4.6	4.8	—	—
2	Isooctane	2.4	3.8	4.9	6.6
3	Isooctane	2.4	4.1	5.6	7.0
4	Isooctane	2.4	3.9	5.4	7.4
5[a]	Cyclohexane–benzene–ethyl acetate (105 : 1 : 1) Benzene–cyclohexane (40 : 1)	2.0	4.0	6.0	9.6

[a] Developed with two solvent combinations in sequence; first development to 18 cm, second to 4 cm.

Eluotropic series of solvents for resins

When heavy oil samples are separated by TLC into the various component fractions (*e.g.*, via cyclohexane–benzene–ethyl acetate (105 : 1 : 1)), the resin fraction is compact and usually extends over only a relatively small area of the plate near the starting point even though the other component types develop suitably. To avoid some of the problems associated with compacted areas, a number of developer combinations has been examined for their ability to spread the resin fraction over a larger area of the plate. Ideally, all of the other fractions present would be oriented with the new developer in the upper portion of the plate, *i.e.*, either in the silica or alumina stages for the case of a three-stage plate, much as they were with cyclohexane–benzene–ethyl acetate. When only migration of the resin portion of a sample on a three-stage plate was considered, the classification shown in Table IV for various solvents was obtained. A high rating in the eluotropic series does not necessarily mean that a particular solvent combination is eminently suited for development of the resins in a multi-component application. An example is the development of the re-

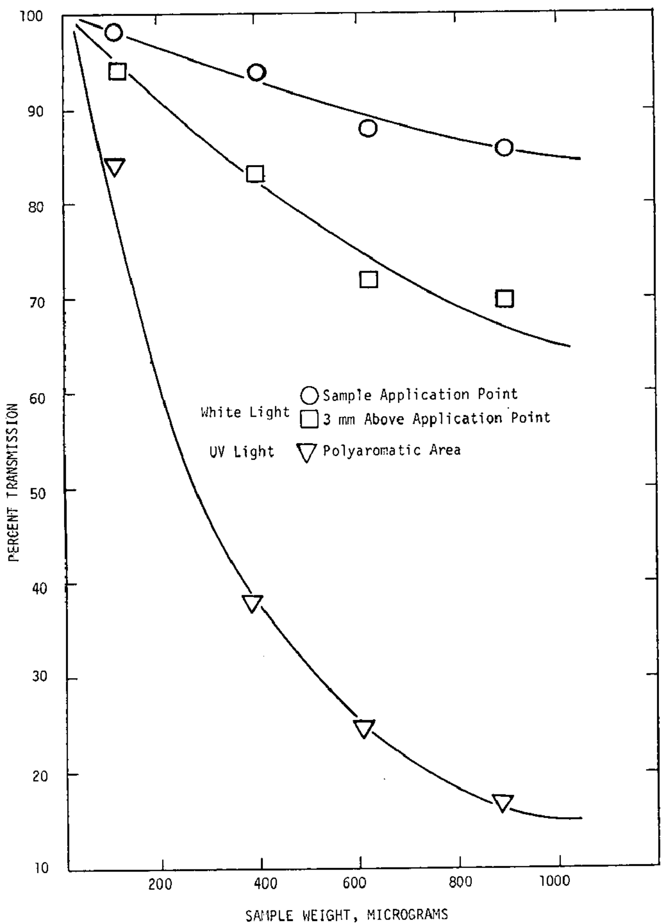


Fig. 6. Effect of sample weight on photometer readings.

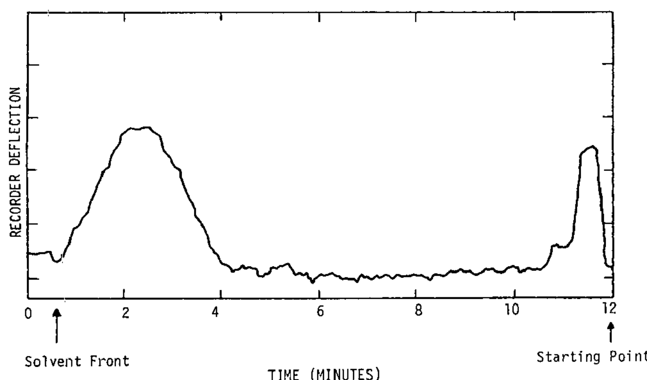


Fig. 7. Thin-layer chromatogram of hydrotreated flashed distillate.

TABLE IV

ELUOTROPIC SERIES OF SOLVENTS FOR RESINS

Increasing elutive power—
—Water
—Benzene–petroleum ether (4 : 6)
—Methylene dichloride
—Chloroform–petroleum ether (6 : 4)
—Methanol
—Acetone
—Benzene–acetone (1 : 1)
—Benzene–ethyl acetate–dimethyl sulfoxide (10 : 10 : 1)
—Benzene–ethyl acetate (1 : 1)

spective monoaromatic, polyaromatic, and resin cuts from the 373 molecular weight fraction with a solvent mixture composed of 10 : 10 : 1 benzene–ethyl acetate–dimethyl sulfoxide (DMSO). This solvent retarded the monoaromatics relative to the polyaromatics and resins. With a solvent of methanol–DMSO–benzene–ethyl acetate (16 : 3 : 1 : 1), the polyaromatics and monoaromatics were both retarded relative to the resins. Alteration of the elutive power of benzene with cyclohexane, at a ratio of 19 : 1 for example, will approach the desired development of resins from the 373 molecular weight fraction relative to the other cuts.

Photoelectric scanners

Different amounts of a deasphalted oil sample were developed with cyclohexane on a three-stage plate and scanned by the manual technique. Fig. 6 shows the data obtained from measurements made at three levels on this plate; namely, at the sample application point, at 3 mm above the sample application point, and in the polyaromatic area (alumina) about 10 cm above the starting point. The results obtained are reasonably consistent and suggest the possibility that, with good repeatability of zone development, a photometer with fixed apertures might be used to provide quantitative data.

The technique of marking off a plate in narrow columns was attempted with Eastman Chromagram Silica Gel Sheets, but the results were not as good as those obtained with the laboratory-prepared glass plates. The principal reason for this difference is that the layer on the Chromagram sheet is somewhat brittle, which results in a ragged edge. The ragged edge permits sample to "leak" out of the column, thus adding to the measuring difficulty.

Better results were obtained with Chromagram sheets by first developing samples on an 8 × 8-in. sheet and then cutting developed sample strips with scissors. The second scanner arrangement was adapted with guides over the slit so that these small strips could be pulled across the slit with the timer motor. Since much of the resin material is colored, a tungsten lamp was used for most of the work. A recorded chromatogram that was developed with cyclohexane–benzene–ethyl acetate (105 : 1 : 1) is shown in Fig. 7. This strip (silica gel) was scanned from the solvent front to the starting point. The large peak near the solvent front represents the less polar compounds and the peak near the starting point is due to strongly adsorbed polar materials. This was a sample blended in the laboratory to contain 2 wt. % resins and 98 wt. % deresined oil. Preliminary results indicate that over a limited concentration

range, the resin peak height is proportional to concentration and perhaps can be employed for the rapid quantitative measurement of resin content of oils.

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CHROM. 4825

A TWO-DIMENSIONAL THIN-LAYER CHROMATOGRAPHIC TECHNIQUE FOR THE RESOLUTION OF MONOCARBONYL DINITROPHENYLHYDRAZONES

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(Received May 11th, 1970)

SUMMARY

Carbonyl dinitrophenylhydrazones have been separated by class (alkanone, alkanal, alk-2-enal and alka-2,4-dienal) using adsorption thin-layer chromatography and on the basis of chainlength using partition techniques. A two-dimensional technique is now described in which the two separations can be effected on one plate. The problem that initial class separation demands a small sample to prevent overloading, yet secondary chainlength separation following Carbowax 400 impregnation demands a large sample to allow facile detection, has been solved by employing a novel chromatographic concentration step between separations.

A complete separation can be achieved during one working day.

INTRODUCTION

Since carbonyl compounds, formed as a result of autoxidation, contribute significantly to the flavour and aroma of fats and fat-containing foods, their generation, isolation and analysis in such systems has been extensively studied. Amongst the methods used in these studies, thin-layer chromatography (TLC) of the 2,4-dinitrophenylhydrazones (DNPs) has been extensively exploited as it offers the possibility of quick and easy fractionation of extremely small quantities of the isolated derivatives.

Although ONOE[1] and later DHONT AND DE ROOY[2] had used TLC to separate the DNPs of a limited range of carbonyl compounds, it was not until 1963 that SCHWARTZ AND PARKS[3], BADINGS AND WASSINK[4] and URBACH[5] employed TLC for the examination of the wide variety of carbonyls formed in autoxidizing lipids.

SCHWARTZ AND PARKS[3] proposed the use of magnesium oxide to resolve DNPs into classes by adsorption chromatography. BADINGS AND WASSINK[4] proposed class separation by a zinc carbonate adsorption technique or complex formation by silver

nitrate impregnated kieselguhr; for chainlength separation they proposed partition between Carbowax 400 and petroleum ether, amongst other systems. For resolution of complex mixtures into individual components, they used a three stage separation, recovering groups of components by solvent elution of selected bands for re-running in the subsequent systems. URBACH[5] achieved class separation by adsorption chromatography on alumina or on silver nitrate impregnated plates, and chainlength separation by partition on 2-phenoxyethenol impregnated plates. She employed two-dimensional systems for resolution of complex mixtures.

In addition to these papers, many others have appeared advocating different adsorbents or partition systems[6], application to other carbonyl types[7–13] and improvements in technique[14], but fundamentally, it remains true that classes are separated by adsorption and chainlengths by partition chromatography.

In this laboratory, the most satisfactory techniques investigated have been magnesia adsorption chromatography for class separation, and Carbowax 400 partition chromatography for chainlength separation.

A two-dimensional technique is described in which separation is effected first by class and then, after impregnation with Carbowax, on a chainlength basis. The problem that a small sample must be applied in the first dimension (to prevent overloading) and that a large sample is required for the second dimension (to allow facile detection) has been solved by employing a chromatographic concentration step between separations.

EXPERIMENTAL

Materials

Magnesium oxide (B.D.H.)
Microcell T 38 (Johns Manville)
Carbowax 400.
Solvents: petroleum ether 40–70°, SVR and chloroform, distilled to remove non-volatile residue.
TLC Sample Streaker (Applied Science Laboratories)
TLC spreader and associated apparatus (Desaga)

Preparation of TLC plates

The magnesium oxide used for the adsorbent layer is dried overnight at 110°. Magnesium oxide (20 g) and Microcell T 38 (20 g) are slurried with water (120 ml) and rolled in a ball mill for ½ h to ensure intimate mixing and grinding of coarse particles. The slurry so prepared is sufficient to coat five plates 200 × 200 mm. The plates are air-dried for 1 h, activated for 1 h at 60° and then stored until required in a desiccator containing freshly dried silica gel.

Development of chromatogram

Fig. 1 represents the various stages of development of the chromatogram. The sample is applied as a streak running longitudinally in the direction of spreading of the adsorbent layer, *i.e.*, the first solvent is run at right angles to the spreading direction. The sample streak is applied at least 5 cm from the bottom of the plate, commencing 4 cm from the left hand edge and terminating 3 cm from the right hand edge. The

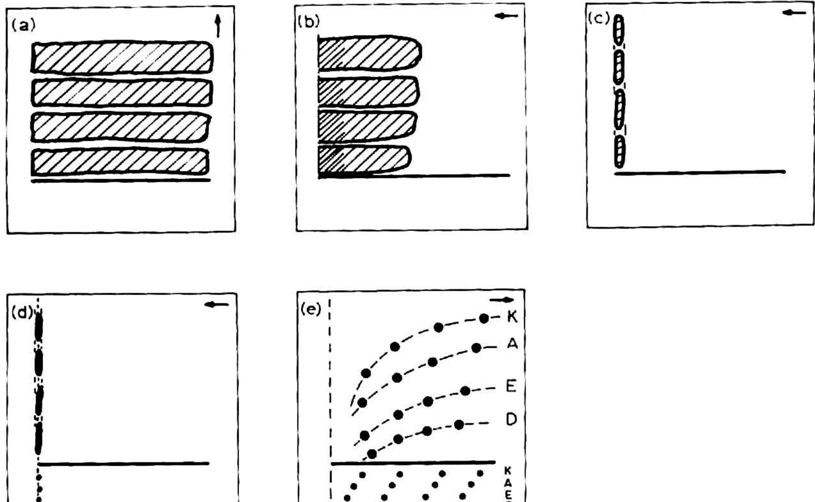


Fig. 1. Diagrammatic representation of the two-dimensional separation of dinitrophenylhydrazones (direction of solvent travel indicated by arrow). (a) Stage 1, class separation; (b) Stage 2, first concentration; (c) Stage 3, second concentration; (d) Stages 4 and 5, impregnation and marker application; (e) Stage 6, chain length separation. K = ketones; A = anals; E = enals; D = dienals.

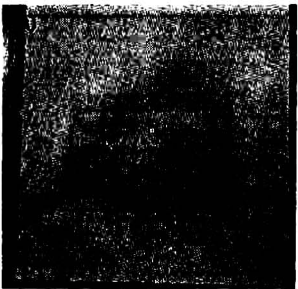
plate is immediately developed in a tank lined with filter paper saturated in the developing solvent (petroleum ether–chloroform (85:35). The solvent is allowed to rise for approximately 12 cm after which the plate is removed and the solvent front marked by small nicks at the edges.

For the second development, the right hand edge becomes the bottom of the plate and the separated bands are chromatographed in a solvent consisting of chloroform–SVR (80:20). Development is continued until the solvent front just reaches the top limit of the original streak. After brief drying, the plate is developed a second time in the same direction in the same solvent. As all DNPs have nearly unit R_F value in this solvent, they are now all concentrated into fairly compact bands near the top of the plate. A third development is given in the same direction in a solvent consisting of 20% Carbowax 400 in chloroform, development being continued until the solvent front moves just past the line of DNPs. This treatment serves to impregnate the plate with stationary phase and further compacts the DNP bands.

After brief air drying, mixtures of known DNPs are spotted on to the 5 cm section below the original sample streak, the plate is turned through 180° and the chainlength resolution effected by development in petroleum ether. Fig. 2 shows a typical separation achieved.

DISCUSSION

Whilst the technique described appears complex, there is really little actual work involved; the whole fractionation can be completed within one working day and, provided certain precautions are observed, the separations achieved are remarkably clear.



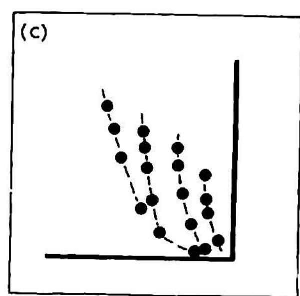


Fig. 2. TLC separation of dinitrophenylhydrazones. (a) Stage 1, class separation; (b) Stage 6, final chromatogram of 19 DNPs; (c) diagram of (b). From left to right: ketones, $C_{3,5,7,11}$; anals, $C_{1,3,5,7,9,11}$; enals, $C_{3,5,7,9,11}$; dienals, $C_{6,8,9,11}$.

The main requirement for success is that the DNP classes must be well separated in the first dimension, and the bands produced must be as near to linear as possible. The most important factors involved in the class separation are careful preparation of the absorbent, even coating of the plate and sample application in a narrow streak of uniform concentration along the length of the streak. This latter factor is important since the R_F value of the various classes is influenced by concentration, a high concentration leading to a higher R_F value. Quite spectacular improvement in clarity of class resolution can be achieved by applying a thin (1 mm) even line of sample and, to achieve this ideal, the use of a mechanical streaker has been found to be virtually mandatory.

The maintenance of linear bands during the first development appears to be dependent upon two factors, *viz.* the use of a filter paper lining to the solvent tank to ensure rapid equilibration of the plate with the solvent atmosphere prior to development, and application of the sample streak at least 5 cm above the bottom of the plate. Such sample applications ensure an appreciably lower rate of flow of solvent by the time it reaches the sample, and thus allow a more even development. When the sample was applied closer to the plate edge, the separated bands always were curved, and usually of higher R_F value near the edges of the plate. Moreover, the 5 cm sample-free band is useful in that it is later used for the application of known DNPs to assist identification of the unknown spots.

Usually adequate class separation can be achieved with one solvent development. Occasionally, the adsorbent activity tends to be too high with resultant lower R_F values and lesser separation. A second development can serve to improve resolution.

For reasons unknown, overnight drying of the magnesium oxide was found to reduce the tendency of the spots to tail during the chainlength separation. With this exception, there appears to be no other critical factor which must be observed to effect clean fractionation once the class separation has been achieved.

In the interest of speed, it has been found convenient to use a large volume of Carbowax solution during the impregnation step and so start the solvent travel from well up the plate. This is possible because two developments with chloroform–alcohol move the DNPs nearly to the desired starting line.

Separation into classes is not absolute. Whilst, in general, the R_F values decrease in the order ketones, alkanals, alk-2-enals and alka-2,4-dienals, the shortest chain-

length members have R_F values approximately equal to that of one class lower, *i.e.* acetone DNP runs with the alkanals etc. Likewise, the chainlength separation is to some extent influenced by the class. However, after two-dimensional separation there are no compounds inseparable within the chainlength limitations of the Carbowax plate. Homologues fall along a smooth curve.

With the impregnation technique described it is possible to separate compounds differing by one carbon atom up to 9 for alkanones, 10 for alkanals, 12 for alk-2-enals and 12 for alka-2,4-dienals. No doubt the limit for chainlength separation could be increased somewhat by applying a more concentrated Carbowax solution. At the other end of the scale, the resolution of the short chain members can be increased if necessary by running the plate a second time in petroleum ether. Qualitative identification remains facile because the markers on the side of the plate receive the same treatment.

Sensitivity of detection can be increased by spraying with a solution of 10% potassium hydroxide in 80% aqueous ethanol. This offers the further advantage that the classes develop different colours, *viz.* alkanones—grey-brown to brown, alkanals—brown to reddish brown, alk-2-enals—pink-brown and alka-2,4-dienals—mauve.

ACKNOWLEDGEMENTS

We are grateful for the discussions with Mrs. C. SZONYI; the technical assistance by Mrs. J. VAN WEIJE and Mrs. K. DEAKIN is gratefully acknowledged.

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CHROM. 4815

QUANTITATIVE ANALYSIS OF NITRO COMPOUNDS IN THE MICRO- TO PICOGRAM RANGE BY A COMBINATION OF THIN-LAYER AND VAPOR PHASE CHROMATOGRAPHY WITH THE NICKEL-63 ELECTRON CAPTURE DETECTOR

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(Received May 4th, 1970)

SUMMARY

A method has been developed for the quantitative analysis of nitro compounds in the micro- to picogram range by a combination of thin-layer and vapor phase chromatography employing the nickel-63 electron capture detector. The relative electron absorptivities of ten nitro compounds have been measured with 1,3,5-trinitrobenzene as a standard. Experimental variables affecting the nickel-63 electron capture detector are presented.

INTRODUCTION

Recently we reported a method for the quantitative analysis of polynitroaromatic compounds in complex mixtures by combination of thin-layer chromatography (TLC) and visible spectrometry[1]. The method is based on the formation of a colored "Meisenheimer" complex between the polynitroaromatic compound and ethylenediamine (EDA) in dimethylsulfoxide (DMSO) solution[2] after separation and extraction from thin-layer plates. This method has proved particularly useful in those cases where stable colored complexes were formed which possessed relatively high molar extinction coefficients (15 to 20 $\times$ 10^4) and whose solutions obeyed Beer's law. However, there are instances where the method fails. Cyclotrimethylenetrinitramine (RDX), for example, forms no colored complex with EDA in DMSO solution[2]. In addition, Beer's law was found not to hold for both the *meta*- and *para*-dinitrobenzenes[2], while variable results were obtained with picryl chloride in EDA–DMSO solutions[3]. In other cases the development of the colored complex was quite slow as with 1,3,5-trimethyl-2,4,6-trinitrobenzene[2]. Rationales for these results have been presented[2] in terms of chemical interactions involving both displacement of nitro groups and irreversible reactions between the polynitroaromatic compound and the nucleophilic base, ethylenediamine.

It appeared that quantitative analysis of nitro compounds in amounts usually

encountered in TLC separations (10^{-5} to 10^{-6} g) might be possible by vapor phase chromatography (VPC) with an extremely sensitive detector. In this regard, the nickel-63 electron capture detector has been most valuable in pesticide residual analysis where amounts as low as 10^{-12} g of halogen containing pesticide have been detected[4-7]. Furthermore, LOVELOCK AND LIPSKY[8] found, for example, that the electron affinity of nitrobenzene was about 35% that of hexachlorobenzene, C_6Cl_6, a compound which was detectable at the picogram (10^{-12} g) level. We wish now to report a method for the quantitative analysis of nitro compounds by a combination of TLC and VPC employing the nickel-63 electron capture detector.

EXPERIMENTAL

Preparation of Silica Gel HF-254 thin-layer plates

Thirty grams of fluorescent Silica Gel HF-254 (Brinkman Inst. Co.) was vigorously slurried for 2 min in 65 ml of distilled water in a 500 ml erlenmeyer flask to make 11 to 12 10 × 20 cm TLC plates with a Camag applicator. The plates were allowed to dry partially at room temperature for 3–4 h, then in a drying oven at 110° for 1.5 h. The dried plates were stored at room temperature in a closed container.

Developing solvents

Nitro compounds were developed with (a) benzene–hexane–pentane (50:40:10), and (b) benzene–hexane–pentane–acetone (50:40:10:10) depending on the separation desired (see Table I).

TABLE I

R_F VALUES OF VARIOUS NITRO COMPOUNDS ON SILICA GEL HF-254 PLATES VISUALIZED UNDER 2537 Å UV LIGHT

Compound	*R_F value*[a]	*R_F value*[b]
Cyclotrimethylenetrinitramine (RDX)	0.02	0.06
2,3,4-Trinitrotoluene	0.13	0.24
1,2-Dinitrobenzene	0.18	0.45
2,4,6-Trinitroanisole	0.20	0.74
1,3,5-Trinitrobenzene	0.21	0.76
2,4,6-Trinitrotoluene	0.28	0.83
1,3-Dimethyl-2,4,6-trinitrobenzene	0.34	1.00
2,4,6-Trinitro-1-chlorobenzene	0.34	0.84
1,3,5-Trimethyl-2,4,6-trinitrobenzene	0.51	1.00
1,3-Dichloro-2,4,6-trinitrobenzene	0.56	0.90
1,3,5-Trichloro-2,4,6-trinitrobenzene	0.84	0.95

[a] Developing solvent (a).
[b] Developing solvent (b).

Zone visualization on TLC plates

Developed zones containing the nitro compounds were visualized under a 2537 Å UV light source with a Chromato-Vue Cabinet (Ultra Violet Products Inc., San Gabriel, Calif., U.S.A.) and appeared as dark spots against a yellowish-green background. Limits of detection were found to be approximately 4×10^{-7} g/spot.

Vapor phase chromatography

An F & M Model 5754A Research Gas Chromatograph equipped with a Model 5763A electron capture (EC) Nickel-63 detector and pulser kit together with a Model 7128A Moseley Dual Channel Recorder was used for all the compounds studied.

Septa were cleaned by thoroughly washing with distilled water, acetone, and hexane (GC spectrophotometric quality solvent, Baker Chem. Co.) and finally heated at 250° overnight under a vacuum of approximately 1 mm Hg.

Column preparation

The following general procedure is recommended to provide an even coating of the liquid phase on the solid support and to avoid the formation of fines encountered in any mechanical grinding of the solid support and is illustrated for the preparation of a 2.53% Apiezon M liquid hydrocarbon phase on 60/80 mesh Diatoport-S. A total of 0.26 g of Apiezon M was dissolved in 50 ml of boiling benzene (special quality recommended for pesticide residual analyses, No. 1043, Mallinckrodt Chem. Works) and then added to a boiling mixture of 10.0 g of 60/80 mesh Diatoport-S (Hewlett Packard ST-120-1) and 50 ml benzene. The mixture was then boiled fairly vigorously in a fume hood until most of the benzene had been removed, and the damp cake was stirred occasionally with a clean plastic spatula until no vapors of benzene were detected. The dry, coated support was then vacuum loaded by means of a water aspirator into a clean 4 ft. × $\frac{1}{4}$ in. glass column, and conditioned at 200° with a flow of Ar–CH_4 (95:5) at 70 ml/min for 3 h. During the conditioning period the column was disconnected from the nickel-63 detector to avoid contamination.

The following columns were used for all the nitro compounds studied: 4 ft. × $\frac{1}{4}$ in. glass, packed with 1.18%, 2.04%, 2.92%, 3.51%, and 5.33% Apiezon M liquid hydrocarbon and 3.75% silicone grease DC-11 on 60/80 mesh Diatoport-S. Methylene chloride was used as solvent in the preparation of the silicone coated column, while benzene was used to prepare the Apiezon M columns.

Analytical procedure

Since the nickel-63 detector is extremely sensitive, it is also quite easily contaminated or overloaded. As little as 1×10^{-6} g of 1,3,5-trinitrobenzene was found to overload the detector and resulted in almost complete loss of sensitivity. The contaminant was completely removed only after baking out the detector at 300° with a flow of Ar–CH_4 (95:5, v/v) also at 300° for a period of 12 h. Therefore, it is necessary to use two sets of Hamilton syringes: (1) one for concentrated solutions and spotting TLC plates, and (2) another for VPC analyses. These syringes were kept thoroughly clean by washing with acetone followed by high purity benzene between injections.

Stock solutions containing known amounts of nitro compounds in concentrations of approximately 2×10^{-6} g/μl in benzene or benzene–acetone (90:10) were accurately spotted by means of a 10 μl (No. 701) or 50 μl (No. 705) Hamilton syringe onto Silica Gel HF-254 TLC plates. The volume spotted in each case was no more than 5 μl/spot and the total number of spottings for a 10 × 20 cm plate was no more than 8. 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. After the solvent front had traveled a distance of between 10 and 14 cm, the plate was removed from the developing chamber and

allowed to dry in a vacuum hood for about 10 min, before visualization under 2537 Å UV light.

The zones appeared as dark spots against a yellow-green background under UV light and were marked and scraped off into a small beaker prior to extraction as previously described[1]. This procedure was modified with equally good results by scraping the zones directly into a 10 ml volumetric flask and extracting with benzene or benzene–acetone (90:10) without physically removing the silica gel support. The volume occupied by the silica gel is negligible and is compensated for by the addition of an internal standard. For zones with low R_F values, for example RDX, it was found best to add 1 ml of acetone to the silica gel in the volumetric flask, swirl for about 1 min, then dilute with benzene to make 10 ml of solution. To this solution was added 24 μl of a solution of 1,2-dinitrobenzene (1.804×10^{-7} g/μl) as internal standard (IS) to make a final concentration of 4.33×10^{-10} g/μl. Concentrations of nitro compounds in solution varied from 4 to 70×10^{-10} g/μl depending on the relative responses of the individual compound (see Table VIII). Injections of 1.0 to 2.8 μl of these solutions were made with 2.8 μl solvent "back flush". "Back flush" was accomplished by drawing 2.8 μl of solvent into a 10 μl syringe, followed by about 0.2 μl air, and finally by 1.0 to 2.8 μl of sample solution. Sample injection was followed by injection of a standard solution containing a known concentration of the nitro compound with the same concentration of internal standard as in the sample solution.

The grams, g_{tlc}, of nitro compound extracted from the TLC plate into 10 ml of solvent were found from the expression,

$$g_{tlc} = (nh_{tlc}/h_{std})(C_{std})(10^4) \quad (1)$$

where nh_{tlc}, h_{std}, and C_{std} are the normalized sample peak height, standard peak height, and standard concentration of nitro compound in 10 ml of solution expressed in g/μl.

The normalized sample peak height, nh_{tlc}, is readily found by,

$$nh_{tlc} = (h'_{IS}/h''_{IS})(h_{tlc}) \quad (2)$$

where h'_{IS} and h''_{IS} are the internal standard peak heights for the standard and sample (TLC extract) solutions, respectively.

The total number of grams of nitro compound, g_{total}, in volume, V, from which the TLC spottings were made, may be calculated from the expression,

$$g_{total} = g_{tlc}(V/\mu l_{tlc}) \quad (3)$$

where μl_{tlc} is the total volume of solution spotted on the TLC plate. In practice, it is not necessary to know the precise volume of the TLC extraction, as long as known aliquots of internal standard are injected into the TLC extract and standard solutions (see Table II). The concentrations of internal standard and standard nitro compound in 10 ml of solution must, however, be known exactly.

RESULTS AND DISCUSSION

Thin-layer and vapor phase chromatographic analysis of 1,3,5-trinitrobenzene (TNB)

The vapor phase chromatographic analysis of TNB after development and

TABLE II

ANALYSIS OF 1,3,5-TRINITROBENZENE (TNB) BY A COMBINATION OF THIN-LAYER AND VAPOR PHASE CHROMATOGRAPHY

Solution	*Peak heights*[a]		*Normalized peak height TNB (mm)*	*μg TNB*[b] *found*
	IS (mm)	*TNB (mm)*		
TLC extract	211[c]	163	164 (nh_{tlc})	17.4[d]
VPC standard[e]	213[f]	167	167 (nh_{std})	

[a] IS = internal standard, 1,2-dinitrobenzene.
[b] Calculated from: (μl IS_{sample})/(μl $IS_{standard}$) $\times$ (nh_{tlc})/(nh_{std}) $\times$ C_{std} $\times$ 10^4.
[c] μl IS_{sample} = 12; that is, 12 μl of internal standard (1.804 $\times$ 10^{-7} g/μl) were injected into about 5 ml of benzene TLC extract.
[d] Compared to 17.7 $\times$ 10^{-6} g of TNB actually spotted on the plate.
[e] C_{std} = 3.536 $\times$ 10^{-9} g/μl.
[f] μl $IS_{standard}$ = 24; that is 24 μl of internal standard were injected into 10.00 ml of TNB standard.

extraction from a TLC plate is illustrative of the general methcd of analysis (see Table II). A total of 17.68 $\times$ 10^{-6} g of TNB was applied in two spots on a fluorescent TLC plate. The plate was developed with benzene–hexane–pentane (50:40:10) and zones were located under 2537 Å UV light, scraped off and extracted with 4 to 5 ml of benzene without filtering. To this solution was added 12 μl of stock (1.804 $\times$ 10^{-7} g/μl) 1,2-dinitrobenzene as internal standard (IS). The concentration of standard TNB solution in 10 ml benzene was 3.536 $\times$ 10^{-9} g/μl to which had been added 24 μl of 1,2-dinitrobenzene internal standard. Comparison of the extract and standard solutions was made by injecting 1.8 μl of each solution together with 2.8 μl benzene solvent "back flush" into the chromatograph. A 2.53% Apiezon M on 60/80 mesh Diatoport-S column was used isothermally at 150° with a flow/purge rate of 143 ml/min with Ar–CH_4 (95:5, v/v), an injection temperature of 160°, a detector temperature of 275°, a pulse interval of 150 μsec, attenuation of 64, and a chart speed of 6.35 mm (0.25 in.)/min. The results of the analysis (see Table II) indicate 17.4 $\times$ 10^{-6} g of TNB present in the TLC extract as compared to 17.7 $\times$ 10^{-6} g actually spotted on the plate.

The results of analyses of several other nitro compounds after development on TLC plates may be found in Table III. The accuracy of the method is of the order of 2–3% under optimum conditions. Since ratios of the chromatographic peak heights are used to analyze the nitro compounds, serious errors may be introduced when the peak heights are small, or when the peak heights of internal standard, sample and standard are widely different. The maximum reading with the recorder chart paper used (precision chart paper, Hewlett-Packard No. 927–1010) is 10 in. or 254 mm. Since drift and noise from the combined sources of column bleed, detector, electrometer, and recorder introduce an uncertainty of about 0.1 in. or 2.5 mm for a particular reading, best analytical results will be obtained when

$$h_{IS} \cong h_{std} \cong h_{sample} \cong 127 \text{ to } 254 \text{ mm.}$$

Concentrations of standard and internal standard can be adjusted readily from standard concentrated solutions (10^{-5} to 10^{-6} g/μl) by diluting with 10 or 50 μl Hamil-

TABLE III

ANALYSES OF VARIOUS NITRO COMPOUNDS BY A COMBINATION OF THIN-LAYER AND VAPOR PHASE CHROMATOGRAPHY

Compounds	*μg applied to TLC plate*	*μg found*[a]
2,4,6-Trinitro-1-chlorobenzene	66.9; 66.9	67.4; 65.6
2,4,6-Trinitrotoluene	6.84; 6.84	6.62; 6.82
1,3,5-Trinitrobenzene	17.7	17.4
1,3-Dimethyl-2,4,6-trinitrobenzene	17.5	17.3
1,3,5,-Trimethyl-2,4,6-trinitrobenzene	13.3	13.0
2,4,6-Trinitrotoluene[b]	10.6	10.8
Cyclotrimethylenetrinitramine (RDX)[b]	49.7; 66.2	46.8; 66.6[c]

[a] By VPC analysis after development of TLC plate and extraction into 10 ml of solvent. Benzene was used as the extraction solvent in all cases except for RDX where benzene–acetone (90 : 10) was used.

[b] A mixture of RDX and 2,4,6-trinitrotoluene in acetone solutions with concentrations of 3.31 and 2.65 x 10^{-6} g/μl, respectively.

[c] RDX and 2,4,6-trinitrotoluene were analyzed on separate TLC plates since the height response (gram basis) was only about 1/4 that of 2,4,6-trinitrotoluene (see Table VIII).

ton syringes into 10 ml volumetric flasks to make standard VPC solutions (10^{-8} to 10^{-10} g/μl) to meet the above criteria. Concentrations of sample in the TLC extract can be controlled by (a) size of the aliquot spotted on the TLC plate, and (b) the volume of the TLC extraction solvent.

The solvents of choice for TLC zone extraction are (a) benzene, or (b) benzene-acetone (90:10). Methanol and ethanol were found to be unsatisfactory since reaction between solvent and nitro compound was detected in some instances. For example, no peak for RDX was observed at all when a solution of RDX in methanol was injected at 180°. *n*-Hexane is a solvent ideally suited for work with the nickel-63 detector. However, it has the disadvantage of not being polar enough to extract quantitatively the relatively polar nitro compounds from silica gel layers.

TABLE IV

NICKEL-63 DETECTOR RESPONSE AND LINEAR RANGE FOR 1,3,5-TRINITROBENZENE

ng on column[a]	*Detector response (mm/ng)*[b]	
3.18	17.2	
6.36	17.6	
12.7	17.1	
15.9	17.5	
18.9	15.1	detector saturation
22.3	13.1	detector saturation
25.5	11.7	detector saturation

[a] 4 ft. × 1/4 in. glass; 2.53% Apiezon M on 60/80 mesh Diatoport-S. Conditions in order: flow rate, ml/min, Ar–CH_4 (95 : 5, v/v); column temp.; injection temp.; detector temp.; pulse interval; injection solvent: 250 ml/min; 150°; 155°; 275°; 150 μsec; benzene, respectively.

[b] Calculated for attenuation 64; retention time, 4.0 min.

TABLE V

VARIATION OF NICKEL-63 DETECTOR RESPONSE TO 1,3,5-TRINITROBENZENE WITH GAS FLOW THROUGH COLUMN

Flow rate (ml/min)[a]	*Retention time (min)*[b]	*Dectector response (mm/ng)*[c]
67	10.2	25.9
113	6.7	26.7
143	5.6	26.6
167	4.8	22.5
200	4.2	20.2
250	4.0	17.9

[a] 4 ft. × 1/4 in. glass column; 2.53% Apiezon M on 60/80 mesh Diatoport-S. Conditions in order: column temp.; injection temp.; detector temp.; pulse interval; injection solvent: 150°; 160°; 275°; 150 μsec; benzene, respectively.
[b] Measured from solvent-pressure peak.
[c] Attenuation 64.

Nickel-63 detector response

The upper limit for linear detector response was found to be about 1.6×10^{-8} g of TNB for injected samples operating with a pulse interval of 150 μsec; flow/purge rate of 250 ml/min with Ar–CH_4 (95:5, v/v); detector temperature of 275°; and column temperature of 150° (see Table IV). This upper limit or saturation point will vary somewhat depending on the particular operating conditions employed. For example, this limit would be expected to decrease on raising the column temperature to 160° since a greater concentration of the TNB would be in the detector for a shorter period than would be true with a column temperature of 150° (see Tables VI and VII). On the other hand, within certain limits, the detector response appears to be independent of carrier gas flow (see Table V). However, it is always best to check detector response for linearity by making several injections of a particular nitro compound at different concentrations containing identical internal standard concentrations and normalizing sample peak heights. A constant value of height response in mm/ng within ± 3% indicates linearity. A lower value for the height response in going to a more concen-

TABLE VI

VARIATION OF NICKEL-63 DETECTOR RESPONSE TO 1,3,5-TRINITROBENZENE WITH COLUMN TEMPERATURE

Column temperature (°C)[a]	*Retention time (min)*[b]	*Detector response (mm/ng)*[c]
150	9.7	12.5
160	4.4	18.2

[a] 4 ft. × 1/4 in. glass; 3.51% Apiezon M on 60/80 mesh Diatoport-S. Conditions in order: flow rate, ml/min; injection temp.; detector temp.; pulse interval; injection solvent: 168 ml/min; 160°; 275°; 150 μsec; benzene, respectively.
[b] Measured from solvent-pressure peak.
[c] Attenuation 64.

TABLE VII

VARIATION OF RETENTION TIME AND NICKEL-63 DETECTOR RESPONSE TO 1,3,5-TRINITROBENZENE WITH DIFFERENT COLUMNS

Percent liquid phase on column[a]	*Column temp. (°C)*	*Flow rate (ml/min)*	*Retention time (min)*[b]	*Detector response (mm/ng)*[c]
1.18% Apiezon M	170	200	1.3	67.8
3.75% Silicone Grease, DC-11	160	190	1.8	26.2
2.92% Apiezon M	160	250	3.7	27.6
2.53% Apiezon M	150	250	4.0	17.3
Mixture (3.75% Silicone Grease, DC-11 and 3.51% Apiezon M, 50 : 50, w/w)	150	184	4.7	16.2
2.04% Apiezon M	150	168	5.8	17.9
5.33% Apiezon M	160	182	9.3	14.4
3.51% Apiezon M	150	168	9.7	12.5

[a] 4 ft. × 1/4 in. glass; solid support, 60/80 mesh Diatoport-S. Conditions in order: injection temp.; detector temp.; pulse interval; injection solvent: 165°; 275°; 150 μsec; benzene, respectively.

[b] Measured from solvent-pressure peak.

[c] Attenuation 64.

trated solution of the nitro compound indicates saturation of the detector (see Table IV). Erratic detector responses may indicate decomposition of the nitro compound either in the injection port or on the column or may indicate a defective septum.

Tables IV through VIII are useful for predicting detector height responses for

TABLE VIII

RELATIVE ELECTRON ABSORPTIVITY RESPONSES OF VARIOUS NITRO COMPOUNDS

Compound	*Retention time (min)*[a]	*Relative electron absorptivity*[b,d]	
		Gram basis	*Mole basis*
Cyclotrimethylenetrinitramine (RDX)	6.3	0.70	0.73
2,4,6-Trinitro-1-chlorobenzene	4.0	0.67	0.78
1,3,5-Trinitrobenzene	3.6	1.0	1.0
1,3-Dichloro-2,4,6-trinitrobenzene	4.4	0.95	1.3
2,4,6-Trinitroanisole	3.2	1.4	1.6
2,4,6-Trinitrotoluene	2.9	2.9	3.1
2,3,4-Trinitrotoluene	3.6	3.2	3.4
1,3,5-Trichloro-2,4,6-trinitrobenzene	3.1	3.2	4.8
1,3-Dimethyl-2,4,6-trinitrobenzene	6.4[c]	4.9	5.5
1,2-Dinitrobenzene	0.64	10	8.2
1,3,5-Trimethyl-2,4,6-trinitrobenzene	4.2	8.2	9.8

[a] Measured from solvent-pressure peak, 4 ft. × 1/4 in. glass column, packed with 2.53% Apiezon M on 60/80 mesh Diatoport-S. Conditions in order: flow rate, ml/min, Ar–CH_4 (95 : 5, v/v); column temp.; injection temp.; detector temp.; pulse interval; injection solvent: 250 ml/min; 155°; 155°; 275°; 150 μsec; benzene, respectively.

[b] Relative height response: 1.0 = 21.2 mm/ng for attenuation 64.

[c] Same conditions as in (a), except: 4 ft. × 1/4 in, glass column packed with 2.92% Apiezon M on 60/80 mesh Diatoport-S with a flow rate of 130 ml/min.

[d] For conditions as in (c), 1.0 = 30.9 mm/ng for attenuation 64.

a number of nitro compounds relative to 1,3,5-trinitrobenzene under a variety of conditions.

Injection temperatures up to 200° were found to be acceptable for the quantitative analysis of nitro compounds; however, column temperatures greater than 170° should be avoided. Presumably interaction between the nitro compound and the liquid phase occurs readily at column temperatures near 180°. There is also the further possibility that decomposition of the nitro compound itself takes place at these elevated temperatures especially for those compounds with long retention times.

ACKNOWLEDGEMENTS

The author wishes to express his sincere appreciation for several helpful discussions with D. J. GLOVER, C. W. DICKINSON, and C. BOYARS of this laboratory. The encouragement and interest of J. M. ROSEN was especially helpful throughout all phases of the work. Thanks are also due to B. O. WILKERSON, who carefully prepared all the thin-layer plates used in this study.

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CHROM. 4827

IDENTIFICATION AND DETERMINATION OF IMPURITIES IN PENTAERYTHRITOL TETRANITRATE

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(Received May 14th, 1970)

SUMMARY

Thin-layer chromatography (TLC) combined with photometry has been used successfully for the simultaneous determination of PETriN, DiPEHN, and TriPEON in PETN samples. The impurities are separated by one-dimensional TLC on Silica Gel G–Zn–sodium sulfanilate plates with an acetone–benzene mixed solvent. When the chromatogram is sprayed with N,N-dimethyl-1-naphthylamine (DMNA)–acetic acid reagent, the separated components are converted to a diazo dye to produce red spots on an off-white background. The intensities of the spots are measured by visible reflectance and are related to concentration by calibration curves. The standard deviations obtained in the analysis of various synthetic mixtures and authentic samples are less than 0.2%. Identification of the impurities is accomplished by their R_G values, measured relative to PETN.

INTRODUCTION

Pentaerythritol tetranitrate (PETN) commonly contains several impurities that can alter its physical and chemical properties. Dipentaerythritol hexanitrate (DiPEHN), for example, can cause a measurable change in the firing characteristic of exploding-bridgewire, PETN detonators. Thus a convenient procedure for identifying and determining the impurities in PETN is of considerable practical interest.

IR spectroscopy and thin-layer chromatography (TLC) are two of the most common techniques used for the analysis of PETN impurities. With IR it is difficult to detect the impurities at low concentration unless some sort of concentration step is incorporated into the procedure[1]. Furthermore, analytical bands become scarce if DiPEHN and tripentaerythritol octanitrate (TriPEON) are both present in the sample. TLC combined with radiometric scanning has been employed successfully to determine the hydrolysis products of tagged PETN, but DiPEHN and TriPEON were not included in the study[2].

TLC is an excellent method for separating mixtures, but the ultimate success of the method lies in the detection method used to locate the separated components.

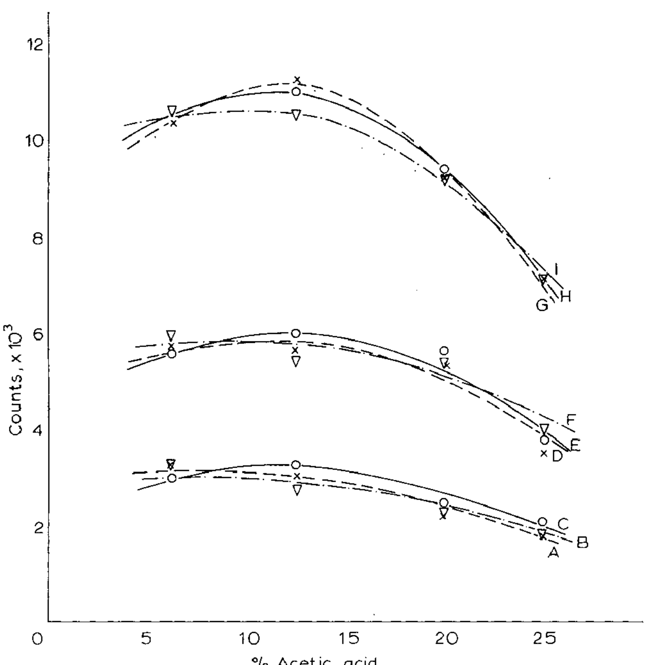


Fig. 1. Effect of acetic acid concentration (vol.%) on the color intensity of the diazo dye; 1% N,N-dimethyl-1-naphthylamine. (———), PETriN; curve C = 2.1 μg; curve E = 4.2 μg; curve H = 8.4 μg. (– – – –), TriPEON; curve A = 2.1 μg; curve D = 4.1 μg; curve G = 8.2 μg. (—·—·—), DiPEHN; curve B = 2.0 μg; curve F = 3.9 μg; curve I = 7.9 μg. Each point represents an average of 2–4 determinations.

In the work reported here we describe a TLC method for the separation of PETN impurities and a diazotization reaction to detect the components. The impurities are identified by measurement of their relative migration rates (R_G values). For quantitative analysis, the intensities of the developed spots are measured photometrically and related to concentration by means of calibration curves. In this manner pentaerythritol trinitrate (PETriN), DiPEHN, and TriPEON, commonly found in PETN, are determined simultaneously with a single sample.

MATERIALS AND METHODS

Equipment and reagents

Applicator, chromatojar, Silica Gel G, and glass plates (200 × 200 mm) were purchased from Brinkmann Instruments, Inc. Zinc metal dust, AR grade, was from Mallinckrodt Chemical Works. A stock solution of 0.04 *M* sodium sulfanilate was prepared by dissolving 7.6484 g of sulfanilic acid monohydrate (Matheson, Coleman, and Bell) in about 800 ml of distilled water, neutralizing the solution with dilute sodium hydroxide solution to pH 7, and diluting the solution to 1 l with distilled water. White-label N,N-dimethyl-1-naphthylamine (DMNA) (Eastman Organic Chemical) was distilled at 115–116° and 50 μm pressure. The spray reagent consisted of 5 g of DMNA and 62.5 ml of glacial acetic acid diluted to 500 ml with absolute ethanol.

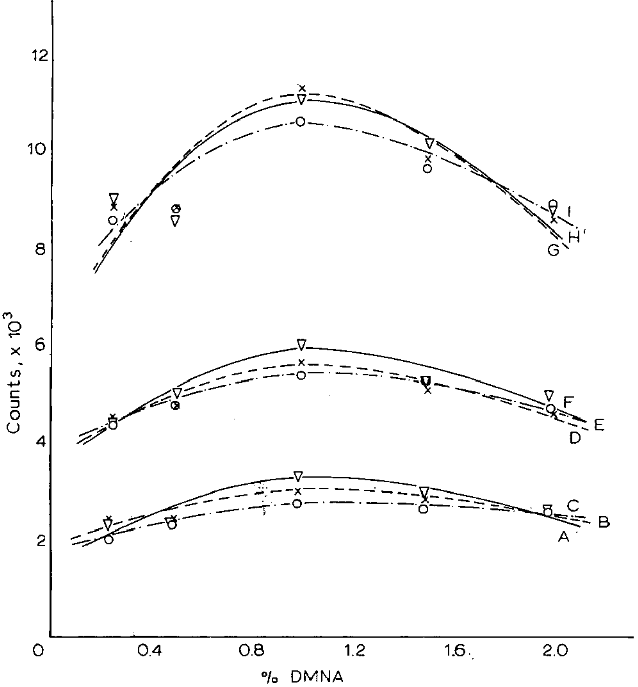


Fig. 2. Effect of DMNA concentration (w/v) on the color intensity of the diazo dye; 12.5 vol.% acetic acid. (———), PETriN; curve A = 2.1 μg; curve F = 4.2 μg; curve H = 8.4 μg. (– – – –), TriPEON; curve B = 2.1 μg; curve D = 4.1 μg; curve G = 8.2 μg. (—·—·—), DiPEHN; curve C = 2.0 μg; curve E = 3.9 μg; curve I = 7.9 μg.

An Agla micrometer syringe was used to deliver aliquots of the sample onto the TLC plates. Stopwatch, 250 ml Kensco atomizer, and a cylinder of nitrogen equipped with pressure reducer valve were used for reagent application. A Nester-Faust Uniscan 900 photometer with Summatic integrator and 10-mV recorder was used for the photometric measurements on the developed spots.

Experimental

Several essential modifications were incorporated into the Uniscan 900. A new base plate, 1/2-in. thick and flat to within 1 mil, was substituted for the original plate. The guide arm for the scanning head was aligned in such a manner that the distance of the scanning head from the thin-layer surface remained constant over a distance of at least 8 in. A micrometer adjustment screw was installed on the scanning head to control precisely the distance between the head and the thin-layer surface. These changes produced a considerable improvement in the performance of the instrument.

The chromatographic conditions were studied for the purpose of developing a method for the complete separation of PETriN, DiPEHN, and TriPEON from PETN. Silica Gel G–Zn–sodium sulfanilate plates activated at 110° for 2, 4, 6, and 16 h were tested with a four-component mixture and with acetone–benzene of varying composition as the mobile phase. The best results were obtained with plates activated at 110° for 16 h and chromatographed with acetone–benzene (4:96).

The color development of each impurity was investigated as a function of acetic acid and DMNA concentrations in the spray reagent. Holding DMNA constant and varying the acetic acid concentration, we found a broad maximum at or near 12.5 vol.% acetic acid for all three impurities (see Fig. 1). By varying the DMNA concentration while maintaining the concentration of acid constant at 12.5 vol.%, we found a maximum in color intensity at 1% DMNA (Fig. 2). From this study we conclude that a spray reagent consisting of 1% DMNA and 12.5% acetic acid in absolute ethanol provides the best compromise for photometric measurements.

Zinc dust in the amount of 0.5 g/30 g of Silica Gel G worked satisfactorily. Increasing the amount of zinc in the thin layer leads to a more rapid color development as well as a more rapid fading of the developed color.

The color stability of the diazo dye was studied. The intensity of the spots remained constant for from 10 to 25 min after the chromatogram has been sprayed. This provides ample time to scan three PETN samples on a single plate.

Preparation of Silica Gel G–Zn–sodium sulfanilate plates. A slurry of 30 g of Silica Gel G and 0.5 g of zinc dust in 65 ml of 0.02 M sodium sulfanilate solution is used to coat five glass plates (8 $\times$ 8 in.) by pulling an applicator across the plates at a constant speed. The coated plates are air-dried for 15 min and activated at 110° for 16 h before use.

Procedure. Three samples, each containing 200 μg of PETN, are applied 2 in. apart and 1 in. from the lower edge of an activated plate. The diameter of the sample is maintained at 1/4 in. The plate is developed in 250 ml of acetone–benzene (4:96) for 50 min. It is then air-dried for 5 min and sprayed uniformly with DMNA–acetic acid reagent for 35 sec, using 3 p.s.i. N_2 to propel the reagent. The chromatogram is allowed to develop in the dark for 7 min and is then dried with cold air from a hair dryer. 12 min after the chromatogram has been sprayed, the intensities of the spots are measured by visible reflectance with a dual-channel scanning head. The Summatic integrator of the Nester-Faust photometer, operated manually, registers a number of counts proportional to the intensity of a spot. A previously prepared calibration curve for each component is used to obtain the desired concentrations.

TABLE I

R_G VALUES OF PETN AND IMPURITIES

Compound	*R_G value*
PETN (ref. compound)	1.00
DiPEHN	0.70
TriPEON	0.47
PETriN	0.24

RESULTS AND DISCUSSION

For identification purpose, the R_G value of each impurity was measured relative to PETN. Table I lists the average values for three measurements.

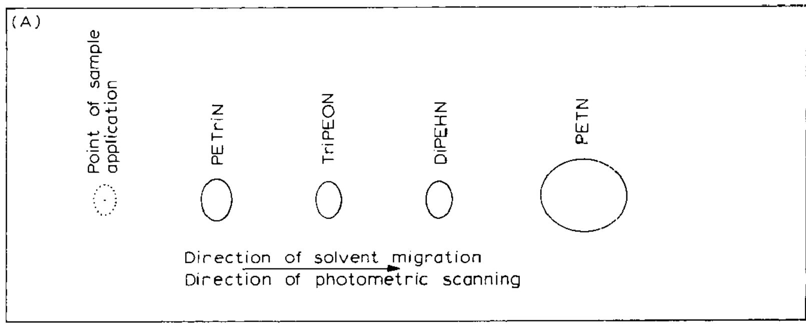
Table II summarizes the data obtained on synthetic mixtures and authentic samples. It is evident that the results are excellent, with the standard deviations being

TABLE II

RESULTS OBTAINED ON VARIOUS PETN SAMPLES

Sample	*Composition*	*No. of determinations*	*PETriN found (%)*	*Std. dev.*	*TriPEON found (%)*	*Std. dev.*	*DiPEHN found (%)*	*Std. dev.*
Synthetic mixture	PETriN–TriPEON–DiPEHN–PETN (0.3:0.2:0.4:99.1)	6	0.4	0.07	0.3	0.06	0.5	0.08
Synthetic mixture	PETriN–TriPEON–DiPEHN–PETN (0.8:0.8:1.0:97.4)	6	0.8	0.13	0.8	0.18	1.0	0.13
Synthetic mixture	PETriN–TriPEON–DiPEHN–PETN (1.9:1.8:2.2:94.1)	5	2.1	0.07	1.6	0.12	2.2	0.11
Recrystallized mixture	TriPEON–PETN[a] (1:99)	6	<0.1	—	1.0	0.10	—	—
MD-2 recrystallized PETN	—	6	0.2	0.10	<0.1	—	0.7	0.13
Trojan special PETN	—	5	0.2	0.00	—	—	0.2	0.00
PETN, local sample	—	6	<0.1	—	—	—	<0.1	—
DuPont PETN	—	5	<0.1	—	<0.1	—	<0.1	—

[a] Contains about 0.1% tetrapentaerythritol decanitrate.



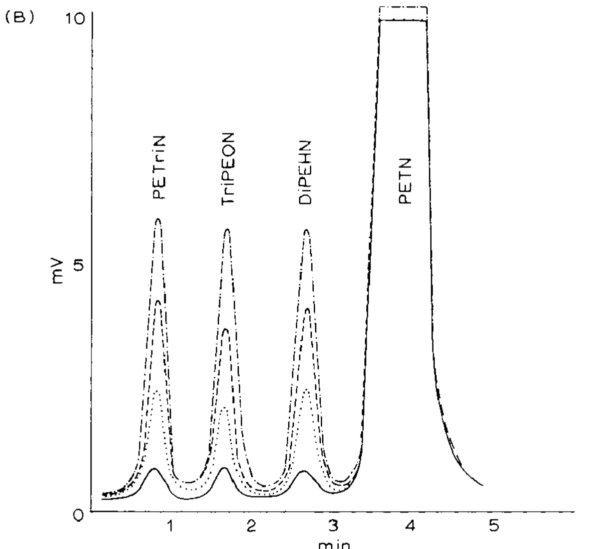


Fig. 3. (A) Tracing of a one-dimensional thin-layer chromatogram. Chromatographic conditions: thin layer, Silica Gel G–Zn–sodium sulfanilate activated at 110° for 16 h; solvent, 250 ml of acetone–benzene (4:96); development time, 50 min; development temp., 25°; spray reagent, 1% DMNA and 12.5 vol.% acetic acid in absolute ethanol. (B) Photometric tracings of thin-layer chromatograms. Instrumental settings for Uniscan 900: mode of operation, visible reflectance; scanning head, dual beam; filter, green; no collimator; clearance of scanning head from thin-layer surface, 1/16 in.; slit, 3/4 × 1/16 in.; span, 500; gain, × 100; rate of travel of scanning head, 1.25 in./min. Summatic integrator, operated manually. 10-mV Varian G-14A-1 recorder, recorder speed 0.8 in./min. 200 μg per sample. (———), 0.2% PETriN, 0.2% TriPEON, and 0.2% DiPEHN in PETN; (.........), 1.0% PETriN, 0.9% TriPEON, and 1.0% DiPEHN in PETN; (– – – –), 2.0% PETriN, 1.9% TriPEON, and 2.0% DiPEHN in PETN; (—.—.—), 3.0% PETriN, 3.0% TriPEON, and 3.0% DiPEHN in PETN.

less than 0.2% in all cases. With the exception of MD-2 recrystallized PETN, only small amounts of impurities were found in the various commercial and local samples.

Fig. 3B shows several scans of synthetic mixtures as displayed on a 10-mV recorder. A tracing of a typical chromatogram is shown in Fig. 3A. The horizontal displacement of the components on the recorder tracing is due to the difference in the rates of travel of the scanning head (1.25 in./min) and the recorder (0.8 in./min). However, complete resolution of each peak is obtained on the recorder tracing.

The development of the red spot on the chromatogram is based on a diazotization reaction; the chemical equations may be written

$$R(ONO_2)_x + x\,Zn^0 + 2x\,H^+ \rightarrow R(OH)_x + x\,Zn^{2+} + x\,HNO_2$$

$$x\,HNO_2 + x\,H^+ \rightarrow x\,H_2O + x\,NO^+ \text{ (nitrosonium ion)}$$

$$x\,NO^+ + x\,NaO_3S\text{—}C_6H_4\text{—}NH_2 \rightarrow x\,NaO_3S\text{—}C_6H_4\text{—}N_2^+ + x\,H_2O$$

$$x\,NaO_3S\text{—}C_6H_4\text{—}N_2^+ + x\,C_{10}H_7\text{—}N(CH_3)_2 \rightarrow$$

$$x\,NaO_3S\text{—}C_6H_4\text{—}N{=}N\text{—}C_{10}H_6\text{—}N(CH_3)_2 + x\,H^+$$

the net result of which is

$$R(ONO_2)_x + x\,Zn^0 + 2x\,H^+ + x\,NaO_3S\text{—}C_6H_4\text{—}NH_2 + x\,C_{10}H_7\text{—}N(CH_3)_2 \rightarrow$$

$$R(OH)_x + x\,Zn^{2+} + 2x\,H_2O + x\,NaO_3S\text{—}C_6H_4\text{—}N{=}N\text{—}C_{10}H_6\text{—}N(CH_3)_2$$

(red diazo dye)

Here

$R = [HOCH_2\text{—}C(CH_2\text{—})_2\text{—}CH_2\text{—}]$ and $x = 3$ for PETriN,

$R = [\text{—}CH_2\text{—}C(CH_2\text{—})_2\text{—}CH_2\text{—}]$ and $x = 4$ for PETN,

$R = [\text{—}CH_2\text{—}C(CH_2\text{—})_2\text{—}CH_2\text{—}O\text{—}CH_2\text{—}C(CH_2\text{—})_2\text{—}CH_2\text{—}]$ and $x = 6$ for DiPEHN,

and

$R = [\text{—}CH_2\text{—}C(CH_2\text{—})_2\text{—}CH_2\text{—}O\text{—}CH_2\text{—}C(CH_2\text{—})_2\text{—}CH_2\text{—}O\text{—}CH_2\text{—}C(CH_2\text{—})_2\text{—}CH_2\text{—}]$ and $x = 8$ for TriPEON.

In the absence of the sample, no color is produced on the chromatogram. With sodium nitrite or nitrate, an intense red spot is formed identical to that produced by PETN. Therefore, PETN and its impurities must hydrolyze and produce nitrite ion in the presence of zinc and acetic acid. The formation of nitrosonium ion diazotizes the sodium sulfanilate, and the diazonium ion couples with DMNA to produce the red spots.

The color stability of the diazo dye is superior to that produced by the Griess reagent. The latter utilizes 1-naphthylamine as the coupling agent. The color develops rapidly, but it also fades rapidly, making it unsuitable for quantitative TLC work. DMNA as a coupling agent has been used in the determination of nitrite ion in aqueous solution[3].

The shelf lives of the sodium sulfanilate solution, the spray reagent, and the TLC plates were studied. The sodium sulfanilate solution can be stored for a month, but the spray reagent must be prepared weekly. The activated TLC plates, which are stored in a desiccator, cannot be stored for more than two days, as the color intensity of the spot for a given weight of sample decreases with the age of the plate. All reagents and plates must be stored in the dark.

ACKNOWLEDGEMENTS

The author thanks D. L. UPHAM for the modification of the Uniscan 900. This work was done under the auspices of the United States Atomic Energy Commission.

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CHROM 4828

DETERMINATION OF TRACE AMOUNTS OF EASTMAN 910 ADHESIVE IN CERTAIN PLASTIC-BONDED EXPLOSIVES

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(Received May 14th, 1970)

SUMMARY

A pyrolytic gas chromatographic method for determining small amounts of Eastman 910 polymer in certain plastic-bonded explosives is described. The Eastman 910 polymer is separated from the plastic-bonded explosives by solution in nitromethane–chloroform, and an aliquot of the solution is converted to the monomer, methyl 2-cyanoacrylate, by controlled pyrolysis for gas chromatographic analysis. The peak area of the monomer is related to polymer concentration by means of a calibration curve. It is shown how the method may be used to study the penetration of the adhesive into glued pellets of the explosive.

INTRODUCTION

When adhesives are used with porous substrates, questions frequently arise concerning the extent to which the adhesive penetrates the substrate. Such penetration may affect the strength of the bond and may alter the properties of the substrate in the region adjacent to the bond. In this paper we describe an application of pyrolytic gas chromatography to a particular problem of this type, namely, the determination of small amounts of Eastman 910 adhesive (methyl 2-cyanoacrylate polymer) in certain plastic-bonded explosives (PBX). Eastman 910 is frequently the adhesive of choice for use with explosives, as, for example, when it is desired to glue a pressed PBX charge to a metal plate to obtain intimate contact at the interface.

A recent publication by the author[1] showed that cured Eastman 910 pyrolyzes to methyl 2-cyanoacrylate monomer as its characteristic product. Under controlled pyrolysis, it was found that the monomer yield was proportional to the polymer concentration. However, when PBX is present, the explosive component interferes. Interference is minimized by preferentially dissolving the Eastman 910 in a nitromethane–chloroform mixture and taking an aliquot of the solution for analysis. Good results were obtained with synthetic samples and with PBX 9010 and PBX 9407 samples contaminated with the adhesive.

MATERIALS AND METHODS

Materials, equipment and reagents

PBX 9010 is 90/10 RDX (hexahydro-1,3,5-trinitro-s-triazine)/Kel-F 3700 elastomer (3 M Co.). PBX 9407 is 94/6 RDX/Exon 461 (Firestone Plastics Co.). Composition B-3 is 60/40 RDX/TNT (trinitrotoluene).

A Perkin–Elmer pyrolysis unit, an Aerograph hi-fi Model 600-D gas chromatograph, and a Varian Model G-14 recorder were used.

A 1-ft.-long by 1/8-in.-O.D. (0.094-in.-I.D.) stainless-steel tube was packed with 25% (by weight) of diisodecyl phthalate on 60–80 mesh, acid-washed Chromosorb W, using a Matronic XL-300 column packer. The packed column was preconditioned with helium carrier gas at 125° for 24 h before use.

Liquid Eastman 910 adhesive, purchased from Eastman Chemical Products, Inc., was distilled twice under vacuum and the distillate was polymerized in water. The dried polymer had an indicated purity of 99.2% from C, H, and N analyses.

Six 1-ml and 10-ml volumetric flasks, two Hamilton microsyringes (10 μl capacity), and twelve micro porcelain combustion boats (No. 00000) were required. An ultrasonic shaker and a centrifuge were used for sample preparation.

Experimental

Several partitioning columns were prepared and tested for their ability to separate methyl 2-cyanoacrylate monomer from other pyrolysis products. The 1-ft. 25% diisodecyl phthalate column was found to work satisfactorily. At a column temperature of 80° and a flow rate of 32–33 cm^3/min of hydrogen carrier gas, methyl 2-cyanoacrylate elutes completely from the column in about 11 min as a reasonably symmetrical peak acceptable for area measurement. The conditions necessary to optimize the yield of the monomer with this column were investigated.

Fig. 1 shows the dependence of the monomer yield on the pyrolysis temperature. The maximum occurs between 250 and 270°, which is much lower than the optimum ranges for polystyrene (500 to 525°) and Estane 5740 X2 (600°), which we have studied previously[2,3].

The influence of the hydrogen flow rate on the monomer yield is shown in Fig. 2 for two different pyrolysis temperatures. At flow rates of 25–35 cm^3/min, a broad maximum is observed; however, at about 25 cm^3/min peak broadening occurs. Therefore, a flow rate of 32–33 cm^3/min was chosen for our work.

Three explosives (PBX 9010, PBX 9407, and Composition B-3) were studied in mixtures with Eastman 910. At a pyrolysis temperature of 260° and with the gas chromatographic conditions given in Fig. 1, mixed samples were pyrolyzed to study monomer recovery. With increasing amounts of explosive, the recovery of the monomer decreased markedly. Consequently, a solvent was sought to dissolve the polymer preferentially so that the explosive would not interfere with the analysis. As a further precaution, the pyrolysis temperature was lowered. Treatment of the mixed sample with nitromethane and chloroform and the lowering of the pyrolysis temperature to 200° worked with PBX 9010 and PBX 9407 containing trace amounts of Eastman 910. Mixtures of Composition B-3 and Eastman 910, however, could not be analyzed by this method, because TNT dissolves readily in the solvent and interferes with the analysis.

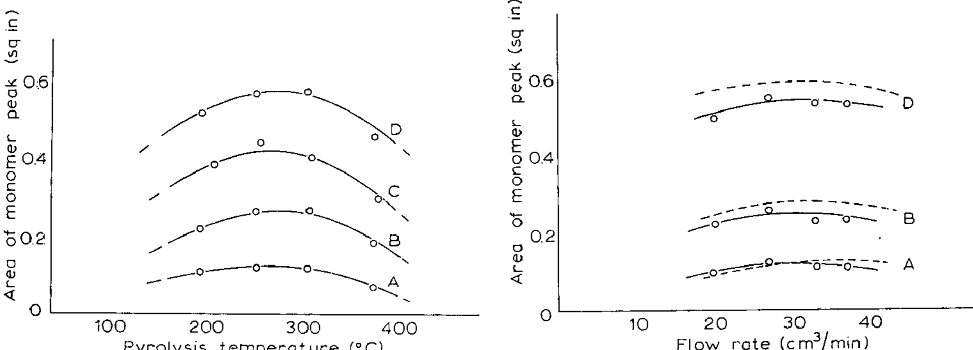


Fig. 1. Effect of pyrolysis temperature on the yield of Eastman 910 monomer. Chromatographic conditions: Column, 1 ft. long by 1/8 in. O.D. (0.094 in. I.D.); 25% diisodecyl phthalate on 60–80 mesh, acid-washed Chromosorb W. Column temp., 80°; inlet temp., 100°; hydrogen carrier gas, flow rate 32–33 cm^3/min; recorder speed, 16 in./h; electrometer range 0.1; attenuation × 16. Sample size: curve A = 0.5 μg Eastman 910 polymer; curve B = 1.0 μg Eastman 910 polymer; curve C = 1.5 μg Eastman 910 polymer; curve D = 2.0 μg Eastman 910 polymer. Each point represents an average of three runs.

Fig. 2. Effect of hydrogen flow rate on yield of Eastman 910 monomer. Chromatographic and pyrolysis conditions: chromatographic conditions same as in Fig. 1, except variable flow rates. Pyrolysis temp., – – – – = 260°; ——— = 200°. Sample size: curve A = 0.5 μg Eastman 910 polymer; curve B = 1.0 μg Eastman 910 polymer; curve D = 2.0 μg Eastman 910 polymer. Each point represents an average of three runs.

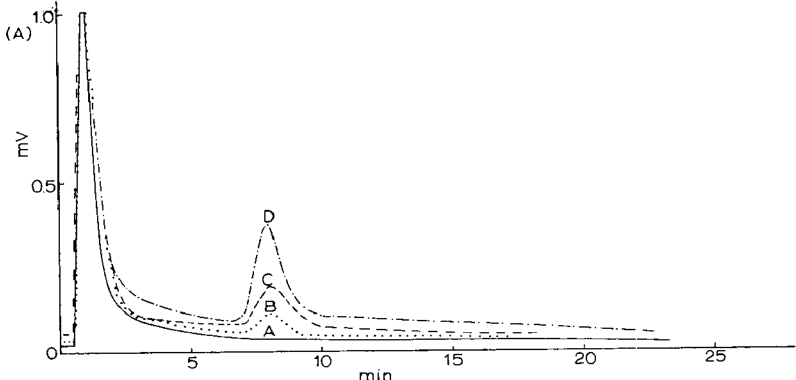
Procedure

A 0.05–0.1 g sample of explosive contaminated with Eastman 910 is dispersed in 0.25 ml of nitromethane with an ultrasonic shaker for 10–15 min. The solution is diluted to 1 ml with chloroform and then centrifuged to separate the undissolved explosive. With the Hamilton microsyringe, 2–5 μl of the solution is transferred into a micro porcelain combustion boat and the sample is evaporated to dryness at room temperature. Up to six boats with samples are loaded into the pyrolysis unit, and the flow rate of the hydrogen carrier gas is adjusted to 32–33 cm^3/min at a column temperature of 80°. After the recorder is turned on and the electrometer is balanced, the flame detector is ignited. The sample is moved into the pyrolysis chamber, maintained at 200°, with a magnet and boat-pusher. The pyrolysis products are separated on a 1-ft. diisodecyl phthalate column. The area of the methyl 2-cyanoacrylate peak is measured by the (peak height) × (peak width at half-height) method and related to polymer concentration by means of a calibration curve.

RESULTS AND DISCUSSION

Typical pyrograms of synthetic PBX samples are shown in Figs. 3A and B. With mixed-sample pyrolysis, the methyl 2-cyanoacrylate peak appears on the trailing edge of the PBX pyrolysis products; however, area measurements by planimeter and by the (peak height) × (peak width at half-height) method gave identical results.

The results obtained with synthetic samples and authentic glued samples of PBX 9010 and PBX 9407 are summarized in Table I. The standard deviations are



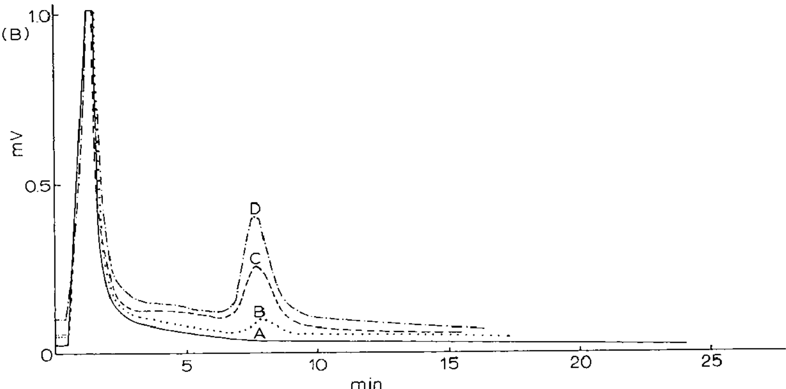


Fig. 3. (A) Pyrograms of synthetic samples of Eastman 910–PBX 9010. Chromatographic and pyrolysis conditions: Column, 1 ft. long by 1/8 in. O.D. (0.094 in. I.D.); 25% diisodecyl phthalate on 60–80 mesh acid-washed Chromosorb W. Column temp., 80°; inlet temp., 100°; hydrogen carrier gas, flow rate 32–33 cm^3/min; recorder speed, 16 in./h; electrometer range 0.1; attenuation ×8; pyrolysis temp., 200°. Sample size: curve A = 5 µl CH_3NO_2–$CHCl_3$ solution saturated with PBX 9010; curve B = 5 µl 0.026% synthetic sample = 0.13 µg 910 polymer; curve C = 5 µl 0.052% synthetic sample = 0.26 µg 910 polymer; curve D = 5 µl 0.090% synthetic sample = 0.45 µg 910 polymer. Peaks B, C, and D are methyl 2-cyanoacrylate monomer.
(B) Pyrograms of synthetic samples of Eastman 910–PBX 9407. Chromatographic and pyrolysis conditions same as for (A). Sample size: curve A = 5 µl CH_3NO_2–$CHCl_3$ solution saturated with PBX 9407; curve B = 5 µl 0.022% synthetic sample = 0.11 µg 910 polymer; curve C = 5 µl 0.053% synthetic sample = 0.27 µg 910 polymer; curve D = 5 µl 0.100% synthetic sample = 0.50 µg 910 polymer.

excellent, though the average values appear to be systematically low by a small amount for the synthetic samples. The glued PBX 9407 pellets (1/16-in. thick by 1/4-in. diam.), which were simply pried off the surface to which they were glued, contain 0.47 to 0.78% Eastman 910. The variation in the polymer content is due to the varying amounts of adhesive originally applied at the glue-bond surface. PBX 9010

TABLE I

ANALYSIS OF EASTMAN 910 POLYMER IN PBX MIXTURES

Sample	*Ratio of composition*	*No. of det'ns*	*910 polymer found (%)*	*Std. dev.*
Synthetic mix	0.40/100–910 polymer/PBX 9010	5	0.37	0.02
Synthetic mix	0.09/100–910 polymer/PBX 9010	5	0.08	0.01
Synthetic mix	0.05/100–910 polymer/PBX 9010	5	0.05	0.00
Synthetic mix	0.03/100–910 polymer/PBX 9010	5	0.02	0.00
Synthetic mix	0.50/100–910 polymer/PBX 9407	5	0.50	0.03
Synthetic mix	0.10/100–910 polymer/PBX 9407	5	0.10	0.01
Synthetic mix	0.05/100–910 polymer/PBX 9407	5	0.05	0.01
Synthetic mix	0.02/100–910 polymer/PBX 9407	5	0.02	0.01
PBX 9407, glued pellet	—	5	0.47	0.04
PBX 9407, glued pellet	—	7	0.78	0.04
PBX 9407, glued pellet	—	5	0.58	0.02
PBX 9407, glued pellet (top half away from glue bond)	—	6	0.15	0.02
PBX 9407, glued pellet (top half away from glue bond)	—	5	0.06	0.00
PBX 9407, glued pellet (top half away from glue bond)	—	5	0.09	0.01
PBX 9010, glued slab	—	5	0.07	0.01
PBX 9010–Sample A (first 1/16 in.)	—	6	0.43	0.04
PBX 9010–Sample B (next 1/8 in.)	—	5	0.00	0.00
PBX 9010–Sample C (remainder of pellet)	—	5	0.00	0.00

(about 3/8-in. thick) glued to an aluminum shell shows 0.07% of the Eastman 910 polymer.

To study the penetration of Eastman 910 into the PBX, glued samples were sectioned for analysis. With PBX 9010, sample A consisted of a 1/16-in.-thick layer adjacent to the glue-bond surface, sample B the next 1/8-in. layer, and sample C the remaining portion of the PBX 9010. With PBX 9407, three pellets were sectioned, and the top half of the pellet, furthest from the glue bond, was analyzed. The results are shown in Table I.

It can be seen that, with PBX 9010, the Eastman 910 penetrated no more than 1/16 in. into the sample. With PBX 9407, the amount of Eastman 910 found in the top half of the pellets varied from 0.06 to 0.15%, so that the glue penetrated at least 1/32 in. into the pellets.

ACKNOWLEDGEMENT

This work was done under the auspices of the U. S. Atomic Energy Commission.

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CHROM. 4838

AN IMPROVED TECHNIQUE FOR THE ANALYSIS OF AMINO ACIDS AND RELATED COMPOUNDS ON THIN LAYERS OF CELLULOSE

III. DETECTION AND IDENTIFICATION BY SELECTIVE STAINING

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(Received May 15th, 1970)

SUMMARY

A simple and rapid method is described for the detection and unambiguous identification of seventy-six nitrogen-containing metabolites which are commonly found in biological fluids. Following the separation of these compounds by thin-layer chromatography they are identified by means of selective staining reagents.

INTRODUCTION

In earlier papers[1,2] (Parts I and II) the separation and quantitative determination of the common amino acids have been described. Nevertheless because of the large number of amino acids and related nitrogen-containing metabolites which occur in biological fluids, their unambiguous identification after separation by thin-layer chromatography (TLC), continues to present many difficulties within the concentration range of 5×10^{-4} to 2×10^{-2} μmole. The simplest approach[3,4] aims at ensuring that each amino acid occupies a unique position on the thin-layer chromatogram. This approach fails in the case of complex biological materials which may contain over seventy nitrogen-containing compounds as well as sugars and inorganic salts. Another approach favours the use of multiple solvent systems or repeated development[5-7]. It is our opinion that such methods are tedious and possess few attributes for general use.

A different approach to the problem involves the use of selective staining reagents in order to identify each amino acid unambiguously. This technique can be very effective in conjunction with solvent systems[1,2] which are capable of resolving many amino acids. The two-dimensional solvent system of HAWORTH AND HEATHCOTE[1] clearly resolved some forty amino acids and the positions of some twenty-three additional amino acids of less frequent occurrence were also noted. Ninhydrin is still the most widely used sensitive reagent for amino acids and there have been many variations of reagent composition, including the incorporation of metal salts into the

ninhydrin solution in order to enhance the stability of the resulting amino acid complex and to give some measure of specificity. Extensive lists of amino acid staining reagents have been given by STAHL[8], DAWSON *et al.*[9], and by KRAUSS[10].

The present paper describes the chromatographic behaviour of seventy-six compounds of biochemical interest and also lists selective staining reagents which permit their unambiguous identification. The compounds include the sixty-three substances listed by HAWORTH AND HEATHCOTE[1], and their sequence of numbering is retained.

EXPERIMENTAL

Materials and equipment

The TLC equipment, glass tanks, and 1 μl "microcaps" capillary pipettes were supplied by Shandon*.

Preparation of the cellulose layers. The purified cellulose powder MN300** (15 g) was spread over five plates (20 × 20 cm) at a thickness of 400 μ. After coating the plates with the cellulose slurry, the plates were allowed to dry horizontally overnight before use. The conditions for the purification of the powder, spreading the slurry, application of the sample solutions (1 μl) and development were those described previously[1].

Standard solutions. Stock solutions (0.025 *M*) of amino acids and other common nitrogen-containing metabolites were prepared using aqueous 2-propanol (10% v/v) as the solvent. In the case of tyrosine and other sparingly soluble amino acids, the minimum quantity of dilute hydrochloric acid was added to effect solution. These stock solutions and a range of solutions (0.025–0.0005 *M*) prepared by dilution were kept refrigerated when not in use.

Chromatographic solvent systems. The solvent systems which were used for the separation of the nitrogen-containing metabolites were those described by HAWORTH AND HEATHCOTE[1]. These were, for the first dimension (Solvent No. 1): 2-propanol–butanone–1 *N* hydrochloric acid (60:15:25, v/v), and for the second dimension (Solvent No. 2): 2-methylpropanol-2-butanone–propanone–methanol–water (0.88) ammonia (40 : 20 : 20 : 1 : 14 : 5, v/v).

Application of samples and development of plates

In general the conditions of development which were followed were those previously described[1] but the first solvent front was not isolated when indoles of high R_F values were suspected to be present. Briefly the conditions were as follows:

Standard solutions (1 μl) were applied at a position 1.5 cm from both the bottom and left hand edges of the coated plate. The plates were then placed in chromatographic tanks containing 100 ml of Solvent No. 1, or 170 ml of Solvent No. 2. The atmosphere in each tank had been pre-saturated with the appropriate solvent before use, as described by SANKOFF AND SOURKES[11]. After the ascending solvent front due to Solvent No. 1 had travelled 13 cm from the origin, the plate was removed and dried in a stream of cold air for 15 min. Traces of acid impair the subsequent identification of the amino acids and, in order to remove all traces of hydrogen chloride, the air-dry

* Shandon Scientific Co., 65 Pound Lane, London, N.W. 10, Great Britain.
** Macherey, Nagel and Co. Ltd., Agents Camlab (Glass) Ltd., Cambridge, Great Britain.

plate must be heated to 60° for a minimum of 15 min, or until no odour remains. The dry plate was then developed in Solvent No. 2 until the solvent front had reached a height of 13 cm from the origin, at which point the plate was removed and dried at 60° for 15 min.

Detection of amino acids and related compounds

The initial detection of the amino acids was effected by spraying the developed and dried plate with the cadmium acetate–ninhydrin reagent of HEATHCOTE AND WASHINGTON[12]. This reagent (designated Reagent A), was prepared from cadmium acetate (0.5 g), water (50 ml), glacial acetic acid (10 ml), and propanone to a final volume of 500 ml. Sufficient solid ninhydrin was added to suitable portions of this stable stock solution to give a ninhydrin concentration of 0.2% (w/v) for immediate

TABLE I

COMPOSITION AND CONDITIONS OF USE OF THE STAINING REAGENTS

Reagent	*Composition of the reagent*	*Reference*
A	Cadmium acetate–ninhydrin reagent.	12
B	Solution (1) was made from ethanol (50 ml), collidine (2 ml), glacial acetic acid (10 ml) and sufficient ninhydrin to give a 0.2% (w/v) solution. Solution (2) was a solution of copper nitrate (1% w/v) in ethanol. Mix solution (1) (50 ml) and solution (2) (3 ml). Spray the plate with the reagent and heat to 105° for 2 min.	13
C	This reagent was formulated as for Reagent A, except that isatin (0.2% w/v) replaced ninhydrin. After spraying, heat the plate at 90° for 10 min.	2
D_1	This was a solution of O-phthalaldehyde (0.2% w/v) in propanone.	14
D_2	A solution of potassium hydroxide (1% w/v) in ethanol. Spray with Reagent D_1 and heat at 50° for 20 min. Re-spray with Reagent D_2. Examine the plate at each stage of spraying under visible and UV light.	15
E	Solution of vanillin (2% w/v) in 1-propanol. Heat to 110° for 5 min and then re-spray with Reagent D_2. Examine the plate at each stage of spraying under both visible and UV light.	16
F	Reagent consists of *p*-dimethylaminobenzaldehyde (1 g) dissolved in conc. hydrochloric acid (5 ml) and then ethanol added to a final volume of 100 ml. Use at room temperature.	17
G	A solution of isatin (0.2% w/v) in a copper uranyl acetate solution. The latter was prepared from copper acetate (0.227 g), uranyl acetate (0.273 g), glacial acetic acid (20 ml), water (50 ml), and propanone to a total volume of 500 ml. After spraying, heat the plate to 90° for 10 min, examine under visible and UV light and then re-spray with Reagent F.	18
H	Aqueous solutions of sodium hydroxide (10% w/v), potassium ferricyanide (10% w/v) and sodium nitroprusside (10% w/v) are prepared. Equal volumes of each of the above solutions are mixed at 0°, allowed to stand at 0° for 30 min. Water (360 ml) is then added, and the reagent is stable at 0° for 1 month. Spray plate; colours appear within 5 min at room temperature.	19
I_1	Solution of pyridine (2% v/v) in methanol, to which add sufficient periodic acid to give a concentration of 0.03 *M*.	20
I_2	Ammonium acetate (15 g), glacial acetic acid (0.3 ml), acetylacetone (1 ml) and methanol to a total volume of 100 ml. After spraying the plate with Reagent I_1, leave at room temp. for 10 min; examine under visible and UV light. Re-spray plate with Reagent I_2 and examine as before.	
J	Solution (1): sodium nitroprusside (5% w/v) in methanol. Solution (2): piperidine (20% v/v) in methanol. Mix equal volumes of solutions (1) and (2) to form reagent which is unstable. Spray plate with Reagent I_1 and leave at room temp. for 5 min; examine under visible and UV light and then re-spray with Reagent J.	20

TABLE II

R_F VALUES OF ADDITIONAL[a] METABOLITES ON THIN LAYERS OF CELLULOSE

No.	*Compound*	R_F (× 100) *Solvent No. 1*	R_F (× 100) *Solvent No. 2*
64	Saccharopine	73	0
65	Indican	89	91
66	5-Hydroxytryptophan	43	26
67	3-Indolyllactic acid	96	60
68	Tryptamine	73	97
69	3-Indolylacetic acid	98	56
70	Hippuric acid	71	0
71	Urea	70	50
72	Creatinine	47	41
73	Guanidine	50	36
74	Thiourea	71	70
75	Carnosine	9	21
76	Allantoin	49	9

[a] Additional to the 63 previously reported[1].

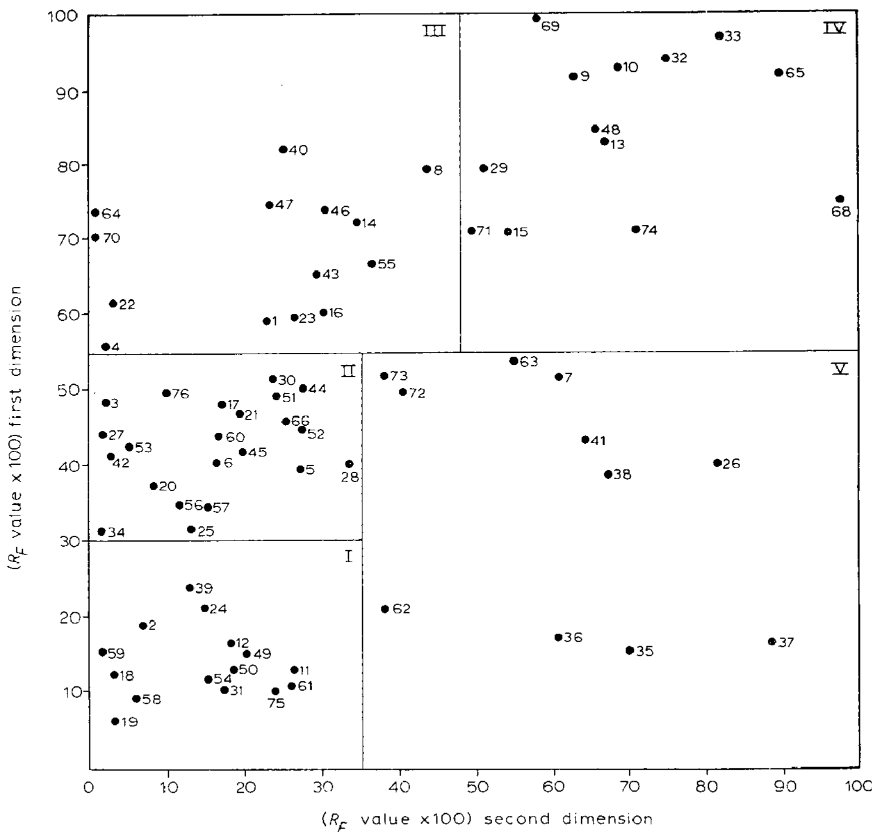

Fig. 1. Positions of metabolites on a TLC plate.

use. After the plates had been sprayed, they were heated to 60° for 15 min or left overnight at ambient temperature as convenient.

The final characterisation of the separated compounds was accomplished by the use of one or more staining reagents (Reagents B–J) listed in Table I. This table gives the composition of the reagents and the conditions under which they were used.

RESULTS AND DISCUSSION

The R_F values of thirteen compounds (Nos. 64–76) additional to the sixty-three reported by HAWORTH AND HEATHCOTE[1], are given in Table II. After separation by TLC, all the seventy-six compounds were arbitrarily arranged into five groups (I–V) on the one (20 × 20 cm) plate. See Fig. 1. The staining reactions of the individual metabolites are given for each group in Tables III–VII.

TABLE III

COLOUR REACTIONS OF METABOLITES OF GROUP I

The conditions which produce a positive response with Reagents D, E, G, I, J are indicated by superscripts: visible light and first spray reagent^{1}; UV light and first spray reagent^{2}; visible light and second spray reagent^{3}; UV light and second spray reagent^{4}. The colours produced are indicated by the following letter codes: Pink, P; Blue, Bl; Brown, Bn; Black, Bk; Yellow, Y; Orange, O; Red, R; Green, G; Grey, Gy; Purple, Pu; White, W; Yellow-Green (mixed colour), Y-G; faint, f; no reaction, —.

Compound	*No.*	*Colours produced by staining reagents*									
		A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*
Arginine	2	R	Pu	R-Pu	Pu^{1}	Y^{3}	—	Pu^{1}	P-O	—	—
Histidine	11	R	Pu	Bl-Gy	Pu^{1} G^{3}	Y^{3}	—	Pu^{2,3}	Pu	Y-G^{4}	—
Lysine	12	R	Pu	Pu	Pu^{1}	Y^{3}	—	Pu^{1}	—	—	—
Cysteine	18	Pu	Gy	Gy	Pu^{1}	Y^{3}	—	Pu^{1}	—	—	—
Cystine	19	Pu	Bl	Pu	Pu^{1}	Y^{3}	—	Pu^{1}	—	—	—
Asparagine	24	Bn	Bn	Pu	Pu^{1}	Y^{3}	—	Pu^{1}	—	—	—
Hydroxylysine	31	R	Pu	Pu	Pu^{1}	Y^{3}	—	Pu^{1}	—	Bl^{4}	Bl^{3}
Homoarginine	39	R	Pu	Pu	Pu^{1}	Y^{3}	—	Pu^{1}	P-O	—	—
1-Methylhistidine	49	R	Pu-Gy	Pu	Pu^{1}	Y^{3}	—	Pu^{1}	—	—	—
3-Methylhistidine	50	R	Gy-Y	Bl-Gy	P^{1}	Y^{3}	—	Pu^{1}	—	—	—
Ornithine	54	R	Pu	Pu	Pu^{1} P^{4}	Y^{3}	—	P^{1} Pu^{3}	Bn	—	—
Djenkolic acid	58	Pu	Bn	Pu	R^{1}	Y^{1,3} Bl^{4}	— Y	Bl^{1} G^{3}	—	—	—
2,6-Diaminopimelic acid	59	R	G	R-Pu	Pu^{1}	Y^{3}	—	Pu^{1}	—	—	—
2,4-Diaminobutyric acid	61	R	Y	Pu	Pu^{1} W^{3} P^{4}	Y^{3}	—	Pu^{1}	P-O	Y-G^{4}	—
Carnosine	75	Bn	Bn	R-Pu	Gy^{1} Pu^{2} Gy^{3} Bn^{4}	—	P^{1} f	—	—	—	—

In the TLC of amino acids, the low concentration levels (5×10^{-4} μmole) often encountered sometimes affect the response to a particular reagent. For example O-phthalaldehyde (Reagent D) gave a specific green colour with glycine on paper chromatography but a non-specific purple colour on thin layers of cellulose. Nevertheless, most N-containing metabolites can be identified unambiguously by one or

TABLE IV

COLOUR REACTIONS OF METABOLITES OF GROUP II

Conditions and abbreviations as in Table III.

Compound	*No.*	*Colours produced by staining reagents*									
		A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*
Aspartic acid	3	R	Bl	Pu	Pu^{1}	Y^{3}	—	Pu^{1}	—	—	—
Serine	5	R	Gy	P	Pu^{1}	Y^{3}	—	Bn^{1,3}	—	Y-G^{4}	—
Glycine	6	Bn	O	P	Pu^{1}	Y^{3}	—	Pu^{1}	—	—	—
Hydroxyproline	17	Y	Gy	Bl	Bn^{1}	Y^{3}	—	Pu^{1,3}	—	—	—
Cysteic acid	20	R	Pu	P	Pu^{1}	Y^{3}	—	Pu^{1}	—	—	—
β-Alanine	21	P	Bl	Pu	Pu^{1}	Y^{3}	—	Bn^{1,3}	—	—	—
Glutamine	25	R	Gy	R-Pu	Pu^{1}	Y^{3}	f-Y	P^{1}	—	—	—
Phosphoethanolamine	27	R	Pu	Gy-Pu	Pu^{1}	Y^{3}	—	Pu^{1}	—	—	—
Taurine	28	R	Gy	P	Bn^{1}	Y^{3}	—	Pu^{1}	—	—	—
γ-Amino-*n*-butyric acid	30	R	Gy	R-Pu	Pu^{1} P^{4}	Y^{3}	—	Pu^{1}	—	—	—
δ-Aminolaevulinic acid	42	Y	Y	O	Pu^{1}	Y^{3}	—	O^{1} Pu^{3}	—	P^{3}	P^{3}
Methionine sulphone	44	R	Y-G	R-Pu	Pu^{1}	Y^{3}	—	Pu^{1}	—	—	—
Methionine sulphoxide	45	R	Y-G	P	Pu^{1}	Y^{3}	—	Pu^{1}	—	—	—
Sarcosine	51	Pu	Bn	Gy	Bn^{1}	Y^{3}	—	Pu^{1}	—	—	—
Homoserine	52	R	Pu	P	Pu^{1}	Y^{3}	—	Pu^{1}	—	Y-G^{4}	—
3,4-DOPA	53	Pu	Bn	Bl-Gy	Bn^{1}	Bn^{3} Gy-P^{4}	—	Bn^{1} Gy^{3}	Bn	Bn^{1,3}	Bn^{1,3}
Citrulline	56	R	Pu	R	Pu^{1}	Y^{3}	Y	Pu^{1} Y^{3}	—	—	—
Penicillamine	57	R	Bl	Pu	Pu^{1}	Y^{3}	—	Pu^{1}	P	Y-G^{4}	—
Formiminoglycine	60	Bn	Y	Pu	Pu^{1}	Y^{3}	—	Pu^{1}	Pu	—	—
5-Hydroxytryptophan	66	R	G	Bl	Bn^{1,2,3} Y^{4}	Y^{1,3} Pu^{4}	Pu	Bl^{1,3}	G	G^{1,3} Bn^{2}	G^{1,3} Bn^{2}
Allantoin	76	—	—	—	—	Y^{3}	Y f	Y^{1}	—	—	—

TABLE V

COLOUR REACTIONS OF METABOLITES OF GROUP III

Conditions and abbreviations as in Table III.

Compound	*No.*	*Colours produced by staining reagents*									
		A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*
Alanine	1	R	Pu	Pu^{1}	Pu^{1}	Y^{3}	—	Pu^{1}	—	—	—
Glutamic acid	4	R	Pu	Pu^{1}	Pu^{1}	Y^{3}	—	Pu^{1}	—	Y-G^{4}	—
Valine	8	R	Pu	R	Bn^{1}	Y^{3}	—	Pu^{1}	—	—	—
Tyrosine	14	R	Bn	Pu^{1}	Pu^{1}	Y^{3}	—	Bn^{1} Gy^{3}	—	—	—
Proline	16	Y	Y	Bl	Bn^{1}	Y^{3}	—	—	—	—	—
α-Aminoadipic acid	22	R	Pu	Bl-Gy	Pu^{1}	Y^{3}	—	—	—	—	—
β-Aminoisobutyric acid	23	Pu	Y	Bl-Gy	Pu^{1}	Y^{3}	—	Bn^{1}	—	—	—
p-Aminohippuric acid	40	—	—	Y	Pu^{1}	Y^{1,3} Pu^{2} G^{4}	Y	Pu^{1} Y^{3}	Pu	Y-G^{4}	—
α-Aminoisobutyric acid	46	R	—	Gy-P	Pu^{1}	Y^{3}	—	Pu^{1}	—	—	—
ε-Aminocaproic acid	47	R	Pu	Bl-Gy	Bn^{1}	Y^{3}	—	Pu^{1}	—	—	—
Pipecolic acid	55	Pu	Pu	G	Bl^{1}	Y^{3}	—	Bl^{1}	—	—	—
Saccharopine	64	R	—	P	—	Pu^{2,4}	—	—	—	—	—
Hippuric acid	70	—	—	—	—	Pu^{2} Bl^{4}	—	—	—	—	—

TABLE VI

COLOUR REACTIONS OF METABOLITES OF GROUP IV

Conditions and abbreviations as in Table III.

Compound	*No.*	*Colours produced by staining reagents*									
		A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*
Isoleucine	9	R	Pu	R	Pu^{1}	Y^{3}	—	Pu^{1}	—	—	—
Leucine	10	R	Pu	R	Pu^{1}	Y^{3}	—	Pu^{1}	—	—	—
Phenylalanine	13	R	Bn	Bl-Pu	Pu^{1}	Y^{3}	—	Pu^{1}	—	—	—
Tryptophan	15	R	Gy	Gy-Bl	Pu^{1}	Pu^{2} Y^{3}	Pu	P^{1} Bl-G^{3}	Bl-G	—	—
Methionine	29	R	Gy	R	Pu^{1}	Y^{3}	—	Pu^{1}	—	—	—
Norleucine	32	R	Pu	R	Bn^{1}	Pu^{2} P^{3}	—	Pu^{1}	—	—	—
2-Aminooctanoic acid	33	R	Pu	Pu	Pu^{1}	Y^{1,3}	—	Pu^{1}	—	—	—
Ethionine	48	R	—	Pu	Pu^{1} W^{3}	Y^{1,3}	—	Pu^{1}	—	—	—
Indican	65	Bl	Pu	Pu	Pu^{1} Y^{2} Pu^{3,4}	Pu^{1,3} Y^{2} R^{4}	Pu	G^{1,3}	Pu f	P^{1,3} Y^{2} Pu^{4}	P^{1} Y^{2} Pu^{3,4}
Tryptamine	68	Gy	Bn	Pu	Gy^{1} Y^{2,3} Pu^{4}	Y^{1,2} Y^{3} Y^{4}	O to Pu	O^{1} Pu^{3}	Bl-G	Y-G^{3} Pu^{4}	f-Y^{3} O^{4}
3-Indolylacetic acid	69	—	Gy-Bn	Y	Bn^{1} Y^{2,3} Bl^{4}	O^{1,3} Y^{2,4}	P to Pu-Bn	O^{1}Bl-G Pu^{3}	—	Y^{1,2,3} Bn^{4}	Y^{1,2,3} O^{4}
Urea	71	—	—	—	—	—	Y f	—	—	—	—
Thiourea	74	—	—	—	Pu^{2} Bl^{4}	—	Y f	P^{4}	Bl-Pu	—	O^{3,4}

TABLE VII

COLOUR REACTIONS OF METABOLITES OF GROUP V

Conditions and abbreviations as in Table III.

Compound	*No.*	*Colours produced by staining reagents*									
		A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*
Threonine	7	R	Gy	Pu	Pu^{1}	Y^{3}	—	Pu^{1}	—	—	Bl^{3}
Ethanolamine	26	R	Gy	P	Pu^{1} P^{4}	Y^{3}	—	Bn^{1}	—	Y-G^{4}	—
Cadaverine	35	R	Pu	Bl-Gy	Pu^{1}	Y^{3}	—	Pu^{1}	—	—	—
Putrescine	36	R	Pu	Bl	Bl^{1} W^{3} O^{4}	Y^{1} Y^{3}	Y	Bn^{1} Pu^{3}	P	Y-G^{4}	—
Histamine	37	R	Bn	Bl-Gy	Pu^{1} G^{3}	Y^{3}	—	Pu^{1}	Pu	Y-G^{4}	—
Kynurenine	38	R	O	R	Bl^{1} Bl^{4} Y^{3}	Y^{1} Y^{3} Bl^{2} Bl^{4}	O	Bn^{1} O^{3}	Pu	Y^{1,3} Bl^{4}	Y^{1,3} Bl^{4}
4-Amino-5-imidazole-carboxylic acid	41	—	—	—	Y^{1} Y^{3} Y^{4}	Y^{3} G^{4}	Y	Pu^{1} Y^{3}	Y	O^{1} O^{3} O^{4}	O^{1,3,4}
Glucosamine	62	R	Gy	—	Pu^{1}	Y^{3}	—	—	—	—	—
Epinephrine	63	R	Gy	Gy	Bn	Y^{3}	Y	Bn^{1} G^{3}	Y	P^{1} Bn^{3}	P^{1} Bn^{3}
Creatinine	72	—	—	—	Pu^{2} f Pu^{4} f	—	—	—	P	—	—
Guanidine	73	—	—	—	—	—	—	—	P	—	—

TABLE VIII

RESOLUTION OF A MIXTURE OF METABOLITES BY MEANS OF STAINING REAGENTS

Conditions and abbreviations are those indicated in Table III.

Compound	*No.*	*Colours produced by the reagents*					
		A	B	C	D	G	H
Aspartic acid	3	R	Bl	Pu	Pu	Pu[1]	—
Penicillamine	57	R	Bl	Pu	Pu	Pu[1]	P
β-Alanine	21	P	Bl	Pu	Pu	Bn[1,3]	—
Asparagine	24	Bn	Bn	Pu	Pu	Pu[1]	—
Sarcosine	51	Pu	Bn	Gy	Bn	Pu[1]	—
Carnosine	75	Bn	Bn	R-Pu	Gy[1,3] Pu[2], Bn[4]	P[1]	—
3,4-DOPA	53	Pu	Bn	Bl	Bn	Bn[1], Gy[3]	Bn
β-Aminoisobutyric acid	23	Pu	Y	Bl-Gy	Pu	Bn	—
Proline	16	Y	Y	Bl	Pu	Bl[1], Pu[3]	—
δ-Aminolaevulinic acid	42	Y	Y	O	Pu	O[1], Pu[3]	—

TABLE IX

SPECIFIC COLOUR REACTIONS OF SOME METABOLITES

Conditions are those indicated in Table III.

Compound	*No.*	*Reagent*	*Colour reaction*
Glycine	6	B	orange
Histidine	11	D	purple[1], green[3]
Tryptophan	15	G	pink[1], bluegreen[3]
Proline	16	G	blue[1], purple[3]
Hydroxyproline	17	G	purple[1], purple[3]
Glutamine	25	G	pink[1]
Hydroxylysine	31	I	blue[4]
Putrescine	36	D	blue[1], white[3], orange[4]
		G	brown[1], purple[3]
Histamine	37	D	purple[1], green[3]
Kynurenine	38	D	blue[1], yellow[3], blue[4]
		G	brown[1], orange[3]
		I or J	yellow[1], yellow[3], blue[4]
p-Aminohippuric acid	40	E	yellow[1], purple[2], yellow[3], green[4]
4-Amino-5-imidazole-carboxylic acid	41	D	yellow[1], yellow[3], yellow[4]
		I or J	orange[1], orange[3], orange[4]
δ-Aminolaevulinic acid	42	C	orange
		I or J	pink[3]
Ethionine	48	D	purple[1], white[3]
1-Methylhistidine	49	D	pink[1]
3,4-DOPA	53	E	brown[3], grey-pink[4]
		I or J	brown[1], brown[3]
Ornithine	54	G	pink[1], purple[3]
Pipecolic acid	55	C	green
Djenkolic acid	58	G	blue[1], green[3]
2,6-Diaminopimelic acid	59	B	green
2,4-Diaminobutyric acid	61	D	purple[1], white[3], pink[4]
Epinephrine	63	G	brown[1], green[3]

Continued on p. 275.

TABLE IX (*continued*)

SPECIFIC COLOUP REACTIONS OF SOME METABOLITES

Conditions are those indicated in Table III.

Compound	*No.*	*Reagent*	*Colour reaction*
Indican	65	D	purple^1, yellow^2, purple^3, purple^4
		G	green^1, green^3
		I	pink^1, yellow^2, pink^3, brown^4
		J	pink^1, yellow^2, purple^3, purple^4
5-Hydroxytryptophan	66	D	brown^1, brown^2, brown^3, yellow^4
		E	yellow^1, yellow^3, purple^4
		G	blue^1, blue^3
		I or J	green^1, brown^2, green^3
Tryptamine	68	D	grey^1, yellow^2, yellow^3, purple^4
3-Indolylacetic acid	69	I	yellow^1, yellow^2, yellow^3, brown^4
		J	yellow^1, yellow^2, yellow^3, orange^4
Carnosine	75	D	grey^1, purple^2, grey^3, brown^4
Allantoin	76	G	yellow

two of the reagents which we have listed in Table I. Even the most difficult mixture of compounds to analyse such as we have collected together in Table VIII can be distinguished with certainty after TLC by the use of six reagents.

Although it is feasible to identify all of the seventy-six compounds investigated by means of one or more reagents, twenty-seven of them may be identified specifically by one reagent.

This is demonstrated in Table IX where each reagent is capable of producing a unique colour (or fluorescence) sequence when the spot is viewed under successive conditions.

Several well-known specific reagents such as, *e.g.*, the Pauli test for histidine have been omitted from the present work because of their inadequate response at the low levels of concentration which have been used. The fragile nature of the cellulose layer also precludes the use of many other reagents.

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CHROM. 4824

A MICRO-METHOD FOR THE DETERMINATION OF HYDROXYLYSINE AND ITS GLYCOSYLATED DERIVATIVES

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(Received May 6th, 1970)

SUMMARY

A simple method is described, using high voltage electrophoresis for the estimation of hydroxylysine, hydroxylysine galactoside and hydroxylysine glucosidogalactoside in alkaline hydrolysates of proteins.

Hydroxylysine and their glycosylated derivatives have characteristic migration properties; high amounts of salts (even 10–15 times the weight of protein) do not interfere with their separation and quantitative estimation.

INTRODUCTION

Collagen contains small quantities of carbohydrates[1] in the form of galactose and 2-O-α-D-glucopyranosyl-O-β-D-galactopyranose, linked by an O-glycosidic bond to the hydroxylysine[2] of the polypeptide chain. The ratio of the mono- and disaccharide units may be involved in the tissular organisation, as can be concluded from the variations of this ratio in collagens from different organs or species representing different levels of evolution[3–9]. It may also be characteristic for the pathological alterations of collagen[10,11].

The methods described for the detection and determination of hydroxylysine galactoside and hydroxylysine glucosidogalactoside involve the alkaline hydrolysis of the tissue[2,7] or its enzymatic digestion[4,12].

The O-glycosidic linkage is quite stable during the alkaline hydrolysis. After 24 h at 100° in 2 *N* NaOH about 90% of the hexoses can be recovered[13]. Consequently, a quantitative estimation of the hydroxylysine glycosides is possible in these hydrolysates. The methods reported imply the use of automated ion-exchange techniques, but in the case of alkaline hydrolysates the Hyl-Gal peak overlaps with other amino acids[7]. Therefore, for the determination of the Hyl-Gal-Glc/Hyl-Gal ratio it is necessary to perform a second analytical run on an acid hydrolysate[7].

A semi-quantitative estimation of the Hyl-Gal-Glc/Hyl-Gal ratio has been obtained by high voltage electrophoresis of the hydrolysate followed by photodensitometry of the ninhydrin positive spots or by determination of the weight of the

eluted substances[4,5]. All the methods described require previous desalting of the hydrolysate.

In the present study a quantitative paper electrophoretic method is described for the separation and determination of the ratio of lysine, hydroxylysine, hydroxylysine-galactoside and hydroxylysine-glucosido-galactoside in a crude alkaline hydrolysate of the proteins.

Electrophoretic techniques, although less accurate than the ion-exchange methods for the determination of amino acid derivatives, have the advantage of rapidity and permit several simultaneous determinations to be carried out.

MATERIALS AND METHODS

Material

Hydroxylysine-galactoside and hydroxylysine-glucosido-galactoside were prepared from the low molecular weight glycopeptide fractions of the calf corneal stroma[3] and pig aorta[14] by a preparative electrophoretic separation of the desalted alkaline hydrolysates. The substances were analysed for sugars and amino acids. One of the substances was composed of galactose, glucose and hydroxylysine, the other one of galactose and hydroxylysine, in the ratios 1:1:1 and 1:1, respectively. The electrophoretic and chromatographic mobilities of these compounds were identical to those of an authentic sample[2,4].

Hydrolysis of the samples

Samples of 1–2 mg of protein or tissue containing about 40–60% collagen were hydrolysed in 50–100 μl 2 *N* NaOH at 105° for 24 h in sealed pyrex or capped polypropylene tubes. The hydrolysate was acidified with 2.2 volumes of 1 *N* acetic acid and centrifuged. The supernatant was evaporated to dryness *in vacuo* over KOH pellets and dissolved in 15 μl water/1 mg protein. If the hydrolysis was carried out in glass tubes, the precipitated hydrated silica renders the quantitative recovery of the hydrolysis products difficult, but the determination of the Hyl-Gal/Hyl-Gal-Glc ratio is still possible.

Electrophoretic separation

Whatman 3 MM paper strips, 120 cm long and 5 cm wide, were used in a Gilson Electrophoretic Model D. 15–20 μl of the solutions prepared as described above were deposited as an 8–10 mm starting line, about 40 cm from the anodic end of the strips. Two samples were applied on each strip. Up to seven strips were used in a single run. The paper was sprayed with the buffer pyridine–acetic acid–water (1:10:89) pH 3.8, and 4000–5000 V were applied for 1.5–2 h.

Staining of the spots and quantitative evaluation

After electrophoresis, the air-dried paper strips were dipped in the ninhydrin–cadmium reagent[15] (cadmium acetate 0.1 g, ninhydrin 1 g, water 10 ml, acetic acid 5 ml, acetone 50 ml; diluted 1:5 with acetone before use). The colour was developed by heating the strips at 50° for 30 min. The intensities of the developed spots were either evaluated by photodensitometry or by elution of the spots with 1.5 ml of methanol and determination of their optical density at 500 mμ.

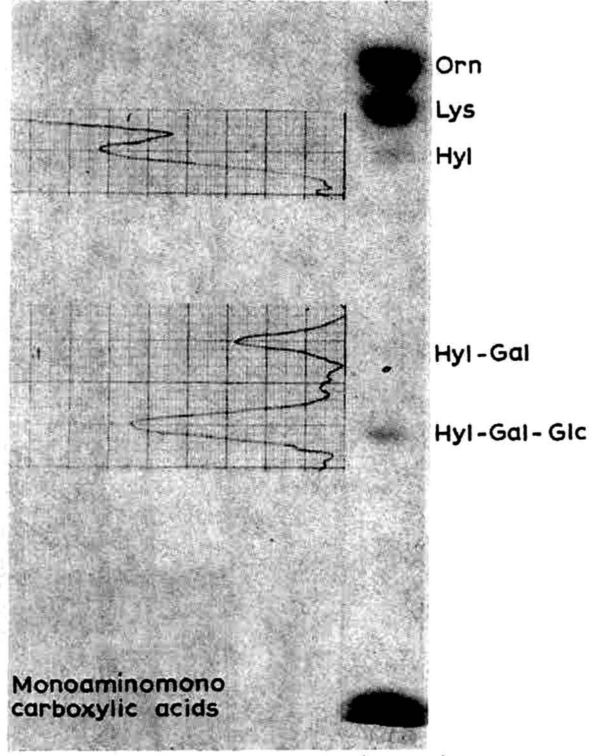

Fig. 1. Electropherogram and photodensitometric recording of hydroxylysine, galactosylhydroxylysine and glucosyl-galactosylhydroxylysine visualised by the ninhydrin–cadmium reagent[15] from an alkaline hydrolysate of 150 μg of calf corneal stroma.

RESULTS AND DISCUSSION

Photodensitometry may be less accurate but more sensitive than the elution of the coloured spots. Samples corresponding to 0.1–0.2 mg of collagen equivalent to 0.5–1 μg (4–6 μM) of the Hyl-glycoside gave readily reproducible densitometric recordings. For the colorimetric determination of the methanolic eluates, samples corresponding to about 0.3–0.6 mg should be used.

However the ratio of hydroxylysine to lysine could not be evaluated by photodensitometry, because of the big difference in the colour intensity of the spots. To obtain comparable optical densities the elution method had to be used, the lysine spot being eluted with 3 times more methanol than the volume used for the elution of the hydroxylysine derivatives.

An electropherogram and the corresponding densitometric recording of the hydrolysate of the corneal stroma of the calf embryo[8] are illustrated in Fig. 1.

The presence of the sodium salts under the conditions used does not interfere with the separation; however higher NaOH–protein ratios should be avoided.

Arginine has the same electrophoretic mobility as hydroxylysine in the buffer used. Arginine is, however, quantitatively hydrolysed during the alkaline treatment

TABLE I

DETERMINATION OF THE RATIO OF THE HYDROXYLYSINE AND ITS GLYCOSYLATED DERIVATIVES IN THE ALKALINE HYDROLYSATES OF THE HUMAN CORNEAL STROMA

Experimental details, see text.

	Run	*Hyl-Gal-Glc*	*Hyl-Gal*	*Hyl*
(a) Photodensitometric recording. Peak areas are related to Hyl-Gal-Glc = 100				
Hydrolysate No. 1	1	100	56	108
(1 mg dry substance)	2	100	48	109
	3	100	55	104
$\bar{m} \pm \sigma$			53 ± 2.5	107 ± 1.6
Hydrolysate No. 2	1	100	50	100
(1.2 mg dry substance)	2	100	56	105
	3	100	58	103
$\bar{m} \pm \sigma$			54.7 ± 2.4	102.7 ± 1.3
(b) Spectrophotometric readings of the eluted spots (1.5 ml methanol) at 500 mμ				
Hydrolysate No. 3 (0.8 mg dry substance) optical density		0.18	0.085	0.175
Ratio related to Hyl-Gal-Glc = 100		100	47	97
Hydrolysate No. 4 (1.2 mg dry substance) optical density		0.29	0.14	0.27
Ratio related to Hyl-Gal-Glc = 100		100	48	93

giving ornithine and urea[16]. The complete decomposition of the arginine during the alkaline hydrolysis of several samples of collagen-containing tissues was checked by eluting the hydroxylysine spot from the electropherograms and submitting this eluate to thin-layer chromatography (microcrystalline cellulose, developed by: (1) pyridine–ethyl acetate–acetic acid water (5:5:1:3) and (2) *n*-butanol–acetic acid–water (12:3:5)). By this method no amino acid other than hydroxylysine could be detected.

Those spots migrating with average electrophoretic mobilities of 0.65 and 0.52, relative to the lysine spot, are the hydroxylysine glycosides: the Hyl-Gal and Hyl-Gal-Glc, respectively, present in the alkaline hydrolysates. The colour developed with the ninhydrin- cadmium reagent at 50° for 30 min[11,15,17] gave the same intensity as the usual method (24 h at room temperature)[15,17]. The two hydroxylysine glycosides behave like other amino acids in this colour reaction[7,13,15,17-19].

Table I presents the results of the photodensitometric and colorimetric evaluation of the electropherograms of an alkaline hydrolysate of human corneal stroma. The peak areas were calculated by multiplying the height of the peak by its width at midheight. Densitometric evaluation always gives somewhat higher hydroxylysine galactoside values related to the hydroxylysine disaccharide and hydroxylysine than spectrophotometric estimation of the intensity of the colour eluted. This may be attributed to the slight irregularity of the densitometric tracings, due to irregular paper structure.

The standard error of the means from 3 determinations is usually of the order of 1.5–3% for the glycosylated hydroxylysine derivatives, and 2–7% for the free hydroxylysine due to the proximity of the strong lysine peak.

It is interesting to compare the value of 70:30 for the Hyl-Gal-Glc/Hyl-Gal ratio obtained by us from the calf corneal stroma to that of 65:35 reported by SPIRO[7] for the citrate soluble collagen of calf cornea.

This method is recommended for the determination of the ratio of hydroxylysine and its glycolylated derivatives in collagen and connective tissue constituents. It has the advantage of a direct estimation, of rapidity, and requires little starting material.

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CHROM. 4839

DETECTION OF MERCURY AND ALKYLMERCURY COMPOUNDS BY REVERSED-PHASE THIN-LAYER CHROMATOGRAPHY

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(Received May 25th, 1970)

SUMMARY

A reversed-phase thin-layer chromatographic method for separation and detection of the dithizonates of inorganic mercury compounds and a series of alkylmercury compounds (C_1–C_{18}) was investigated. The specificity of this method was that all the compounds tested were distinctly separated and detected with high sensitivity. The influence of some of the other inorganic metals and excess dithizone was also examined. The method is applicable to the separation and identification of mercury compounds in foods and sewages.

INTRODUCTION

The contamination of foods by mercury compounds from our environment has been observed. There is no doubt that the mercury compounds in shellfish and fish which caused the Minamata disease and a kind of nervous disease in the Agano River area in Japan was a methylmercury compound[1]. Since then, mercury compounds have toxicologically been evaluated; the organic mercury compounds have longer biological retention times in several tissues and have a greater specific effect on nervous systems than do inorganic mercury compounds. In the field of health sciences, therefore, a simple and rapid method for the analysis of mercury compounds in foods and sewages is needed.

Several authors have presented methods for the detection and determination of organic mercury compounds[2,3]. The compounds are generally separated by extraction from the acidified samples with a suitable solvent such as benzene, concentrated by shaking the extract with a small portion of a cysteine solution, and subsequently extracted with benzene. With or without concentration, the compounds are detected and determined by thin-layer chromatography (TLC) and gas chromatography (GC). Especially in the separation of inorganic and organic mercury compounds, ISHIKURA AND YOKOTA[4] reported that these dithizonates were satisfactorily separated by chromatography on an alumina column. WESTÖÖ[5] reported that using a combined TLC and GC method, the mercury compound found in fishes was identified as a methylmercury compound and quantitatively determined. TANABE[6] investigated

the metabolic fate of mercury compounds in plant tissues applying a similar method. However, using these methods it was impossible to separate a series of alkylmercury compounds which may be found in waste water.

In the present paper, methods are given for the detection of trace amounts of a series of alkylmercuric dithizonates by reversed-phase thin-layer chromatography using corn starch or Avicel SF coated with liquid paraffin.

EXPERIMENTAL

Materials and methods

Adsorbents. Corn starch (obtained from Wako Pure Chemical Ind., Ltd.) and Avicel SF (obtained from FMC American Viscose Division) were used as the adsorbents.

Reagents. Liquid paraffin, chloroform, benzene, ethanol, methyl cellosolve and dithizone were of analytical reagent grade. Mercuric chloride was obtained from Tokyo Kasei Kogyo Co., Ltd. All the alkylmercuric chlorides listed in Table I were synthesized and purified according to the method reported by SLOTTA AND JACOBI[7].

TABLE I

CHEMICAL FORMULAS AND MOLECULAR WEIGHTS OF MERCURY AND ALKYLMERCURY COMPOUNDS

Compound	*Chemical formula*	*Molecular weight*	
		X = Dithizone	*X = Chloride*
Mercury	$Hg–X_2$	711.23	271.50
Methylmercury	$CH_3–Hg–X$	470.96	251.08
Ethylmercury	$CH_3–CH_2–Hg–X$	484.98	265.11
n-Propylmercury	$CH_3–(CH_2)_2–Hg–X$	499.01	279.14
n-Butylmercury	$CH_3–(CH_2)_3–Hg–X$	513.04	293.17
n-Amylmercury	$CH_3–(CH_2)_4–Hg–X$	527.07	307.20
n-Octylmercury	$CH_3–(CH_2)_7–Hg–X$	566.12	349.28
Stearylmercury	$CH_3–(CH_2)_{17}–Hg–X$	696.31	489.55

Dithizonates of all the compounds were prepared as follows. 10 mg of mercuric chloride or methylmercuric chloride were dissolved in 20 ml of water and 10 mg of the other compounds were dissolved in 20 ml of benzene. After a portion of a benzene solution of dithizone (0.4%, w/v) had been added to each solution with stirring until a green coloration took place, the benzene solution was shaken with 10 ml of 1 *N* sulfuric acid. It was then shaken with 30 ml of dilute aq. ammonia and subsequently washed twice with 20 ml of water to remove excess dithizone. The solution was then evaporated to dryness under reduced pressure.

By dissolving 10 mg of the dithizonates in 20 ml of benzene, test solutions were prepared. Aliquots (µl) of these solutions were used for TLC.

Apparatus. The thin-layer applicator and accessories were obtained from Yasawa Seisaku Co., Ltd. Glass plates were 20 cm × 5 cm. The chromatographic chamber was 9 cm in diameter and 27 cm in height.

Preparation of chromatoplates. (a) Corn starch–liquid paraffin: According to the general method, glass plates were evenly coated with a homogeneous slurry composed of 30 g of corn starch, 3.0 ml of liquid paraffin and 40 ml of chloroform by using

an applicator giving a thin layer approximately 300 μ thick. The plates were dried in air at room temperature and stored overnight before use in a desiccator containing calcium chloride.

(b) Avicel SF–liquid paraffin: The chromatoplates were prepared in the same manner as (a) with a homogeneous slurry composed of 30 g of Avicel SF, 6 ml of liquid paraffin and 60 ml of chloroform. The plates were stored overnight before use in a desiccator containing calcium chloride as were the corn starch–liquid paraffin plates.

Solvent systems for TLC. The solvent systems used in the experiments were prepared as follows. To ethanol and methyl cellosolve, respectively, water was added at ratios from 0 to 30%.

Application of samples and development of chromatoplates. 0.2–0.5 μl of the test solutions of dithizonate of each mercury compound were spotted with a micropipette on the starting line 2 cm from the lower edge. The plate was then placed inside a chamber containing the solvent system to a depth of about 1 cm and the sample was developed by the ascending method until the solvent front had travelled a distance of 10 cm from the starting line. The spots were observed under visual light.

RESULTS AND DISCUSSION

Reproducibility of R_F values of the dithizonates of mercury compounds

It is well known that the nature of the adsorbent, the thickness and activity of the plates and the solvent saturation in the chromatographic chamber are factors that control the reproducibility of R_F values in TLC. In the separation of the dithizonates of mercury and alkylmercury compounds tested, the most important factor that controls the reproducibility of R_F values, except those listed above, was the preparation of the layers. The content of liquid paraffin in the layers of corn starch, and Avicel SF, and the amount of moisture which adhered to the layers while chloroform was vaporizing from the layers, after the glass plates had been coated with both slurries, were found critical. In order to resolve the latter problem, the layers prepared were dried overnight in a desiccator containing calcium chloride before use.

The content of liquid paraffin in corn starch– and Avicel SF–liquid paraffin layers

In order to find the content of liquid paraffin in corn starch– and Avicel SF–liquid paraffin layers most suitable for separation of the dithizonates of these mercury compounds, each layer containing liquid paraffin at a ratio of 1.5, 3.0, 4.5 and 6.0 ml, respectively, for 30 g of each adsorbent was prepared according to the sequence and steps described above. The test solutions were applied to the layers which were then developed with a solvent system composed of ethanol and water.

The patterns of the distribution of the spots of the mercuric dithizonates on the chromatograms of both layers were similar when developed with the same solvent system. R_F values of the compounds decreased as the length of the carbon chain increased; and when increasing the content of liquid paraffin in the layers, R_F values of the compounds decreased relatively with good distribution of the spots. From the results of this experiment, the content of liquid paraffin in corn starch– and Avicel SF–liquid paraffin layers was adjusted to that described in EXPERIMENTAL.

Solvent systems

In order to obtain solvent systems which would be able to separate all the mercuric and alkylmercuric dithizonates tested on corn starch– and Avicel SF–liquid paraffin layers, the compounds were applied to each layer and developed with either ethanol–water or methyl cellosolve–water, the water content of which was 0, 10, 20, 25 and 30%, respectively.

As shown in Fig. 1a and 1b, the distribution of spots of the mercury compounds on corn starch–liquid paraffin layers was better using methyl cellosolve–water systems than using ethanol–water systems. It was found from Fig. 1b and 1c that there was

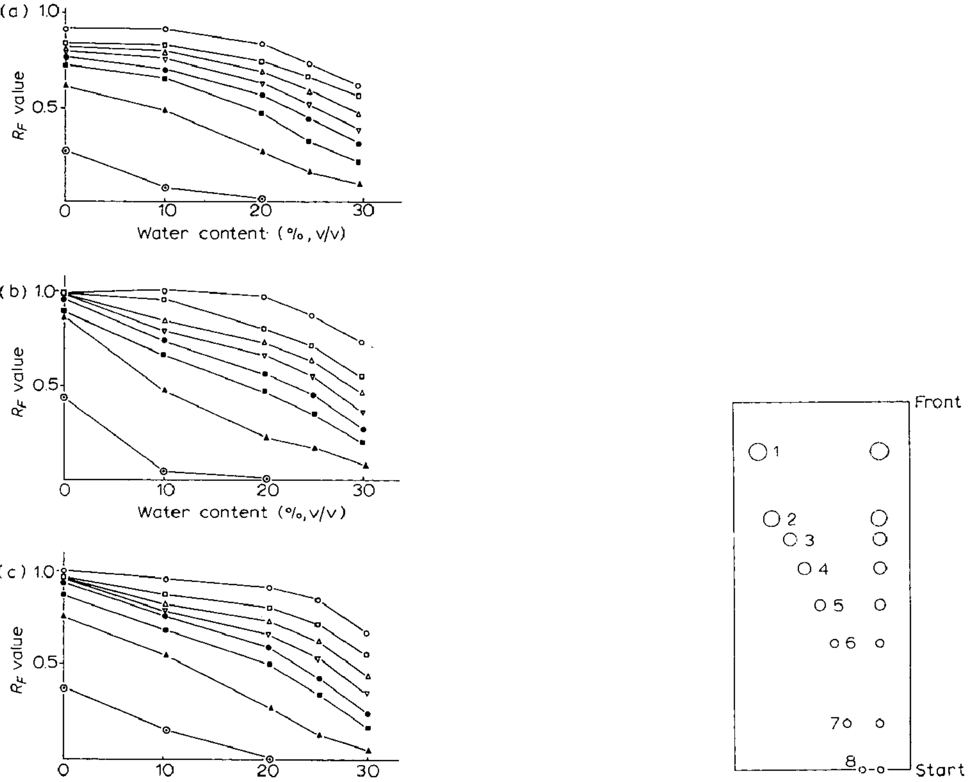

Fig. 1. Relationship between the distribution of mercuric and alkylmercuric dithizonates and the water content in the solvent systems. Mercuric dithizonate (○—○); ethylmercuric dithizonate (□—□); *n*-butylmercuric dithizonate (△—△); *n*-octylmercuric dithizonate (▽—▽); methylmercuric dithizonate (●—●); *n*-propylmercuric dithizonate (■—■); *n*-amylmercuric dithizonate (▲—▲); stearylmercuric dithizonate (⊙—⊙). (a) solvent system: ethanol–water; layer: corn starch–liquid paraffin; (b) solvent system: methyl cellosolve–water; layer: corn starch–liquid paraffin; (c) solvent system: methyl cellosolve–water; layer: Avicel SF–liquid paraffin.

Fig. 2. Chromatogram of mercuric and alkylmercuric dithizonates and the mixture of them on Avicel SF–liquid paraffin layer. Solvent system: methyl cellosolve–water (75:25). 1 = mercuric dithizonate; 2 = methylmercuric dithizonate; 3 = ethylmercuric dithizonate; 4 = *n*-propylmercuric dithizonate; 5 = *n*-butylmercuric dithizonate; 6 = *n*-amylmercuric dithizonate; 7 = *n*-octylmercuric dithizonate; 8 = stearylmercuric dithizonate.

some difference in the distribution of spots between both layers when using methyl cellosolve–water systems.

On corn starch–liquid paraffin layers, the dithizonates of inorganic mercury and alkylmercury compounds with a carbon chain from C_1–C_8 were distinctly separated with round spots when developed with the solvent systems having a water content of 15–25%, and it was assumed that the spots of dithizonates of alkylmercury compounds with a carbon chain from C_8–C_{18} might be sufficiently separated from those of octyl- and stearylmercuric dithizonates when developed with a solvent system having a water content of 0–10%.

On Avicel SF–liquid paraffin layers, the dithizonates of mercury and alkylmercury compounds with a carbon chain from C_1–C_5 were separated with the above solvent systems having a water content of 25–30%, and the dithizonates of alkylmercury compounds with a carbon chain from C_5–C_{18} with the solvent systems containing 0–20% water. In addition, the distribution and sharpness of spots on Avicel SF–liquid paraffin layers were superior to those on corn starch–liquid paraffin layers.

A chromatogram of the compounds on Avicel SF–liquid paraffin layers when using methyl cellosolve–water (75:25) is shown in Fig. 2.

The running time varied with the type of layer used and the water content in the solvent system. On corn starch–liquid paraffin layers 1–1.5 h and on Avicel SF-liquid paraffin layers 3 h were generally required at 20 ± 1°.

TABLE II

DETECTION LIMITS OF MERCURY AND ALKYLMERCURY COMPOUNDS

Solvent system: Methyl cellosolve–water (75:25). Layer: Avicel SF–liquid paraffin layer.

Compound	*Dithizonate (μg)*	*Chloride (μg)*
Mercury	0.01	0.004
Methylmercury	0.1	0.053
Ethylmercury	0.1	0.055
n-Propylmercury	0.1	0.056
n-Butylmercury	0.1	0.057
n-Amylmercury	0.05	0.029
n-Octylmercury	0.01	0.006
Stearylmercury	0.01	0.007

Sensitivity test for the detection of mercury and alkylmercury compounds

When spotting each 0.01–1.0 μg of the mercuric and alkylmercuric dithizonates on Avicel SF–liquid paraffin layers and developing them with methyl cellosolve–water (75:25), each spot was detected under visual light. The detection limits of mercuric and alkylmercuric dithizonates and of their chlorides calculated from the corresponding dithizonates are shown in Table II. Detection was so sensitive that the compounds were found at the 0.005–0.057 μg level when calculated as the chloride forms. The lower sensitivities of alkylmercury compounds having a shorter carbon chain in the molecules might be dependent upon any dispersion brought by their higher R_F values. In contrast to this assumption, mercuric dithizonates showed higher sensitivity than dithizonates of methyl, ethyl, propyl, *n*-butyl and amylmercury compounds. This was, however, explained from the results that mercuric dithizonate was

observed as a red spot, which is most sensitive, while the other dithizonates were seen as orange-yellow spots.

Influence of other metals and excess dithizone

As mercury and alkylmercury compounds form the dithizonates with dithizone over an extensive pH range and even at a comparatively low pH, other metals except copper and some rare metals could be removed without formation of the dithizonates in the procedure described above. The metals, however, which may sometimes contaminate subsequent procedures and may be present in the layers, will react with small amounts of dithizone that may remain in the reaction mixture even after treatment with aq. ammonia or be formed by breakdown of the dithizonates during concentration of the reaction mixture and exposure under helio light. It is, therefore, necessary to know whether any dithizonates of the common metals and residual dithizone influence the detection of these mercuric dithizonates during TLC.

50 mg each of lead nitrate, copper sulfate ($CuSO_4 \cdot 5H_2O$), stannous chloride ($SnCl_2 \cdot 2H_2O$) and zinc sulfate ($ZnSO_4 \cdot 7H_2O$) were dissolved in 20 ml of water and neutralized with a small portion of diluted sodium hydroxide. To the solution, a portion of benzene solution of dithizone (0.4%, w/v) was added until the color of the mixture remained green. Treated with aq. ammonia and water, as in the preparation of mercuric dithizonates, the solution was taken to dryness under reduced pressure. After 10 mg each of the dried dithizonates and dithizone had been dissolved in 20 ml of benzene, aliquots of the solutions were spotted on Avicel SF–liquid paraffin layers and developed with methyl cellosolve–water (7:3).

On the chromatogram, all the compounds examined were found close to the solvent front. It is of interest that dithizone was observed as a pink spot. The spot might be of zinc dithizonate formed by the reaction between dithizone and trace amounts of the zinc compounds which had been present in the layer. Thus it was found that all the compounds examined did not have an influence on the detection of the mercury and alkylmercury compounds.

CONCLUSION

On either the corn starch– or Avicel SF–liquid paraffin layers, the inorganic mercury and a series of alkylmercury compounds were submitted to reversed-phase thin-layer chromatography. The distribution and sharpness of the spots of the compounds on the Avicel SF layers were superior to those on the corn starch layers, although the running time on the former was longer than on the latter layers. The best pattern of separation of all the compounds was obtained using development on the Avicel SF layers with methyl cellosolve–water (7:3). No interference of the other metals and the residual dithizone was observed.

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CHROM. 4832

CRYSTALLINE INSOLUBLE ACID SALTS OF TETRAVALENT METALS

XI. SYNTHESIS AND ION-EXCHANGE PROPERTIES OF TIN(IV) PHOSPHATE AND TIN(IV) ARSENATE

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(Received May 25th, 1970)

SUMMARY

The syntheses of a crystalline tin(IV) phosphate and tin(IV) arsenate are reported. These two new inorganic ion exchangers have been characterised on the basis of their X-ray powder patterns, chemical analysis, chemical stability and thermal dehydration behaviour.

Their ion-exchange properties were investigated with alkali metal ion uptake curves. Tin(IV) phosphate and tin(IV) arsenate have a high ion-exchange capacity for Li^+ (7.9 and 6.6 mequiv./g, respectively) while they are extensively hydrolysed by the other alkali metal ions.

INTRODUCTION

The good ion-exchange properties of zirconium phosphate have recently stimulated interest in the preparation of insoluble acid salts of other tetravalent metals such as titanium, cerium(IV), tin(IV) and thorium[1]. In regard to tin(IV) phosphate, various products exhibiting some ion-exchange properties have been obtained by INOUE[2–4] and PIRET *et al.*[5]. All these products are amorphous and very hydrolysable materials and their P:Sn ratio is lower than 2. WINKLER AND THILO[6] have recently obtained a crystalline tin(IV) phosphate with a P:Sn ratio of 2.4. However the ion-exchange properties of this material were only investigated using a titration curve with NaOH, and data on its chemical stability at various pH values and on its affinity for the various cations are not available.

The results obtained in our laboratory on the synthesis of crystalline ion exchangers of the insoluble acid salts of tetravalent metals[7–12] have encouraged us to continue studies in this field and also to investigate the synthesis of crystalline tin(IV) phosphate and arsenate. Among the various products obtained, a crystalline tin(IV) phosphate (P:Sn ratio $\simeq$ 2) and a crystalline tin(IV) arsenate (As:Sn ratio $\simeq$ 2) showed good chemical stability and interesting ion-exchange properties. Some details on the synthesis and ion-exchange properties of these new ion-exchange materials are reported and discussed.

EXPERIMENTAL

Reagents

All reagents used were Carlo Erba R.P. $3As_2O_5 \cdot 5H_2O$ was a Merck "pro analyse" product.

Analytical procedure

The P:Sn and As:Sn ratios in tin(IV) phosphate and tin(IV) arsenate, respectively, were determined by the following procedure. About 100 mg of sample were dissolved in 10 ml of hot 1 *M* NaOH. This solution was acidified with 8.5 ml of conc. HCl and then diluted to 100 ml with distilled water. The phosphate or arsenate content was determined colorimetrically in 2 ml of this solution as previously described[7,12]. Tin(IV) was determined in the remaining solution by precipitation with a 6% solution of Kupferron and calcination to SnO_2.

The pyrophosphate content in heated tin(IV) phosphate was determined as follows. 100 mg of sample were dissolved in 10 ml of hot 1 *M* NaOH, and the solution was diluted to 100 ml with distilled water. 2 ml of this solution were directly analysed for the orthophosphate content, while 10 ml of hot conc. HCl were added to 25 ml to hydrolyse the pyrophosphate. The solution was then diluted to 50 ml with distilled water and analysed for orthophosphate. The pyrophosphate content was calculated as described[13]. Titration, hydrolysis, ion uptake and weight loss curves were obtained as previously described[9]. The equilibrium time allowed for titration experiments was 4 days. X-ray photographs of powder patterns were taken using CuK_α radiation.

RESULTS AND DISCUSSION

Preparation of materials

Attempts to obtain crystalline materials by refluxing amorphous stannic phosphate (or arsenate) in conc. phosphoric (or arsenic) acid containing 1 *M* HCl or 3 *M* HNO_3 were unsuccessful even after 200 h of refluxing. On the contrary, by refluxing solutions containing tin(IV) chloride, nitric acid and phosphoric (or arsenic) acid in different ratios, microcrystalline products began to precipitate after some hours of refluxing while NO_2 was evolved during the precipitation.

The crystalline tin(IV) phosphate described in this paper was prepared as follows: To 1 l of 8 *M* H_3PO_4 and 3 *M* HNO_3 solution anhydrous tin(IV) chloride was added until the ratio P:Sn was 30. Although the precipitation was complete in 5–7 h, refluxing was continued until no further modification in the X-ray diffraction patterns of this material was observed ($\sim$ 100 h).

The product was then filtered, washed with distilled water until reacting about pH 4, and dried over P_4O_{10} under vacuum to constant weight. Tin(IV) arsenate was prepared by a similar procedure. In this case arsenic acid was 6 *M* while the As:Sn ratio was 10.

Composition, thermal and chemical stability of tin(IV) phosphate and arsenate

Chemical analysis of tin(IV) phosphate and tin(IV) arsenate gave a P:Sn and As:Sn ratio of 2.05 and 1.97, respectively. Weight loss curves for the H^+ form of

TABLE I

d-VALUES (A) FROM X-RAY POWDER PATTERNS OF CRYSTALLINE TIN(IV) PHOSPHATE IN H^+, Li^+, Na^+ AND K^+ FORMS DRIED OVER P_4O_{10}

H^+ *form* (*a*)	Li^+ *form* (*b*)	Na^+ *form* (*c*)	K^+ *form* (*d*)
7.76 s	7.76 m	8.13 m	9.13 m
4.21 m	4.39 vw	4.20 m	4.29 vw
3.46 vs	4.22 w	3.72 w	3.90 m
3.16 w	3.46 m	3.51 w	3.43 vw
2.64 w	3.18 vw	2.51 m	3.14 m
2.54 m	2.65 vw	1.86 vw	2.48 m
2.41 vw	2.56 vw	1.64 vw	2.09 vw
2.36 vw	2.41 vw		1.95 vw
2.09 w	2.12 vw		
2.01 w	2.02 vw		
1.95 w	1.53 vw		
1.91 w			
1.80 vw			
1.73 w			
1.62 w			
1.56 m			
1.43 w			

these materials are reported in Fig. 1, while their X-ray powder patterns are listed in Table I(a) and Table II(a).

Since tin(IV) phosphate and arsenate behave in a similar way, this paper is concerned chiefly with tin(IV) phosphate. X-ray diffraction patterns of this material heated to 800° were found to correspond to that reported on A.S.T.M. cards and referred to SnP_2O_7 (ref. 14). Thus the total weight loss can be related to elimination

TABLE II

d-VALUES (A) FROM X-RAY POWDER PATTERNS OF CRYSTALLINE TIN(IV) ARSENATE IN H^+, Li^+ AND Na^+ FORMS DRIED OVER P_4O_{10}

H^+ *form* (*a*)	Li^+ *form* (*b*)	Na^+ *form* (*c*)
7.77 m	9.16 w	8.50 m
4.31 m	7.77 m	4.33 m
4.10 vw	4.33 w	3.83 m
3.90 vw	3.68 m	3.59 m
3.51 vs	3.55 w	2.91 vw
3.20 vw	2.75 w	2.75 w
2.67 w	2.65 w	2.66 w
2.53 m	2.54 vw	2.53 w
2.43 w	2.43 vw	2.07 vw
2.18 vw		1.90 vw
2.06 w		
1.99 vw		
1.92 vw		
1.84 vw		
1.76 vw		
1.65 w		
1.60 vw		
1.47 m		
1.44 w		

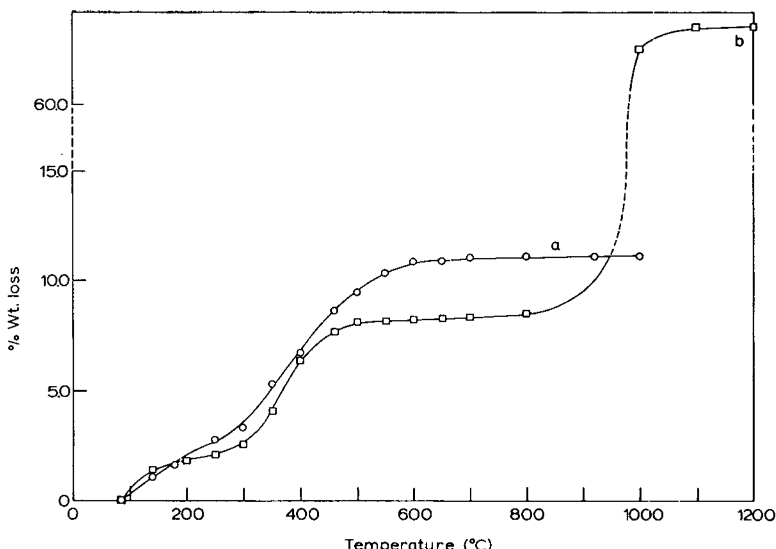

Fig. 1. Per cent weight loss of crystalline tin(IV) phosphate (curve a) and tin(IV) arsenate (curve b) at different temperatures.

of water and, from considerations similar to those reported in previous papers of this series[7,8], the empirical formula $SnO_2 \cdot P_2O_5 \cdot 2H_2O$ was ascribed to tin(IV) phosphate dried over P_4O_{10} under vacuum*.

Since one mole of water must be related to the phosphate condensation, the dehydration process can be represented by the following two possibilities:

$$\left.\begin{array}{l} SnO(H_2PO_4)_2 \searrow \\ Sn(HPO_4)_2 \cdot H_2O \nearrow \end{array}\right. Sn(HPO_4)_2 + H_2O\uparrow \rightarrow SnP_2O_7 + 2H_2O\uparrow$$

However, contrary to other crystalline exchangers of this series, the weight loss curve does not distinctly show the two dehydration steps, and this fact could be explained by assuming that the two processes overlap.

To corroborate this hypothesis, the pyrophosphate content in a tin(IV) phosphate sample heated to 350°, where the first mole of water is lost, has been determined. It was found that 31% of the phosphate groups was converted to pyrophosphate; this indicates that the condensation begins before the first water molecule is completely lost. Further it was found that tin(IV) phosphate first heated at 350° and then suspended in water for 5 days again takes up only 0.65 moles of water per formula weight. Thus the water not readsorbed (0.35 moles) is in good agreement with that calculated

* It is interesting to note that tin(IV) phosphate on standing in a desiccator until constant weight first over a NaCl satd. solution (~75% rel. humidity at room temperature) and then over a $Na_2HPO_4 \cdot 12H_2O$ satd. solution (~95% rel. humidity at room temperature) absorbs, respectively, 0.5 and 1.2 moles of water without appreciable modification in its X-ray powder patterns.

using the pyrophosphate content. Unfortunately it was not possible to determine at which temperature the first dehydration process is complete*. However this temperature being higher than 350°, the water held in the exchanger can be assumed to be constitution water.

In tin(IV) arsenate the sharp weight loss observed between 800° and 1000° must be related to loss of arsenic pentoxide, since the material heated to 1200° was found to correspond to SnO_2. From considerations similar to those made for tin(IV) phosphate, it was possible to represent the dehydration process by two possibilities:

$$\left.\begin{matrix} SnO(H_2AsO_4)_2 \searrow \\ Sn(HAsO_4)_2 \cdot H_2O \nearrow \end{matrix}\right. Sn(HAsO_4)_2 + H_2O\uparrow \rightarrow SnO_2 \cdot As_2O_5 + 2H_2O\uparrow \rightarrow SnO_2 + As_2O_5\uparrow + 2H_2O\uparrow$$

Chemical stability of tin(IV) phosphate and arsenate was evaluated by equilibrating 100 mg of sample with 50 ml of 8 *N* and 12 *N* HCl, 8 *N* H_2SO_4, 8 *N* HNO_3, and 8 *N* HF solutions at room temperature for one week. Phosphate ions (< 0.01 mmoles of phosphate or arsenate per gram of exchanger) were not found in the supernatant liquid. Qualitative tests have shown that the materials are not soluble in concentrated mineral acids also at boiling temperature. These exchangers are not very stable in an alkaline medium and even are soluble in alkaline solutions at concentrations higher than 1 *M*.

It can be noted that the stannic phosphate prepared according to INOUE[3] is

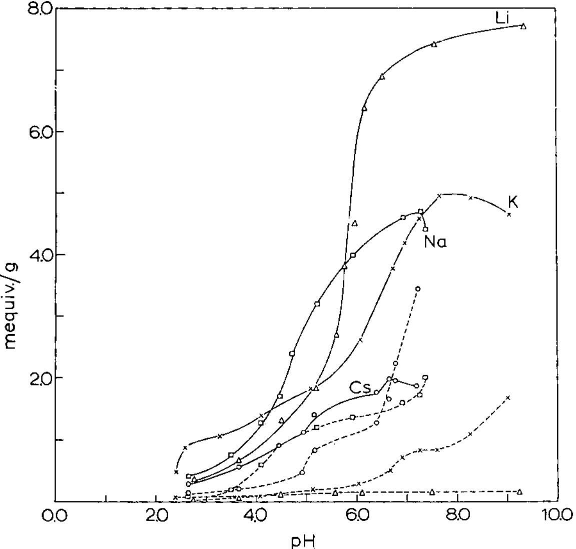

Fig. 2. Ion uptake and hydrolysis curves for crystalline tin(IV) phosphate titrated with 0.1 *N* (MCl + MOH). Ordinates: solid lines, ion uptake (mequiv./g of tin(IV) phosphate dried over P_4O_{10}); dashed lines, mmoles of phosphate released to solution by 1 g of material.

* At temperatures higher than 350°, the sample was not completely soluble in hot NaOH, and therefore pyrophosphate could not be determined.

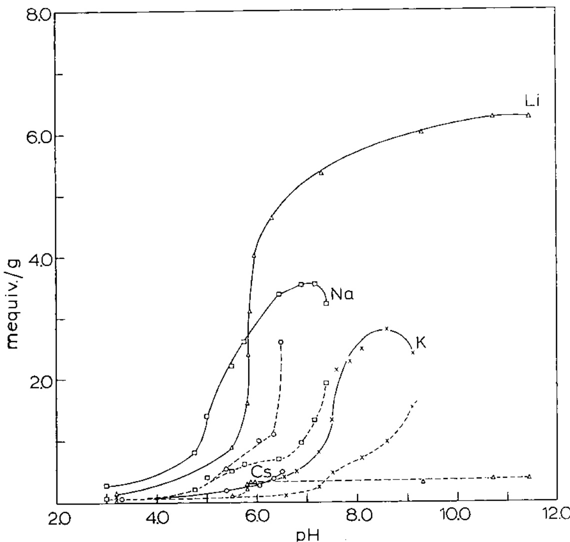

Fig. 3. Ion uptake and hydrolysis curves for crystalline tin(IV) arsenate titrated with 0.1 N (MCl + MOH). Ordinates: solid lines, ion uptake (mequiv./g of tin(IV) arsenate dried over P_4O_{10}); dashed lines; mmoles of phosphate released to solution by 1 g of material.

soluble in 6 M HCl and dissolves or peptises in a 0.1 N NaOH solution. Thus the crystallisation process increases the chemical stability of the products.

Ion exchange properties

Uptake curves for Li^+, Na^+, K^+ and Cs^+ (mequiv./g) and phosphate or arsenate (mmoles/g) released to the external solution as a function of pH are plotted in Figs. 2 and 3 for tin(IV) phosphate and tin(IV) arsenate, respectively.

From these figures the following can be noted. (a) The shape of uptake curves depends on the nature of the exchanging ion and inversions in the selectivity order can be observed. At low salt conversion the selectivity sequence for tin(IV) phosphate is $K^+ > Na^+ > Li^+ > Cs^+$ but increasing the equilibrium pH the sequence becomes $Na^+ > K^+ > Li^+ > Cs^+$, then $Na^+ > Li^+ > K^+ > Cs^+$ and finally $Li^+ > Na^+ > K^+ > Cs^+$. In tin(IV) arsenate we have only the inversion Na^+–Li^+ while K^+ and Cs^+ are excluded in an acid medium. This behaviour, already observed in other exchangers of this class, can be related to steric effects and is difficult to explain without knowledge of the structure of the materials. In any case, the exclusion effects are more evident in tin arsenate than in tin phosphate, and this fact, probably due to the larger size of arsenate groups, was also found from the comparison of ion-exchange properties of phosphate and arsenate of zirconium, titanium and cerium(IV) (refs. 7–11).

(b) Both exchangers are extensively hydrolysed when titrated with Na^+, K^+ and Cs^+ also in neutral or weakly alkaline solutions. Furthermore above a certain pH value the apparent capacity can even decrease, as already found for titanium phosphate[7] and titanium arsenate[11], and this can be related to the fact that the uptake of ions is less than the amount lost on hydrolysis of the exchanger.

In the case of Li^+ uptake, the exchangers are not appreciably hydrolysed, and

it seems possible to relate the extent of hydrolysis not only to an alkaline medium but also to the mechanical stresses involved in the exchange process with ions of large ionic radius. In this regard, it is interesting to note that exchangers peptise when titrated with KOH and especially with CsOH.

(c) The experimental value of the ion-exchange capacity is markedly influenced by the nature of the exchanging ion. An approximate value of the total ion-exchange capacity can be calculated by adding the alkali metal ions exchanged (mequiv./g) to the phosphate or arsenate released to the external solution (mmoles/g)*.

For the exchange Na^+–H^+ and K^+–H^+ in tin(IV) phosphate and for the exchange Na^+–H^+ in tin(IV) arsenate this calculation brings a value close to the theoretical capacity calculated assuming two exchangeable hydrogen equivalents per formula weight of the two materials (6.08 mequiv./g and 4.80 mequiv./g, respectively). For Cs^+ in tin(IV) phosphate and K^+ and Cs^+ in tin(IV) arsenate, the value of the exchange capacity is much lower than the theoretical values, showing the screening effects of these materials for ions of large ionic radius. On the other hand, the total ion-exchange capacity for Li^+ is higher than the theoretical one (7.9 and 6.6 mequiv./g, respectively). This experimental result can be accounted for by assuming the partial precipitation of trilithium phosphate (or arsenate) inside the exchangers or the presence of more than two exchangeable hydrogen equivalents per formula weight of exchanger.

The increase in ion-exchange capacity due to trilithium phosphate (or arsenate) precipitation cannot be excluded but is unlikely. This phenomenon was never observed in other crystalline ion exchangers of this class, such as titanium phosphate[7] and arsenate[11] which are more hydrolysable than tin(IV) phosphate (or arsenate). Moreover the shape of the uptake curves does not show any inflexion, while, in the case of trilithium phosphate (or arsenate) precipitation, a sharp increase in Li^+ uptake should be observed[9]. Thus the high capacity for Li^+ could be related to the presence of more than two exchangeable hydrogen ions inside the exchangers.

Taking into account the dehydration process of both exchangers and the fact that tin(IV) phosphate heated to 350° partially reabsorbs its water (see above), it seems possible to account for the high Li^+ uptake with the existence of the following tautomeric equilibrium, already suggested by MOUNIER AND WINAND[15] for zirconium phosphate.

$$\begin{array}{ccc} HPO_4 & & H_2PO_4 \\ \| & & | \\ Sn \leftarrow H_2O & \rightleftarrows & Sn-OH \\ \| & & \| \\ HPO_4 & & HPO_4 \end{array}$$

The existence of this equilibrium seems more probable for tin(IV) phosphate (or arsenate) than for zirconium phosphate, but much more data must be collected to confirm this.

X-ray analyses of tin(IV) phosphate and arsenate at various degrees of salt conversion were also carried out. Except in the case of a Li^+–H^+ exchange, the conversion in the salt form causes a strong degradation in the degree of crystallinity and in the case of Cs^+ in tin phosphate and K^+ and Cs^+ in tin arsenate, the structure is

* It was assumed that there is only one mequiv. of exchangeable H^+ per mmole of phosphate or arsenate.

almost completely destroyed. In Table I (*b*, *c*, *d*) and Table II (*b*, *c*) are listed the X-ray powder patterns of the same salt forms of the exchanger, obtained by titration. While X-ray patterns of the Li^+ forms are not appreciably changed, compared with the H^+ forms, in the other salt forms the first *d*-value increases, increasing the ionic radius of the exchanging ion. This fact was already found in other crystalline exchangers of this series[7,10].

CONCLUSION

Crystalline tin(IV) phosphate and tin(IV) arsenate compare favourably with amorphous products, but they show low resistence to hydrolysis when titrated with ions having a crystalline radius larger than that of lithium. While in some respect their behaviour is like that of other crystalline ion exchangers of this series, tin(IV) phosphate and arsenate show some peculiar features in the dehydration and Li^+–H^+ exchange processes. More data are thus necessary to establish the formulae of these materials and to characterise their ion-exchange properties.

ACKNOWLEDGEMENTS

The authors wish to thank Prof. G. ALBERTI for his valuable guidance and for many discussions and helpful suggestions.

This work has been supported by the Istituto di Ricerca sulle Acque of the Consiglio Nazionale delle Ricerche.

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Notes

CHROM. 4819

Separation of mono- and dibasic fatty acids by gas chromatography

The separation and quantitative determination of complex mixtures of mono- and dicarboxylic acids by gas chromatography (GC) is not simple when the carbon number of the components varies over a large range and when branched acids and straight-chain acids are present together.

Many GC procedures have been reported in the literature for a single class of acids or for mixtures having a limited number of components. GC separation of the free fatty acids or of their heavier esters (butylesters) is preferred when low carbon number components are present, while a separation as their methyl esters is chosen with acids heavier than C_8–C_{10} (refs. 1–3).

APPLEBY AND MAYNE[4] have recently suggested the separation of C_4–C_{20} fatty acids and C_3–C_{12} dicarboxylic acids as their *n*-propyl esters, using 3% polyethylene glycol or 3% Embaphase silicone oil on 100–120 mesh Celite as the stationary phase.

In our present work, it was necessary to analyze complex mixtures of oxidized

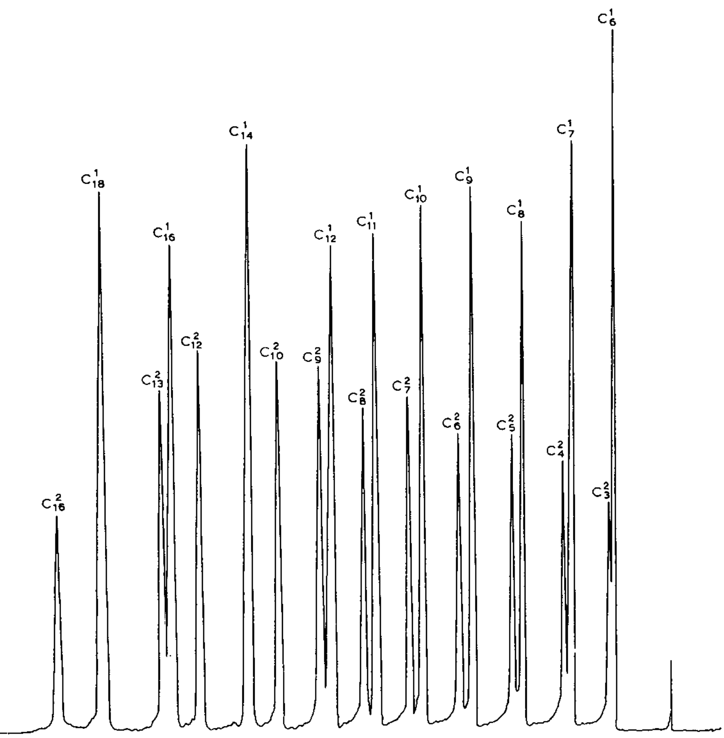

Fig. 1. Chromatogram of the calibration mixture of the methyl esters of the C_6–C_{18} monoacids and C_4–C_{16} diacids. See Table I for GC conditions.

TABLE I

CHROMATOGRAPHIC ANALYSIS CONDITIONS

Apparatus:	Perkin Elmer Model 900 gas chromatograph
Detector:	Flame ionization
Column:	4 ft. × 1/8 in., 5% SE-52 on Chromosorb G AW, 60–80 mesh
Temperature:	programmed 4°/min from 50° to 330°
Carrier gas:	helium 50 ml/min
Recorder:	5 mV span
Chart speed:	4 mm/min
Sample:	1 µl

products containing C_6–C_{27} monocarboxylic acids and C_4–C_{16} dicarboxylic acids. It was found that a GC procedure using 5% of silicone gum rubber, SE-52, on 60–80 mesh Chromosorb G AW as the stationary phase was a possible approach. This

TABLE II

RETENTION TIMES AND RESPONSE FACTORS, RELATIVE TO C^1_{10}, OF THE METHYL ESTERS OF MONO- AND DICARBOXYLIC ACIDS

Component		*Purity*[a]	*Retention time (min)*	*Response factor*	
				FID	*TCD*
C^1_6		p	2.8		0.90
	C^2_3	pp	3.6	4.55	1.06
C^1_7		99.9 p	4.9	1.17 ± 0.05	0.91
	C^2_4	p	5.3	2.69 ± 0.10	1.05
C^1_8		99.8 p	7.5	1.08 ± 0.02	1.00
	C^2_5	p	8.1	2.34 ± 0.10	1.07
C^1_9		p	10.6	1.06 ± 0.04	0.97
	C^2_6	99 p	11.4	1.86 ± 0.06	1.05
C^1_{10}		99.9 p	13.7	1.00	1.00
	C^2_7	p	14.6	1.74 ± 0.06	1.07
C^1_{11}		99.8 p	16.9	0.96 ± 0.01	1.01
	C^2_8	p	17.7	1.70 ± 0.05	1.09
C^1_{12}		99.7 p	19.8	0.96 ± 0.01	1.03
	C^2_9	pract.	20.7	(1.41 ± 0.10)	1.07
C^1_{13}		—	22.8	—	—
	C^2_{10}	—	23.5	1.40 ± 0.10	1.13
C^1_{14}		99.9 p	25.4	0.95 ± 0.02	1.05
	C^2_{11}	—	26.2	1.30	—
C^1_{15}		—	28.0	—	—
	C^2_{12}	p	28.8	1.20 ± 0.07	1.13
C^1_{16}		p	30.5	0.86 ± 0.02	1.03
	C^2_{13}	techn.	31.2	(1.48 ± 0.07)	1.28
C^1_{17}		—	32.7	—	—
	C^2_{14}	—	33.4	—	—
C^1_{18}		p	35.1	0.86 ± 0.02	1.08
	C^2_{15}	—	35.8	—	—
C^1_{19}		—	37.3	—	—
	C^2_{16}	—	38.3	1.17	—
C^1_{20}		—	39.3		

[a] p = pure; pp = very pure.

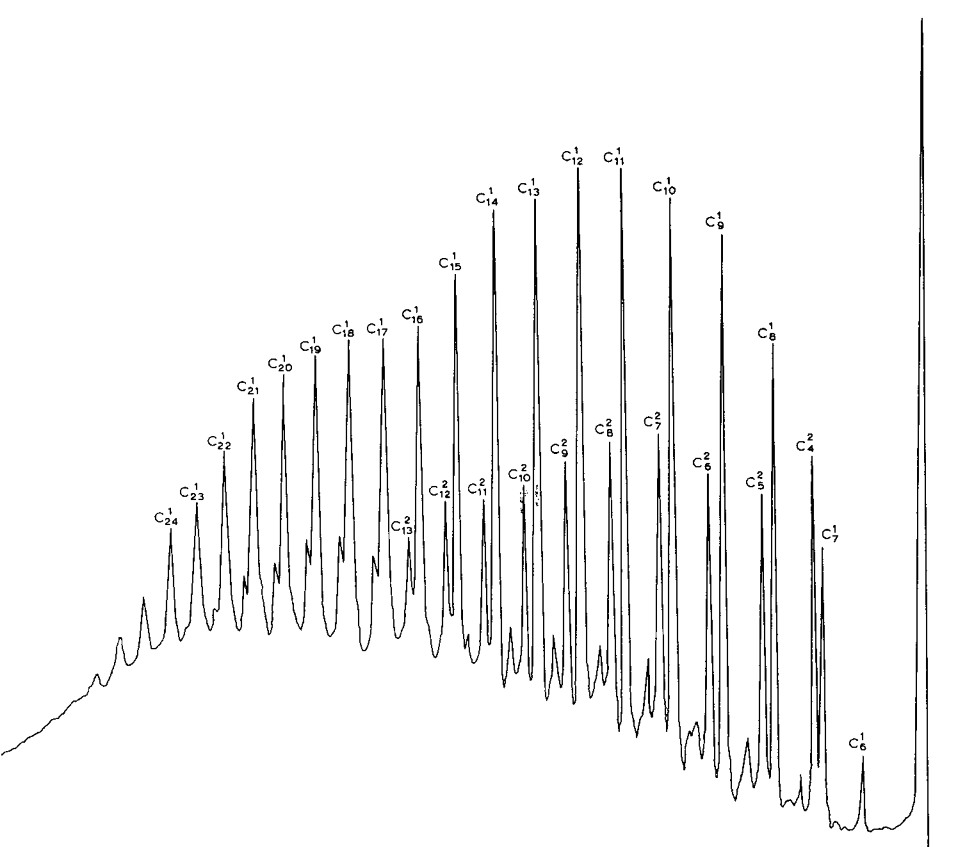

Fig. 2. Chromatogram of methylated acids separated from an oxidation product. See Table I for GC conditions.

stationary phase is stable at high temperatures and allows a final programmed temperature as high as 350°. Therefore a determination of the components with a carbon number higher than C_{20} is possible.

The gas chromatographic conditions used are given in Table I. Table II shows the retention times of the straight-chain mono- and dicarboxylic acids and the response factors for the flame ionization and for the thermal conductivity (filament) detectors (TCD).

A mixture of the methyl esters of C_6–C_{18} monoacids and C_4–C_{16} diacids was prepared from pure components supplied by Merck or Fluka and used for calibration purposes; a typical chromatogram of this mixture is given in Fig. 1.

Fig. 2 is a chromatogram of a mixture of acids separated from an oxidized product methylated according to the methanol–BF_3 method of METCALFE AND SCHMITZ[5]. This chromatogram shows a high background which is mainly due to the methyl esters of branched acids, above which the normal components emerge as well-defined peaks. Normal alkanes mixed with branched and cyclic hydrocarbons give a similar chromatogram when analyzed on the same column.

Some individual branched acids—if present in a comparatively large quantity—could contribute to the area of the *n*-acid peaks; furthermore, when the dicarboxylic ester content is 4–5 times less than that of the monocarboxylic esters, their determination is impossible as is the case of the C_{13} diacid in chromatogram 2, where its peak is overlapped by the C_{16} monoacid.

Flame ionization allows higher sensitivities than thermal conductivity but the relative FID response factors for low molecular weight components are rather high. This is because of the considerable influence of the carboxylic acid group in the lighter components as stated by ACKMAN AND SIPOS[6]. For this reason TCD is to be preferred for the analysis of low carbon number components when no sensitivity problems are foreseen.

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First received March 12th, 1970; revised manuscript received May 15th, 1970

CHROM. 4831

Gas-liquid chromatographic determination of dimethylnitrosamine as dimethylnitramine at picogram levels

In view of the extreme carcinogenicity of some of the nitrosamines in different animal species[1,2] and their reported occurrence in foodstuffs[3-6] considerable interest has developed, during the past few years, in chromatographic and other procedures for the detection of these compounds[7-13]. Polarographic[14], thin-layer (TLC) and gas-liquid chromatographic (GLC) methods have been used for their detection in foods with varying degree of success[7-13]. The polarographic method is only sensitive to 1 p.p.m. levels and it lacks specificity[12,13]. The TLC and GLC methods are more sensitive and specific than the polarographic technique but they require a large sample size (0.2-1 kg), and the clean-up steps are quite laborious and lengthy. These methods are unsuitable for analyzing small amounts of biological samples that are encountered during the metabolic studies of nitrosamines in laboratory animals. There is a need, therefore, for a more sensitive method of detecting these compounds.

An attempt to increase the sensitivity of the method by reducing the nitrosamines to hydrazines, prior to GLC, has proven unsuccessful[8]. The detection limit of the hydrazines was no better than that of the nitrosamines. During the course of our investigation we have observed that dimethylnitramine (DMNA), the oxidation product of dimethylnitrosamine (DMN), is extremely sensitive to electron capture detection, and this technique can be used to detect DMN at minute quantities. This communication reports the results of our findings.

Experimental

Reagents and solvents. All reagents were of analytical grade. Ethyl acetate, methylene chloride, *n*-pentane and *n*-hexane were distilled in all-glass apparatus.

Preparation of DMNA. Crystalline DMNA was prepared by nitrolysis of N,N-dimethylformamide according to the method of ROBSON[15]. The product was recrystallized from diethyl ether and *n*-pentane, and the final material had a m.p. of 52° (literature value, 54-56°). The IR spectrum (KBr pellet) showed peaks at 1470, 1437, 1385 and 1310 cm^{-1} indicating the presence of a nitro group. The compound was dissolved in ethyl acetate to give a concentration of 1 mg/ml and subsequent dilutions were carried out in *n*-pentane.

Conversion of DMN to DMNA. The method of EMMONS AND FERRIS[16] was used with minor modifications. About 1-5 μg of DMN in 2-10 μl of methylene chloride was added to 9 ml of trifluoroacetic acid and 50% hydrogen peroxide mixture (5:4) and the solution was allowed to stand at room temperature for 12-24 h. The mixture was poured on 10-15 g ice, made alkaline (pH 10-11) by careful addition of 30-40 ml 20% potassium carbonate, and extracted with two 50 ml portions of methylene chloride. The methylene chloride extract was dried over anhydrous sodium sulfate, filtered, and concentrated to *ca.* 5 ml by evaporating on a steam bath. The concentrated extract was quantitatively transferred into a glass-stoppered graduated test tube and 1 ml *n*-hexane was added. The solution was then concentrated to 0.2 ml on a hot water bath in a stream of nitrogen (care should be taken to avoid complete drying).

The solution was made up to 5–10 ml with *n*-pentane and a 1–4 μl aliquot was used for GLC analysis.

Isolation of DMN from nitrite-treated fish. The sample was prepared and extracted as described by us earlier[17]. The only difference was that all the concentration steps were carried out by evaporation through Snyder columns (macro and micro) instead of in a stream of nitrogen. The spot corresponding to DMN on the TLC plate was eluted with methylene chloride using a micro-Soxhlet apparatus. About 1 ml water was added to the extract and the mixture heated on a water bath (Snyder column) until all the methylene chloride was driven off. The aqueous solution was cooled to room temperature and the DMN present in the solution was converted to DMNA by the method described above. The preparation was passed through an alumina column (Woelm basic, cationotropic, activity grade 1; 2.5 cm $\times$ 1 cm diameter) and the interfering materials were removed by washing with 50 ml *n*-pentane. Finally, the adsorbed DMNA was eluted with 100 ml diethyl ether, the eluate concentrated to 0.5 ml, and a 0.2μl aliquot used for GLC analysis.

GLC analysis. A Varian Aerograph gas chromatograph, Model 1200, equipped with an electron capture detector (^{3}H) and a 1 mV recorder was used. Conditions:

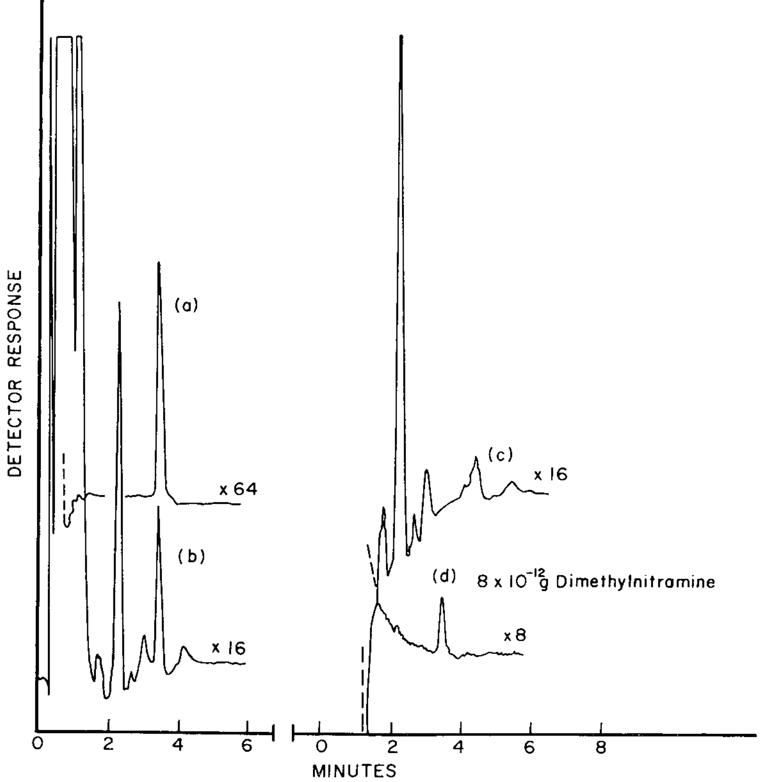

Fig. 1. GLC diagrams: (a) DMNA from nitrite-treated smoked hake (after clean-up through the alumina column), (b) DMNA prepared from DMN by pertrifluoroacetic acid oxidation, (c) pertrifluoroacetic acid oxidation blank and (d) DMNA prepared by nitrolysis of dimethylformamide. Range setting, 1; attenuator setting as shown in the diagrams. For other conditions see text.

10% Carbowax 20 M on 60–80 mesh chromosorb W (HMDS treated), 6 ft. × 1/8 in. stainless-steel column; column 152°, injector 225°, detector 210°, nitrogen flow 24 ml/min.

Results and discussion

The use of an electron capture detector made the GLC method very sensitive; about 8 pg of DMNA (or 16 pg DMN) could be detected under the conditions used (Fig. 1). This is about a thousand times more sensitive than the detection limit of nitrosamines by the hydrogen flame detector[12,17]. The peak heights were proportional to the amount of DMNA at least up to 100 pg (Fig. 2). A blank carried out through all the steps did not show the corresponding peak for DMNA (Fig. 1). The DMNA prepared by nitrolysis of dimethylformamide was undistinguishable from that prepared by pertrifluoroacetic acid oxidation of DMN.

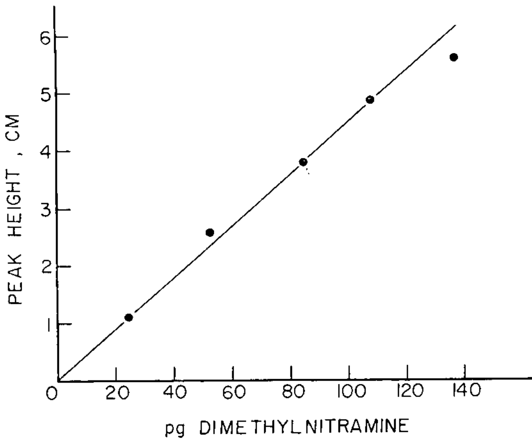

Fig. 2. Standard curve for DMNA.

The percentage conversion of DMN to DMNA varied with the conditions used but the results were reproducible. About 60% of the theoretical yield was obtained when the reaction mixture was allowed to stand for 24 h. An overnight reaction period at room temperature gave about 50% yield. The amount of DMNA formed from 5 μg DMN was proportional to that obtained from a 1 μg sample. The use of 90% hydrogen peroxide would be expected to produce a higher yield[16]. Due to relative unavailability of 90% hydrogen peroxide and the hazard involved in its use no attempt was made to use the concentrated reagent.

Thus far, we have shown that DMN can be oxidized to DMNA on a micro scale and detected by GLC at picogram levels. A great deal of work remains to be carried out before it can be directly applied to food extracts. Proper clean-up procedures need to be developed and applied to different foods. However, the technique, as it stands now, can be used to confirm the identity of nitrosamines isolated from foods by the existing procedures. To demonstrate this, we isolated DMN from a sample of nitrite-

treated smoked hake which was found to contain DMN in our previous studies[17]. A strong peak corresponding to DMNA (Fig. 1) thus confirms the identity of DMN.

Although we have only used the technique for the determination of DMN, it is anticipated that other volatile nitrosamines could be similarly converted to the nitramines and detected by GLC. It is hoped that the extreme sensitivity of the technique will be useful for studying the metabolism of various nitrosamines as well as for detecting their presence in the environment.

The author wishes to thank Mrs. BARBARA DONALDSON for technical assistance and Mr. R. C. O'BRIEN for taking the infrared spectrum.

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Received May 4th, 1970

J. Chromatog., 51 (1970) 301–304

CHROM. 4846

Separation of carcinogenic nitrosamines on Sephadex LH-20

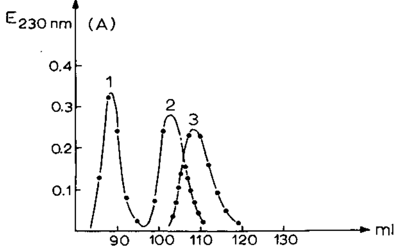
Chromatographic separations are well-established methods for the analysis of N-nitroso compounds. Thin-layer chromatography on silica gel or alumina[1] and a variety of gas chromatographic methods have been successfully utilised for the separation and identification of these substances[2]. During our investigations of suitable methods for trace analysis of N-nitroso compounds[3–5] we examined whether gel chromatography could be employed as a separation technique for nitrosamines and possibly also for the removal of interfering contaminants.

Materials and methods

All nitrosamines investigated were synthesised in our laboratory and purified by known methods; their purity was established by a gas chromatographic examination. Fifty percent aqueous methanol, analytical grade, was the solvent system of choice even for highly lipophilic N-nitroso compounds such as di-*n*-hexylnitrosamine.

A wheat flour concentrate was prepared by extraction of 1000 g of commercial wheat flour with dichloromethane in a Soxhlet for 12 h. The solvent was evaporated (rotatory) and the residue was partitioned in *n*-heptane–acetonitrile[3]. The acetonitrile phase was evaporated together with 100 g of alumina (neutral). The alumina with the adsorbed residue was filled in a column and eluted with 50% aqueous methanol; 50 ml were collected.

Sephadex LH-20 was allowed to swell for 12 h in the solvent and was then packed into a 1 × 100 cm column. The gel was washed with the solvent until the eluate showed no absorption in UV light at 230 nm. To prevent photolytic cleavage



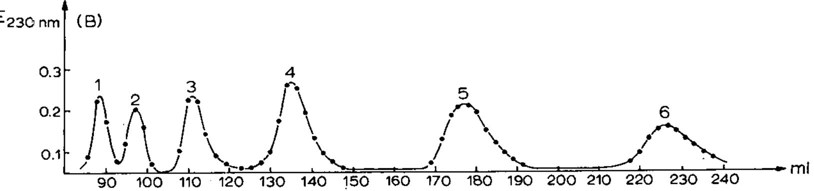


Fig. 1. Gel chromatography on Sephadex LH-20. Column, Sephadex LH-20, 100 × 100 mm; eluent, methanol–water (1 : 1), 4.3 ml/h; sample volume, 0.5 ml. (A) 1 = 19.4 µg methylethylnitrosamine; 2 = 26.4 µg methylbutylnitrosamine; 3 = 25.3 µg methylpentylnitrosamine. (B) 1 = 10.5 µg dimethylnitrosamine; 2 = 14 µg diethylnitrosamine; 3 = 21.5 µg di-*n*-propylnitrosamine; 4 = 50 µg di-*n*-butylnitrosamine; 5 = 74 µg di-*n*-pentylnitrosamine; 6 = 52 µg di-*n*-hexylnitrosamine.

of nitrosamines during chromatography, the column was screened from direct light with black paper. The compounds were applied in a sample volume of 0.5 ml, elution was performed at a rate of 4.3 ml/h and fractions of 2 or 3 ml were collected. Nitrosamine concentrations in the fractions were determined by UV spectroscopy at 230–235 nm, using a Zeiss DMR 21 recording spectrophotometer.

Results and discussion

As can be seen from Fig. 1, gel chromatography on Sephadex LH-20 resulted in an efficient separation of all six symmetrical di-*n*-alkylnitrosamines under the con-

ditions described above. Dimethylnitrosamine was eluted first, followed by diethylnitrosamine, di-*n*-propylnitrosamine and the higher homologues.

The elution sequence of the methylalkylnitrosamine homologues follows the same principle; thus methylethylnitrosamine is eluted before methylbutylnitrosamine which is followed by methylpentylnitrosamine. A similar separation as that shown in Fig. 1 was obtained with the application of 140–270 μg of symmetrical dialkylnitrosamines using a 2 × 100 cm column.

Whilst dialkylnitrosamine homologues which differ in two methylene groups were invariably well separated, the separation of nitrosamines with a difference of only one methylene group was incomplete (methylbutyl- from methylpentylnitrosamine).

The above results indicate that the separation is not based upon a gel filtration process. Apparently the elution behaviour of symmetrical and unsymmetrical nitrosamines under these conditions is determined primarily by absorption effects. The affinity of a given nitrosamine to the Sephadex LH-20 matrix increases with its lipophilic character and can be correlated with the distribution coefficient of the compounds in the system *n*-hexane–aqueous buffer (ref. 6, p. 115).

The usefulness of gel chromatography for the separation of micro-amounts of nitrosamines from biological materials was also investigated. A similar mixture of nitrosamines was dissolved in a 2-ml aliquot of the wheat flour concentrate and applied on the 2 × 100 cm column. Over the whole elution range in which nitrosamines were to be expected, UV-absorbing contaminants from the extract prevented the determination of the substances in the eluate by UV spectroscopy.

Although mixtures of pure nitrosamines can be successfully separated by gel chromatography on Sephadex LH-20, the method as described cannot be used for the separation of nitrosamines from interfering biological contaminants.

This investigation was carried out under contract with the International Agency for Research on Cancer.

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Received May 26th, 1970

CHROM. 4811

The effect of temperature on the interaction of phenols with Sephadex gels

In gel chromatography, mixtures of substances are separated according to their molecular sizes. The mixture is eluted through a column of the gel and, since the structure of the gel is a three-dimensional network of meshes, the molecules of largest size are eluted first, as they are least able to diffuse into the pores of the gel. Separations of substances on this principle would not be expected to be dependent on temperature, and, indeed, it has been convincingly shown that elution volumes are unaffected by changes of temperature[1].

However, certain substances are strongly adsorbed by gels, and, in such cases, it is probable that elution volumes will vary with temperature. Many aromatic and heterocyclic compounds are adsorbed by cross-linked dextran[2] and other gels. Mono-substituted phenols interact particularly strongly with Sephadex G-10, a dextran gel which has a high degree of cross-linking[3]. The phenol is adsorbed onto the glyceryl cross-links[4], and it was suggested that the hydroxyl group of the phenol interacts with the cross-links through hydrogen bonds[3]. The interaction can be represented by the equilibrium shown in eqn. (1),

$$\mathrm{P} + \mathrm{D} \overset{K_D}{\rightleftharpoons} \mathrm{P{-}D} \qquad (1)$$

where P is the substituted phenol, D is the dextran gel, and P–D the phenol–dextran gel hydrogen-bonded complex. The equilibrium constant is obtained from eqn. (2),

$$K_D = \frac{V_e - V_0}{V_i} \qquad (2)$$

where V_e is the elution volume of the phenol, V_0 the void volume of the column, and V_i the internal aqueous volume of the gel. For normal gel filtration, K_D lies between 0 and 1. When a substance is adsorbed by the gel, K_D is greater than 1.

Equilibrium constants, and hence elution volumes, for reactions of the type shown in eqn. (1) usually depend on temperature. Consequently, the elution volumes of a series of monosubstituted phenols from columns of Sephadex G-10 were measured at various temperatures. In addition, an investigation of the temperature dependence of K_D should lead to values for the free energy (ΔG), enthalpy (ΔH) and entropy (ΔS) by using eqns. (3), (4) and (5).

$$\Delta G = -RT \log_e K_D \qquad (3)$$

$$\mathrm{Log}_e K_D = -\frac{\Delta H}{RT} + I \qquad (4)$$

where I is a constant, and

$$\Delta G = H - T\Delta S \qquad (5)$$

Materials and methods

The column, of diameter 1.5 cm, was packed with Sephadex G-10 gel to a height of 11.5 cm as described by DETERMANN[1]. The column was surrounded by a

water-jacket through which water at constant temperature was passed. The temperature of the column was constant to ± 0.1°C.

Samples, 0.5 ml of eluent containing 1 to 5 mg of the substituted phenol and 0.1 mg of Blue Dextran 2000 as internal standard, were introduced onto the column with a hypodermic syringe. The eluent was acetate buffer of pH 4.0.

The effluent from the column was continuously monitored with an L.K.B. Uvicord I flow analyser which measured absorbance at 254 nm. The flow analyser was connected to a Leeds and Northrup "Speedomax H" strip-chart recorder. Elution volumes (V_e) were measured from the recorder chart.

TABLE I

THE VARIATION OF K_D WITH TEMPERATURE OF SUBSTITUTED PHENOLS

Phenol	*K_D value*			
	5° C	*15° C*	*25° C*	*35° C*
Phenol	10.1	9.6	9.1	8.4
m-Fluorophenol	16.5	15.7	14.7	13.5
p-Chlorophenol	36.2	33.4	30.3	26.7
p-Bromophenol	55.0	49.9	45.3	38.7
p-Iodophenol	101.8	89.2	75.6	64.0
p-tert.-Butylphenol	23.9	24.0	24.5	25.1

Results and discussion

The variation of K_D with temperature of a series of substituted phenols is shown in Table I. It was assumed in the calculation of K_D that the internal volume of the gel (V_i) was independent of temperature.

As expected, there was a large variation of K_D with temperature over the range investigated, particularly in the case of the halogen-substituted phenols. Hence, temperature programming should prove to be useful for the separation of mixtures of phenols.

However, *p-tert.*-butylphenol is a notable exception. Although it is strongly adsorbed by the gel, K_D is virtually independent of temperature. This implies that the mechanism of adsorption of *p-tert.*-butylphenol is different from the other substituted phenols. The adsorption effect cannot therefore be due to hydrogen-bonding of the phenolic hydroxyl group to the cross-linking of the gel. It is possible that the interaction of *p-tert.*-butylphenol with the gel is caused by steric effects associated with the *p-tert.*-butyl group. The side chain may be a particularly good fit in the pores of the gel. Alternatively, alkyl groups may have some specific interaction with the gel, since MARSDEN[5] has shown that for a series of alcohols, K_D increases with the length of the alkyl chain. A similar effect may be operating in the case of *p-tert.*-butylphenol.

A plot of log K_D for the substituted phenols against the reciprocal of absolute temperature is shown in Fig. 1. The curves are approximately linear and the slopes of the lines, calculated by a least-squares treatment, gave the enthalpies (ΔH) for the reaction between the substituted phenols and the dextran gel (eqn. 1). The thermodynamic parameters at 25°C are shown in Table II.

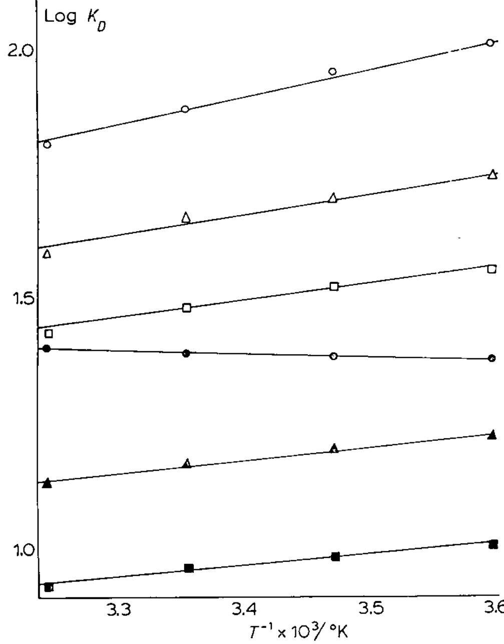

Fig. 1. A plot of log K_D against the reciprocal of absolute temperature for a series of substituted phenols. ■, phenol; ▲, *m*-fluorophenol; ●, *p*-*tert*.-butylphenol; □, *p*-chlorophenol; △, *p*-bromophenol; ○, *p*-iodophenol.

The value of ΔH should give some idea of the strength of the bond between the phenol and the dextran gel. However, this will not be an exact measure of bond dissociation energy of the complex as solvation effects cannot be taken into account since ΔH is the sum of all bonds formed and all bonds broken. In addition to the formation of the bond of the phenol–dextran gel complex, bonds will be broken between the phenol and its solvation sheath, and between the dextran gel and solvent hydrogen-bonded to the cross-linking.

For phenol, values of ΔH are in reasonable agreement with the suggestion that the phenol is hydrogen-bonded to the dextran gel. However, for the formation of such a complex, ΔS would be expected to be large and negative. In fact, except

TABLE II

THERMODYNAMIC PARAMETERS FOR THE ADSORPTION OF A SERIES OF SUBSTITUTED PHENOLS ON SEPHADEX G-10 AT 25° C

Phenol	$-\Delta G$ *(kJmol⁻¹)*	$-\Delta H$ *(kJmol⁻¹)*	ΔS *(Jmol⁻¹deg⁻¹)*
Phenol	5.4	4.3	3.7
m-Fluorophenol	6.6	4.9	5.8
p-Chlorophenol	8.4	7.1	4.4
p-Bromophenol	9.4	8.2	3.9
p-Iodophenol	10.7	11.1	−1.3
p-*tert*.-Butylphenol	7.9	−1.2	30.9

for *p*-iodophenol and *p-tert.*-butylphenol, ΔS is small and positive. This must be due to desolvation of the phenol and gel.

Again, *p-tert.*-butylphenol is an exception. The decrease in free energy is entirely associated with an increase in entropy, which suggests that the adsorption of *p-tert.*-butylphenol is controlled by some steric process.

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Received May 4th, 1970

J. Chromatog., 50 (1970) 307–310

CHROM. 4818

Tetrazole analogues of amino acids and peptides

II. Paper and thin-layer chromatography of tetrazole analogues of amino acids

Like amino acids (I), tetrazole analogues of amino acids (II)[1-3]—the compounds in which the carboxyl group is replaced by a 5-tetrazolyl group—exist in a zwitterion form.

$H_3\overset{+}{N}$—CHR—C(O)(O)$^-$ (I) $H_3\overset{+}{N}$—CHR—C(N=N)(N=N)$^{\ominus}$ (tetrazolyl anion) (II)

A number of tetrazole analogues of amino acids were prepared and characterised in the first paper[3] of this series. As a further part of the study the chromatographic behaviour of these compounds was investigated, and the sensitivity of the reaction with ninhydrin was determined.

The physical and chemical properties of tetrazole analogues of amino acids are very similar to the properties of amino acids. They are soluble in water, alkalis, aqueous acids, and sparingly soluble or insoluble in organic solvents. All tetrazole analogues of amino acids have high melting points, and all decompose at the melting temperatures. The dissociation constants of the tetrazole analogues of the amino acids are comparable to the respective constants for the amino acids.

We propose that the tetrazole analogues of amino acids, as a group, should be

called aminoalkyltetrazoles and abbreviated to AT. In order to emphasise the analogy between a particular aminoalkyltetrazole and its corresponding amino acid we suggest that the common name of the amino acid followed by the word tetrazole is used. For example, the tetrazole analogue of alanine would be called "alaninetetrazole", and for shorthand notation written as AlaT.

Experimental

Three adsorbents were used: (A) Whatman paper No. 1; (B) pre-coated cellulose TLC plates (Merck DC Fertigplatten Cellulose F); and (C) silica gel TLC plates (Merck Kieselgel G).

Silica gel TLC plates were prepared by the following method: 6 g Silica Gel G were shaken vigorously in an erlenmeyer flask with 12 ml of distilled water for about 2 min. The slurry was spread evenly on a dry plate (20 × 20 cm) and then the plate was dried at room temperature for about 24 h.

The solvent systems used were: S_1, methanol–water (7:3)[4]; S_2, *n*-propanol–water (4:1)[5]; S_3, *n*-butanol–acetic acid–water (4:1:1)[6,7]; S_4, pyridine–isoamyl alcohol–

TABLE I[a]

CHROMATOGRAPHIC DATA FOR AMINO ACIDS AND THEIR TETRAZOLE ANALOGUES

Solvents: S_1, methanol–water (7 : 3); S_2, *n*-propanol–water (4 : 1); S_3, *n*-butanol–acetic acid–water (4 : 1 : 1); S_4, pyridine–isoamyl alcohol–water (7 : 7 : 6); S_5, phenol–water (3 : 1, w/w). Adsorbents: A, Whatman paper No. 1. B, cellulose TLC plates (Merck DC – Fertigplatten Cellulose F); C, silica gel TLC plates (Merck Kieselgel G). Technique: ascending; length of development: 24 cm (Whatman paper) or 10 cm (TLC plates).

No.	Amino acid	Tetrazole analogue of amino acid	R_F values in solvent systems														
			S_1			S_2			S_3			S_4			S_5	
			Adsorbent			Adsorbent			Adsorbent			Adsorbent			Adsorbent	
			A	B	C	A	B	C	A	B	C	A	B	C	A	B
1	Gly		0.55	0.52	0.46	0.11	0.15	0.25	0.09	0.11	0.22	0.16	0.13	0.20	0.38	0.30
2		GlyT	0.57	0.54	0.56	0.18	0.23	0.47	0.14	0.13	0.39	0.27	0.25	0.36	0.40	0.33
3	β-Ala		0.64	0.62	0.38	0.16	0.20	0.17	0.19	0.22	0.31	0.16	0.14	0.21	0.64	0.55
4		β-AlaT	0.64	0.61	0.50	0.25	0.29	0.38	0.19	0.19	0.40	0.27	0.26	0.34	0.60	0.55
5	Ala		0.71	0.71	0.50	0.21	0.27	0.32	0.20	0.22	0.30	0.21	0.19	0.28	0.55	0.49
6		AlaT	0.72	0.73	0.58	0.30	0.38	0.53	0.27	0.28	0.47	0.34	0.33	0.47	0.57	0.50
7	Pro		0.72	0.71	0.38	0.27	0.30	0.17	0.25	0.26	0.19	0.23	0.22	0.17	0.88	0.89
8		ProT	0.73	0.71	0.49	0.34	0.40	0.33	0.31	0.31	0.33	0.36	0.35	0.29	0.87	0.87
9	Abut		0.76	0.78	0.52	0.31	0.37	0.39	0.32	0.32	0.35	0.26	0.26	0.33	0.67	0.63
10		AbutT	0.78	0.80	0.59	0.44	0.54	0.62	0.45	0.42	0.55	0.44	0.43	0.50	0.69	0.63
11	Val		0.78	0.80	0.53	0.42	0.46	0.40	0.46	0.44	0.42	0.34	0.32	0.34	0.73	0.71
12		ValT	0.80	0.81	0.59	0.54	0.62	0.63	0.55	0.54	0.63	0.52	0.47	0.58	0.74	0.69
13	Nva		0.78	0.81	0.54	0.46	0.51	0.40	0.49	0.51	0.48	0.36	0.37	0.36	0.77	0.75
14		NvaT	0.79	0.83	0.59	0.61	0.66	0.62	0.59	0.63	0.60	0.54	0.52	0.58	0.76	0.73
15	Ile		0.80	0.85	0.55	0.55	0.60	0.43	0.58	0.61	0.50	0.43	0.43	0.48	0.80	0.80
16		IleT	0.81	0.86	0.60	0.66	0.72	0.66	0.65	0.70	0.65	0.60	0.59	0.60	0.79	0.78
17	Phe		0.72	0.72	0.58	0.49	0.54	0.55	0.53	0.51	0.59	0.49	0.47	0.52	0.87	0.83
18		PheT	0.74	0.75	0.63	0.61	0.68	0.71	0.62	0.62	0.70	0.64	0.63	0.58	0.81	0.79
19	Leu		0.81	0.82	0.55	0.55	0.63	0.54	0.61	0.60	0.61	0.48	0.44	0.49	0.82	0.81
20		LeuT	0.83	0.83	0.60	0.67	0.75	0.75	0.71	0.69	0.72	0.65	0.60	0.66	0.80	0.78
21	Cys/BZL/		0.71	0.71	0.59	0.61	0.70	0.63	0.58	0.66	0.62	0.65	0.63	0.62	0.86	0.89
22		CysT/BZL/	0.73	0.73	0.70	0.69	0.77	0.72	0.66	0.73	0.72	0.73	0.72	0.68	0.83	0.85

[a] Symbols in this paper are according to SCHWYZER *et al.*[11].

water (7:7:6)[8]; S_5, phenol–water (3:1, g/g)[6,7,9]; and were prepared according to the literature.

1% solutions of the amino acids (AA) and their tetrazole analogues (AT)[3] in 0.1 *N* hydrochloric acid were used for spotting. Sample spots were applied in amounts of 10–30 μg (Whatman paper) or 1–10 μg (TLC plates). A 0.25% solution of ninhydrin in acetone was used for the detection of the AA and AT on the chromatograms.

Results and discussion

R_F values of AA and AT from comparative runs in five solvents are given in Table I.

The highest R_F values for AT were observed in solvents S_1 and S_5. Both these systems gave approximately the same R_F values for each pair involving the amino acid and the corresponding tetrazole analogue of the amino acid. In the other solvent systems studied (S_2, S_3, S_4), the observed differences of R_F values for such corresponding pairs were in range 0.05 to 0.24, the R_F value of the AT being, in general, higher than that of the AA.

Lower AA and AT (glycine, alanine, β-alanine, α-aminobutyric acid and their tetrazole analogues) developed with S_2, S_3 or S_4 on silica gel TLC plates usually gave higher R_F values than those obtained for the other two adsorbents.

S_4 (pyridine–isoamyl alcohol–water) appeared to be the best system for the

TABLE II

SENSITIVITY OF THE TETRAZOLE ANALOGUES OF AMINO ACIDS TO THE NINHYDRIN REACTION ON WHATMAN PAPER NO. 1

No.	*Amino acid*	*Tetrazole analogue of amino acid*	*Sensitivity (μg)*		*Colour of spot*[a]
1	Gly		0.1	(0.2)[b]	violet-brown
2		GlyT	0.2		brown-violet
3	β-Ala			(0.4)[b]	violet
4		β-AlaT	0.4		grey-blue
5	Ala		0.2	(0.2)[b]	violet
6		AlaT	0.3		violet
7	Pro			(0.5)[b]	yellow-brown
8		ProT	3.0		yellow-orange
9	Abut			(0.1)[b]	violet
10		AbutT	0.2		violet
11	Val		0.2	(0.1)[b]	violet
12		ValT	0.4		violet
13	Nva				violet
14		NvaT	0.4		violet
15	Ile			(0.1)[b]	violet
16		IleT	0.5		violet
17	Phe		0.5	(0.7)[b]	violet
18		PheT	1.0		violet
19	Leu			(0.1)[b]	violet
20		LeuT	0.4		violet
21	Cys/BZL/				violet
22		CysT/BZL/	0.5		violet

[a] After 18 h.
[b] Reported by SAIFER AND ORESKES[10].

separation of aminoalkyltetrazoles not only because of differences in R_F values but also because the spots after spraying with ninhydrin were dense and intensely coloured. Development of AT by phenol–water solvent system (S_5) resulted in a low sensitivity for the ninhydrin reaction. In spite of thorough removal of phenol from the chromatograms it was difficult to calculate the R_F values on Whatman paper and cellulose TLC plates and very difficult on silica gel TLC plates, though the spots of amino acids could be easily detected with ninhydrin on these plates.

After spraying the chromatograms with ninhydrin the tetrazole analogues of amino acids gave a yellow colour. The spots changed to grey-brown and finally to violet.

The reactivity and sensitivity of the various aminoalkyltetrazoles with ninhydrin was determined by the SAIFER AND ORESKES method[10]. The limits of the sensitivity of the tetrazole analogues of amino acids (Table II) are comparable with the sensitivity values of the respective amino acids.

The author is grateful to Dr. B. LIBEREK for his interest in this work and for help with the preparation of the English manuscript.

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Received April 22nd, 1970

CHROM. 4821

An improved method of separating glucosaminitol from galactosaminitol and their amino sugars on an amino acid analyzer

In a recent report on the separation of galactosaminitol from glucosaminitol using the amino acid analyzer, DONALD[1] obtained a good resolution between the two glycitols in about 10 h and with their corresponding amino sugars in 15 h. In the method reported by WEBER AND WINZLER[2] the separation between the two glycitols was not adequate. This latter method therefore is good only if one glycitol was present together with the amino sugars and permits identification but not quantitation of the glycitols.

The method described in the present paper is an improvement of the method of DONALD[1] wherein a complete analysis of hexosamines and their glycitols could be done in the standard 56 cm column of the amino acid analyzer in less than 4 h.

Experimental

Glucosamine·HCl and galactosamine·HCl, A.G., were purchased from Calbiochem. Chromatography on the amino acid analyzer showed that each had only one component. Glucosaminitol and galactosaminitol were prepared by borohydride reduction using the method of CRIMMIN[3].

A Beckman 120C amino acid analyzer equipped with a 0.9 × 56 cm column of Beckman UR-30 resin was used in the separation. The column was equilibrated and eluted with citrate–borate buffer pH 5.06 at a flow rate of 40 ml/h. Ninhydrin flow rate was 20 ml/h. The column temperature was kept at 65°.

The 0.35 M sodium citrate buffer pH 5.28 (ref. 4), a standard buffer for basic amino acid analysis, was used for preparing the citrate–borate buffer. To this buffer was added 18.55 g/l boric acid (0.3 M) to give a final pH of 5.06.

Results

The separation of galactosaminitol, glucosaminitol, glucosamine and galactosamine is shown in the chromatogram in Fig. 1. The retention times relative to glucosamine are: galactosaminitol 0.72; glucosaminitol 0.76; galactosamine 1.12.

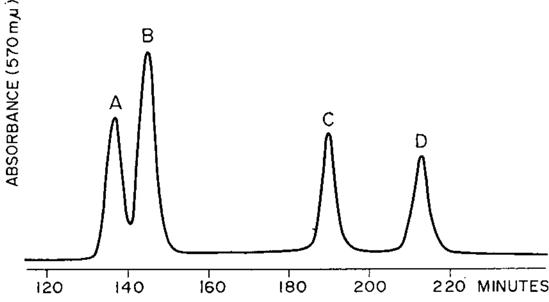

Fig. 1. Chromatographic separation of galactosaminitol (A), glucosaminitol (B), glucosamine (C) and galactosamine (D), on a 0.9 × 56 cm column of Beckman UR-30 resin eluted with 0.35 M citrate buffer pH 5.06 containing 0.3 M borate.

The Beckman 120C analyzer used in this separation is equipped with a 4–5 mV range recorder bridge[5]. With this expanded scale a normal load of 10–100 nmoles of each sugar base could be determined. The standard 0–5 mV range recorder bridge provided with most instruments allows analysis of 0.14–1.5 μmoles.

An increase in temperature to 65° provides an improved resolution of galactosaminitol from glucosaminitol. To accelerate the analysis by increasing the rate of buffer elution would mean a sacrifice in resolution. The flow rate of 40 ml/h is the ideal condition for the separation of the glycitols with this method. If only one of the sugar alcohols is present the analysis can be accelerated to 68 ml/h.

Chromatograms obtained from Beckman AA-15 resin also produce a similar satisfactory separation of galactosaminitol, glucosaminitol, glucosamine and galactosamine. Resins of similar grade to Beckman UR-30 or AA-15 available from other commercial sources would probably behave in the same manner.

This work was supported by VA RE TR-48 and G. D. Searle Grant 444918-58005.

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Received May 12th, 1970

CHROM. 4569

Microdetermination of molar ratios of neutral sugars on thin layers of borohydride-reduced cellulose*

An important problem in structural determination of the oligosaccharide units of glycoproteins is the availability of a sensitive, accurate and convenient analytical method for determining molar ratios of the constituent sugars. While paper chromatography of hydrolysates of oligosaccharides, followed by elution and colorimetric determination, can give good results[1,2], it is time consuming and requires relatively large amounts of material. Gas chromatography (GC) has also been used for quantitative determinations[3,4] and is extremely sensitive. However, unless the alditol acetate derivatives are prepared, one encounters problems with isomerization during sample preparation which could be serious if several different sugars are present. Thus, the preparation of suitable derivatives imposes a restriction on the sample size for GC analysis, even though a given analysis may require only 1% of the total sample. Recently, ion-exchange column chromatographic methods have been introduced[5,6] and have been found to give good separations with sensitivity in the 10 μg range. Both the GC and the ion-exchange methods require expensive instrumentation for quantitative results.

The method described in this communication requires only equipment for thin-layer chromatography (TLC) and for colorimetric determination. It combines the excellent separations of neutral sugars on thin layers of cellulose with a modification of the PARK–JOHNSON reducing sugar method[7] and enables the reproducible determination of molar ratios on as little as 0.5 μg of each sugar.

Materials and methods

Crystalline cellulose (Avicel SF, American Viscose Corp.) was reduced by a modification of the method of WOLFROM *et al.*[8]. The cellulose was suspended in 1.0 *M* aqueous sodium borohydride and vigorously stirred for 12 h. After this time, the once-reduced cellulose was filtered off and the above treatment repeated. The final reduced product was washed thoroughly with 0.1 *N* acetic acid and then with water and was dried in a desiccator. This treatment was found to yield a cellulose having chromatographic properties not significantly differing from those of native cellulose and a reducing content diminished to a point suitable for determination by the PARK–JOHNSON method. The dried, reduced cellulose was ground to a powder, and a slurry was prepared by homogenizing 5 g in 20 ml of distilled water in an Omnimixer blender. The slurry was rolled onto glass plates by hand, using a plastic rod. Plates prepared in this manner could be stored indefinitely with no special precautions regarding moisture or temperature and could be used with no pretreatment.

Standard sugars and neutralized hydrolysates of glycoprotein oligosaccharides were applied to the plates in 1–2 mm spots with a Hamilton microsyringe. The plates were developed to a distance of 10 cm. After development, the inner lanes were protected with aluminium foil and outer lanes were sprayed with *o*-aminobiphenyl reagent[9], and the plate was heated at 100° for 1 min to develop the fluorescent color. The sugars were removed from the plate by scraping free the area corresponding to

* Contribution No. 596 from the McCollum-Pratt Institute.

the standard sugar and collecting it by suction into a glass-wool-plugged Pasteur pipette connected to a water aspirator. For a given plate, the same size areas were removed for each sugar. The reducing sugars were then eluted from the cellulose with distilled water and determined by the PARK–JOHNSON procedure scaled down to a final volume of 1.5 ml. In this modification, color development was linear with reducing sugar content over a range of 0.5–5 μg for D-mannose, D-galactose and D-xylose. Blanks for the determinations consisted of non-sugar containing areas of the plates.

Results and discussion

Blank values for the reduced cellulose layers were found to be low when the reduction was carried out as described. In an experiment in which ten areas were scraped from a plate to which no sample had been applied, the "plate blank" was found to give an absorbance of 0.112 ± 0.007 relative to water, as compared to a PARK–JOHNSON reagent blank of 0.085 ± 0.005. In the PARK–JOHNSON assay employed here, the color yield was $A_{690\ nm} = 0.267$ per μg reducing sugar as D-mannose against the reagent blank. Thus, when even as little as 0.5 μg of sugar is measured, the difference between the plate blank and the reagent is only 20% of the value for the sugar.

The recovery of reducing sugars from the reduced cellulose layers was found to be quantitative (Table I). Replicate aliquots of standard D-mannose solution were applied to a plate, chromatographed, eluted and determined colorimetrically. Using the same microsyringe and the same solution, the same volume of standard D-mannose solution was delivered to test tubes for direct colorimetric analysis. The results of both sets of reducing power determinations show that recovery of the standard sugar was virtually 100%. Recoveries were also shown to be quantitative when as much as 10 μg of neutral sugar was applied. In addition, mixtures of standard sugars were spotted on plates, separated, and recovered as described. The molar ratios of the isolated sugars did not differ significantly from those of the starting mixture.

TABLE I

QUANTITATION PARAMETERS FOR MICRODETERMINATION OF SUGARS ON REDUCED CELLULOSE LAYERS

	Volume of standard D-mannose solution (1 μg/μl)	*$A_{690\ nm}$ after Park–Johnson reaction*	*% recovery (A/B × 100)*
(A) Plate	1 μl (7 spots)	0.263 ± 0.006[a]	99
(B) Test tubes	1 μl (7 tubes)	0.267 ± 0.007	100.0
	Molar ratios relative to D-mannose as 1.00		
	D-Mannose	*D-Fucose*	*D-Xylose*
Standard sugars mixed	1.00	0.506	0.585
Standard mixture after TLCseparation			
A	1.00	0.500	0.579
B	1.00	0.498	0.606

[a] After subtraction of plate blank.

TABLE II

MOLAR RATIOS OF GLYCOPROTEIN NEUTRAL SUGARS

Glycoprotein	*Neutral sugar constituent*	*Molar ratios of neutral sugar constituents*	
		TLC	*Automated borate chromatography*
α-Amylase	Mannose	3.90[a,b,c]	3.70
	Galactose	1.00	1.00
Bromelain II[d]	Mannose	2.00[e,f]	2.00
	Fucose	1.00	1.07
	Xylose	1.00	0.88
Bromelain III[d]	Mannose	2.00	2.00
	Fucose	1.03	1.12
	Xylose	0.93	0.95

[a] The neutral sugars in this glycoprotein show non-integral molar ratios due to microheterogeneity of the carbohydrate group[10].
[b] Ratios expressed relative to galactose as 1.00.
[c] Solvent system for separation was ethyl acetate–(isopropanol–water, 2:1), 60:40. Plates were developed twice.
[d] Commercial bromelain was fractionated by ion-exchange chromatography into several similar proteases with identical carbohydrate content.
[e] Ratios expressed relative to mannose as 2.00.
[f] Solvent system for separation was *n*-butanol–pyridine–ethyl acetate–acetic acid–water (10:3:3:3:4). Plates were developed three times.

The molar ratios of the major neutral sugar constituents of two glycoproteins, *Aspergillus oryzae* α-amylase and pineapple stem bromelain, determined by this method, agreed well with the ratios determined by automated borate complex anion-exchange chromatography[6]. The results (Table II) show that the present method gives values in good agreement with the sophisticated automated system.

In summary, microdetermination of molar ratios of reducing sugars by the present method provides an inexpensive valuable alternative to existing methods of quantitative sugar estimation.

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Received November 19th, 1969; revised manuscript received April 27th, 1970

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CHROM. 4816

One-stage solvent system and one-dimensional thin-layer chromatographic separation of milk simple lipid classes on 20 × 20 cm plates

MANNERS *et al.*[1] reported that no one-stage TLC solvent system was available for separating the main simple lipid classes. But STORRY AND TUCKLEY[2] used a one-stage, one-dimensional system on 20 × 34 cm plates for separating blood plasma simple lipids of a lactating cow. Several two-stage, one-dimensional systems have been used with 20 × 20 cm[1,3] and 20 × 34 cm[4,5] plates. These techniques were used to separate simple lipid classes of gut contents[1], serum enzyme digest[3], pig intestines and plasma[4] and rat liver[5]. It is well known that the amount of each lipid class from any biological extraction will vary with the source, and efficient separation by TLC will require a particular solvent system. Because the simple lipids in cow's milk contain *ca.* 98% triglyceride, it is difficult to separate free fatty acids and cholesterol esters. This was found when the techniques of KELLEY[3] and STORRY AND TUCKLEY[2] were studied in our laboratory.

We believed that a one-stage solvent system for separating the main simple lipid classes with standard TLC equipment would be helpful to other investigators. Such a system was developed for the simple, rapid and efficient separation of simple lipid classes in milk.

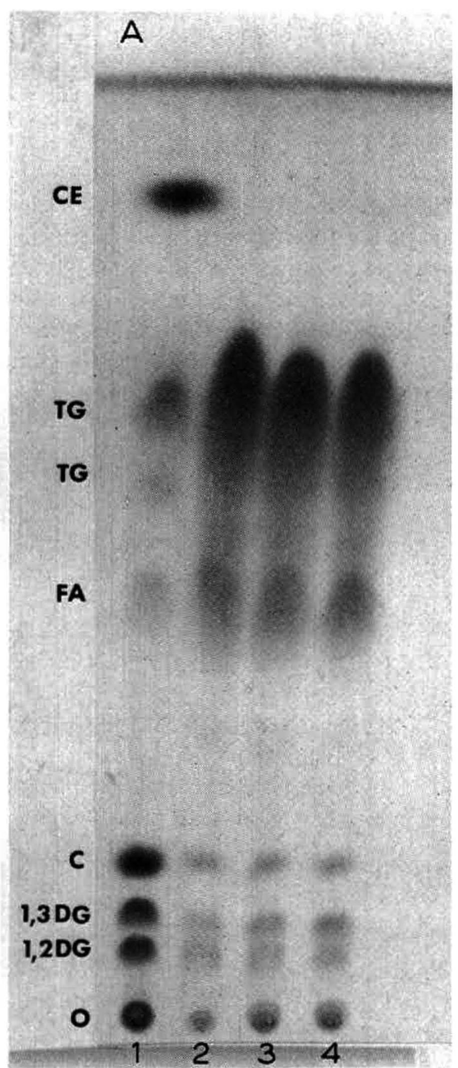
Methods

All solvents were ACS grade and used as obtained from the manufacturer. Silica Gel G (according to Stahl) was purchased from Brinkman Instruments, Inc., N.Y. Plates were activated for 1 h at 120° and stored in a desiccative cabinet until used. Approximately 1 h before use, a developing chamber was saturated with vapors of its solvent. The solvent systems moved 15 cm from the point of spotting. The spots were observed by spraying the plates with H_2SO_4–H_2O (1:1) and heating in an oven at 120° until the organic material charred.

The solvent system of VOGEL *et al.*[6], utilizing petroleum ether–diethyl ether–formic acid (180:20:2) with plates 0.25 mm thick (Fig. 1A), was compared with a new solvent system of hexane–ethyl acetate–formic acid (175:25:2) with plates 0.50 mm thick (Fig. 1B). Plates were prepared with a Desaga adjustable applicator using a suspension of Silica Gel G in 0.01 *M* sodium carbonate[7].

The reference mixture of simple lipid classes (Fig. 1A and B, No. 1) contained *ca.* 10 μg of each of the following compounds: monoolein, 1,2- and 1,3-dioleins, cholesterol, free fatty acid (C_4–C_{18}, C_{18-}, $C_{18=}$), triglycerides (trilaurin, tripalmitin, tristearin, tripalmitolein and triolein) and cholesterol acetate. The monoglyceride, diglycerides and unsaturated triglycerides were purchased from Applied Science Laboratories, Inc., State College, Pa.; the others from Fisher Scientific Co., Medford, Mass. They were dissolved in chloroform–methanol (2:1) and the mixture applied to the plates with a disposable micropipet.

An application of the technique was witnessed in a lipolysis study of cow's milk obtained from the University Dairy Plant. Lipid hydrolysis was induced by mixing equal portions of raw and homogenized milks, heating the mixture to 30°, and placing it in a refrigerator overnight at 4°. Free fatty acid values (FFA) were determined by



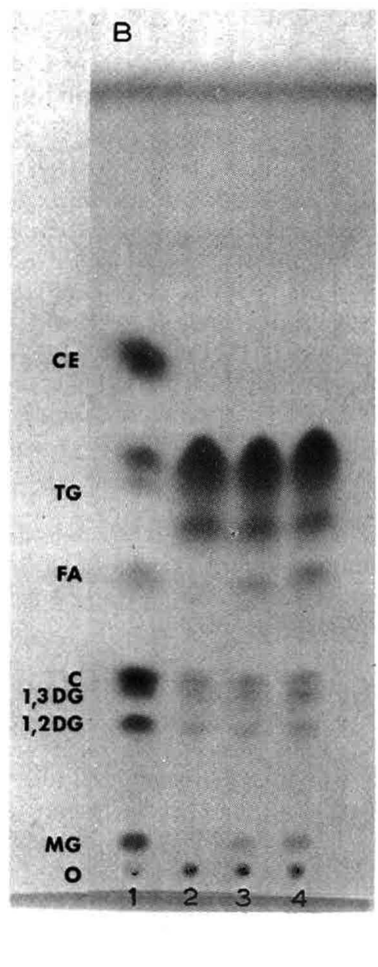


Fig. 1. Separation of simple lipid classes in two solvent systems. A, VOGEL *et al.*[6] solvent system of petroleum ether–diethyl ether–formic acid, 180 : 20 : 2, and B, our solvent system of hexane–ethyl acetate–formic acid, 175 : 25 : 5. Abbreviations: O = origin; MG = monoglyceride; 1,2DG = 1,2-diglyceride; 1,3DG = 1,3-diglyceride; C = cholesterol; FA = free fatty acid; TG = triglyceride; CE = cholesterol ester. Samples: (1) reference mixture of simple lipid classes; (2) total milk lipid extract, FFA 0.58; (3) total milk lipid extract, FFA 7.86; (4) total milk lipid extract, FFA 18.50.

the method of THOMAS *et al.*[8], as modified in our laboratory[9]. Three samples with specific FFA were prepared by mixing appropriate volumes of pasteurized hydrolyzed milk with pasteurized milk. Milk lipid (Fig. 1A and B, No. 2–4) was extracted by the method of MOJONNIER AND TROY[10], dissolved in chloroform–methanol (2:1), and spotted on the plates at a concentration of *ca.* 175 μg with disposable micropipets.

Results and discussion

VOGEL *et al.*[6] developed a one-stage TLC solvent system for rapid separation of most simple lipid classes on 20 × 20 cm plates. It has been the common solvent system used in our laboratory. But when reference simple lipid classes are analyzed (Fig. 1A, No. 1), the monoglycerides remain at the origin. And when milk lipid extracts are analyzed (Fig. 1A, No. 2–4), the free fatty acids trail with the triglycerides and the monoglycerides remain at the origin with complex lipids. The influence of lipid class concentration on separation ability is seen on this plate.

Our new solvent system overcomes these problems (Fig. 1B). With reference simple lipids (No. 1), the monoglycerides move off the origin. And the separation of free fatty acids from milk lipid triglycerides is distinct (No. 2–4). Excellent separation of the main simple lipid classes except 1,3-diglyceride and cholesterol is seen in Fig. 1B.

An application of the differences in solvent systems is witnessed in Fig. 1A and B, No. 2–4. As lipolysis proceeds, the amount of monoglyceride and free fatty acids increases. This is clearly seen only with the new solvent system (Fig. 1B).

This study was supported by the U.S. Department of Agriculture, Federal Grant HA-143; University of Vermont Agricultural Experiment Station Journal Article No. 239.

We thank MRS. LLOYD L. HOWARD, JR. for her technical assistance.

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Received April 23rd, 1970

CHROM. 4817

Determination of steroids by densitometry of derivatives

I. Assay of estrogens as azobenzene-4-sulfonates

The assay of steroids in biological extracts, as carried out in most clinical routine laboratories, usually depends on more or less specific color reactions, the resulting chromogens being submitted to photometry or fluorometry for evaluation[1-3]. Increased specificity of such methods may be obtained by introduction of additional chromatographic procedures. On the other hand, the isolation of individual steroids prior to the color reaction requires their elution from the chromatogram, thus adding to the losses and limiting the capacity of the particular method. In order to overcome such drawbacks, the direct photometry of colored steroid derivatives has been proposed[4-6]; this not only allows easy quantitation but may also increase the sensitivity of the assay.

The present communication describes the estimation of estrogens after conversion into their azobenzene-4-sulfonic acid esters and densitometry of the derivatives on chromatoplates.

Methods

0.1 ml 0.1% azobenzene-4-sulfonyl chloride (ABS chloride) in dry acetone, 0.85 ml acetone and 0.05 ml 0.02 *N* sodium hydroxide are added to the dry residue containing 0.05–10.0 μg estrogen in a glass stoppered centrifuge tube. The reaction mixture is kept at 50–55° for 30 min, diluted with 20 ml ether and then extracted twice with 5 ml 0.1 *N* sodium hydroxide and twice with 5 ml water, before being filtered through anhydrous sodium sulfate and evaporated to dryness under nitrogen.

The residue is quantitatively transferred on to the chromatoplates (20 × 20 cm) coated with Silica Gel G (0.25 mm thickness; Merck AG, Darmstadt, G.F.R.) by means of chloroform or benzene. Ascending chromatography is performed in one of the solvent systems indicated in Table I, using 1 cm wide bands for the samples with empty bands in between. After drying the chromatograms thoroughly, the sample lanes are subjected to densitometry at 313 nm in a spectrodensitometer (SD 3000; Schoeffel Instruments Corp., Westwood, N.J., U.S.A.). The peaks registered can be evaluated on the basis of their height or area, as determined by triangulation, and by comparing them to corresponding values of standard material.

Results and discussion

When six 1.0 μg samples of 6,7-^{3}H-estrone (3-hydroxy-1,3,5-estratrien-17-one) or 6,7-^{3}H-estriol (1,3,5-estratriene-3,16α,17β-triol) with 6,270 c.p.m. and 12,770 c.p.m. ^{3}H, respectively, were subjected to the above reaction, the recovery of ^{3}H-activity from the final ether extracts amounted to 96.9 ± 2.8% for estrone and 94.2 ± 3.3% for estriol. After subsequent thin-layer chromatography of such extracts and elution of the derivatives, recoveries of between 85.4% and 87.1% of the original ^{3}H-activity were obtained, indicating significant losses during the additional steps. However, it may be concluded from these recovery experiments with labeled estrogens, that the reaction of the phenolic steroids with ABS chloride proceeded practically to completeness.

TABLE I

TLC R_F VALUES OF ESTROGENS AND THEIR ABS DERIVATIVES

Solvent systems: A = chloroform–benzene-ethanol (18 : 2 : 1); B = chloroform–dioxan (94 : 6); C = cyclohexane–ethyl acetate (3 : 1).

Solvent system	*Estrone*		*Estradiol*		*Estriol*	
	Free	*ABS*	*Free*	*ABS*	*Free*	*ABS*
A	0.48	0.67	0.34	0.51	0.05	0.14
B	0.55	0.62	0.29	0.35	0.02	0.08
C	0.44	0.57	0.17	0.28	0.02	0.07

The absorption spectra of the ABS derivatives of estrone, estradiol (1,3,5-estratriene-3,17β-diol) and estriol all exhibited absorption maxima at 323 nm and 446 nm, characteristic for the azobenzene group. The molar extinction coefficients ε at 323 nm were found to be between 28,300 and 28,400 for the three derivatives, whereas the ε values at 446 nm were only approximately 720. The almost identical ε values at 323 nm, corresponding to that of the reagent, obviously suggest that for all three derivatives tested one common hydroxy group, *viz.*, the phenolic 3-hydroxy group was esterified under the experimental conditions specified. This could be confirmed by the failure of ABS estradiol or ABS estriol to react with BARTON's reagent[7] or Fast Blue B salt. The R_F values of the free estrogens and their derivatives in different solvent systems are listed in Table I. Apparently, the replacement of the 3-hydroxy group by the azobenzene-4-sulfonate group does not cause a substantial change in polarity.

Multiple assays of 0.1–10.0 μg of estrone or estriol by densitometry of their ABS derivatives on chromatoplates yielded the results compiled in Table II. While the accuracy of the assay ranged between 92 and 95%—comparable to the data of the recovery experiments with labeled estrogens—its precision did not exceed 6.1%

TABLE II

THE RELATIONSHIP BETWEEN PEAK AREA AND CONCENTRATION OF ESTRONE OR ESTRIOL AS THEIR ABS DERIVATIVES

s = sensitivity setting of the instrument; n = number of determinations.

Amount of estrogen (μg)	*s*	*Peak area in cm² and standard deviation from the mean*			
		n	*ABS estrone*	*n*	*ABS estriol*
0.10	0.2	5	0.99 ± 0.06	5	0.93 ± 0.05
0.25		5	2.34 ± 0.14	5	2.40 ± 0.14
0.50		5	4.55 ± 0.26	5	4.53 ± 0.27
0.75		5	6.80 ± 0.36	5	7.02 ± 0.38
1.00		4	9.12 ± 0.47	4	9.04 ± 0.49
1.0	1.0	6	1.24 ± 0.07	5	1.21 ± 0.08
2.5		5	2.94 ± 0.15	5	2.90 ± 0.14
5.0		5	5.72 ± 0.26	5	5.45 ± 0.23
7.5		5	8.30 ± 0.37		
10.0		5	10.15 ± 0.43		

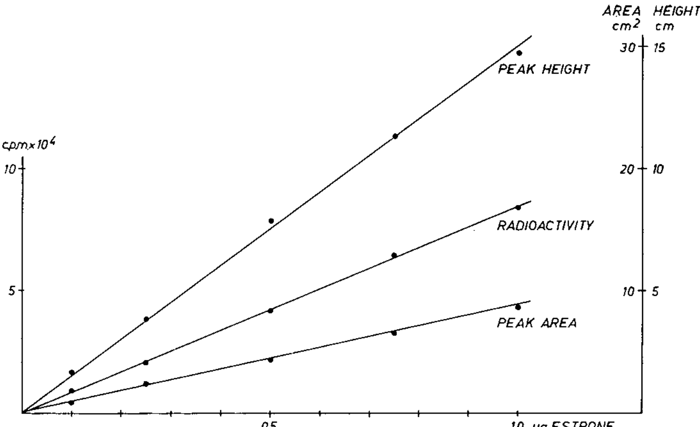

Fig. 1. Calibration curve for the estimation of estrones as their ABS derivatives, using 6,7-^{3}H-estrone. Densitometry at 313 mμ; slit width 0.2 mm; sensitivity 0.2, paper speed 4 in./min).

at the lower levels and 4.7% at the higher concentrations. A typical calibration curve, set up for the estimation of estrone, is shown in Fig. 1. Although the absorption maximum of the ABS derivatives lies near 323 nm, densitometry at 313 nm led to slightly higher values than the same measurements at 323 nm, due to the intensity of the mercury line of the light source at 313 nm. The sensitivity of the method was estimated as approaching 50 ng from the ε values of the derivatives and the height or the area of the peak per μg of estrogen. By performing the densitometry at the highest sensitivity of the instrument (sensitivity setting: 0.1 instead of 0.2) the peak height or area per unit weight of estrogen can be almost doubled. However, unless uniform layers and dividing lines between the bands are obtainable, the background at such a sensitivity is apt to interfere with reproducible measurements. Likewise, the evaluation of the peaks by their height depends as much on adequate chromatographic techniques as on the speed of the recorder.

In view of the experimental data obtained, the present assay appears to be suitable for the analysis of estrogens like estrone, estradiol or estriol in pregnancy urine as will be shown in a forthcoming publication.

This investigation was supported by the Deutsche Forschungsgemeinschaft, 532 Bad Godesberg, G.F.R.

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Received April 27th, 1970

CHROM. 4841

Determination of steroids by densitometry of derivatives

II. Direct fluorometry of DANSYL estrogens

In a preceding communication[1] the determination of estrogens by direct densitometry of their azobenzene-4-sulfonates has been reported. The sensitivity of this assay was found to approximate 50 ng. Since estrogens are known to react also with 1-dimethylaminonaphthyl-5-sulfonyl chloride (DANSYL chloride)[2], as evidenced by a recent publication on the estimation of estriol in pregnancy urine[3], it seemed of particular interest to attempt the direct fluorometry of DANSYL estrogens on chromatoplates for quantitation of ng amounts of estrogens.

Methods

Studies on the reaction between estrogens and DANSYL chloride have revealed, that in a 10^{-3} N sodium hydroxide solution in aq. acetone the velocity of derivative formation depends on the water content of the reaction mixture[4]. Higher concentrations of water not only increased the rate of formation of derivatives but also favored their hydrolysis within a certain period of time. Based on these experiences, the following procedure was adopted for the conversion of estrogens into their DANSYL derivatives.

The dry residue with up to 1000 ng of estrogen is dissolved in 0.9 ml of acetone. Then 0.1 ml of 0.01% DANSYL chloride in dry acetone and 0.01 ml of 0.1 N sodium hydroxide are added. The reaction mixture is kept at 50° for 30 min, diluted with 20 ml of benzene and extracted once with 5 ml of 0.1 N sodium hydroxide and twice with 5 ml of water before being filtered through anhydrous sodium sulfate and evaporated to dryness under nitrogen.

By means of chloroform or benzene the residue is quantitatively transferred onto chromatoplates with silica gel (No. 1500, acid stable, without binder; Schleicher & Schüll, Dassel, G.F.R.). Ascending chromatography is performed in chloroform–benzene–ethanol (18:2:1). Finally, the dry chromatogram is submitted to direct fluorometry in the TLD 100 (Vitatron, Dieren, The Netherlands), using the appropriate filter combination.

Results and discussion

Recovery experiments with 10–1000 ng of [6,7-^{3}H]estrone (3-hydroxy-1,3,5-estratrien-17-one) or [6,7-^{3}H]estriol (1,3,5-estratriene-3,16α,17β-triol) showed that the final benzene extracts contained 93.4–95.2% of original ^{3}H-activity, indicating an almost complete conversion of estrogens into their DANSYL derivatives. At concentrations between 1 and 10 μg, the recovery was found to range from 95.0 to 98.7%.

In the solvent system chloroform–benzene–ethanol (18:2:1), used for separation of DANSYL estrogens, the following R_F values were obtained: 0.65 $\pm$ 0.02 for DANSYL estrone, 0.50 $\pm$ 0.02 for DANSYL estradiol (1,3,5-estratriene-3,17β-diol) and 0.11 $\pm$ 0.01 for DANSYL estriol. The R_F values of the corresponding free estrogens amounted to 0.48 $\pm$ 0.2 for estrone, 0.34 $\pm$ 0.02 for estradiol and 0.05 $\pm$ 0.01 for

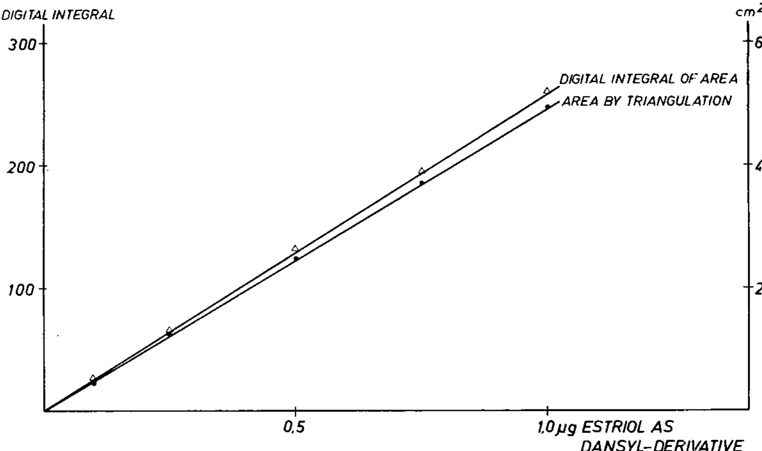

Fig. 1. Direct fluorometry of DANSYL estriol on chromatoplates.

estriol. Hence, the separation of these three estrogens and their DANSYL derivatives by ascending chromatography in the above solvent system can be considered satisfactory.

The absorption spectra of DANSYL estrone, DANSYL estradiol and DANSYL estriol exhibited maxima at 273 and 362 nm, the latter wavelength being used for excitation of fluorescence. In the emission spectra of the estrogen derivatives a common maximum near 513 nm was observed. By direct fluorometry of 100–1000 ng of estriol as DANSYL derivative, a linear relationship between the intensity of fluorescence, recorded in digital integrals of the peak area, and the amount of steroid could be established (Fig. 1). The multiple analysis of 250 ng of estriol and 750 ng of estrone by the DANSYL method revealed an accuracy of $\pm$ 5.3% for the lower concentration and $\pm$ 4.8% for the higher levels, n being 8 and 6 respectively. The evaluation of recorded peaks by triangulation led to comparable results, as demonstrated in Fig. 1. Since these measurements were performed at a rather low sensitivity of the instrument (settings: span = 78.6 and c = 5), the sensitivity of the foregoing assays did not exceed 50 ng. When the sensitivity of the instrument was increased (settings: span = 100 and c = 6), as little as 5 ng of either estrogen could be estimated (Fig. 2). At such low levels, however, the accuracy of the method still varied between 10.2 and 13.0%. According to preliminary findings, improvements in chromatographic techniques,

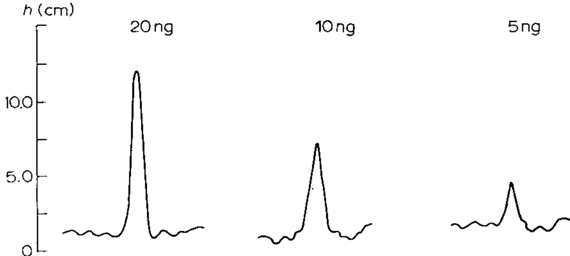

Fig. 2. Estimation of 5–20 ng of estriol as DANSYL derivative.

such as the use of uniform and extremely thin layers or the automated application of derivatives, appear to raise the accuracy considerably. A successful increase in the accuracy of the method eventually may extend its application to the analysis of estrogens in peripheral human plasma or non-pregnancy urine.

This investigation was supported by the Deutsche Forschungsgemeinschaft, 532 Bad Godesberg, G.F.R.

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Received May 5th, 1970

J. Chromatog., 51 (1970) 325–327

CHROM. 4829

Direct bioautography on thin-layer chromatograms as a method for detecting fungitoxic substances

A simple bioautographic technique according to WELTZIEN[7], and modified by DEKHUIJZEN[1] for detection of fungitoxic substances has been in use for many years in this laboratory. Chromatograms on Whatman No. 3MM paper are developed with propanol–water (85:15) and after drying are sprayed with a conidial suspension of *Glomerella cingulata*. After incubation, clearly visible inhibition zones indicate the presence of fungitoxic compounds. Chromatography thus permits not only the detection of fungitoxic substances *per se*, but also makes the study of the conversion reactions and of decomposition of such compounds possible.

Although this method is elegant for many reasons (high sensitivity, possibility of keeping records), it has the disadvantage of paper chromatography in general, the development being rather time consuming (16 h). This proved to be especially inconvenient in the study of fungitoxic compounds which are gradually converted non-enzymatically into other compounds either by oxidation (*e.g.* phenylthiosemicarbazide and derivatives[2]) or hydrolysis (*e.g.* benomyl[3]). The rather slow development did not give a satisfactory separation of the various components, confluent spots being obtained instead. Therefore, silica gel thin-layer chromatography on DC-Alufolie Kieselgel F_{254} plates (Merck) was considered as an alternative to the paper chromatographic technique.

With the widespread usage of TLC, many bioautographic methods have been introduced which make use of this technique for the more rapid separation of antimicrobial substances. On perusing the literature we did not really come across a very simple bioautographic technique based on TLC. Moreover several additional manip-

ulations had to be performed for the bioassay to be carried out. General routine normally includes either pressing the thin-layer plate on agar seeded with a suitable sensitive microorganism[4] or pouring a molten nutrient agar on the thin-layer chromatogram, which after solidification is then seeded with the test organism[5]. Neither method is very appropriate if one wishes to save the plates; moreover, with the first technique at least some skill is required to handle the thin-layer plates properly. To overcome some of the difficulties WAGMAN AND BAILEY[6] introduced the use of Chrom-AR® silicic acid/glass fiber sheets for bioautography of antimicrobial compounds, although according to this method the material to be investigated again has to be transferred from the sheet to agar.

We found that direct spraying of the thin-layer chromatograms with a spore suspension of the test fungus in a glucose–mineral salts medium was by far the easiest technique, and also gave the most reliable results. The thin-layer plates are developed for 20–60 min depending on room temperature and the solvent system employed. Ether and ethyl acetate proved the most suitable solvents because no trace of these remains in the silica gel after drying, as opposed to other systems, as for instance butanol–acetic acid–water (4:1:1) where even prolonged drying does not completely remove the acetic acid present. After locating the UV-absorbing spots, the chromatograms are usually sprayed with a conidial suspension of *Cladosporium cucumerinum* in a medium prepared as follows. A stock solution contains: 7 g KH_2PO_4, 3 g $Na_2HPO_4 \cdot 2H_2O$, 4 g KNO_3, 1 g $MgSO_4 \cdot 7H_2O$, 1 g NaCl per l of tap water. The solution is autoclaved at 120° for 20 min. Just before making the conidial suspension 10 ml of a 30%

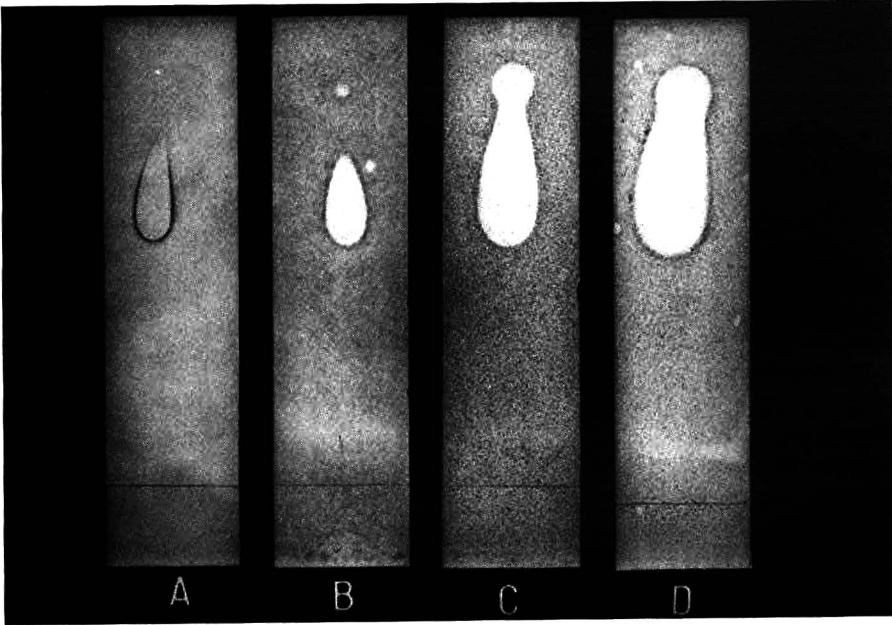

Fig. 1. Differential sensitivity for benomyl (= 1-(butylcarbamoyl)-2-benzimidazole carbamic acid, methyl ester) of: (A) *Ascochyta pisi*; (B) *Fusarium culmorum*; (C) *Penicillium expansum*; and (D) *Colletotrichum lindemuthianum*. The quantity spotted was in each case 1.25 µg active ingredient.

aqueous solution of glucose is added per 60 ml of this solution. During spraying, care should be taken to avoid the plates becoming too wet. After spraying the thin-layer plates are incubated in a moist atmosphere[1] for 2–3 days at 25°. Inhibition zones indicate the presence of the original fungitoxic product plus, if present, conversion or decomposition products, which are fungitoxic. It should be realized, that not all UV-absorbing spots will be fungitoxic as well, nor will all fungitoxic spots absorb at 254 nm.

This technique has been successfully used in the study of phenylthiosemicarbazide and various derivatives[2] and benomyl and its conversion products[3], among others fungicides, and is presently being used to investigate the non-enzymatic and metabolic conversion of a fungitoxic piperazine derivative, W 524 (= N,N'-bis-(1-formamido-2,2,2-trichloroethyl)piperazine) (Boehringer Sohn, Ingelheim am Rhein, G.F.R.). In addition to *Cladosporium cucumerinum*, many other fungi proved to be excellent test organisms for the detection of fungitoxic compounds. For instance, benomyl and its conversion products could be bioassayed by using, among others, *Aspergillus niger*, *Ascochyta pisi*, *Botrytis cinerea*, *Colletotrichum lindemuthianum*, *Fusarium culmorum* and *Penicillium expansum*, as is shown for four of these in Fig. 1; *Glomerella cingulata*, however, proved not to be sensitive to benomyl.

All results obtained so far show, that direct spraying of thin-layer plates with conidial suspensions of fungi is a most useful, easy and rapid technique for the detection of fungitoxic substances. The pliable aluminum thin-layer plates have the additional advantage of being easily cut with scissors, and they also can be preserved without the risk of loosening the silica gel layer.

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Received May 13th, 1970

CHROM. 4851

Galenik der Vitamine

21. Mitt. Dünnschichtchromatographische Strukturaufklärung des Pyridoxol–Borsäure-Komplexes*

Pyridoxol bildet mit Borsäure einen Komplex[1], der für die Gewinnung, Analyse und Verarbeitung des Vitamins Bedeutung besitzt. Der Wirkstoff kann in Form dieses Komplexes aus Rohmaterial isoliert und rein dargestellt werden[2].

Am häufigstens wird die Komplexbildung zur Bestimmung des Pyridoxols herangezogen[3]. In Gegenwart von Borsäure verläuft die Farbreaktion des Pyridoxols mit 2,6-Dichlorchinonchlorimid[4] negativ. Pyridoxal und Pyridoxamin (sowie weitere störende Phenole) reagieren unter diesen Bedingungen dagegen unverändert positiv. Dadurch kann Pyridoxol quantitativ neben anderen Komponenten des Vitamins B_6 erfasst werden. Da der positive Verlauf der Farbreaktion voraussetzt, dass am aromatischen bzw. quasiaromatischen Ringsystem eine phenolische Hydroxylgruppe und ein in *p*-Stellung dazu unsubstituiertes C-Atom vorliegen, wird die analytische Reaktivität des Pyridoxols im Borsäure-Komplex wahrscheinlich durch Blockierung dieser Phenolfunktion aufgehoben.

Daneben ist das Verhalten des Pyridoxols in Gegenwart der Borsäure auch für die Arzneimitteltechnologie von Interesse. Unter bestimmten Bedingungen bilden sich in Pyridoxollösungen bekanntlich schwerlösliche dimere oder trimere Derivate[5]. Nach Zugabe von Borsäure erfolgt jedoch selbst beim Erhitzen unter ungünstigen pH-Verhältnissen keine Polymerisation. Borsäure erweist sich als ein Stabilisator dieser Zersetzungsreaktion[1]. Da die Polymerisation unter Beteiligung der 4-Hydroxymethylgruppe des Pyridoxols verläuft[5–9], kann man auf Grund der Beobachtung weiterhin annehmen, dass auch diese Funktion bei der Komplexbildung beansprucht wird.

Die beiden Reaktivitätsänderungen am Pyridoxolmolekül verweisen somit darauf, dass sich der Borsäure-Komplex unter Beteiligung der phenolischen und der benachbarten primären Hydroxylgruppe bildet. Nach den ersten Untersuchungen werden dabei zwei Moleküle Pyridoxol an ein Molekül Borsäure gebunden[1].

HO N CH_3 O O B⊖ O O OH H_3C N⊕ H

Diese Struktur wurde später auch bei der Darstellung analoger Komplexe vorausgesetzt[10–12]. Einige Unklarheiten in der Literatur führten dazu, die Problematik erneut zu bearbeiten[13]. Obgleich aus den Ergebnissen eine Bestätigung der angenommenen Konstitution hervorgegangen ist, haben wir unsere gleichzeitig begonnenen Arbeiten fortgeführt. Wir versuchten, bei der Klärung der Frage eine Methodik an-

* 20. Mitt.: *Pharmazie*, 24 (1969) 761.

zuwenden, die auch für die Strukturaufklärung anderer phenolischer Verbindungen[14,15] als Modell dienen kann.

Aus papierchromatographischen Untersuchungen war bekannt, dass Borsäure-Komplexe geringere Polarität und höhere R_F-Werte als die Ausgangsverbindungen besitzen. Die Änderung des R_F-Werts stellt einen Masstab für die Anzahl der zur Komplexbildung fähigen Funktionen dar[16]. Es war zu erwarten, dass sich auch in der Dünnschichtchromatographie eine Komplexbildung durch eine signifikante Verschiebung der Laufstrecke nachweisen lässt. In Gegenwart von Borsäure mussten sich die reagierenden von den nichtreagierenden Verbindungen unterscheiden lassen. Wir versuchten, den Effekt nicht nur an Kieselgel G Schichten zu beobachten, sondern auch Polyamid als Sorbent einzusetzen und dadurch die selektive Adsorption bzw. Trennfunktion des Polyamids gegenüber Phenolen auszunutzen[17–19]. Wir nahmen an, dass bei Verwendung von Polyamid die Maskierung der für die Sorption massgebenden (phenolischen) Funktion besonders deutlich hervortritt.

Bei der Komplexbildung zwischen Pyridoxol und Borsäure war zu entscheiden, ob die Borsäure an den beiden alkoholischen Gruppen oder an einer alkoholischen und einer phenolischen Funktion angreift. Wir ermittelten daher den Einfluss der Borsäure auf den R_F-Wert folgender Verbindungen:

I II III V VI

oder

IV VII

Die Chromatographie wurde jeweils an normalen Kieselgel-G- bzw. Polyamidschichten und vergleichsweise an Schichten mit einem Zusatz von 5% bzw. 2% Borsäure durchgeführt. Die Entwicklung der zu vergleichenden Chromatogramme erfolgte in demselben Gefäss, so dass übereinstimmende Bedingungen vorlagen[19,20]. Zur Detektion dienten der Zusatz eines Fluoreszenzindikators und die Betrachtung im UV-Licht oder das Besprühen mit einer 1% wässrigen 2,6-Dichlorchinonchlorimid-Lösung.

An Kieselgel G wurden die in Tabelle I zusammengestellten Ergebnisse erhalten. Die Werte stellen Mittelwerte aus mindestens drei, im allgemeinen fünf Bestimmungen dar.

Die Gegenüberstellung zeigt, dass von den Komponenten des Vitamin-B_6-Komplexes erwartungsgemäss nur Pyridoxol durch Borsäure eindeutig beeinflusst wird. Eine wesentliche Änderung des chromatographischen Verhaltens bewirkt Borsäure ausserdem bei Salicylalkohol, Phthalylalkohol und Salicylsäure. Folglich verhalten sich Pyridoxal und Pyridoxamin gegenüber Borsäure indifferent, die übrigen Verbindungen reagieren evtl. unter Komplexbildung. Ein Hinweis auf die Struktur

TABELLE I

CHROMATOGRAPHIE AN KIESELGEL-G-SCHICHTEN

Fliessmittel A = Wasser; Fliessmittel B = Aceton–Dioxan–25% Ammoniaklösung (45 : 45 : 10).

	hR_F-Werte			
	Fliessmittel A		*Fliessmittel B*	
	—	*Borsäure*	—	*Borsäure*
Pyridoxol (I)	49	0	41	0
Pyridoxal (II)	42	42	54	45
Pyridoxamin (III)	21	20	56	45
Salicylalkohol (V)	87	54	82	13
Phthalylalkohol (VI)	—	—	81	48
Salicylsäure (VII)	100	68	45	30

des Pyridoxol–Borsäure-Komplexes kann aus diesen Ergebnissen nicht abgeleitet werden.

Wir führten daher gleiche Untersuchungen am Polyamidschichten durch. Nach einer allgemeinen Regel kommen Unterschiede in der Sorption eines Phenols an Polyamid nur dann voll zur Geltung, wenn zwischen stationärer und mobiler Phase ein möglichst grosser Polaritätsunterschied besteht, die Substanzen im Fliessmittel nicht zu gut löslich sind und die Verdrängungswirkung des Fliessmittels in bezug auf die zu untersuchenden Substanzen mittlere Stärke besitzt[18]. Hydrophobe Lösungsmittelkomponenten erhöhen die Affinität eines Phenols zum Polyamid. Die gleichen Substanzen wurden deshalb erneut in weniger polaren Systemen untersucht. Die mit dem Fliessmittel Cyclohexan–Äthanol (50 : 50) erzielten Ergebnisse gehen aus Tabelle II hervor.

Da in der Papierchromatographie ein stärkerer Einfluss des pH-Wertes auf das Verhalten der Vitamin-B_6-Komponenten nachgewiesen worden ist[21], berücksichtigten wir ausserdem die Einstellung der Wasserstoffionenkonzentration. Die Polyamid-Suspensionen mit 2% Borsäure besassen pH 3.6. Die borsäurefreien Ver-

TABELLE II

CHROMATOGRAPHIE AN POLYAMID-SCHICHTEN

Fliessmittel: Cyclohexan–Äthanol (5 : 5).

	hR_F-Werte		
	Ohne Imprägnierung	*Mit Puffer*	*Mit Borsäure*
Pyridoxol (I)	83	88	54
Pyridoxal (II)	90	86	83
	64	66	62
Pyridoxamin (III)	42	49	34
Dimeres Pyridoxol (IV)	77	79	25
Salicylalkohol (V)	77	77	57
Phthalylalkohol (VI)	88	87	84
Salicylsäure (VII)	24	22	20

gleichsplatten wurden deshalb durch Anwendung eines Natriumacetat–Natriumhydroxid-Puffers auf den gleichen pH-Wert eingestellt.

Tabelle II lässt erkennen, dass in Gegenwart von Borsäure der hR_F-Wert der Verbindungen Pyridoxol, dimeres Pyridoxol und Salicylalkohol (*o*-Methylol) deutlich herabgesetzt wird. Für die Beeinflussung ist folglich neben einer primären Hydroxylgruppe eine β-ständige phenolische Hydroxylgruppe erforderlich. An die gleichen strukturellen Voraussetzungen ist die Komplexbildung mit Borsäure gebunden. Damit wird die bisher angenommene Struktur des Pyridoxol–Borsäure-Komplexes erneut bestätigt. Einen analogen Komplex bildet dimeres Pyridoxol. Von den beiden Wirkungsantagonisten des Pyridoxols (Antivitaminen), Desoxypyridoxol (VIII) und 5-Desoxymethyl-pyridoxol (IX)[22], besitzt Verbindung IX die für die Komplexbildung erforderlichen Funktionen. Die Wechselwirkung zwischen Phthalylalkohol bzw. Salicylsäure[23] und Borsäure tritt an Polyamidschichten mit nur 2% Borsäure nicht hervor.

VIII IX

Die Borsäure-Komplexe erreichen in beiden Systemen niedrigere hR_F-Werte als die freien Verbindungen. Für diesen Effekt kommt die Änderung der Molekülgrösse in Betracht. Durch Komplexbildung mit Borsäure wird das Molekulargewicht der organischen Komponenten etwa verdoppelt. An Polyamidschichten ist ausserdem die Maskierung der Phenolfunktion von Bedeutung.

Durch das Ergebnis wird erneut deutlich, dass sich die drei elektronenspendenden Substituenten des π-Mangel-Heteroaromaten Pyridoxol nicht gleichwertig verhalten. Die Sauerstoffunktionen in 3- und 4-Stellung sind durch erhöhte Reaktivität ausgezeichnet. So wie im Falle der Komplexbildung werden bekanntlich auch bei der Acetalisierung[24–26] und Acylierung[27–29] bevorzugt die Hydroxylgruppen in Position 3 und 4 umgesetzt, obgleich die Bildung von 4,5-Derivaten oder 3,4,5-Derivaten (Triacylaten) durchaus möglich ist.

Für die Untersuchung fanden Verwendung: Kieselgel-G- und Polyamid-Pulver Merck, Polyamid Woelm DC und Leuchtstoff Grün Woelm (Detektion im UV-Licht). Phthalylalkohol wurde über Xylylendibromid nach STEPHENSON[30] dargestellt.

Herrn U. OLTHOFF danke ich für die Herstellung dieser Vergleichsverbindung. Herrn H. THIEME, Universität Leipzig, schulde ich Dank für die Überlassung einer Probe Saligenin (Salicylalkohol). Frau M. DINGLER bin ich für die Hilfe bei der Durchführung der chromatographischen Arbeiten zu Dank verpflichtet.

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Eingegangen am 8. Dezember 1969; geänderte Fassung am 3. Juni 1970

J. Chromatog., 51 (1970) 330–334

CHROM. 4847

Argentation thin-layer chromatography with silver oxide

I. Separation of pyridine homologues*

During the last few years argentation TLC, initially developed for lipid separation, has found extensive use in the separation of fatty acids, neutral lipids, phenylhydrazones of aldehydes and ketones, and several classes of unsaturated compounds, capable of complexing the silver ion (for a good review see ref. 1). Argentation chromatography has also been used for column and GLC separations. $AgNO_3$ is the main compound, but not the only one[1], used and the principle of true argentation chromatography is the complexation of the olefinic bond with silver ions[2].

We have tried to extend argentation TLC to pyridine and other nitrogen heterocyclics that are known to complex silver ions giving crystalline complexes[3–5] with both $AgNO_3$ and $AgClO_4$. Some nitrates[4,6] are soluble in $CHCl_3$ and other organic solvents, and some of the heterocyclic bases give complexes with a 3:1 proportion of

* This work was supported by a grant from the Fundação de Amparo à Pesquisa do Estado de São Paulo.

ligand to metal[3-5], which could be explained by partially covalent properties of the NO_3–Ag bond, giving tetrahedral sp^3 instead of digonal sp complexes[4-6]

Chromatography was first tried with 5% $AgNO_3$ on SiO_2 plates, with several solvents, and some separation was obtained. But all the solvents in which there was some separation, leached some $AgNO_3$, some of them removing it almost completely from the lower part of the plate.

This is why we decided to try Ag_2O on SiO_2 as the "adsorbent". Ag_2O has recently been shown to complex pyridine and its homologs[7], when a sufficient excess of water is used, giving strong complex bases.

First, we tried an "activated" adsorbent, obtaining good separations, and then thin-layer plates equilibrated in a water saturated atmosphere, expecting to have true complexation chromatography with the participation of the water retained by the silica gel. Both methods gave good results, as can be seen below, and we are extending them to other classes of compounds. The activated adsorbent is of more general use, giving separations where the water equilibrated plates are useless.

Experimental

Preparation of the plates. The 5% $AgNO_3$ plates were prepared as usual[1]. For Ag_2O plates, 5% $AgNO_3$ solution was added to the Silica Gel G, to give 5% Ag, in the total solids and enough 5% NaOH solution was slowly added to react stoichiometrically with all the silver ions, while mixing thoroughly. After drying 1 h, some of the plates were activated in an oven at 105–110° for 1 h (activated Ag_2O plates) and the others were equilibrated for 24 h in a desiccator with water.

Activated Ag_2O on Silica Gel H plates were prepared by the same technique, giving some good separations. Ag_2O-on-alumina plates were prepared by the same method but preliminary results were bad, so we did not further study the possibility of their use.

Identification reagents. (1) Iodine vapour gives brown spots only with large amounts of the pyridines. (2) The Dragendorff reagent for alkaloids[8] was the best reagent in our experience, giving red or orange spots on a light orange background. (3) Sulphuric acid did not reveal all the spots. (4) Formaldehyde gave black spots on a dark grey background with $AgNO_3$ plates, but with Ag_2O plates it did not give good results.

Solvents. Benzene, petroleum ether, and similar non-polar solvents did not elute the pyridines. Alcohols elute the pyridines near the front, but do not separate them. *tert.*-Butyl alcohol reacts with the Ag_2O and leaves a black band on the immersed part of the plate.

Discussion

We tried 36 solvent systems, of which 19 are given in Tables I and II, for activated and non activated plates, equilibrated in a water saturated atmosphere of Ag_2O on Silica Gel G.

For the last 5 of these solvents, comparison is made with Silica Gel G plates treated in the same way (activated or equilibrated with water), and the results are seen in Tables III and IV.

The R_F values are the mean of 3 runs, and the difference was less than 0.01 R_F units, for plates prepared from the same slurry. For plates prepared from different

TABLE I

$R_F \times 100$ ON ACTIVATED SILICA GEL G + Ag_2O PLATES

Solvents: (1) Me_2CO–C_6H_6(2:3); Me_2CO–$CHCl_3$(2:3); (3) Me_2CO–*sec.*-BuOH–C_6H_6(4:3:3); (4) Me_2CO–*n*-BuOH–$CHCl_3$(4:3:3); (5) Me_2CO–*n*-BuOH(3:2); (6) $CHCl_3$–MeOH(3:2); (7) MeCOEt–$CHCl_3$–*n*-BuOH(4:3:3); (8) MeCOEt–*n*-PrOH(7:3); (9) MeCOEt–*iso*-PrOH (4:1) (10) AcOEt; (11) AcOEt–Me_2CO(1:1); (12) *tert.*-BuOH–$CHCl_3$(3:2); (13) Me_2CO–*tert.*-BuOH–$CHCl_3$(4:3:3); (14) *iso*-PrOH; (15) Me_2CO–AcOEt–C_6H_6(4:3:3) (16) AcOEt–MeCOEt(1:1); (17) *tert.*-BuOH–AcOEt–C_6H_6(4:3:3); (18) *tert.*-BuOH–$CHCl_3$–C_6H_6(4:3:3); (19) Me_2CO–$CHCl_3$–C_6H_6(4:3:3). A preliminary selection of the solvents was made from the STAHL eluotropic series[13] followed by combination of the more common solvents that gave good separation.

Compounds: (I) 2-picoline; (II) 3-picoline; (III) 4-picoline; (IV) 2,4-lutidine; (V) 3,5-lutidine; (VI) 2,4,6-collidine; (VII) 2-ethylpyridine; (VIII) 4-ethylpyridine; (IX) 4-propylpyridine; (X) 2,6-lutidine; (XI) 5-ethyl-2-methylpyridine; (XII) pyridine.

Solvent	*Compound*											
	I	*II*	*III*	*IV*	*V*	*VI*	*VII*	*VIII*	*IX*	*X*	*XI*	*XII*
1	40	47	38	31	54	26	64	49	57	40	52	53
2	47	55	46	42	65	34	67	55	63	50	64	58
3	68	77	71	68	83	55	82	75	76	55	75	67
4	65	71	67	64	80	61	83	78	50	63	79	69
5	80	76	67	67	80	53	85	80	85	63	83	—
6	85	87	84	87	93	84	92	91	94	85	93	86
7	48	56	46	40	61	21	—	53	61	41	60	—
8	59	67	59	55	75	43	79	—	81	62	75	—
9	85	74	83	60	83	42	85	75	83	56	80	—
10	28	36	26	18	42	20	47	39	42	26	40	34
11	56	63	54	46	70	44	77	71	74	54	69	60
12	54	68	62	51	78	33	69	73	74	45	66	62
13	60	69	61	55	77	38	84	83	85	60	50	70
14	—	62	58	56	81	50	—	69	72	41	68	—
15	47	55	48	34	60	25	75	62	66	47	57	56
16	43	50	40	30	56	24	62	60	58	38	54	50
17	42	58	48	36	69	20	38	56	46	39	74	24
18	60	71	64	51	78	37	74	84	75	41	70	62
19	51	58	45	41	64	31	72	54	58	43	60	57

slurries with the same composition, we observed some larger differences in R_F (about 0.05 to even 0.1 units).

Comparing Tables I and III, and Tables II and IV, it can be seen immediately that the separations are better on Ag_2O–Silica Gel G plates than on pure Silica Gel G alone, both on the activated and on water-equilibrated plates, for the given solvents. On the plates containing Ag_2O, the R_F values are lower, because complexation slows down the pyridines.

We could not always find a simple correlation between the order of the R_F values and the basicity[10] (inductive effect of substituents) of the pyridines, but on taking into account the dipole moments and stability constants, the picolines and lutidines travel up the plate in all our solvents in an order that can be explained.

That the dipole moments of the substituted pyridines are in good agreement with those expected from the polar effects of the substituents on the different positions in the pyridine ring can be seen well from the literature data[10,12]. Dipole moments and pK_a values of some of the compounds of interest are given in Table V.

With respect to the picolines[10] we have for the dipole moment $2 < 3 < 4$ as would be expected from the direction of the substituent moment vectors.

TABLE II

$R_F \times 100$ ON SILICA GEL G + Ag_2O PLATES EQUILIBRATED IN A WATER SATURATED ATMOSPHERE
For key to solvents and compounds see Table I.

Solvent	Compound											
	I	*II*	*III*	*IV*	*V*	*VI*	*VII*	*VIII*	*IX*	*X*	*XI*	*XII*
1	57	68	60	54	65	41	75	63	71	57	67	65
2	72	85	77	71	90	65	84	72	79	68	80	69
3	66	74	69	68	78	48	77	69	77	60	77	70
4	70	79	77	78	87	72	87	85	90	76	89	75
5	—	82	71	74	86	51	89	86	91	80	80	—
6	84	85	83	84	89	82	87	85	88	80	88	82
7	47	55	45	43	65	31	62	52	63	43	59	—
8	67	78	73	72	85	60	84	79	64	70	89	—
9	—	88	62	68	87	47	88	82	88	58	83	60
10	21	32	24	17	46	19	53	44	49	25	45	28
11	69	70	72	73	87	71	88	85	91	66	88	65
12	66	79	63	65	88	45	79	83	87	60	85	72
13	63	75	68	62	82	49	78	81	82	56	77	64
14	—	69	—	72	88	74	—	77	90	55	85	—
15	50	59	47	41	60	33	65	58	71	55	68	62
16	27	41	29	20	44	13	60	47	42	24	33	36
17	30	50	32	25	55	14	57	56	53	21	37	38
18	50	70	60	48	75	23	81	82	86	47	71	62
19	44	51	42	33	55	27	66	63	58	41	48	47

TABLE III

$R_F \times 100$ ON SILICA GEL G ACTIVATED PLATES
For key to solvents and compounds see Table I.

Solvent	Compound											
	I	*II*	*III*	*IV*	*V*	*VI*	*VII*	*VIII*	*IX*	*X*	*XI*	*XII*
15	84	88	85	82	87	82	91	85	96	90	95	83
16	82	87	81	80	86	79	92	87	97	91	96	84
17	74	81	86	85	91	82	91	87	86	74	94	—
18	95	97	91	92	99	93	95	95	98	92	98	89
19	81	81	87	88	94	89	99	96	99	97	91	81

TABLE IV

$R_F \times 100$ ON SILICA GEL G PLATES EQUILIBRATED IN A WATER SATURATED ATMOSPHERE
For key to solvents and compounds see Table I.

Solvent	Compound											
	I	*II*	*III*	*IV*	*V*	*VI*	*VII*	*VIII*	*IX*	*X*	*XI*	*XII*
15	87	87	95	96	97	94	97	96	100	97	96	89
16	88	94	80	78	100	90	100	100	100	92	100	90
17	72	77	84	86	81	74	95	96	93	85	96	94
18	86	90	89	92	95	86	95	91	93	89	96	87
19	75	78	67	69	87	87	88	79	78	67	78	80

TABLE V

pK_a VALUES[9] AND DIPOLE MOMENTS[10] OF ALKYLPYRIDINES

Compound	*pK_a*	*μ (benzene)*
Pyridine	5.17	2.21
2-Picoline	5.97	1.97
3-Picoline	5.68	2.40
4-Picoline	6.02	2.60
2-Ethylpyridine	5.97	—
4-Ethylpyridine	6.02	2.65
4-Isopropylpyridine	6.02	—
2,6-Lutidine	6.75	1.66
2,4-Lutidine	6.79	2.30
3,5-Lutidine	6.20	2.58
2,4,6-Collidine	7.59	—

The order of the pK_a values is $3 < 2 < 4$ and the order of R_F values is, in some of the solvents, $3 > 2 > 4$. The inverse order càn be explained as stronger complexation of the more basic compounds, slowing down the rate of travel on the plate. In some other solvents the order of R_F values of the picolines is $3 > 4 > 2$, which could possibly be the result of steric hindrance making more difficult the release of 2-picoline from the complex and thus slowing it. The stability constants of Py_2AgNO_3 (ref. 3) and Py_2AgClO_4 (ref. 11) type complexes are in the order $2 > 4 > 3$.

For the lutidines, the order of dipole moments[11] is $2,6 < 2,4 < 3,5$ and pK_a values $2,4 < 2,6 < 3,5$; the order of the R_F values varies somewhat with the solvent, but is always the same as the pK_a values or as the dipole moments (polarity). The obvious conclusion is that basicity and polarity differences of pyridine and its homologues and, in some cases, the stability of the complexes play a major role in their separation in argentation TLC with silver oxide.

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Received May 21st, 1970

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CHROM. 4848

Isotachophoresis*

Experiments in methanol

Volatile liquids can be used for electrophoretic separations by means of capillaries and closed systems, however, not any liquid can be used as the solvent. The mean properties should be about the same as those of water. This means that all substances to be separated should reasonably dissolve and be ionised in it, and the solvent must solvate the ions. It is also important that the dielectric constant E as well as the dipole moment μ have about the same values as those of water.

Methanol was chosen because it is similar to water in that the molecule is small, the boiling point is 64.7°, the dielectric constant $E = 32.63$ (25°), the dipole moment $\mu = 1.70$ (gas phase) and it has an amphoteric character.

Whereas the mobility (cm²/Vsec) of K^+ and NH_4^+ in water is about the same, in methanol their mobility differs considerably. The mobility of the hydroxyl ion in water has the highest value of all anions; in methanol however the hydroxyl ion can be a zone sandwiched between, for instance, nitrate and acetate.

TABLE I

THE MOBILITIES OF SOME ION SPECIES

The mobilities are calculated as average values and are taken from Ref. 5. The step-heights are measured from our electropherograms.

Ion	*Water*		*Methanol*	
	$m \times 10^5$ (cm²/Vsec)	*h (mm)*	*$m \times 10^5$ (cm²/Vsec)*	*h (mm)*
OH^-	204.6	—	54.8	158
Br^-	81.3	105	58.6	153
I^-	79.8	106	66.1	138
Cl^-	79.0	107	54.1	164
NO_3^-	74.0	111	63.9	148
F^-	56.5	134	42.2	196
$HCOO^-$	56.6	136.5	51.7	176
CH_3COO^-	42.6	162	40.8	191
H^+	362.2	34	149.7	94
Cs^+	81.3	132	62.5	163
Rb^+	80.3	131	58.4	174
NH_4^+	76.9	138	58.7	170
K^+	76.7	138	54.4	181
Na^+	52.8	182	46.8	206
Li^+	40.2	220	40.4	236

Table I shows the mobility of some ion species in water and methanol and their corresponding step-heights measured in our electropherograms.

* In earlier papers the name Displacement Electrophoresis" was used. Ref. 1 gives the reason why the name is changed to "Isotachophoresis".

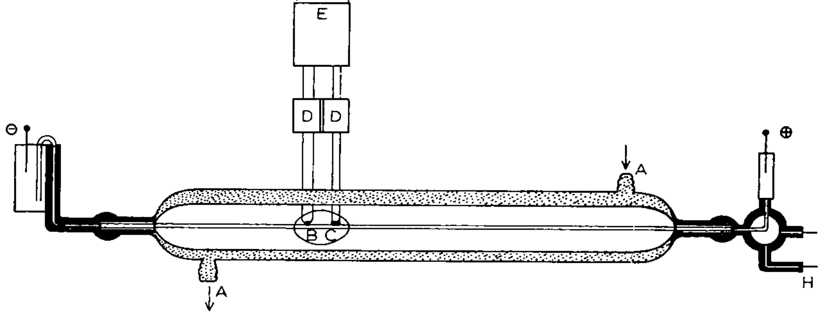

Fig. 1. Apparatus for isotachophoresis. A = thermostated water, B = integral thermocouple, C = differential thermocouple, D = preamplifier, E = two-pen recorder, H = flat four-way tap.

Apparatus

For the experimental work an apparatus similar to that described in ref. 2 was used, because methanol will destroy our injection systems made of perspex[3]. Fig. 1 shows the apparatus schematically. A teflon tube mounted in a double walled glass vessel (like a Liebig-condenser), leads to a flat four-way tap H. The tap can be used

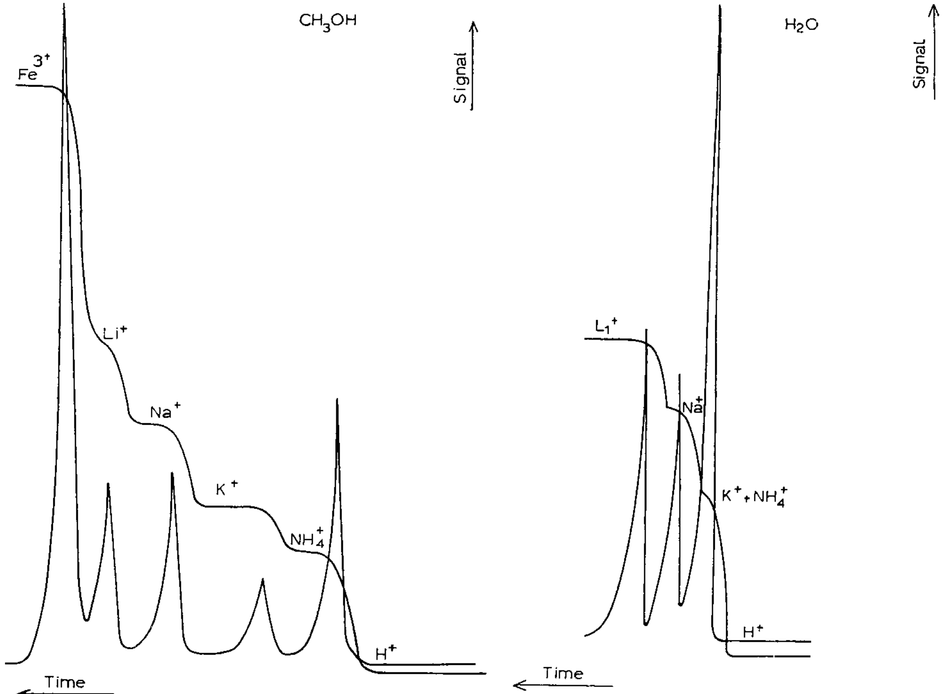

Fig. 2. Electropherogram of the separation of cations where methanol instead of water is used as a solvent.

Fig. 3. Electropherogram of the separation of cations with water as solvent. Under the conditions described no separation between K^+ and NH_4^+ was found.

as a sample tap and connects the capillary and a compartment filled with the terminal electrolyte.

Because it was difficult to make the flat tap fit tightly, the reproducibility of the analysis was the same as that in earlier experiments (2%). At the other end, the capillary was dipped into a reservoir containing the same electrolyte as that filling the capillary. Thermocouples were again mounted as the detectors[3,4].

The signals from these thermocouples are led to a preamplifier and from these to a two-pen recorder. It should be mentioned that the electropherogram must be interpreted as follows[2]: The step height of the integral curve is a measure for the ion species present in the sample. The length of the step or the distance between two successive peaks is a measure of the amount of an ion species injected.

Separation of cations

Fig. 2 shows an electropherogram of a separation of cations. The conditions for the analysis were as follows: The leading electrolyte was HCl ($1 \cdot 10^{-2}$ M) in methanol. No polymer for stabilisation or buffer was added. As intermediate ions[1] 50 μl of a mixture of NH_4Cl ($5 \cdot 10^{-4}$ M), KCl ($7 \cdot 10^{-4}$ M), NaCl ($5 \cdot 10^{-4}$ M) and LiCl ($5 \cdot 10^{-4}$ M) in methanol was used. The terminator[1] was $FeCl_3$ ($1 \cdot 10^{-2}$ M) in methanol. The current was stabilised and was 70 μA. The starting-voltage was about 5 kV; the end-voltage was about 13 kV. The capillary tube (teflon) was 100 cm long, the outside diameter was 0.7 mm and the inside diameter was 0.45 mm. The analysis time was 45 min. The speed of the recorder paper was 10 mm/min.

Fig. 3 shows the same electropherogram if water is used as a solvent. Instead of

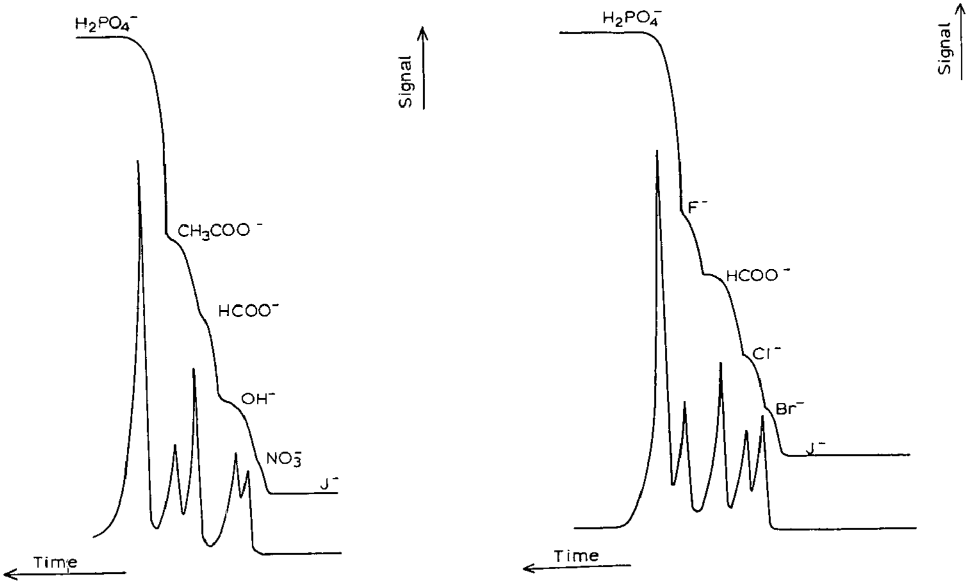

Fig. 4. Electropherogram of the separation of anions. The hydroxyl ion in methanol is not any longer the fastest moving anion.

Fig. 5. Electropherogram of the separation of the halides. Formic acid was added to make it possible to compare the electropherogram with the one in Fig. 4.

Fe^{3+}, Li^+ was used as the terminator. The analysis time was 30 min. The speed of the recorder paper was 5 mm/min.

It will be clear from Figs. 2 and 3 that K^+ and NH_4^+ are not difficult to separate if methanol is used as a solvent.

Separation of anions

Fig. 4 shows an electropherogram of a separation of anions. The conditions for this analysis were as follows: The leading electrolyte was NaI ($1 \cdot 10^{-2}$ *M*) in methanol. No polymer for stabilisation or buffer was added. As intermediate ions 50 μl of a mixture of $NaNO_3$ ($5 \cdot 10^{-4}$ *M*), NaOH ($2 \cdot 10^{-4}$ *M*), HCOONa ($5 \cdot 10^{-4}$ *M*) and CH_3-COONa ($5 \cdot 10^{-4}$ *M*) in methanol was used. The terminating electrolyte was H_3PO_4 (1.10^{-2} *M*) in methanol. The analysis time was about 50 min. The speed of the recorder paper was 5 mm/min. The current was stabilised and was 70 μA. The starting-voltage was about 7 kV; the end-voltage was 13 kV. The hydroxyl ion is sandwiched between nitrate and formate.

Fig. 5 shows an electropherogram of the separation of the halides. The conditions of this analysis were about the same as those mentioned above. The terminator was H_3PO_4.

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Received May 21st, 1970

CHROM. 4852

Thin-layer chromatography of inorganic sulphur compounds

Procedures have been described for the separation of inorganic sulphur compounds by paper[1–5] and ion-exchange[6–8] chromatography, but few systems using thin-layer chromatography have been described[9,10]. Owing to the high reactivity of thiosulphate and polythionates in the mixtures encountered in metabolic studies of such compounds[1,7,11], a method for the rapid analysis of these mixtures was desirable. The procedures described below enable separations of sulphate, thiosulphate, trithionate, tetrathionate and thiocyanate to be made.

Experimental

Stationary phases were sheets of Gelman instant thin-layer chromatography (ITLC) media, types SA and SG (20 × 20 cm). Chromatograms were run in Gelman ITLC chambers at 22–25°. Marker compounds (usually 10 μg in 10 μl water) were applied 3 cm from the edge of the sheets and the solvent allowed to run 13 cm beyond the origin line. Sources of marker compounds were as follows: AnalaR grade KSCN and $K_2S_2O_3$ from the British Drug Houses; ^{35}S-labelled sulphate and thiosulphate from The Radiochemical Centre (Amersham, Great Britain); ^{35}S-labelled thiocyanate was synthesised by the cyanolysis of labelled tetrathionate; and potassium tetrathionate[12] and trithionate[13] were synthesised. Solvents were AnalaR or best commercial grades and were not further purified. Sulphur compounds were detected by spraying dried chromatograms with 8% (w/v) silver nitrate in acetone containing 10% (v/v) water, and by radioautography and liquid scintillation counting when ^{35}S-labelled materials were used.

Results and discussion

A number of solvents, including several suitable for paper chromatography, were generally unsatisfactory for TLC separations in this study (Table I). Solvent S_2 is very useful for paper chromatography[2], and did separate the four compounds tested. The rather large spots produced did, however, decrease the efficiency of sepa-

TABLE I

TLC OF THIONATES AND THIOCYANATE ON SA MEDIUM USING MIXED SOLVENTS

Solvents used were: S_1 1-butanol–acetone–water (40:40:30)
S_2 1-butanol–pyridine–acetic acid–water (30:20:6:24)
S_3 1-butanol–pyridine–acetic acid–water (90:3:1:6)
S_4 1-propanol–pyridine–water (50:35:50)
S_5 ethanol–pyridine–water (80:10:5)
S_6 ethanol–pyridine–water (60:30:10)
S_7 2-heptanol–methanol–water (85:10:5)

Compound	R_F						
	S_1	S_2	S_3	S_4	S_5	S_6	S_7
Thiosulphate	0.68	0.50	0.02	0.84	0.45	0.54	0.05
Trithionate	0.76	0.61	0.26	0.86	0.72	0.74	0.05
Tetrathionate	0.79	0.67	0.39	0.86	0.78	0.76	0.05
Thiocyanate	0.84	0.78	0.62	0.82	0.76	0.75	0.22

TABLE II

TLC OF INORGANIC SULPHUR COMPOUNDS ON SA MEDIUM USING ALCOHOLS AS SOLVENTS

Solvents used were: S_8 methanol
S_9 99% ethanol
S_{10} 5% (v/v) water in 1-propanol
S_{11} 5% (v/v) water in 1-butanol
S_{12} 5% (v/v) water in 1-pentanol
S_{13} 5% (v/v) water in 1-hexanol
S_{14} 1-octanol saturated with water
S_{15} 1-propanol–methanol (1:1)
S_{16} 5% (v/v) water in 2-butanol

Compound	R_F								
	S_8	S_9	S_{10}	S_{11}	S_{12}	S_{13}	S_{14}	S_{15}	S_{16}
Sulphate	0[a]	0	0	0	—	—	—	0[a]	—
Thiosulphate	0.78	0.25	0.06	0.02	0.10	0	0	0.49	—
Trithionate	0.84	0.73	0.54	0.35	0.09	0.03	0.03	0.73	0.27
Tetrathionate	0.84	0.80	0.63	0.46	0.19	0.10	0.04	0.77	0.37
Thiocyanate	0.83	0.79	0.71	0.64	0.47	0.38	0.36	0.77	0.60
Approx. time for standard run (min)	45	85	140	180	300	390	520	90	240

[a] See text.

ration of thiocyanate and the polythionates from each other. Thiocyanate was separated effectively from all the other compounds only by solvent S_7.

In contrast, all four compounds and sulphate could be separated from each other using pure alcohols or alcohols containing small quantities of water (Table II). No single solvent effected perfect separation of all five compounds, but 5% (v/v) water in 1-butanol (S_{11}) gave complete separation of thiosulphate, trithionate, tetrathionate and thiocyanate, while methanol (S_8) or methanol–propanol (S_{15}) completely resolved sulphate from the other compounds. Solvent S_{15} allowed isolation of sulphate, thiosulphate and thiocyanate or polythionates from each other. Resolution of a mixture of all five compounds can thus be achieved by two-dimensional chromatography in solvents S_{15} and S_{11}. The behaviour of sulphate in solvents S_{15} and S_8 indicated S_{15} to be preferable. Sulphate showed no significant movement in ethanol or longer-chain alcohols, but tended to streak from the origin in solvents S_{15} and S_8. This streaking was more pronounced with S_8 and the distance streaked increased with the amount of sulphate on the thin layer. When 30 μg of sodium sulphate was applied, 80% remained within 2 cm of the origin, but the remainder streaked a further 7 cm in S_8. With methanol–propanol (S_{15}), 80–85% of the sulphate remained within 2 cm of the origin, most not migrating at all, and none moved further than 3.5 cm from the origin. The "tail" of the sulphate thus remained 1.5–2 cm behind the rear of a 20 μg spot of potassium thiosulphate.

Pure 1-propanol was an unsatisfactory solvent, as sulphate and thiosulphate remained at the origin, while trithionate and tetrathionate gave spots of R_F 0.36 and 0.54 but streaked from the origin.

Gelman ITLC SG medium was not generally suitable for these separations, although with solvent S_{14} thiocyanate (R_F 0.78) was separated from all the other compounds, which did not migrate.

In conclusion, chromatography on Gelman ITLC SA media using 5% water in 1-butanol and a 1:1 mixture (v/v) of methanol–1-propanol effects complete separation of sulphate, thiosulphate, trithionate, tetrathionate and thiocyanate in not more than 5 h. This technique is of use in analysing mixtures such as are encountered during bacterial sulphur metabolism[14,15] or during reaction of thionates with cyanide[16].

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First received May 12th, 1970; revised manuscript received June 4th, 1970

CHROM. 4820

The separation of the valency states of rhenium in HCl solutions

While the separation of the valency states of technetium has been studied extensively in this laboratory no separations have so far been reported in the literature of the rather similar valency states of rhenium. Preliminary results in this direction are reported in this note.

Experimental

Chromatograms were made on the following papers and thin layers: Whatman 3MM, Whatman DE-20, Macherey, Nagel strongly basic ion-exchange cellulose paper, Amberlite SA-2 paper, Amberlite SB-2 paper, MN Polygram Cel 300 DEAE.

Ammonium perrhenate (Fluka) and hexachlororhenate (IV) (Fluka) in 2 *N* HCl were used. Pentavalent rhenium was freshly prepared each time by reducing perrhenate in 4 *N* HCl with two equivalents of stannous chloride.

All chromatograms were developed (usually on strips 20 × 3 cm) by the ascending method.

Re(VII) and Re(V) were visualised by spraying with HCl solution of $SnCl_2$–NH_4CNS. The rather stable $Re(IV)Cl_6^{2-}$ was visualised with Methylene Blue as it does not give a colour with stannous chloride–ammonium thiocyanate.

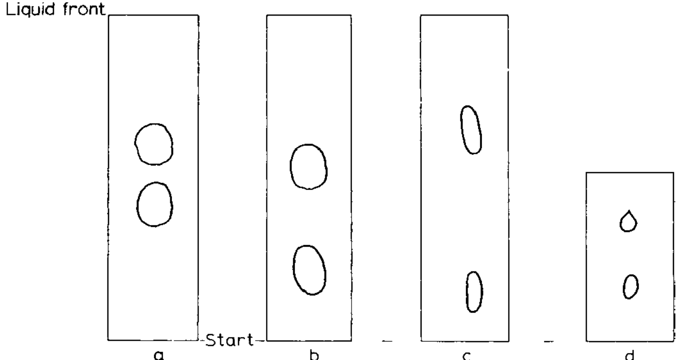
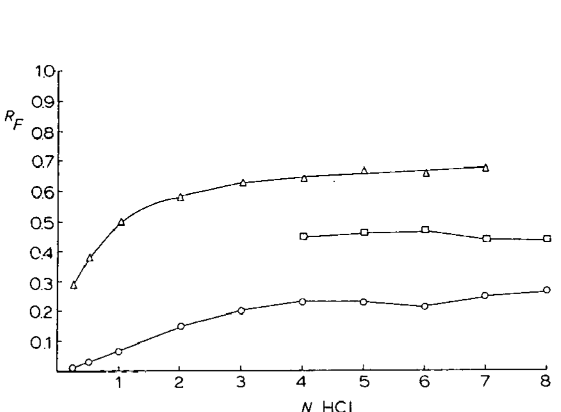


Fig. 1. Chromatograms of mixtures of ReO_4^- and $Re(IV)Cl_6^{2-}$. (a) Macherey, Nagel strongly basic cellulose anion-exchange paper, eluent 2 *N* HCl; (b) Whatman AE-30 paper, eluent 2 *N* HCl; (c) Whatman DE-20 paper, eluent 2 *N* HCl; (d) MN-Polygram Cel 300 DEAE, eluent 6 *N* HCl.

Results

The separation of Re(VII)–Re(IV) on various papers is shown in Fig. 1. The best separation was obtained on Whatman DE-20 paper and this paper was therefore selected for the separation of all three valency states. The R_F values of these are shown in Fig. 2. No separation of Re(VII) and Re(V) (oxidation?) could be obtained below 4 *N* HCl; above 8 *N* HCl perrhenate gives a very diffuse zone.

A good separation of Re(VII), Re(V), Re(IV) and Sn(IV) is obtained with 4 *N* HCl as eluent on Whatman DE-20 paper operating at 0° (in a refrigerator). The lower



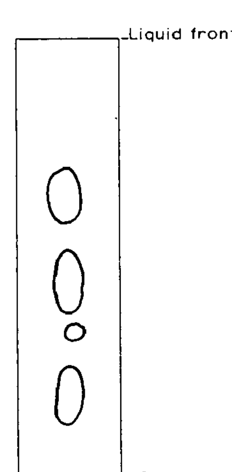


Fig. 2. R_F value–HCl concentration diagram for various valencies of rhenium on Whatman DE-20 paper developed at room temperature. ReO_4^- (△—△); Re(V) (□—□); $ReCl_6^{2-}$ (○—○).

Fig. 3. Separation of Re(VII), Re(V), Sn(IV) and Re(IV) (top to bottom) on Whatman DE-20 paper developed with 4 N HCl at 0°.

temperature inhibits the decomposition of Re(V) during the development. A typical chromatogram is shown schematically in Fig. 3.

During this work the possibilities of this separation in investigations of the solution chemistry of rhenium have been noted and these will be reported in detail in a subsequent publication.

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Received May 16th, 1970

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CHROM. 4836

Detection of some noble metals by heat coloration on thin layers with direct flame

Hitherto, little work has been carried out on the thin-layer chromatography (TLC) of such noble metals as platinum, palladium, etc. The present studies were undertaken to separate platinum, palladium, gold and copper by TLC with a singular coloration method. The method was suggested considering a long known fact[1] that colloidal solutions of platinum or gold have been easily prepared when the surface of the metal salt solution came in contact with the outer flame of a Bunsen burner. Prior to the experiments with mixtures, detection limits of the individual metals were established using the microsyringe (for gas chromatography) to apply a minute amount of each metal solution onto the thin-layer plate. As a result, 10 γ of Au, 5 γ of Pt, 1 γ of Cu and 2.5 γ of Pd were detected. By this chromatography method, every process of the experiment was simple and rapid.

Experimental

Silica Gel H (Merck, according to STAHL) was used as adsorbent. The powder was treated with HCl (1:1) to remove mainly iron and was washed thoroughly with distilled water using centrifugation repeatedly. The adsorbent was moderately dried in the air and thereafter the suction filter was applied. Finally, it was washed with benzene, and the powder was then dried in an oven at 120° for 2 h.

The thin-layer plates were prepared as follows: 2–2.5 volumes of water were added to one volume of the powder freed from impurities and mixed thoroughly until a slurry was obtained. The slurry was spread onto the glass plates (3.5 × 10 cm) using the applicator to give a layer 0.7 mm thick. (The thickness was not so critical for the experiment.)

After exposing the plates to air for 15 min, they were dried at 100–110° for 1 h. Sample solutions used were chloroauric acid, copper nitrate, chloroplatinic acid and palladium dichloride, containing 10 mg of the individual metallic ion in 1 ml. A minute amount of the solution containing four components was applied with the aid of a capillary tube to a point on the layer surface 1.2–1.5 cm from the lower edge of the plates. The plates containing the spotted samples were left exposed to the air for 15–20 min; the development was then allowed to take place. A small desiccator (base diameter, 11 cm) was used as the chromatographic tank in which the solvent had been placed to a depth of 5 mm. The thin-layer plates were put in the tank, resting against the wall, so that the lower edges were immersed in the solvent. The development was stopped when the solvent front had moved 7–9 cm from the starting point. The development time was 15–20 min.

Of several solvents tried, pure acetone (water content, 0.3–0.4%) gave the most satisfactory result. Dioxane–acetone (1:1) and dioxane–acetone–conc. HCl (200:100:1) were second to acetone for the separation of four components, although slight tailing could be seen in the case of the latter two systems.

The plates removed from the tank after development were then dried in the air. Finally, the whole surface of the thin layer was heated by contacting the oxidizing flame of the aerated Bunsen burner as uniformly (to prevent the glass plates from breaking) as possible for 1–1.5 min.

TABLE I

R_F VALUES AND SPOT COLORS

Metal (spot color)	*R_F values*		
	Acetone	*Dioxane–acetone–conc. HCl (200:100:1)*	*Dioxane–acetone (1:1)*
Au (purplish pink)	0.94	0.84	0.99
Pt (brown)	0.68	0.40	0.49
Pd (brownish black)	0.81	0.29	0.44
Cu (yellowish brown)[a]	0	0.79	0.09

[a] Mixtures probably of Cu_2O and CuO.

Results and discussion

All spots were colored by the heating. The colors of the individual spots and R_F values corresponding to solvents are given in Table I. Of course, before any separation experiment, the colors of the individual components caused by the heating, must be established. In this method, in addition to differences in the colors and locations of the spots, the palladium ion is very quickly reduced and, therefore, also facilitates the identification of those components.

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1 W. OSTWARD, P. WOLSKI AND A. KUHN, *Kleines Praktikum der Kolloidchemie*, Verlag von Theodor Steinkopff, Dresden and Leipzig, 1923, p. 4.

Received May 15th, 1970

Book Reviews

CHROM. 4842

Practical Manual of Gas Chromatography, edited by JEAN TRANCHANT, Elsevier, Amsterdam, 1969, 387 pp., price Dfl. 85.00, U.S. $ 24.

This is the English translation (with some revision) of the second French edition of a very popular manual. The English version will probably not be so popular due to the large number of competitive English books and the rather high price.

The book is written for practicing chromatographers and emphasizes the execution of specific techniques. Theoretical development of equations or concepts is not presented, but there are over 1600 references supplied for students eager to pursue the subject in more depth. Chapters included are: principles and retention values, isothermal–isobaric chromatography, programmed chromatography, apparatus, columns, detectors, qualitative analysis, quantitative analysis, and applications. The coverage is more than adequate and the style is easy to follow.

Unfortunately there are numerous mistakes, of which the following is a small selection:

Programmed temperature resolution of two peaks can never be better than isothermal operation. A lower isothermal temperature will increase resolution at the expense of time (p. 35).

Velocity of carrier gas is *more* (not less) at the exit of the column (p. 93)

Porapak is listed as a support together with Chromosorb. Whereas a small amount of liquid phase can affect its retention characteristics, most work employs Porapak as a unique column packing, its partitioning and adsorptive properties providing the separation (p. 98).

In describing a flame ionization detector, "the response for water is 10^4–10^6 times weaker than for hydrocarbons". In fact the detector does not respond to water. Water may cool the flame and produce a baseline shift but the detector does not measure water (p. 176).

Despite the stated goal (in the preface) of not discussing obsolete equipment, reference is made to several such items: F & M blood gas analyzer which was discontinued in 1965 (p. 81), Aerograph Pestilyzer discontinued in 1965 (p. 82), Aerograph model 900 simulated distillation unit, of which only two prototypes were produced in 1966 (p. 88).

The references cited are disproportionately French, and whereas this is useful in the French edition, it is a disadvantage in the English edition. In many cases references to fundamental work in 1967 and 1968 are not included.

In summary, the editor and his contributors have worked hard to prepare a thorough and well organized manual which is presented in a practical and easily readable style. Unfortunately it is full of many minor mistakes, is not current in the references cited, and is too expensive for the intended audience.

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CHROM. 4797

Techniques and Methods of Polymer Analysis. Vol. 3. Characterization and Analysis of Polymers by Gas Chromatography, by MALCOLM P. STEVENS, Marcel Dekker Inc., New York, 1969, 198 pages, price $ 12.75, £ 6-2.

It is immediately apparent that this eminently readable little book is the work of a man who is interested to the point of involvement in his subject. An appreciation of certain of the problems of polymer analysis is combined with an objective, ordered approach to their solutions and, where pertinent, suggestions are offered as to how a method might be improved.

After a brief introduction wherein the salient features of gas chromatography are defined, the conventionalities of the detection and determination of volatiles in a raw synthetic resin or a composition thereof are considered. Detailed examples are appended and commentary is offered on the significance of specific findings.

Chemical degradation techniques for polymers containing reactive functional groups are then classified, summarised and liberally exemplified. Particular attention is paid to a representative range of natural products that may be encountered as either a primary presence or as an adjunct in a polymer composition.

The ensuing section (which comprises nearly half the text) demonstrates the very wide applicability of the pyrolysis technique. Modes of degradation of a number of polymers derived from ethylenically unsaturated monomers are enunciated and many cases treated mechanistically. Perhaps more emphasis should have been laid upon the criticality of sample size within the paragraphs devoted to an appraisal of pyrolysis and certainly experimental detail would have been reinforced by stringent stipulation of the sample size employed in each of the examples. Differences over four or five orders could well account for certain conflicting elements.

The penultimate chapter deals all too briefly with radiolysis, reaction gas chromatography, oxidative degradation and inverse gas chromatography. It must be conceded that there is little in the literature that can be quoted in the context of polymer chemistry but a little more detail could well provide the stimulus for further work in these fields.

Finally there is a comprehensive survey of the application of gas chromatography to problems of monomer purity. Logically, this should appear before the second chapter on volatiles in resins for such detail could enhance appreciation of the reasons for some of these presences. Moreover, impurities retained in the polymer system frequently point to not only the monomer but also its source.

The index has been both sensibly and competently compiled and the author index is a monument to Mr. STEVENS' diligence. It is regrettable that E. R. ADLARD has been perpetuated as E. E. ADLAND but in general the references appear to be accurate and devoid of ambiguity.

In summary, the author must be commended for producing an informative volume that can effectively double as a laboratory manual, additionally, the publishers deserve mention for the presentation and production which cannot be faulted.

Vinyl Products Ltd., Carshalton, Surrey (Great Britain) C. E. ROLAND JONES

News

Meetings

Joint Meeting of the Chromatography and Electrophoresis Group and the East Anglia Section of the Society for Analytical Chemistry.

A Joint Meeting of the Chromatography and Electrophoresis Group and the East Anglia Section of the Society for Analytical Chemistry will be held at 2.30 p.m. on Friday, September 25th, 1970, in Lecture Theatre No. 2, University Village, University of East Anglia, Norwich, Great Britain.

The subject of the meeting will be: *The Applications of Chromatography in Food and Agriculture.*

Programme

2.30 p.m. Acid Catalysed Reactions of Monoterpenes, by D. A. BAINES, M.Sc., R. ALAN JONES, M.A., B.Sc., Ph.D., T. C. WEBB, M.Sc., and I. H. CAMPION-SMITH, M.Sc. (University of East Anglia, Norwich).

3.15 p.m. Some Applications of Thin-layer Chromatography to Pesticide Analysis, by M. WAKEFIELD, B.Sc., T. TAYLOR, A.I.M.L.T., and J. S. LEAHY, M.A., A.R.I.C. (Huntingdon Research Centre).

4.00 p.m. Tea.

4.30 p.m. Analysis of Food Constituents by Gas Chromatography and Associated Techniques, by T. GALLIARD, B.Sc., Ph.D., A.R.I.C., and P. A. T. SWOBODA, M.Sc., M.A., Ph.D. (ARC Food Research Institute, Norwich).

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Paper Chromatography

8. SUBSTANCES CONTAINING HETEROCYCLIC OXYGEN

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10. CARBOHYDRATES

10a. Mono and oligosaccharides; structural studies

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11. ORGANIC ACIDS AND LIPIDS

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21 . PURINES, PYRIMIDINES, NUCLEIC ACIDS AND THEIR CONSTITUENTS

21a. Purines, pyrimidines, nucleosides, nucleotides

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22. ALKALOIDS

BENEŠOVÁ, V., SAMEK, Z., HEROUT, V. AND ŠORM, F.: Plant substances. XXIX. Isolation and structure of two new indole alkaloids from *Riccardia sinuata* (Hook.) Trev. *Collection Czech. Chem. Commun.*, 34 (1969) 1807–1809—silica gel.

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CHROM. 4857

STATISTICAL PROCESSING OF CALIBRATION DATA IN QUANTITATIVE ANALYSIS BY GAS CHROMATOGRAPHY

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(Received June 2nd, 1970)

SUMMARY

The requirements that have to be met in the processing of calibration data by the linear regression method in quantitative gas chromatography are discussed and documented on four typical variants of the absolute calibration technique.

A mean slope method has been suggested for the precise processing of the data in cases where the statistically dependent variable displays a constant relative error. The effect of the systematic error on the course of the calibration line has been shown.

INTRODUCTION

Methods involving the work with a calibration curve are among the very popular procedures in quantitative analysis by gas chromatography. The most used methods are variants of the absolute calibration and internal standard techniques[1]. The calibration curve is often constructed by merely interlacing the experimental points by rule of thumb; however, when more precise work is required, it is necessary to process the calibration data with the aid of statistics. While in the first case mentioned there are no special limitations, in the second case, it is necessary to consider carefully the character of the problem in hand and to match the procedure of obtaining the most plausible curve with the requirements dictated by statistics[2].

THEORETICAL

The plotting of a calibration line by statistically processing the experimental data may be characterized as the determination of the most plausible linear relation between a number of the values of a random variable y and a nonrandom variable x. Then, the relation obtained provides for the best estimation of a value Y of the variable y for a given precise value X of the variable x. From the statistical viewpoint, the above procedure is characterized as linear regression, and the calibration line obtained corresponds to the regression straight line. Most of the experimental techniques of quantitative gas chromatography (the standard addition technique is an exception[1]) merely produce calibration lines that pass through the origin of the coördinate system.

To follow the correct procedure in the linear regression, it is necessary to decide

which of the variables involved should be considered as a dependent one and to ascertain the character of the variance of the above variable in the calibration range. From the statistical point of view, the variable which is looked upon as dependent is that one the values of which are substantially less precise than those of the other variable, which is considered as independent. However, this statistical concept has nothing in common with the actual relationship between the variables and, furthermore, is in no way connected with the order of obtaining the individual values, *e.g.* in the premise "given–found".

Hence, there are two situations which may occur in reading out the results of an analysis from the calibration line:

(1) The result corresponds to the dependent variable y

In this case, the value of the result required (Y) for a given precise value X is read out from the calibration line with a slope K according to the relation $Y = KX$. Thus, only the variance of the slope, S_K^2, has any effect on the variance of the result, S_Y^2, the latter being given by $S_Y^2 = X^2 S_K^2$.

(2) The result corresponds to the independent variable x

This case represents a situation when the value X is read out for a measured value Y from the calibration line corresponding to the relation $X = Y/K$, so that the variance of the final result of analysis is given by the relation $S_X^2 = (Y^2 S_K^2/K^4) + S_Y^2/K^2$. Hence it follows that the variance of the result comprises both the variance of the slope of calibration line and the variance of the measured value Y.

When considering the properties of the variance of the dependent variable, it is important to know whether the variance is constant within the calibration limits and whether its values are dependent on the value corresponding to the quantity measured. In quantitative gas chromatographic analysis, one is concerned most frequently with the following situations:

(a) The dependent variable has a constant variance

In this case, the calculation of the most plausible slope of the calibration line may be carried out by the least squares method. In order to use this the constancy of the variance of the dependent variable is a necessary condition (*cf.* ref. 2). The estimation of the calibration line slope is given by $K = \sum^n [(Y - \bar{Y})(X - \bar{X})] / \sum^n (X - \bar{X})^2$ where $\bar{Y}$ and $\bar{X}$ are the arithmetic averages of the respective values measured. The variance of the slope is given by $S_K^2 = (\sum^n Y^2 - K\sum^n XY)/(n - 2)\sum^n (X - \bar{X})^2$ where n is the number of experimental points. The expression $(\sum^n Y^2 - K\sum^n XY)/(n - 2)$ gives the variance of the dependent variable, S_Y^2. This variance may be determined independently of the construction of the calibration line (*cf.* ref. 3), the variance of the slope being given by $S_K^2 = S_Y^2/\sum^n (X - \bar{X})^2$.

With constant variance, the half width of the interval of confidence (i) is also constant, and the experimental points cover, with a probability of $1 - \alpha$, an area defined by the parallels $Y = K'X \pm i$ around the calibration line, i being given by $i = t_\alpha(v) S_Y$ where $t_\alpha(v)$ is the Student coefficient for the confidence level α and the number of degrees of freedom corresponding to the number of measurements used

for calculating the independent variance S_Y^2. The value of K' is the correct value of the slope and may be substituted by the value of K, obtained from the above mentioned relation, when dealing with a large number of experimental data.

(b) The dependent variable has a nonconstant variance

In this case, the least square method is not appropriate to the determination of the slope of the regression line. However, if the dependent variable displays a constant relative error, which very frequently occurs in quantitative gas chromatography, it is possible to process the experimental data precisely by the method of mean slope. In this method, the slope of the calibration line plays the role of a random (dependent) variable with a constant variance, and the best estimation of it is the arithmetic mean. Hence, $K = (1/n)\Sigma^{n}(Y/X)$, and the variance of the slope is given by $S_K^2 = \Sigma^{n}[(Y/X) - K]^2/n(n-1)$.

If the relative error of the dependent variable is known, *e.g.*, if this error has been obtained independently of the calibration as the coefficient of variation I_Y, given by $I_Y = 100\ S_Y/Y$ where S_Y is the standard deviation of the value Y, it is possible to write for the variance of the slope $S_K^2 = (1/n)\ (I_Y/100)^2K^2$. In this case, the experimental points cover, with a probability of $1 - \alpha$, a divergent area defined by the straight lines $Y = K'(1 \pm i)X$ where $i = t_\alpha(v)I_Y/100$. The correct value of K' may again be substituted by the value of K calculated from the respective relationship.

When evaluating calibration lines by the methods mentioned in connection with situations (a) and (b), cases frequently occur in which the statistically dependent variable suffers from a significant systematic error, d. Though this error may manifest itself in different ways, two cases are the most frequent.

In the first case, the error manifests itself as a constant absolute error, *i.e.*, a measured value Y and the correct value Y' are related to each other by $Y = Y' + d$. The calibration line is most often obtained by the procedure described under (a) in this case. When following the above procedure, the value of the slope is not modified by the error d and, therefore, the calculated slope, K_1, corresponds to the correct value, K'; the error d will manifest itself as an intercept on the y co-ordinate, *i.e.*, the calibration line does not pass through the origin of the co-ordinate system. The d value may be calculated from the data necessary for constructing the calibration line by using the relation $d = \bar{Y} - K_1\bar{X}$.

In the second case, the error d presents itself as a constant relative error, so that the relation between the measured and correct values may be expressed in the form $Y = Y'(1 + d)$. In this case, the calibration line is most often constructed by the method mentioned in connection with the situation under (b). It is characteristic for this procedure that the error d manifests itself by a modification of the slope of the calibration line; the relation between the calculated slope K_2 and the correct slope K' is as follows: $K_2 = K'(1 + d)$.

EXPERIMENTAL

The validity of the above relations was confirmed by comparing the spread of the experimental points about the regression line with the interval of confidence

calculated on the basis of the independently determined variance of the statistically dependent variable. The procedure was based on measuring the independent variances of all the variables occurring in the calibration; by comparing the relative errors of the given variables, it was ascertained which of the variables had associated with it a greater error and, consequently, would be considered as statistically dependent. This variable was always assigned the perpendicular co-ordinate (y).

In addition, an adequate number (40) of points was measured within the calibration limits investigated and, allowing for the character of the variance of the dependent variable, a suitable method (situations (a) or (b)) was employed to obtain the respective calibration line. By virtue of the independent variance, the respective interval of confidence was constructed around the calibration line and compared with the lay-out of the experimental points. The effect of the systematic error (d) on the calibration line was tested by comparing two calibration procedures in which the systematic error manifested itself either as the intercept on the y co-ordinate or as the change in the slope.

The above procedure has been carried out with the absolute calibration technique, in which the calibration line is obtained by plotting the peak areas or peak heights against the absolute amounts injected of the substance under analysis. The concentrations have been expressed in molarities (M), the volumes injected (v) in μl, the peak areas (A) in cm^2, and the peak heights (h) in cm. The peak area was determined as the product of the peak height and the peak width at its half height (r). The experimental design covers four relevant cases, *i.e,* four combinations of the possibilities that the final result is from the statistical viewpoint, either a dependent (1) or an independent (2) variable, having either a constant (a) or a nonconstant (b) variance. The final results are expressed in the absolute amounts (Mv) of the test substance introduced. The effect of the systematic error has been tested in the case of the systematic error of the volume injected, which is the most frequent error in the method employed; the most usual source of the above error is the evaporation of part of the sample from the needle of the syringe used.

The measurement was carried out on a Becker Multigraph F, Model 410 (Becker Delft, N.V., Delft, The Netherlands) equipped with a Servogor RE 511 recorder (Goerz Electro, G.m.b.H., Austria). 1 m long aluminum columns, 4 mm I.D., with 4 g of 20 wt. % squalane on Celite 545 30–60 mesh were used, and kept at 60°. The N_2, H_2, and air flow rates were 0.80, 1.25, and 10 ml/sec, respectively, as measured at the detector jet nose (24°, 746 mm Hg). The column inlet excess pressure was 0.2 atm. The injection port was kept at 140°. The samples were introduced by a Hamilton 701-N (10 μl) syringe (Hamilton Co., Whittier, U.S.A.). The model mixtures were prepared by weighing chromatographically pure chloroform, benzene, and toluene (Lachema, N.E., Brno, Czechoslovakia) on a Meopta A3/100 analytical balance (Meopta, N.E., Prague, Czechoslovakia) with a precision better than 10^{-2} % of the value weighed.

The principle of the method used makes it necessary to take into account the variables M, h, r, A, and v as well as the respective variances, S_M^2, S_h^2, S_r^2, S_A^2, and S_v^2 (*cf.* ref. 3). The concentration (M) was determined by weighing and, therefore, the respective variance (S_M^2) may be neglected in comparison with the error of the other variables. When measuring the lengths h and r by the same gauge, the variances S_h^2 and S_r^2 are identical and will be designated by S_l^2 from now on. The measurement

of lengths was carried out by a rule; the respective variance (S_l^2) was determined by measuring 15 standard lengths and amounted to 4.0×10^{-4} cm². The variance of the area calculated by $A = hr$ is given by $S_A^2 = (r^2 + h^2)S_l^2$ and its value has to be determined for particular values of h and r. The variance of measuring the volumes injected, S_v^2, was determined, for the given 10 μl Hamilton syringe, by weighing 15 measured volumes of tetrabromoethane, and amounted to 16×10^{-4} μl².

RESULTS AND DISCUSSION

Variant 1(a)

The above variant corresponds to the case when the dependent variable represents the final result of analysis and displays a constant variance. In the absolute calibration technique, this situation occurs when the measurement of the sample charge suffers from the larger error and when the calibration points have been obtained by injecting different volumes of the same sample mixture. In our experiments,

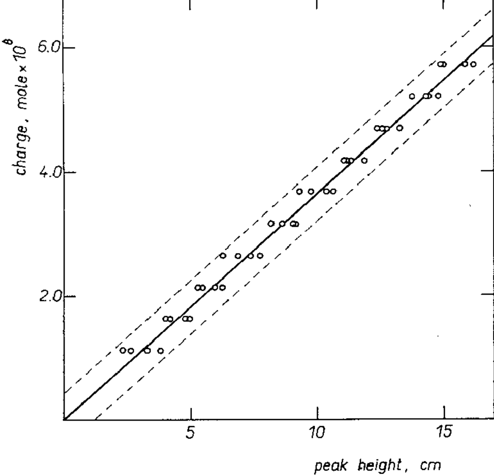

Fig. 1. Variant 1(a). Main source of error — measuring of the sample charge; calibration data obtained by injecting different volumes of a sample of a constant concentration of the substance analyzed; $K = 0.3634 \times 10^{-8}$ mole cm⁻¹.

a 0.05105 M solution of benzene in toluene was injected, the sample size ranging from 0.20 to 1.10 μl. The respective peak heights varied from 3 to 15 cm. The calibration line determined by the least square method is in Fig. 1. The dashed lines depict the interval of confidence calculated from the independent variance by $S_{Mv}^2 = M^2 S_v^2$. Hence, for the half width of the interval of confidence, i, $i = t_{0.05}(14) M S_v$, and the numerical value is 0.44×10^{-8} mole for $t_{0.05}(14) = 2.14$; the respective interval of confidence is within the parallels $Mv = Kh \pm 0.44 \times 10^{-8}$.

Variant 1(b)

This variant is partly analogous to the preceding one. The dependent variable again expresses the final result of the analysis, but the variance of the former is not constant. In this case, the relative error, *i.e.*, the coefficient of variation of the de-

pendent variable, is constant. In the absolute calibration technique, this variant again represents a case when it is the measurement of the volume injected that suffers from the larger error, but the calibration line is constructed from points obtained by injecting equal volumes of sample solutions of different concentrations. In the respective experiments, 1.1 μl charges of different solutions of benzene in toluene were

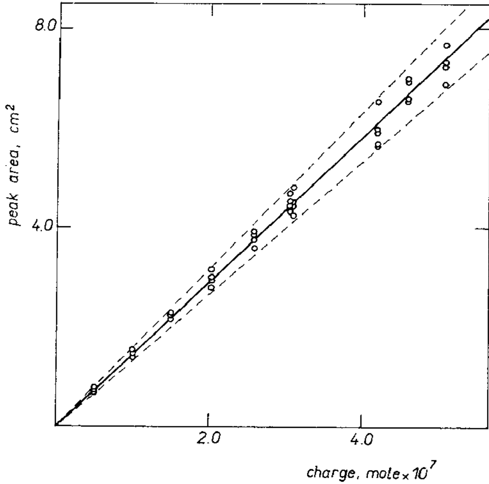

Fig. 2. Variant 1(b). Main source of error — measuring of the sample charge; calibration data obtained by injecting equal volumes of samples of different concentrations of the substance analyzed; $K = 0.3655 \times 10^{-8}$ mole cm^{-1}.

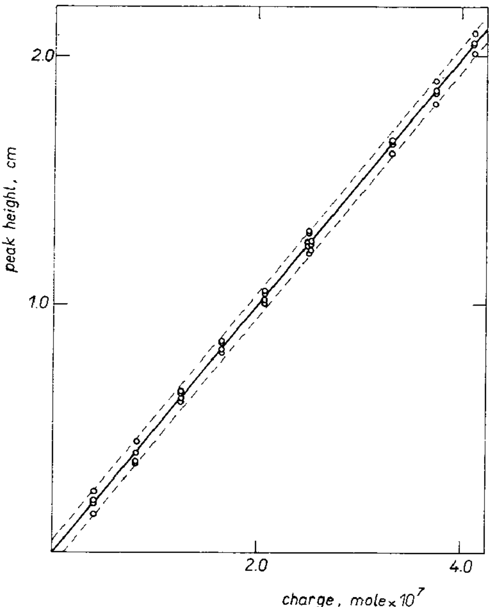

Fig. 3. Variant 2(a). Main source of error — measurement of the chromatogram; peak height is the quantitative parameter; $K = 0.4975 \times 10^{7}$ cm mole^{-1}.

injected, the benzene peak heights varying from 3 to 15 cm. The calibration line obtained by the mean slope method is given in Fig. 2. The interval of confidence, given by the lines $Mv = K(1 \pm i)h$ where $i = t_{0.05}(14)I_{Mv}/100$ and I_{Mv} stands for the coefficient of variation of the amount injected, is designated by the dashed lines. The numerical value of I_{Mv}, calculated from the independent variance S_v^2 and for the volume injected, is 3.6 %. The lines defining the interval of confidence are given by $Mv = K(1 \pm 0.076)h$.

Variant 2(a)

This variant represents a case when the final result of analysis, Mv, is the independent variable, and the dependent one has a constant variance. The corresponding variant of the absolute calibration technique is that in which the measurement of the chromatogram is associated with the larger error and has a constant variance. In our case, 10 μl charges of solutions of chloroform in toluene were injected, the corresponding peak heights varying from 0.2 to 2 cm. The respective calibration line, obtained by the least squares method, is shown in Fig. 3. The interval of confidence is given by the parallels $h = KMv \pm i$ where $i = t_{0.05}(14)S_l$; substitution of the numerical values gives $h = KMv \pm 0.043$.

Variant 2(b)

In this case, the result of the analysis is the independent variable. The dependent variable has a nonconstant variance, but displays a constant relative error within the entire calibration range. In the absolute calibration technique, this variant corresponds to a case when the peak area is determined as the product hr and the measurement of the peak width suffers from the largest error ($r^2 \ll h^2$). Then, the variance of the dependent variable, S_A^2, may be expressed by $S_A^2 = h^2S_l^2$, and the coefficients of variation of the variables A and r are constant and equal to each other. 10 μl sample

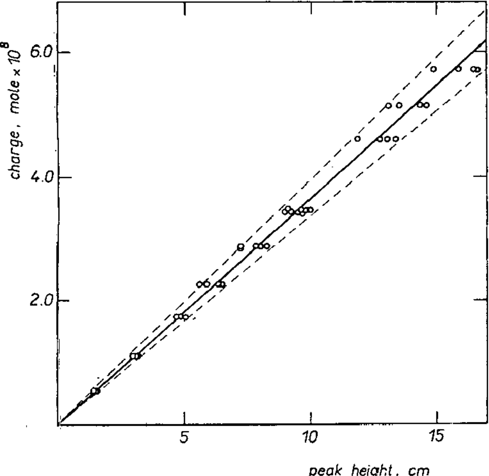

Fig. 4. Variant 2(b). Main source of error — measurement of the chromatogram; the product of the peak height and the peak width at the half height is the quantitative parameter; $K = 1.460 \times 10^7$ cm^2 mole^{-1}.

charges of different benzene-in-toluene solutions were injected. The benzene peak widths amounted to about 0.5 cm and the peak heights varied from 1.5 to 16 cm. The respective calibration line is shown in Fig. 4. The interval of confidence is given by the lines $A = Kmv(1 \pm i)$ where $i = t_{0.05}(14)I_r/100$. The value of I_r, expressed by means of S_l^2 for $r = 0.5$ cm, is 4 %, so that the equations to the lines indicating the interval of confidence are given by $A = KMv(1 \pm 0.086)$.

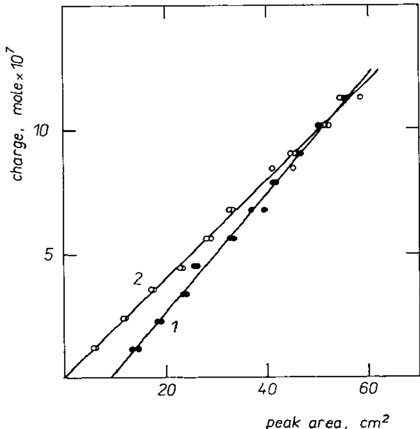

Fig. 5. Effect of the systematic error on the calibration line. Line 1: injection of different volumes of a sample of constant concentration, $K_1 = 1.990 \times 10^{-9}$ mole cm^{-2}, the intercept corresponds to 0.196 μl; line 2: injection of equal volumes of samples of different concentrations, $K_2 = 2.399 \times 10^{-9}$ mole cm^{-2}.

Effect of the systematic error of sample introduction

To demonstrate the above effect, two procedures were employed for constructing the calibration line. In the first case, a 0.1119 M solution of isooctane in toluene was injected, the sample charges varying from 0.10 to 1.0 μl. The statistically dependent variable was the quantity Mv, the independent variable was the isooctane peak height, and the calculation was carried out by the procedure mentioned in connection with variant 1(a). In this case, the error d manifests itself as a constant systematic absolute error of the magnitude Md. The respective calibration line (No. 1) is shown in Fig. 5. The slope of the line is $K_1 = 1.990 \times 10^{-9}$ mole cm^{-2} and the intercept on the x co-ordinate corresponds to 2.20×10^{-8} mole. With the given sample solution, the systematic error incidental to the above intercept amounts to 0.196 μl.

In the second case, solutions of isooctane of different concentrations in toluene were injected, the sample volumes being 1.0 μl. In this case, the error d exerts itself as a constant systematic relative error. The calibration line, constructed according to the above 1(b) variant, is designated by numeral 2 in Fig. 5. The line passes through the origin of the co-ordinate system and its slope is $K_2 = 2.399 \times 10^{-9}$ mole cm^{-2}. The value of the slope K_2 may be calculated using the error d and the correct value of K_1 (equal to K') known from the preceding case; the value calculated ($K_2 = 2.380 \times 10^{-9}$ mole cm^{-2}) is in good agreement with the actual value (the difference is about 0.8%).

CONCLUSION

In processing calibration data by linear regression, it is necessary to proceed with regard to which of the variables involved is associated with the larger error; this variable has to be regarded, from the statistical viewpoint, as the dependent one. Linear regression may be carried out by the method of least squares only in the cases where the dependent variable displays a constant variance within the calibration limits. If the dependent variable with a nonconstant variance has a constant relative error, precise processing of the calibration data is possible by means of the mean slope method. The presence of a systematic error is manifested according to the working procedure used, either as a systematic constant absolute error or as a systematic constant relative error. In the first case, an intercept on the respective co-ordinate is incidental to the error whereas, in the second case, the error is responsible for a deviation in the slope of the calibration line.

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CHROM. 4845

THE DEPENDENCE OF FLAME IONISATION DETECTOR RELATIVE RESPONSE FACTORS ON THE CONFIGURATION OF THE ELECTRODES

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(Received May 26th, 1970)

SUMMARY

Results obtained by quantitative evaluation of chromatograms of a model mixture chromatographed on seven different commercial gas chromatographs were processed statistically by analysis of variance. Flame ionisation detectors with two parallel electrodes and a floating jet were shown to have relative ionisation efficiencies per 1 gram atom of carbon generally dependent on both the construction of the detector and the type of hydrocarbon. No significant variations in the above relative ionisation efficiency were found with detectors in which the jet served as a polarising electrode, even when the detectors differed substantially from each other in overall geometry and in the shape and position of the collecting electrode.

INTRODUCTION

It is already a well established fact that the performance of the flame ionisation detector (FID) is strongly affected by many experimental variables. On the other hand, the FID has excellent properties when operated under properly chosen and stabilised conditions. This explains why the FID has received so much attention since its advent[1,2]. Along with numerous works affording basic information on the constructional and geometrical aspects[3–13], effects of various experimental parameters on the detector performance[4,6,7,14–19], and the behaviour of the FID under varying normal working conditions[20], papers have also been published stating that the relative response factors are dependent on the experimental parameters[19,21]. Furthermore, recent findings by DEANS[22] show that the relative response factors also depend on the type of apparatus.

In the present work, an attempt has been made to find a conclusive cause of the above phenomenon. The essence of the work is a statistical analysis of the results obtained by evaluating the chromatograms of a model mixture obtained on several commercial gas chromatographs under identical conditions.

DESIGN OF THE EXPERIMENTS

The results obtained from the chromatograms were processed by the analysis

of variance, namely, by factorial experiments[23]. In order to make maximum use of the data measured, the latter were expressed in the form of relative ionisation efficiency which is defined as the ratio of the ionisation efficiencies of one gram atom of carbon in the substance under consideration and that in a reference substance. This makes it possible to determine not only whether there are differences in the relative response factors measured with different flame ionisation detectors for a given pair of substances, but also whether the kind of model substance plays some role and whether a change in the nature of the substance brings about the same or different changes in the relative response factor with different detectors.

The ionisation efficiency, q, as quoted above, may be defined by the relation

$$q = AM/K(\Sigma C)w \qquad (1)$$

where A is the peak area of the substance chromatographed, as measured in the chromatogram, w is the corresponding weight of the substance contained in the charge injected, M and (ΣC) are the molecular weight and the number of carbon atoms pertaining to the substance, and K is an apparatus constant. Denoting the quantities corresponding to the substance in question and to the reference substance by subscripts i and s, it is possible to write, in compliance with eqn. 1,

$$q_{is} = q_i/q_s = A_iM_i(\Sigma C)_s w_s/A_sM_s(\Sigma C)_i w_i \qquad (2)$$

where q_{is} designates the above mentioned relative ionisation efficiency. As the corresponding relative weight response, RWR_{is}, is given by

$$RWR_{is} = A_i w_s/A_s w_i \qquad (3)$$

the relationship between RWR_{is} and q_{is} can be written:

$$RWR_{is} = q_{is}\, M_s(\Sigma C)_i/M_i(\Sigma C)_s \qquad (4)$$

In eqns. 2 and 3, the ratios A_i/A_s and w_i/w_s may obviously be substituted by the corresponding ratios of the peak area and weight fractions.

The analysis proper is based on the presumption that the relative ionisation efficiency with a detector a and substance i may be regarded as a sum of additive contributions, *i.e.*,

$$q_{is} = q_{is}^0 + \varepsilon_a + \varepsilon_i + \varepsilon_{ai} + \varepsilon_r$$

where q_{is}^0 is a relative ionisation efficiency value identical for all the detectors tested, ε_a and ε_i are the contributions accounting for the differences in the detectors and kinds of substance, respectively, ε_{ai} is the interaction contribution, and ε_r is the random error. The above contributions were processed in the form of the respective variances, S_a^2, S_i^2, S_{ai}^2, and S_r^2. The statistical significance of the variances S_a^2, S_i^2, and S_{ai}^2 was tested by comparing them with the residual variance S_r^2 by means of the SNEDECOR criterion[23].

EXPERIMENTAL

In order to be able to compare the results, the components of the model mixture as well as the column stationary phase were the same as those used in the work by DEANS. In order to reduce the number of variable factors, one and the same column was used with all the gas chromatographs employed, and it was operated at the same temperature and carrier gas flow rate. In addition, a Servogor RE 512 recorder (Goerz Electro, G.m.b.H., Austria) was used in all cases, thus eliminating the possible variability in peak distortion due to the recorder. Under the above conditions, changes in relative response factors may be considered as incidental only to variations in the detector parameters. The following gas chromatographs were employed in the measurements:

(i) Becker Multigraph, Type 409, Delft, The Netherlands.
(ii) CHROM 2, Laboratory Instruments, n.e., Prague, Czechoslovakia.
(iii) CHROM 4, Laboratory Instruments, n.e., Prague, Czechoslovakia.
(iv) Carlo Erba Fractovap, Model C, Type AID/f, Milan, Italy.
(v) Giede Hochtemperatur Gas Chromatograph, Model GCHF 18.3, Betriebskontrollgeräte KG, Berlin, D.D.R.
(vi) Hewlett-Packard High Efficiency Gas Chromatograph, Model 402, U.S.A.
(vii) Shimadzu Gas Chromatograph, Model GC-4A PTF, Kyoto, Japan.

The column was a 150 cm long stainless steel tube of 3 mm inner diameter, packed with 5.40 g of Chromosorb W 80–100 mesh coated with 10% (by weight) Apiezon L. In all cases, the carrier gas (N_2) flow rate was 0.13 ml/sec, and the column and injection block temperatures were 100 and 200°, respectively. The hydrogen flow rates were set at values giving a maximum sensitivity with each apparatus under the above conditions, the air flow rate amounting to about 600 ml/min.

The model mixture contained 22.92, 29.00, 25.00, and 23.08 wt. % of hexane, benzene, methyl cyclohexane, and octane, respectively. All the components were of an analytical grade purity (B.D.H. Ltd., Great Britain). 0.5 μl samples of the above

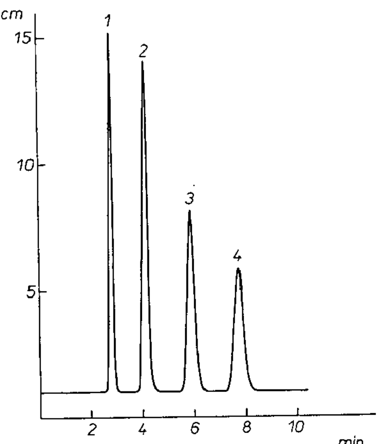

Fig. 1. A chromatogram of the model mixture; 1 = hexane; 2 = benzene; 3 = methyl cyclohexane; 4 = octane.

mixture were injected with a Hamilton 7001 (1 μl) syringe (Hamilton Co. Inc., Whittier, U.S.A.); the sensitivity attenuation necessary to obtain chromatograms of comparable sizes with different chromatographs varied between 1×10^4 and 4×10^4, except with the Giede apparatus where the attenuation had to be 2×10^3. The data such as retention times, intervals of elution, etc., are apparent from the chromatogram in Fig. 1 (obtained on the Becker Multigraph 409). The chromatograms were evaluated by measuring the peak areas as the multiples of the peak height and peak width at half height.

TABLE I

RELATIVE IONISATION EFFICIENCY (q_{is}) VALUES OBTAINED WITH INDIVIDUAL INSTRUMENTS FOR THE COMPONENTS OF THE MODEL MIXTURE

Apparatus	*Relative ionisation efficiency*		
	Benzene	*Methyl cyclohexane*	*Octane*
Becker	1.078	1.040	0.979
CHROM 2	0.996	1.057	1.035
CHROM 4	1.041	0.960	0.895
Carlo Erba	1.113	1.012	0.891
Giede	1.119	0.916	0.703
Hewlett-Packard	1.050	1.024	0.957
Shimadzu	1.085	1.186	1.114

TABLE II

SIGNIFICANCE OF THE INDIVIDUAL FACTORS INVESTIGATED

Factor	$F_{exptl.}$	$F_{crit.}$	*Significance*
Instruments	54.93	2.16	+
Substances	127.7	3.06	+
Interactions	26.83	1.82	+

RESULTS AND DISCUSSION

The experimental values of the relative ionisation efficiencies for the individual chromatographs and substances are summarised in Table I, the hexane carbon ionisation efficiency has been used as a reference throughout. All the data represent the average values of 10 measurements; the standard deviation of the averages varied within approximately 0.005–0.03. A comparison of the significance of the individual factors is shown in Table II where $F_{\text{exptl.}}$ stands for the experimental S_a^2/S_r^2, S_i^2/S_r^2, and S_{ai}^2/S_r^2 ratios and $F_{\text{crit.}}$ represents the tabulated values of the Snedecor distribution $F_\alpha(\nu_1,\nu_2)$ for the confidence level and degrees of freedom ν_1 and ν_2; for the instruments, substances, and interactions, the ν_1 values are given by $k - 1$, $l - 1$, and $(k - 1)(l - 1)$, respectively, where k and l are the number of instruments (7) and the number of substances (3), and the ν_2 is equal to $kl(n - 1)$ where n is the number of replicate chromatographic runs (10). All the data have been expressed for a 0.05 confidence level.

It can be seen from the data in Table II that all the effects investigated are statistically significant, which confirms, in the main, the findings by DEANS[22]. However, the situation affords a further analysis of the data. It is possible to calculate from the residual variance the interval of confidence, I_a, which allows the significance of the differences between the line averages in Table I to be tested. Thus for the above interval:

$$I_a = t_a(\nu_2)S_r(2/nl)^{\frac{1}{2}} = 0.02491$$

where t is the Student coefficient, $\alpha = 0.05$, $\nu_2 = 189$, $S_r = 0.04897$, and $nl = 30$. By applying this test, it is possible to place the individual instruments into several groups in such a way that there is no significant difference between the individual instruments within a group, but significant differences between the groups. Thus, in accordance with the results in Table I, the Giede, CHROM 4, Carlo Erba, and Shimadzu gas chromatographs represent by themselves particular groups in the above sense, while the Hewlett-Packard, Becker Multigraph, and CHROM 2 instruments form a single group.

It should be pointed out that all the instruments falling into the common group have detectors with the burner jet serving as a polarising electrode. In this case, neither a passage from one apparatus to another nor changes in the testing substances used brought about any significant variations in the relative ionisation efficiency, despite the fact that all the three detectors differed appreciably from each other in their geometry. The other groups are formed by instruments having detectors with two parallel electrodes and floating jets; these detectors showed significant differences in the relative ionisation efficiencies with respect to both the kind of detector and the kind of substance.

Provided the detectors with two parallel electrodes are considered as a single group, it is possible to carry out a comparison of the detectors having parallel electrodes and floating jets with those in which the jet serves as an electrode. This may be done by means of the method of linear contrast[23]. In this method, $F_{\text{exptl.}}$, given by $S_{\text{contrast}}^2/S_r^2$, is compared with $F_{\text{crit.}}$; $F_{\text{exptl.}} > F_{\text{crit.}}$ implies a statistically significant difference between the groups under comparison. In our case, $F_{\text{exptl.}}$ amounted to 37.43 ($S_{\text{contrast}}^2 = 0.08975$ and $S_r^2 = 0.002398$) while $F_{\text{crit.}}$ was 3.90, so that the groups differ significantly from each other.

CONCLUSION

Flame ionisation detectors have proved to be classifiable into two groups from the point of view of their performance, detectors with two parallel electrodes situated symmetrically with respect to the flame axis, and detectors in which one of the electrodes consists of the burner jet. In the first case, not only the absolute ionisation efficiency with a given substance, but also relative response factors showed a considerable dependence on the overall detector geometry. Moreover, the ionisation efficiency of a carbon in a hydrocarbon depended on the type of substance used. Detectors with the burner jet as a polarising electrode did not display any of the above unfavourable effects even when the shape and position of the collecting electrode differed appreciably.

Thus, it seems to be of little use listing FID relative response factors measured with detectors of the first type or to employ tabulated relative response factors when working with such a detector. The above problems may be avoided by choosing a detector with the burner jet as a polarising electrode.

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CHROM. 4875

A STUDY OF THE FLAME IONISATION DETECTOR

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SUMMARY

At high concentration levels the linearity of response of the flame ionisation detector is dependent on the hydrogen flow rate, and at high hydrogen–nitrogen ratios marked non-linearity may be observed. Anomalous effects may also occur as a result of contamination of the flame jet. For *n*-heptane an essentially linear response has been obtained up to approximately 60 μg/sec under optimum flow conditions. Under the same conditions toluene exhibits non-linearity at approximately 10 μg/sec, but the linear range for toluene can be extended by increasing the hydrogen–nitrogen ratio. The construction and performance of the detector, amplifier and gas blending system used for the above investigations are discussed. A general expression is given relating current and applied voltage for the flame ionisation detector.

INTRODUCTION

The two most important properties of the flame ionisation detector (FID) are its high sensitivity and its linearity of response. The latter has been established over a wide additive mass flow range, almost 10^8 to 1, by the use of various gas dilution systems[1–4]. However, doubts still remain as to whether exact linearity is achieved, particularly at the higher concentration levels. The present work is concerned mainly with linearity at high concentrations, and was undertaken as a preliminary investigation necessary for the study of peak shapes in gas chromatography.

Deviation from linearity over a wide concentration range should be evident from the slope of a log–log plot of signal output *versus* additive flow rate. The slope of this curve, which has been termed the "response index"[3,4], should be 1.00 if exact linearity is achieved. Typical values reported in practice for the FID are 0.98–1.02 but the results suggest that this deviation is due to experimental error rather than to a basic non-linearity of the detector itself[3,5,6]. The main problem lies in the reliability of methods for generating low concentrations of organic vapours, since there is at present no reference device with which these can be checked. A diffusion dilution system which should give a logarithmic decrease in vapour concentration with time would appear

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to be an ideal reference system, but adsorption problems limit the usefulness of this approach[7,8]. The work reported below employed a direct gas blending system.

EXPERIMENTAL

Apparatus

The apparatus consisted of an insulated jet FID, a variable voltage supply to provide the jet potential, an electrometer amplifier and recorder, and a gas blending system for the introduction of controlled amounts of organic materials into the flame. The study is concerned mainly with a detector burning a mixture of both hydrogen and nitrogen at the jet but some results using hydrogen only are included.

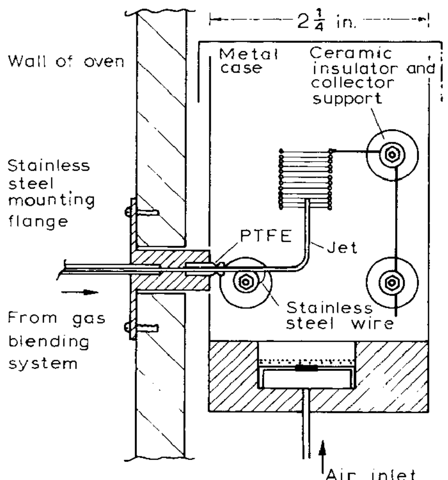

Fig. 1. Flame assembly (cross-sectional view).

The flame assembly is shown in Fig. 1. The main features are the use of a filled PTFE (polytetrafluoroethylene, Teflon) connector to insulate the 20 g stainless-steel jet from earth, the complete separation of the air inlet and jet assembly, and the construction of the collector electrode from heavy gauge wire. The jet, which forms one electrode, is at the axial centre of the cylindrical collector electrode, and the detector can be considered to have cylindrical symmetry. Collector electrodes of different diameters, required for a study of current *versus* voltage relationships, were made by winding 16 g tin-coated copper wire onto suitably sized formers, *e.g.* the shank of a drill. For most of the work reported here an internal diameter of 13 mm was used, an overall length of 15 mm, and the jet projected 2 mm above the bottom of the collector electrode.

The air distributor consisted of two layers of 120 mesh stainless-steel gauze supported on one layer of 18 mesh gauze. A spider support, or baffle, prevented direct flow of air from the centre of the distributor. To avoid disturbing the electrical field within the electrode assembly no internal ignition system was used. The flame was lit with a Toshiba type G-6 battery-operated gas lighter after removing the lid of the flame chamber. Due to the large cross-sectional area of the chamber (2¼ in.

square) a relatively large air flow rate was required to reach the signal maximum. However, the effect of air flow rate was quite flat and there was no evidence of turbulence, the signal stability at a fixed level of addition of organic material being excellent. Using both *n*-heptane and carbon tetrachloride at high concentrations as reference materials, the signal maximum was reached at an air flow rate of 1.0 l/min and remained constant up to at least 2.5 l/min. The detector was normally operated at a flow rate of 1.6 l/min. In comparison, a typical figure at which the maximum is reached with more compact detectors is 0.5 l/min[1,3,6]. At 0.3 l/min the signal obtained with the detector described here was 89 % of the maximum for both *n*-heptane and carbon tetrachloride, and 76 % for *n*-heptane at almost zero air flow rate.

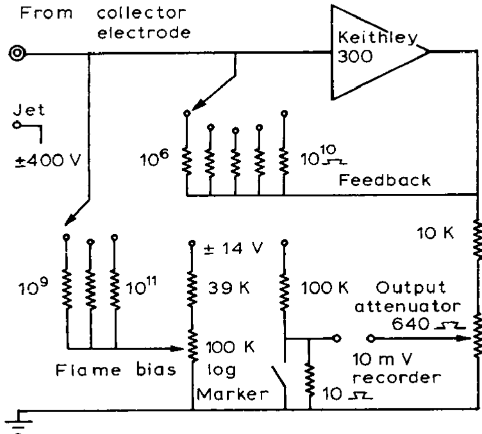

Fig. 2. FID amplifier circuit.

The circuit of the amplifier is shown in Fig. 2. This is based on a Keithley Model 300 operational amplifier (Keithley Instruments Inc., Cleveland, Ohio) which uses electrometer input tubes. The output attenuator resistor chain (640 Ω total) was divided into steps of 1, 2, 4, .. 64, and the feedback resistors provided four further factors of 10, a total range of 6.4×10^5 to 1 for full scale deflection. Overall gain of the circuit was 0.64. With a Varian G-1000 10 mV recorder the noise level was about 0.5 % of full scale at the maximum sensitivity setting (equivalent to 1.6×10^{-11} A full scale) and drift at this sensitivity was less than 1 % per hour. At relatively low gas flow rates (30 ml/min nitrogen, 15 ml/min hydrogen) the background current due to the flame amounted to 1.4×10^{-11} A, this being caused mainly by impurities in the gas supplies[9]. Noise and short term drift (over a period of about 10 min) at an applied voltage of 380 V (positive jet) was less than 3×10^{-13} A. These figures were obtained using combustion air taken directly from the laboratory compressed air supply, with a coarse charcoal filter to remove dust and oil droplets. Provision was made for the jet voltage to be varied over the range ± 380 V in steps of approximately 27 V, or over the range ± 38 V in steps of approximately 2.7 V with an additional voltage divider. This is not shown in Fig. 2. In series with the voltage supply was a 1 MΩ protective resistor which prevents the voltage supply being overloaded in the event of an external short-circuit, and also protects unwary personnel who may ac-

cidently touch the high voltage connections. Also not shown in Fig. 2 is an external zero control which is described in the operating instructions supplied with the Keithley operational amplifier.

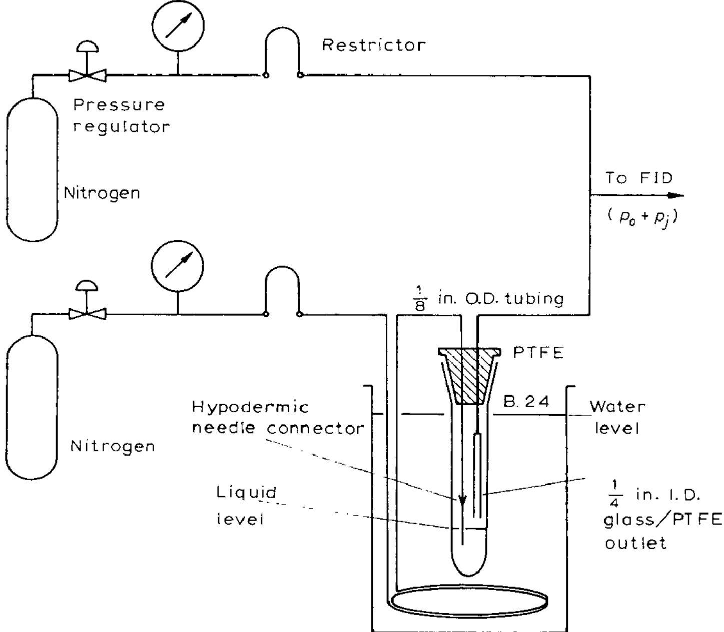

Fig. 3. Gas blending system.

The gas blending system is shown in Fig. 3. It consisted of two separate gas flow control systems and a saturator. The top of the saturator insert was made from PTFE with thin sealing rings to fit the B24 ground glass joint. However, even with careful machining, a thin layer of grease was found necessary to ensure a leak tight seal. By changing the grease when the liquid in the saturator was changed, cross-contamination problems were avoided. The interchangeable restrictors consisted of fixed lengths of 1/8 in. O.D., 1/16 in. I.D., tubing filled with 60–80 mesh glass beads (F & M Scientific Corp., Avondale, Pa.). Typical flow rate figures for nitrogen, for a 5 in. length of 60–80 mesh beads and inlet pressures of 5 and 30 p.s.i.g. are 6 and 60 ml/min, respectively. By calibrating with a soap film flow meter the flow through the saturator could be varied while the total flow was kept constant. A similar flow arrangement has been used in the past for calibrating analysers[10] and in earlier investigations of the FID[2]. More recently the accuracy of the method has been discussed in some detail[11]. The hydrogen flow to the detector is controlled in a similar way, the restrictor this time being a 5 in. length of 1/16 in. I.D. tubing filled with 230–320 mesh glass beads. Flow rate figures in this case are approximately 15 and 155 ml/min for 5 and 30 p.s.i.g. In over eight years use these restrictors have been found to be far more reliable and reproducible than capillaries, being free from blocking problems to which fine capillaries are particularly susceptible. The air flow restrictor consisted of a 5 in. length of 20 g hypodermic needle

tubing with an inlet pressure of 6–10 p.s.i.g. In all cases Watts type 215 low flow low pressure precision regulators (Watts Regulator Co., Lawrence, Mass.) were used for pressure control, the large diaphragm area of these regulators providing precise control and freedom from oscillation.

The main problems encountered with the gas blending system are the saturator efficiency and the backpressure due to the restriction imposed on the gas flow by the detector jet. These are discussed in the next section.

Performance of the gas blending system

The efficiency of the saturator sets the eventual limit for the usefulness of a system of the type described above. TURNER AND CRUM[12] have used a simple saturator for high boiling liquids, and compounds which are solid at ambient temperature, in which the carrier gas passes over the surface of the liquid. They found that in the case of their saturator, and maleic anhydride as the sample, the flow rate of carrier gas was limited to about 12 ml/min for complete equilibration to be achieved.

In attempting to use the same system for samples which are volatile at room temperature, unsatisfactory results were obtained at flow rates much lower than this if the carrier gas merely passed over the surface of the liquid. For this reason the inlet line was terminated with a "Record" taper to which a 25 g hypodermic needle was fitted. The fine needle, positioned below the liquid surface, provided both good contact between the carrier gas and the liquid, and stirring to minimise temperature gradients. At low flow rates the small bubble size also minimises the flow rate variations resulting from bubble formation.

Results obtained with *n*-heptane, *n*-hexane and diethyl ether are shown in Fig. 4, the gas concentration in the effluent gas stream being determined by passing the saturated gas through a gas sampling valve (Carle Instrument Co., Fullerton, Calif.) fitted to a gas chromatograph. As shown by the results for *n*-hexane, the departure from equilibrium is very marked in the absence of the outer water jacket. However, the tendency towards a finite lower limit at high carrier flow rates, and a slow rate of recovery when the flow rate was reduced, suggested that the effect was predomi-

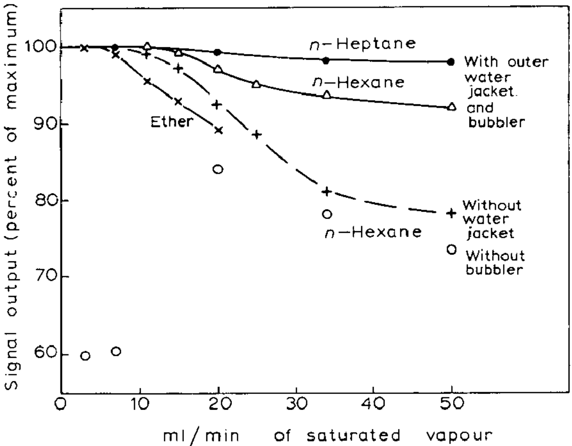

Fig. 4. Variation of saturator efficiency with gas flow rate.

nantly due to evaporative cooling of the liquid sample. Improved temperature stability was obtained with the outer water jacket, and the difference at high and low flow rates could be accounted for by the measured fall in temperature of the sample. Since only low flow rates (less than 11 ml/min) were involved in the present investigation the performance of the saturator was adequate, but it would appear that satisfactory results at higher flow rates and with more volatile samples such as ether (see Fig. 4) could be obtained by further improving heat transfer to the sample.

In the absence of the needle bubbler the results depend on the geometry of the system. In the particular case shown in Fig. 3, removal of the needle results in very inefficient vapour pickup at low flow rates, but there is a marked improvement as the stream of carrier gas impinges more forcibly on the liquid surface. This effect is shown for *n*-hexane by the separate points in Fig. 4.

The error due to the backpressure at the jet can be estimated as follows. The flow through a bed of granular material is related to the inlet pressure p_i and outlet pressure p_o by the expression

$$F_o = \frac{B_0 A}{2\eta L}\left(\frac{p_i^2 - p_o^2}{p_o}\right) \tag{1}$$

where F_o is the flow rate measured at the outlet pressure, B_0 is the specific permeability coefficient which is characteristic of the bed, η is the gas viscosity, A is the cross-sectional area of the bed, and L is its length[13]. Consider now the situation shown in Fig. 3 where there is a constant total flow to the detector, and the backpressure due to the jet is p_j. The mass flow of additive to the detector is determined by the volume flow rate (F_j) of carrier gas passing through the saturator, the molecular weight of the additive (M), and its partial vapour pressure, $p/(p_o + p_j)$, *i.e.*, by the function

$$F_j M\left(\frac{p}{p_o + p_j}\right) = \frac{B_0 A}{2\eta L}\frac{(p_i^2 - (p_o + p_j)^2)}{(p_o + p_j)^2} Mp \tag{2}$$

The error due to the backpressure at the jet can be calculated in terms of the ratio

$$\frac{F_j Mp}{(p_o + p_j)}\bigg/\frac{F_o Mp}{p_o} = \frac{F_j p_o}{F_o(p_o + p_j)} = \left(\frac{p_i^2 - (p_o + p_j)^2}{p_i^2 - p_o^2}\right)\frac{p_o^2}{(p_o + p_j)^2} \tag{3}$$

Since p_o is fixed and p_j is almost constant during each run, it is more convenient to neglect terms in $p_o/(p_o + p_j)$ and consider only the ratio

$$\frac{F'_o}{F_o} = \left(\frac{p_i^2 - (p_o + p_j)^2}{p_i^2 - p_o^2}\right) \tag{4}$$

which reduces to 1.0 for $p_i \gg p_o > p_j$. Values of eqn. 4 are given in Table I for p_o = 14.7 p.s.i.g. Typically the experimentally determined backpressure amounted to 0.2 p.s.i.g.

As a check of the measured flow rates it is convenient to replace eqn. 1 by the form

$$F_o = \frac{B_0 A p_o}{2\eta L}\left(\left(\frac{p_i}{p_o}\right)^2 - 1\right) \tag{5}$$

Values of the term in brackets are given in Table I.

TABLE I

EFFECT OF BACKPRESSURE ON FLOW RATE RATIO F_0'/F_0 (EQN. 4)

p_i (*p.s.i.g.*)	$(p_i/p_0)^2 - 1$	*Backpressure (p.s.i.g.)*				
		0.1	*0.2*	*0.3*	*0.4*	*0.5*
5	0.7960	0.983	0.966	0.948	0.931	0.913
10	1.8233	0.993	0.985	0.977	0.970	0.962
15	3.0820	0.996	0.991	0.987	0.982	0.978
20	4.5722	0.997	0.994	0.991	0.988	0.985
25	6.2937	0.998	0.996	0.993	0.991	0.989
30	8.2466	0.998	0.997	0.995	0.993	0.992

Current–voltage curves for the FID

The form of the current–voltage curves for the FID, at a constant rate of sample addition, is now quite familiar. A steep rise in current is obtained at low voltages and a plateau at higher voltages (Fig. 5), the latter corresponding to collection of all the ions formed in the flame[2]. At still higher voltages the signal may increase again[6,14] and this point will be discussed briefly later. The curves for the jet positive and jet negative differ slightly, the difference being a function of the detector geometry and hydrogen flow rate[15].

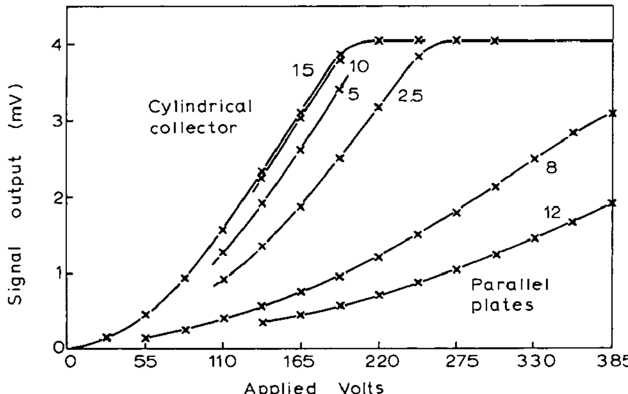

Fig. 5. Current *vs.* voltage curves for cylindrical and parallel plate electrode systems. Numbers on the upper curves represent the cylinder length in mm, on the two lower curves the distance between the plates in mm.

The voltage required to reach saturation is dependent on the saturation ion current, and it has recently been shown[15] that below saturation the current *vs.* voltage (i/V) curves for the cylindrically symmetrical FID and a positive jet can be described by the relationship (given in MKS units)

$$V = \left(\frac{i}{2\pi k_1 \varepsilon_0}\right)^{1/2} (a - b) \left(1 + \frac{b}{a}\left(C \frac{i}{I} - 1\right)\right)^{1/2} \tag{6}$$

where a is the collector radius, b is the outer radius of the ion formation and recombination region (approximately 1 mm for a 20 g jet), C is a constant (approximately

3.0), k_1 is the mobility of the positive ions, and ε_0 is the permittivity of free space (8.854×10^{-12} farad meter^{-1}).

Below the saturation voltage the current is a function of the applied voltage, and only above the saturation voltage can a linear relationship between ion current and additive flow rate be expected. If the applied voltage is sufficiently low the term $C\ (i/I)$ in eqn. 6 will be negligible, and the measured current will be independent of the saturation current and therefore independent of the concentration of organic additive.

In deriving eqn. 6 it is assumed that the measured ion current is small in comparison with the saturation current, but in practice it is found that there is good agreement with the experimental curves up to at least 70% of the saturation level. In fact, the use of the equation can also be extended to the saturation point for which $i = I$. It is then seen that the voltage required to reach saturation will be proportional to the square root of the saturation ion current, or of the mass flow rate of organic additive if the detector response is linear. Experimental results confirming this relationship are shown in Fig. 6. The lines drawn through the points correspond to a square law relationship. Both the coaxial cylinder and single flat plate collector electrode systems[2,16] are seen to obey the square law relationship but the greater ion collection efficiency of the cylindrical electrode is clearly shown. For both hydrocarbons and oxygenated organic compounds the results are independent of the compound itself, and are dependent only on the ion concentration and the geometry of the system[15]. It is also found that saturation is reached at a lower voltage if the jet is positive with respect to the collector electrode than if it is negative.

Eqn. 6 further predicts that the saturation voltage will be a function of the collector diameter, and this relationship has also been confirmed experimentally[15]. It might be thought that the length of the collector electrode would have a pronounced effect on the ion collection efficiency, but this is a secondary consideration as shown by the curves of Fig. 5. In fact, with short collectors precise vertical positioning is more important than length. Also shown in Fig. 5 are some results obtained with parallel plate collectors, of relatively large area and spaced 8 and 12 mm apart. It is

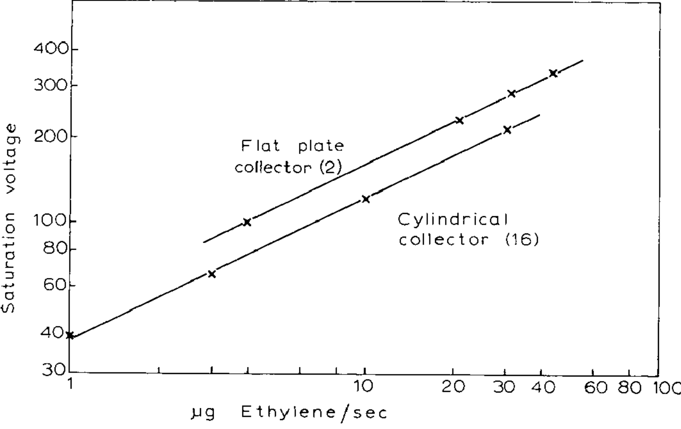

Fig. 6. Relationship between saturation current and mass flow rate of organic additive (log scales).

seen that the collection efficiency obtained with the cylindrical collector is far superior to the parallel plate system.

The effect of the jet temperature

The current *vs.* voltage curves shown in Fig. 5 apply under normal conditions when the jet is relatively cool. At high hydrogen and low nitrogen flow rates the heat released from the flame may cause the jet to become red hot. To avoid these conditions, which result in excessive noise and a very large background signal, relatively massive jets to provide good heat conduction from the jet tip are commonly used with pure hydrogen[1,14].

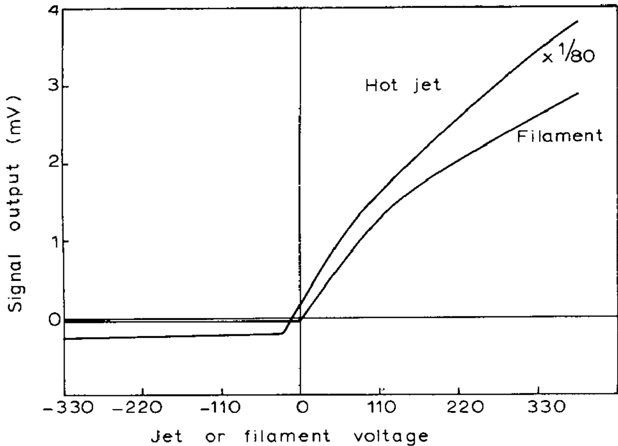

Fig. 7. Current *vs.* voltage curves for the hot jet (hydrogen flow rate 35 ml/min) and for a heated filament suspended in air.

Current *vs.* voltage curves obtained with hydrogen only and without further addition of organic material are shown in Fig. 7. Saturation is reached with the jet negative (corresponding to movement of the negative ions to the outer "collector" electrode) but this saturation current increases rapidly with increasing hydrogen flow rate. At low flow rates (below 40 ml/min) the signal was found to increase at approximately the fourth power of the hydrogen flow rate, but at about 75 ml/min a maxi mum was reached corresponding to a saturation current of 10^{-9} A. Above 25 ml/min the jet glowed a bright red and even at the lowest flow rates a faint red glow was discernable. At higher flow rates the flame was a faint orange colour and completely surrounded the jet tip. This is in marked contrast to a hydrogen–nitrogen flame which cannot be seen without the addition of organic contaminants and is well separated from the jet. It is probable that the observed current can be directly related to the jet temperature, and a visible decrease in temperature occurs at flow rates above that corresponding to the current maximum. With the jet positive there is a steady, almost linear, increase in signal with increase in applied voltage (Fig. 7). These results are similar, but apparently not identical, to those obtained with a heated filament suspended in air, as shown in Fig. 7. Such behaviour has been ascribed to the emission of positive ions by the filament material[17] and extension of this argument to the heated jet of the FID is not unreasonable.

At high concentrations of organic additives a different phenomenon is observed. Thus it has been reported that at high ion currents and high applied fields electron multiplication occurs giving rise to a steep increase in ion current above the saturation level as the voltage is increased. With the present detector and a 13 mm I.D. collector electrode this effect has not been observed under normal operating conditions at voltages up to 380 V and ion currents up to 10^{-6} A, the latter being several orders of magnitude higher than those at which very pronounced effects have been reported[6,14]. However, during some of the early linearity checks it was found possible to obtain this type of response at high hydrogen and low nitrogen flow rates which result in the jet becoming red hot.

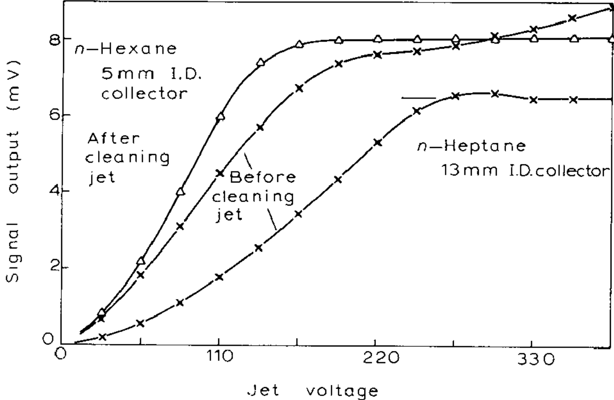

Fig. 8. Current *vs.* voltage curves at high additive concentrations, showing effects of jet contamination.

Subsequently, anomalous results were observed in the current *vs.* voltage curves for *n*-heptane at high ion currents, the current reaching an initial peak above the true plateau level (Fig. 8). This behaviour was accompanied by a sharp rise in the observed current on reducing the voltage, the change being a "metastable" one in that the current again fell to its original level after a short time interval. The latter was critically dependent on both the hydrogen and additive flow rates. It was also found that under these conditions a rapid increase in ion current with applied voltage was obtained with *n*-hexane and a 5 mm I.D. collector electrode (Fig. 8). This rise was independent of the collector material. Further consideration of possible causes of this behaviour led to cleaning of the jet with fine energy paper. As a result both the anomalous metastable response and any further increase above the plateau level disappeared.

Since it was observed that the tip of the jet had been covered with a carbon layer it seemed possible that this behaviour might be related to carbon deposition on the jet. However, depositing carbon on the cleaned jet from a smokey flame did not affect its performance. Oxidation of the metal is also a logical cause in the case of a stainless-steel jet, but this is unlikely to provide an explanation for the similarity in behaviour observed with both stainless-steel[6] and platinum[14] jets. Nevertheless, it seems evident that a surface phenomenon must be involved, even if only as a source of electrons or ions for subsequent multiplication effects. The fact that the increase

is not observed when the jet is negative suggests that it cannot be due to electron emission, but this evidence by itself cannot be regarded as conclusive.

Linearity of response

The main purpose of the present study was to check the linearity of response of the FID at relatively high concentrations of organic additives. Initially, the hydrogen flow rate was adjusted to give the maximum signal output for a given additive flow rate[2]. This was done to ensure that small changes in either the hydrogen or total nitrogen flow would have a negligible effect on the final results. Subsequently, the effect of substantial changes in the hydrogen flow rate was further investigated and these results are also reported below.

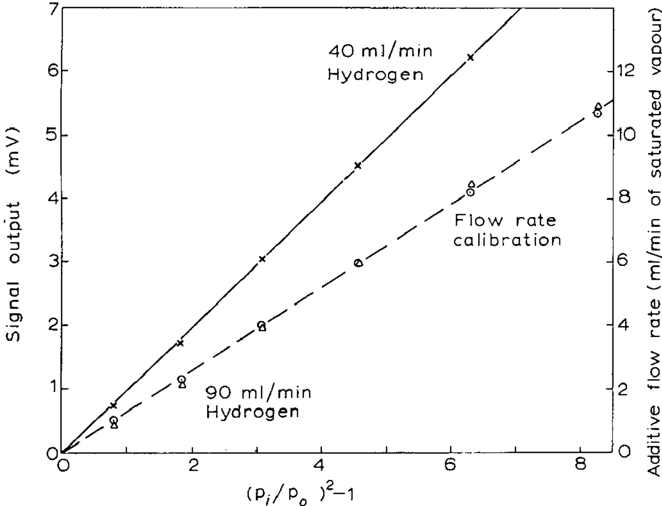

Fig. 9. Linearity of response for *n*-heptane and flow calibration check.

In Fig. 9 the upper line shows the results obtained for *n*-heptane at flow rates through the saturator from 1 to 10 ml/min. The signal output is plotted against the flow term $[(p_i/p_o)^2 - 1]$, eqn. 5, and the flow rate through the saturator (broken line) is also plotted on the same scale. The slight deviations of the experimental points from a straight line are consistent in both sets of results, indicating that this is due to small errors in the pressure gauge calibration. After correction for this and for the backpressure at the jet, it is found that the detector is linear over the range shown to within the accuracy of the experimental results, estimated to be better than $\pm$ 1.5 % overall.

The experimental points shown by the triangles in Fig. 9 are of particular interest since these correspond to a non-linear form of response similar to that reported elsewhere for a detector with a relatively massive jet, designed for use with capillary columns and hydrogen carrier gas[14]. The present results were obtained at a high hydrogen flow rate, although insufficient to cause visible heating of the jet. It is therefore concluded that this non-linearity is associated with a high hydrogen concentration at the jet and is not caused simply by over-heating of the jet. Nevertheless the con-

sequences are self-evident; if hydrogen is used as a carrier gas it is necessary to add nitrogen or a similar diluent to the gas stream entering the detector to preserve linearity at higher concentrations.

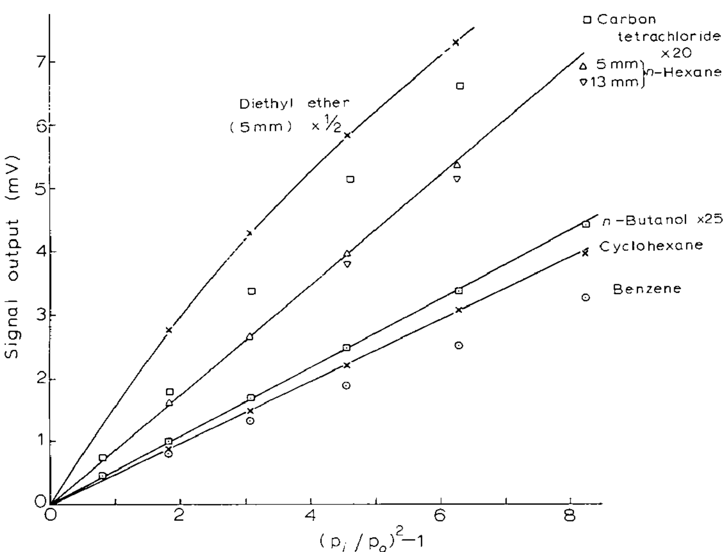

Fig. 10. Linearity of response for various additives.

Further linearity results under near optimum conditions for a number of other compounds are shown in Fig. 10. Benzene and carbon tetrachloride are of interest in that non-linearity becomes evident at much lower concentration levels with these compounds than with aliphatic hydrocarbons[18]. This is evident in comparing the results for benzene and cyclohexane which correspond to a similar signal output. For further comparison and reference purposes Table II gives the approximate mass flow rates corresponding to the second (10 p.s.i.g.), fourth (20 p.s.i.g.) and sixth point (30 p.s.i.g.) on each of the plots in Figs. 9–11. Carbon tetrachloride shows very pronounced non-linearity at ion currents above about 6×10^{-9} A but some improvement may be obtained by increasing the hydrogen flow rate. Thus, it has been found that the linearity of ethyl bromide (not shown in the figures) is improved at higher hydrogen flows, and this may well be a general effect for all halogenated compounds. This point is discussed further below and in the next section.

The results for *n*-butanol are taken from earlier work[15] carried out with the equipment described here, and cover a concentration range approximately twenty times lower than that for cyclohexane. At the other extreme are the results for *n*-hexane and diethyl ether, also from an earlier investigation. The departure from linearity of the *n*-hexane results at medium concentrations, with a 13 mm I.D. collector electrode, was due to failure to reach the saturation current level with an applied voltage of 380 V. This was remedied by using a 5 mm I.D. collector, but a slight residual deviation remains at the higher concentrations. The results for diethyl ether have been corrected for saturator inefficiency (Fig. 4) but they can only be regarded as approximate. Nevertheless the departure from linearity at these con-

TABLE II

REFERENCE DATA FOR FIGS. 9–11

Compound	*Mol. wt.*	*V.p.*[a] (*mm*)	*Fig.*	*Flow rate* ($\mu g/sec$) *corresponding to*		
				10 p.s.i.g.	*20 p.s.i.g.*	*30 p.s.i.g.*
Diethyl ether	74	440	10	(73)	(188)	(341)
n-Hexane	86	121	10	23	60	(109)
Cyclohexane	84	78	10	15	38	(69)
Benzene	78	74	10	(13)	(33)	(60)
n-Heptane	100	35	9	7.9	20	37
Toluene	92	22	11	4.6	11.7	21
n-Butanol	74	5.0	10	0.8	2.1	3.9
Carbon tetrachloride	154	88	10	(31)	(78)	(142)

[a] At 20°: estimated from data given in *Handbook of Chemistry and Physics*, The Chemical Rubber Co., Cleveland, 1969, p.D-148. Figs. in brackets correspond to a non-linear response.

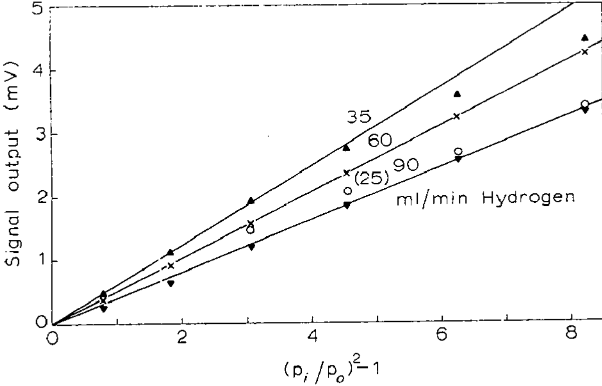

Fig. 11. Linearity of response for toluene at different hydrogen flow rates.

centration levels is quite evident. Once again a 5 mm collector electrode was used and inefficient ion collection did not contribute to these results.

As with benzene (Fig. 10) the linearity of response for toluene is relatively poor at hydrogen flow rates below or at that corresponding to the maximum signal output. This is shown in Fig. 11 by the results corresponding to hydrogen flow rates of 25 and 35 ml/min. However, increasing the hydrogen flow rate still further to 60 ml/min resulted in a considerable improvement in linearity as is shown in the figure. At still higher flow rates (90 ml/min) an alternating effect is observed similar to that obtained with *n*-heptane at high hydrogen flow rates (Fig. 9).

The effect of the hydrogen flow rate

The departure from linearity with a large increase in hydrogen flow rate has already been mentioned in the previous section for the cases of *n*-heptane and toluene. This behaviour has been further investigated by examination of the signal *versus* hydrogen flow rate curves at several concentration levels. This was done by repeatedly injecting fixed volumes of gas sample into a chromatograph with a Carle gas sampling valve and varying the hydrogen flow to the detector. Up till the present time it has

been assumed that the optimum hydrogen–nitrogen ratio is independent of the compound type, and the only reported work in this area supports this view[3]. However this is not always so, although it is generally a sufficiently close approximation at low concentrations (Fig. 12). As the concentration of organic additive increases there is a shift in the maximum to higher hydrogen flow rates, and this is shown for *iso*-pentane in Fig. 13. Also included in this figure are some results for carbon tetrachloride, which show a very pronounced departure from the normal behaviour. At higher concentrations a peak maximum is observed.

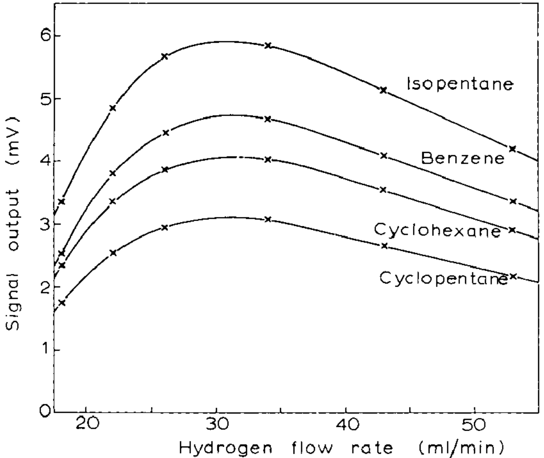

Fig. 12. Effect of hydrogen flow rate on response, for various compounds at low concentrations (by chromatographic analysis).

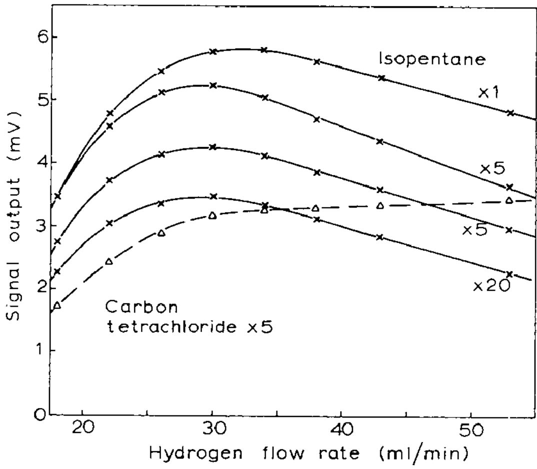

Fig. 13. Effect of concentration and hydrogen flow rate on the response of isopentane (by chromatographic analysis).

Fig. 14 shows the shift in the peak maximum with increased additive concentration for a number of different compounds, each being measured at two concen-

tration levels. The straight connecting line between these two levels does not necessarily imply that a linear shift occurs with increased concentration, but is used merely to simplify the diagram. This shift can of course have a pronounced effect on linearity of response, and may also help to explain the strange changes in relative responses which have been reported for different detectors and different hydrogen flow rates[14, 19]. However, it is evident from Fig. 13 (and from Fig. 12 at low hydrogen flow rates) that even at low concentrations the relative responses may be dependent on the hydrogen flow rate.

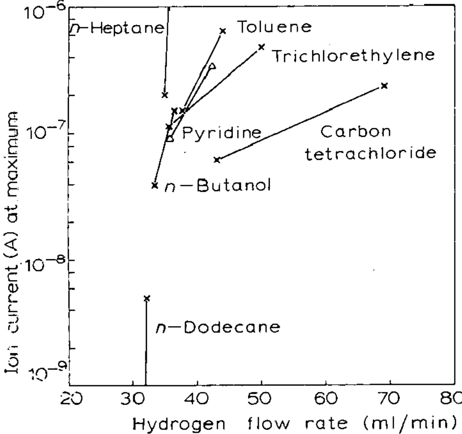

Fig. 14. Effect of compound type and concentration on the hydrogen flow rate required for maximum signal output (nitrogen flow rate 35 ml/min).

It is tempting to try to relate the above shift to the molecular structure of the compound, but insufficient data have been obtained so far for a serious attempt to be made. Nevertheless it is evident that the displacement is most pronounced with aromatic and non-hydrocarbon compounds, and the largest shift so far observed is for carbon tetrachloride. Data obtained at three different total nitrogen flow rates (35, 60 and 90 ml/min) suggest that the shift, measured in ml/min of hydrogen, is almost independent of the nitrogen flow rate over this range. The observed shifts for the two chlorinated compounds are much greater than could be explained on the simple basis of the hydrogen required to convert all the chlorine in the molecule to hydrogen chloride, or indeed for total conversion of the compound to methane and hydrogen chloride. On the other hand, a relationship based on burning velocity considerations[6] also appears to be untenable in view of the observation that carbon tetrachloride has only a small effect on the burning velocity of a hydrogen–air flame, much less than that of saturated hydrocarbons or aromatics[20].

ACKNOWLEDGEMENT

This work was made possible by the award of a Research Fellowship by the Shell Group of Companies in Australia.

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CHROM. 4860

PREPARATIVE GAS CHROMATOGRAPHIC RESOLUTION OF 3,3-DIMETHYL-2-BUTANOL AND OTHER ALCOHOLS*,**

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SUMMARY

The feasibility of preparative gas chromatographic resolution of small quantities of certain alcohols as the N-trifluoroacetyl-L-alanyl diastereomeric esters was demonstrated. The degree of resolution attained and the quantity resolved were strongly affected by the column length. Utilizing a 1 × 1584 cm column, both optical forms of 3,3-dimethyl-2-butanol were obtained with greater than 98 % purity.

INTRODUCTION

In instances where only small amounts (*ca.* 1 ml or less) of both optically active forms of a particular alcohol are required, preparative gas chromatographic resolution would offer certain advantages over the classical method of alcohol resolution[1]. Small amounts of the resolved alcohol could easily be obtained without waste and without the necessity of working with toxic alkaloids. In theory, both optical forms could be obtained in one operation rather than through numerous recrystallizations and the change of the alkaloid base often involved in obtaining both optical forms of a particular alcohol. The optical purity could also be immediately and conveniently determined by analytical gas chromatography.

Recently, correlative to the gas chromatographic resolution of amino acids, the possibility of resolving alcohols by gas chromatography has been demonstrated[2]. Such work has been done predominately towards analytical goals, much of it with long capillary columns.

Motivated by the need for small amounts of both optical isomers of 3,3-dimethyl-2-butanol, the following study was initiated to investigate the possibility of resolving this alcohol, as well as others, by preparative gas chromatography.

MATERIALS AND METHODS

Instruments and equipment

The instrument used in this study was a Model 600 Series Research Specialties gas chromatograph equipped with a dual hydrogen flame detector. The preparative

* Michigan Agricultural Experiment Station Journal Article No. 5095.
** Portion of a thesis submitted by the senior author in partial fulfilment of the requirements for the Doctor of Philosophy degree in entomology.

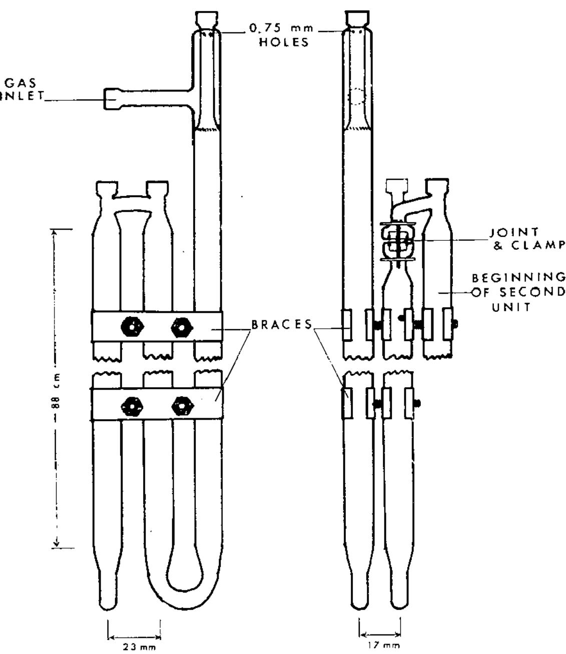

Fig. 1. Diagram of first unit and first section of the second unit of the preparative GLC column used to resolve the various diastereomeric esters.

column (Fig. 1) was constructed of three main units. Each unit consisted of six vertical sections of 10 mm I.D. pyrex tubing *ca.* 88 cm long connected together by 4 mm I.D. horizontal crossovers. Septum sockets were mounted directly above the vertical columns on the top of the crossovers. An all glass condenser type injector head, constructed so the carrier gas would enter the column through four 0.75-mm holes just beneath the injection septum was attached to the initial vertical column of the first unit. The three main units could be connected by means of 5-mm Fischer Porter Solv Seal® joints (Fischer Porter Co., Warminster, Pa., U.S.A.), which were held tightly together by means of a clamp consisting of steel collars (tapped and fitted with two small machine screws) on each joint socket. Thus the column could be operated at three lengths, *ca.* 528 cm, 1056 cm or 1584 cm.

The column was filled through the septum holders and compacted with a vibrator. A 1/8–1/4 in. plug of silanized glass wool was lightly compacted into the junctures of the crossovers and vertical columns.

A splitter consisting of a stopcock and a septum socket (inserted between the terminus of the column and the stopcock) containing a septum pierced by a piece of 1/16 in. stainless steel tubing which led to the hydrogen flame detector was installed in the all glass outlet. The split ratio was coarsely adjusted by the stopcock while fine adjustments were made by adjusting the hydrogen line pressure to the detector. The outlet tube was heated by means of an electrical heating tape.

Two 11 × 1 cm vacuum traps modified with $\mathfrak{S}$ balls to fit the column outlet and cooled with a dry ice–acetone bath were used to collect the material from the column.

Derivative preparation

±3,3-Dimethyl-2-butyl-N-trifluoroacetyl-L-alanine. A mixture of 50 ml of ±3,3-dimethyl-2-butanol containing 1.2 mequiv. of HCl per ml was reacted with 1 g L-alanine at 100° for 1 h in an oil bath-magnetic stirrer. The more volatile components of the mixture were removed from the alanine and alanyl ester by fractional distillation and the alcohol fraction (118–122°) saved and measured volumetrically. An additional 1.2 mequiv. of HCl per ml was added to the alcohol and the mixture was again reacted with 1 g of L-alanine as before. This procedure was continued until four 1-g quantities of alanine had been used. The combined alanine–alanyl ester residues were partitioned between about equal volumes of ether and 1 *M* Na_2CO_3. The carbonate fraction was extracted quantitatively with ether; the ether was removed on a rotating evaporator and the residue dissolved in 10–15 ml methylene chloride. This was reacted at room temperature with about a 1.5 *M* excess of trifluoroacetic anhydride. The more volatile components were removed by a rotary evaporator at 60° and the remaining residue vacuum distilled. A distinct non-viscous fore-run occurred followed by the very pale yellow more viscous N-trifluoroacetyl alanyl ester. In this manner, 10.4 g of the N-trifluoroacetyl alanyl ester was obtained.

±2-Pentyl-N-trifluoroacetyl-L-alanine. 2-Pentyl-N-trifluoroacetyl-L-alanine was prepared in a manner similar to the preparation of the corresponding 3,3-dimethyl-2-butyl derivative except that more amino acid and alcohol–HCl mixture were used initially and only one esterification was carried out.

Other amino acid derivatives. Small amounts of ±3,3-dimethyl-2-butyl esters of valine, phenylalanine and proline were prepared in a manner similar to the preparation of the corresponding alanyl ester. Smaller quantities of alcohol and amino acid were used and only one esterification and no vacuum distillation was carried out. These esters along with small amounts of the corresponding alanyl ester were converted to their N-alkyl derivatives by means of acetic anhydride, trifluoroacetic anhydride, propionyl chloride or trichloroacetyl chloride.

Small quantities of 1,1,1-trifluoro-2-propyl-N-trifluoroacetyl-L-alanine were also prepared in a similar manner.

Effect of liquid phase and particle size

To test the suitability of various liquid phases for resolving ±3,3-dimethyl-2-butanol the following liquid phases were tested in 4 mm I.D. analytical columns: cyclohexanedimethanol adipate, tetramethylcyclobutanediol adipate, 1,2,3-tris(2-cyanoethoxy)propane, OV-1 (dimethyl silicone), OV-17 (phenyl methyl silicone) and OV-25 (phenyl methyl silicone). To test the suitability of various diastereomers for the resolution of 3,3-dimethyl-2-butanol, the N-acetyl, N-trifluoroacetyl, N-trichloroacetyl and N-propyl derivatives of the alanyl, valyl, phenylalanyl and prolyl esters of the alcohol were chromatographed on these columns. The N-trifluoroacetyl ester chromatographed on either the OV-1 or OV-17 columns appeared to give the most favorable results. Columns (1 × 528 cm), packed with either 45/60 or 100/120 mesh DMCS-treated acid-washed Chromosorb W® coated with either 10 % OV-1 or OV-17 were used to determine the effect of particle size and of the two liquid phases on preparative scale separations. The coarser packing appeared to have a slightly higher overload capacity while OV-1 appeared to give resolutions slightly superior to those given by OV-17.

In order to appraise the effect of column length on the quality of separation, the three sections of the preparative column were packed with 10 % OV-1 on 45/60 DMCS-treated acid-washed Chromosorb W. The column was operated at 165° with a 230 ml/min nitrogen flow rate. Injections of 5, 10, 15, and 20 μl of $\pm$3,3-dimethyl-2-butyl-N-trifluoroacetyl-L-alanine were made into the column. This procedure was then repeated for the two shorter lengths.

Resolution of the $\pm$3,3-dimethyl-2-butyl-N-trifluoroacetyl-L-alanine

For the preparative resolution of the $\pm$3,3-dimethyl-2-butyl-N-trifluoroacetyl-L-alanine diastereomers, the 1584-cm column was utilized. The column temperature was 165° and the nitrogen carrier flow rate was 230 ml/min. No flash heater was used. A 15-μl injection was introduced into the column every 6–7 min. Since the injected material had a retention time of approximately 30 min, approximately five injections were on the column at any given time after the fifth injection. Injection timing was varied slightly to facilitate concurrent injection and trapping schedules. The outlet tube to the cold trap was maintained at about 190°. The splitter was adjusted to deliver >99% of the chromatographed material to the cold trap allowing the remainder to go to the hydrogen flame detector. The trapping schedule could thus be synchronized to the actual elution by watching the chart recorder. Trapping for the first peak was initiated after the recorder pen began to rise, and was terminated slightly before it reached the low point between the two peaks. Trapping for the second peak was initiated shortly after the pen began to rise and terminated as the pen reached base line (Fig. 3).

Saponification of the resolved diastereomers

Small portions (0.1–0.3 ml) of the resolved diastereomers were placed in separate 100 × 13 mm screw cap culture tubes. A 20 % (w/v) methanolic NaOH solution made of methanol–water (7:5) was added to each diastereomer until the final NaOH molarity was three times that of the diastereomer. Boiling chips were added and the tubes sealed with Teflon-lined caps and immersed to the level of the contents in a boiling water bath for 1 min. The saponification mixture was cooled under tap water, diluted to twice its volume with water and extracted quantitatively with equal volumes of ether. The pooled ether extracts were then washed with water until neutral and dried over anhydrous Na_2SO_4. The resolved 3,3-dimethyl-2-butanol was conveniently separated from the ether and methanol by preparative gas chromatography using a 1 × 528 cm column packed with 10% OV-17 on 45/60 mesh DMCS-treated acid-washed Chromosorb W.

For larger amounts the saponification time may need to be increased. Trial saponifications may be conveniently monitored on unactivated Silica Gel G thin-layer plates, chromatographed in the top phase of *n*-butanol–acetic acid–water (25:6:25) and developed with ninhydrin. The trifluoroacetyl moiety is removed almost immediately and the resulting ester is readily detected with ninhydrin as is alanine, one of the final hydrolysis products. The R_F value of the alanyl ester is about 4.7 times that of alanine. The disappearance of the alanyl ester may also be monitored by gas chromatography on 3-m 10 % OV-1 analytical columns.

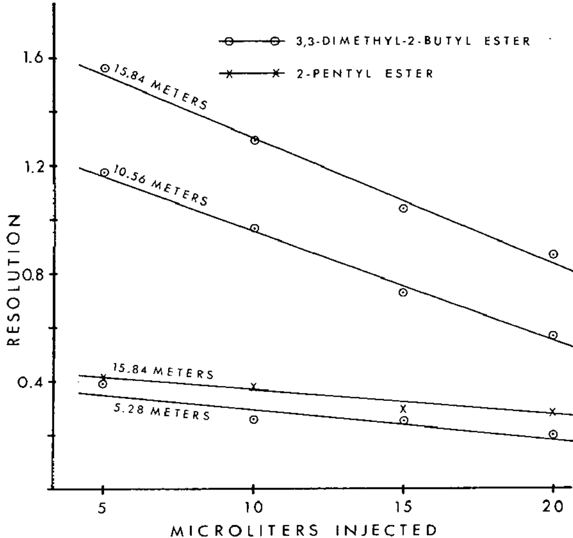

Fig. 2. The effect of length of the preparative column on separation of the two diastereomers of 3,3-dimethyl-2-butyl-N-trifluoroacetyl-L-alanine (⊙—⊙) and 2-pentyl-N-trifluoroacetyl-L-alanine (×—×).

Determination of optical purity

To test the stearic purity of the resolved alcohols the (+) or (—) 3,3-dimethyl-2-butyl-N-trifluoroacetyl-D-alanyl derivative was prepared by the following micro-procedure. D-Alanine (50 mg) was dissolved in 1 ml of trifluoroacetic acid. To this 1 ml of trifluoroacetic anhydride was added. After 15 min at room temperature the solvent was removed under a stream of nitrogen and the N-trifluoroacetyl-D-alanine was dissolved in 1 ml of methylene chloride. To this 1 ml of thionyl chloride was added and allowed to stand for 15 min at room temperature. The solvents were again removed under a stream of nitrogen and the acid chloride dissolved in 1 ml of methylene chloride. From this two 0.1-ml aliquots were taken and reduced to 0.02 ml under a stream of nitrogen in two small conical centrifuge tubes. Separately, 5 μl of the appropriate optical form of the alcohol was added to each tube. After several minutes 0.5 ml of methylene chloride and 0.5 ml of water were added to each of the samples and the aqueous fraction was extracted quantitatively with methylene chloride. The methylene chloride fractions from each sample were dried with a small amount of anhydrous Na_2SO_4, and evaporated to 0.1 ml under a stream of nitrogen. The derivatives were chromatographed on 3-m 10% OV-17/DMCS-treated acid-washed Chromosorb W columns at 118° with a nitrogen carrier gas flow rate of 90 ml/min. No flash heater was used.

RESULTS

The effect of column length on the degree of separation is quite pronounced. This is shown in Fig. 2 by a plot of resolution *versus* the quantity injected. The number of theoretical plates, based on the first peak for 15-μl injections of unresolved material at 165° and a carrier gas flow rate of 230 ml/min, increased with column length,

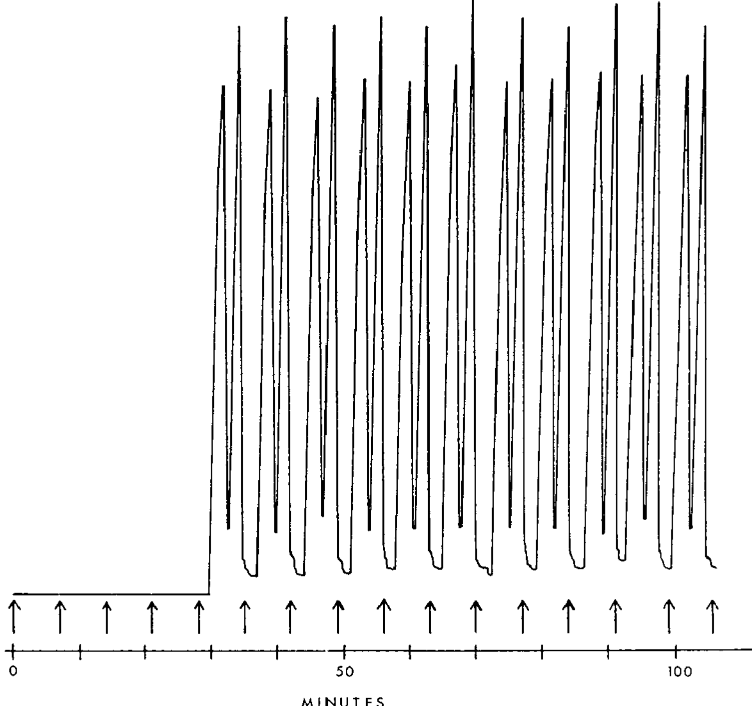

Fig. 3. Preparative separation of ± 3,3-dimethyl-2-butyl-N-trifluoroacetyl-L-alanine. Arrows indicate 15-μl injections. The first peak of each group of two represents the ester containing the levorotatory form of the alcohol and the second peak the dextrorotatory form. Chromatography was done on a 1 × 1584 cm column packed with 10 % OV-1 on 45/60 mesh DMCS-treated acid-washed Chromosorb W at 165° and a nitrogen flow rate of 230 ml/min.

i.e. 553, 1345, and 2277. Fig. 3 shows the separations attained with the 1584-cm column at 165° for 15-μl injections, spaced approximately 6 min apart. Slightly better separations can be obtained at lower temperatures with all three column lengths, but since the peak bases are spread out more, the time interval between injections must be increased and the ratio of the amount injected/unit of time is diminished. As can be seen from Fig. 2, the ability of the 1584-cm column to resolve the 2-pentyl diastereomer is much less than its ability to resolve the 3,3-dimethyl-2-butyl analogue. Slightly better separation was obtained with the 2-octyl analog. Because of difficulties encountered in preparing large quantities of the 1,1,1-trifluoro-2-propyl derivative, only slightly larger than analytical amounts (*ca.* 10 μg) were tested. The degree of separation was approximately the same as that given by a similar load of the 3,3-dimethyl-2-butyl analog.

Fig. 4 shows the chromatograms of the esters formed by esterification of N-trifluoroacetyl-D-alanine with one of the resolved 3,3-dimethyl-2-butanols and thus indicates the degree of resolution achieved by the preparative column. It should be pointed out, however, that the results depicted in Fig. 4 indicate the minimum degree of resolution achieved because of the possibility of small amounts of racimization of the alcohol during the saponification as well as of the alanine during the formation

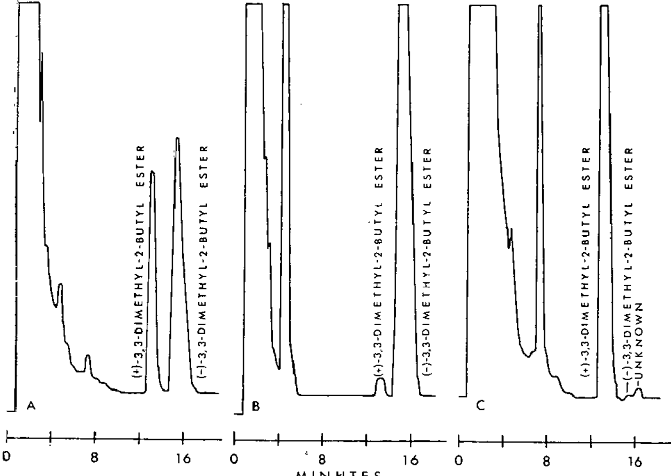

Fig. 4. Gas chromatographic demonstration of the optical purity of the 3,3-dimethyl-2-butanol attained by preparative gas chromatography. The N-trifluoroacetyl-D-alanyl esters were chromatographed on a 3-m 10% OV-17 on 100/120 mesh DMCS-treated acid-washed Chromosorb W column at 118° and a nitrogen flow rate of 90 ml/min. Chromatogram A is from the derivatives made from unresolved alcohol; chromatograms B and C are from derivatives made from alcohols derived respectively from the first and second peaks of the preparative chromatography.

of the N-trifluoroacetyl acid chloride. It is also possible that the D-alanine used for the esterification was contaminated with small amounts of the L-isomer. By triangulating the peaks of the more prevalent diastereomers and comparing the peak areas of the two diastereomers, the optical purities are shown to be $>98\%$.

Polarimetric studies showed the alcohol derived from the first peak from the preparative column to be levorotatory and from the second to be dextrorotatory. The last peak in Fig. 4C is apparently not the result of contamination by the levorotatory form and is of unknown origin, but perhaps represents a structural isomer or analog of 3,3-dimethyl-2-butanol. The contamination due to the levorotatory form is apparently represented by the shoulder preceding the unknown peak. The slight tailing in Fig. 4A probably is also the result of this unknown material.

DISCUSSION AND CONCLUSIONS

With the aid of automatic injection and collecting devices and improved columns, especially longer columns, preparative gas chromatographic resolution of at least certain alcohols would be quite practical. The maximum practical column length for this purpose is not known, but the results indicate that it was not surpassed in this study.

The use of an improved cold trap would also be beneficial. By using the trap described, only about 62% of the amount injected could be accounted for in the trap. Although nearly all the trapped material was condensed on the first 2 or 3 cm of the trap, quantities of smoky-appearing material issued from the cold trap outlet. The appearance of this material coincided with the elution of the two diastereomers. Some of this material could be trapped in a U-tube cooled by liquid nitrogen but even with

several inches of loosely packed glass wool the smoky effluence continued from the trap outlet. A portion of what was trapped in the liquid nitrogen cooled tube was the 3,3-dimethyl-2-butyl derivative, but based on solubility studies, the smoky material also contained a more polar and unknown fraction. The smoky effluent also appeared when trapped material was rechromatographed. This phenomenon was not an artifact of the 3,3-dimethyl-2-butyl derivative alone but also occurred with the other esters tested in this study.

ACKNOWLEDGEMENTS

Funded in part by a National Science Foundation sponsored Michigan State University Breakthrough Research Program and a Biomedical Sciences Support Grant from Michigan State University.

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CHROM. 4850

GAS CHROMATOGRAPHY OF HOMOLOGOUS ESTERS

PART IV. INFLUENCE OF STATIONARY PHASE POLARITY ON RETENTION OF UNSATURATED ESTERS

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(Received June 3rd, 1970)

SUMMARY

The retention behaviour of a number of homologous series of unsaturated esters on polysiloxane stationary phases of increasing polar character is reported.

INTRODUCTION

An earlier report[1] has described the gas chromatography of several series of homologous unsaturated esters on the essentially non-polar Methyl Polysiloxane Stationary Phase SE-30.

Esters may be conveniently represented as

$$\mathrm{R{-}\overset{\overset{\displaystyle O}{\|}}{C}{-}O{-}R'}$$

where the carbon numbers of the acid and alcohol chains are R and R′ respectively. Studies of alkanes[2] and of fatty esters[3–5] show that the introduction of a double bond in the acid chain produces a reduction in retention on a non-polar stationary phase. With simple esters where R = 2, *i.e.* acrylates and propionates, this effect has been observed; similarly where R = 1, 2 or 3 and R′ = 2, *i.e.* vinyl and ethyl esters, decreased retention is also experienced[1].

When the hydrogen of the α-carbon atom of the acrylate ester (R) was replaced by a methyl group (the methacrylate esters) increased retention was observed when compared to the appropriate saturated ester. A greater increase in retention was observed with substitution of the β-carbon atom (R) (the crotonate esters) when compared with the butyrate esters. The replacement of a hydrogen atom in vinyl acetate with a methyl group (isopropenyl acetate) also produced increased retention compared with the saturated ester.

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TABLE I

RETENTION DATA FOR UNSATURATED ESTERS

Ester	*Stationary phase*											
	SE-30			*OV-17*			*OV-25*			*XE-60*		
	V_g[a]	V_R[b]	I_R	V_g[a]	V_R[b]	I_R	V_g[a]	V_R[b]	I_R	V_g[a]	V_R[b]	I_R
Methyl acrylate	0.128	0.194	569	0.140	0.450	743	0.110	0.512	766	0.120	0.78	844
Ethyl acrylate	0.205	0.310	664	0.165	0.550	784	0.155	0.720	831	0.160	1.03	911
Propyl acrylate	—	—	—	0.280	0.930	887	0.260	1.21	939	0.232	1.55	1006
Butyl acrylate	0.595	0.903	878	0.470	1.57	987	0.445	2.02	1040	0.380	2.45	1111
Pentyl acrylate	0.960	1.455	978	0.870	2.80	1108	0.715	3.28	1142	0.580	3.75	1210
Hexyl acrylate	1.595	2.42	1068	1.415	4.55	1200	1.155	5.30	1240	0.895	5.78	1312
Isopropyl acrylate	—	—	—	0.195	0.65	816	0.180	0.835	865	0.165	1.06	914
2-Methylpropyl acrylate	—	—	—	0.410	1.32	953	0.330	1.51	986	0.300	1.94	1055
Methyl methacrylate	0.217	0.329	677	0.210	0.680	829	0.175	0.830	865	0.160	1.02	909
Ethyl methacrylate	0.320	0.482	756	0.249	0.830	863	0.245	1.12	920	0.210	1.40	980
Propyl methacrylate	0.528	0.798	856	0.402	1.34	957	0.375	1.79	1016	0.315	2.10	1085
Butyl methacrylate	0.884	1.34	962	0.690	2.30	1068	0.640	2.94	1118	0.485	3.24	1179
Pentyl methacrylate	1.46	2.212	1064	1.23	3.97	1176	1.035	4.75	1214	0.760	4.90	1272
Hexyl methacrylate	2.40	3.64	1165	2.07	6.68	1281	1.630	7.60	1312	1.115	7.43	1369
Octyl methacrylate	—	—	—	5.10	17.00	1463	3.905	18.45	1489	—	—	—
2-Methylpropyl methacrylate	0.750	1.135	928	0.620	1.94	1032	0.475	2.21	1060	0.410	2.64	1128
3-Methylbutyl methacrylate	—	—	—	4.05	13.51	1416	3.025	14.42	1439	—	—	—
Ethyl crotonate	0.441	0.667	820	0.430	1.39	965	0.395	1.80	1016	0.340	2.12	1086
Propyl crotonate	0.710	1.075	917	0.670	2.23	1065	0.625	2.87	1112	0.535	3.35	1188
Butyl crotonate	1.190	1.804	1021	1.10	3.67	1157	1.005	4.74	1210	0.760	4.90	1272
Pentyl crotonate	1.905	2.89	1119	1.84	6.13	1265	1.515	7.22	1307	1.170	7.55	1372

Isopropyl crotonate	0.540	0.818	863	0.435	1.50	978	0.385	1.77	1015	0.337	2.24	1090
2-Methylpropyl crotonate	0.978	1.481	983	0.900	2.80	1094	0.780	3.58	1156	0.630	4.06	1232
3-Methylbutyl crotonate	1.61	2.44	1082	1.413	4.71	1208	1.255	5.92	1257	0.980	6.32	1332
Vinyl acetate	0.115	0.173	548	0.096	0.320	679	0.070	0.341	688	0.086	0.571	770
Vinyl propionate	0.190	0.286	648	0.150	0.490	763	0.125	0.580	790	0.135	0.870	861
Vinyl butyrate	0.310	0.466	750	0.260	0.810	859	0.200	0.950	892	0.200	1.29	963
Vinyl pentanoate	—	—	—	0.380	1.29	950	0.340	1.56	990	0.280	2.00	1060
Vinyl hexanoate	—	—	—	0.635	2.08	1037	0.565	2.59	1095	0.430	3.07	1162
Vinyl isobutyrate	—	—	—	0.150	0.518	773	0.175	0.815	857	0.145	1.04	912
Vinyl 3-methylbutyrate	—	—	—	0.285	0.950	889	0.250	1.15	928	0.210	1.50	996
Vinyl 4-methylpentanoate	—	—	—	0.500	1.64	998	0.450	2.09	1050	0.350	2.50	1118
Allyl formate	0.130	0.200	571	0.120	0.380	714	0.100	0.455	743	0.120	0.780	844
Allyl acetate	0.210	0.310	670	0.190	0.600	801	0.155	0.720	831	0.144	0.960	892
Allyl propionate	0.340	0.521	770	0.310	0.970	895	0.260	1.18	933	0.230	1.48	995
Allyl butyrate	0.520	0.790	853	0.500	1.60	990	0.400	1.84	1020	0.355	2.29	1096
Allyl pentanoate	0.880	1.33	960	0.830	2.60	1090	0.660	3.07	1123	0.535	3.45	1192
Allyl hexanoate	1.430	2.16	1056	1.375	4.30	1192	1.075	4.89	1219	0.830	5.35	1294
Allyl octanoate	—	—	—	—	—	—	—	—	—	1.960	12.66	1490
Allyl isobutyrate	0.430	0.649	815	0.324	1.08	916	0.295	1.37	961	0.280	1.81	1041
Allyl 3-methylbutyrate	0.710	1.082	915	0.620	1.94	1032	0.500	2.29	1065	0.410	2.64	1130
Allyl 4-methylpentanoate				—	—	—	0.885	4.02	1181	0.640	4.27	1240
Isopropenyl acetate	0.185	0.274	644	0.140	0.460	744	0.130	0.605	782	0.095	0.680	815
Isopropenyl propionate	—	—	—	0.220	0.749	846	0.210	0.970	894	0.165	1.10	921
Isopropenyl butyrate	—	—	—	0.385	1.263	944	0.340	1.58	988	0.225	1.60	1012
Isopropenyl pentanoate	—	—	—	0.600	1.97	1036	0.570	2.60	1095	0.365	2.60	1022
Isopropenyl hexanoate	—	—	—	1.005	3.30	1138	0.895	4.16	1189	—	—	—
Isopropenyl isobutyrate	—	—	—	0.265	0.870	875	0.250	1.17	928	0.200	1.43	988
Isopropenyl 3-methylbutyrate	—	—	—	—	—	—	0.410	1.91	1029	0.330	2.20	1084
Isopropenyl 4-methylpentanoate	—	—	—	0.795	2.61	1092	0.715	3.33	1144	0.510	3.40	1184

[a] Corrected for dead volume.
[b] Relative to nonane.

The present work reports the retention behaviour of unsaturated esters of the types described previously, together with those where R′ = 3 and has both a straight chain (the allyl esters) and also a branched chain (isopropenyl esters). The esters were examined on several stationary phases of increasing polar character. The results are compared with those of fatty esters where decreased and increased retention is observed with the introduction of unsaturation on substantially non-polar and polar stationary phases, respectively.

Retention data of unsaturated esters has been tabulated by SCHUPP AND LEWIS[6]. Allyl esters (C_8–C_{18}) have been examined previously on an Apiezon M column[7] while systematic studies relevant to this work have been previously reviewed[1].

EXPERIMENTAL

Preparation of esters

The esters where available were of commercial quality and of substantial purity. The remainder of the esters were prepared using esterification or transesterification procedures with an acidic ion-exchange resin as catalyst.

Chromatography

The retention data were obtained isothermally at 150° using 12 ft. × $^1/_4$ in. O.D. aluminium columns packed with 10 % stationary phase (Methyl Silicone Polymer SE-30, Methyl Phenyl Silicone Polymers OV-17 and OV-25 and Methyl Cyanoethyl Silicone Polymer XE-60) each on 60–80 mesh acid washed and silanised Celite 560. The equipment, conditions and calibration procedures were as previously reported[1].

The retention data of the esters examined are shown in Table I as net retention (V_g), relative retention (V_R) using nonane as standard, and as retention indices (I_R).

DISCUSSION OF RESULTS

The influence of a double bond in the acid chain (R) is observed by comparison of the homologous acrylate, methacrylate and crotonate esters with the propionate, isobutyrate and butyrate esters as shown in Fig. 1. Figs. 1a and b show plots of relative retention *versus* the number of carbon atoms in the alcohol chain (R′) on SE-30 and XE-60 stationary phases. The slopes of all of the esters on SE-30 were essentially parallel, the minor variations apparent having been previously described[1]. With the XE-60 phase plots representing the acrylate and butyrate esters intercepted (*i.e.* decreased retention due to the double bond being more apparent with the higher alkyl esters) while the plot representing the crotonate esters showed a considerably greater increase in retention on the more polar phase.

London forces, due to the interaction of two rapidly changing instantaneous dipoles, exist to varying degrees between all molecules. With non-polar molecules these are the sole attractive forces. The London forces do not exhibit any special selectivity and the retention behaviour in non-polar systems is determined by the boiling point of the compounds. This effect is observed with simple saturated normal and isoalkyl esters on SE-30 (ref. 8) as is the reduction of retention due to branching as described by JAMES AND MARTIN[3].

The behaviour of fatty esters on non-polar stationary phases was suggested by

JAMES[4,5] to follow that of hydrocarbons. The attractive forces are influenced by the chain length, branching, unsaturation, location of double bond and the presence of conjugation.

Linear relationships between chain length and retention of homologous compounds are usually obtained and greater methylene separation factors are observed

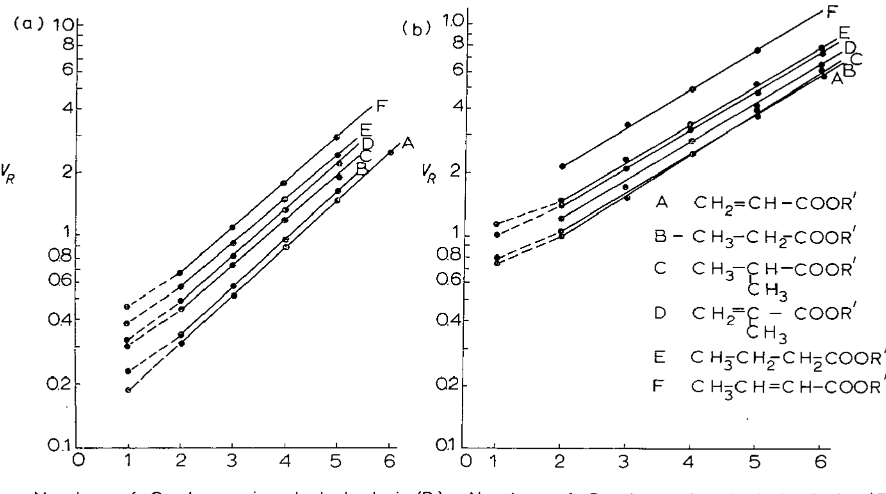

Fig. 1. Plot of logarithm of relative retention *versus* number of carbon atoms in the alcohol chain of unsaturated esters on (a) SE-30 and (b) XE-60 stationary phases.

with non-polar stationary phases where London forces are at their maximum. With polar phases the methylene separation factors are lowered as the London forces are reduced.

The introduction of a double bond into a saturated fatty ester was shown by JAMES AND MARTIN[3] to reduce retention on a non-polar phase, a conclusion now widely apparent. Maximum interaction occurs with methylene groups and this is reduced with the ethylenic group.

With polar stationary phases specific interactions occur between polarisable double bonds and the ester groups in addition to the London interactions which operate in the opposite direction. For each non-interacting double bond an approximately equivalent retention increase is observed on a polar phase conversely on a non-polar phase decreased retention occurs. While conjugated fatty esters have not been extensively studied the limited data available show increased retention occurring on both polar and non-polar phases; conjugation with the ester carbonyl is similarly anomalous[9,10].

Some polar character is exhibited by saturated esters due to the carbonyl structure. Although the dipole moment is not great the degree of polarisability is higher. The dipole moment of esters is little influenced by unsaturation although the polarisability is increased. Increased retention on a polar phase would be expected with an unsaturated ester of similar boiling point to a saturated ester. The reverse effect would be expected on a non-polar stationary phase.

TABLE II

EFFECT OF THE PRESENCE OF UNSATURATION IN THE ACID CHAIN

Esters	*Stationary phase*			
	SE-30	*OV-17*	*OV-25*	*XE-60*
Acrylate	− 15 − 20[a]	0 + 15[a]	0 + 20[d]	0 + 10[d]
Propionate	− 38[b]	+ 27		
Methacrylate	+ 15 + 30[a]	+ 90 + 95[a]	+ 55 + 60[a]	+ 30 + 60[a]
Isobutyrate	+ 8[b]	+ 27[e]		
Crotonate	+ 30 + 40[c]	+ 125 + 130[c]	+ 80 + 95[a]	+ 90 + 100[a]
Butyrate	+ 68[b]	+ 98[b]	+102[b]	

[a] n-C_2–C_6

[b] Methyl ester.

[c] n-C_2–C_4.

[d] n-C_1–C_6.

[e] Ethyl ester.

The effect of unsaturation in the acid chain is shown in Table II. Increased and decreased retention index increments with unsaturation are shown as + and − respectively.

With the acrylate and propionate esters a reduction in retention is observed with unsaturation on the SE-30 phase. Here reduced retention could be expected due to the double bond but conjugation with the ester carbonyl should increase the retention. This system with the stationary phases of increasing polarity shows the anticipated increase in retention. The methacrylate and crotonate esters on the phases show greater retention than the corresponding saturated esters. The methacrylates due to their branched chain have lower retention than the crotonates on each phase.

With fatty esters the introduction of unsaturation produces little relative effect on boiling point but with simple esters variations in boiling point are more apparent especially with the low polarity phase.

With the same total chain length acrylates and propionates have virtually identical boiling points and the boiling points are not of consequence. Methacrylates have slightly higher boiling points than butyrates and some increase in retention could be expected. Crotonates have substantially higher boiling points than butyrates and a greater increase in the retention could be expected. It thus seems that the boiling

TABLE III

EFFECT OF THE PRESENCE OF UNSATURATION IN ALCOHOL CHAIN

Esters	*Stationary phase*			
	SE-30	*OV-17*	*OV-25*	*XE-60*
Vinyl Ethyl	− 20 − 40	− 18 − 30	− 20 − 40	− 20 − 40
Alkyl Propyl	− 30 − 30	+ 3 + 10	+ 3 + 5	− 9 + 25
Isopropenyl	+ 19[a]	+ 15 + 35	+ 45 + 60	− 15 − 20

[a] Isopropenyl acetate.

point of simple unsaturated esters on a non-polar phase plays a very significant role in determining the retention.

The effect of unsaturation in the alcohol chain is shown in Table III, while Figs. 2a–b show plots of the esters obtained using SE-30 and XE-60 stationary phases respectively. With vinyl and ethyl esters reduced retention is experienced with the unsaturated esters on all four stationary phases. Increased retention on the more polar phases is not observed, the reduction being little affected by the four stationary phases. Terminal unsaturation in a chain is responsible for some reduction in retention in comparison to other positions of unsaturation but the results observed would not be expected from studies of fatty esters.

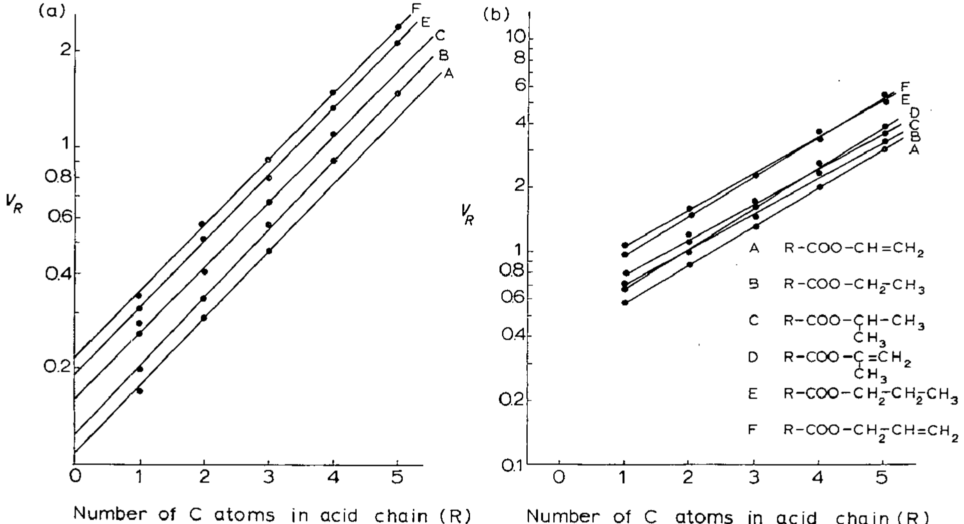

Fig. 2. Plot of logarithm of relative retention *versus* number of carbon atoms in the acid chain of unsaturated esters on (a) SE-30 and (b) XE-60 stationary phases.

Allyl esters exhibited reduced retention on the SE-30 stationary phase and increased retention on the more polar phases. While these results may be as anticipated, terminal unsaturation present as with the vinyl esters and the added methylene group in a non-conjugated environment would not be expected to be significant.

Isopropenyl acetate showed an increase in retention on the non-polar phase similar to that shown by the methacrylate esters.

The majority of the structural arrangements including unsaturation, location of the double bond, *i.e.* terminal or conjugated unsaturation and the presence of branching existing in the simple esters examined have been studied in detail with fatty esters. It is well known that the particular location of a double bond[11,12] or methyl group[13] along a chain will influence retention due to interactions that occur. It is apparent from this work that studies of fatty esters provide little information relevant to the simpler compounds due to the significant effect of interactions that are present. The $CH_2=C(CH_3)-$ structure of the methacrylate and isoprenyl esters is not widely experienced in fatty esters and its behaviour is not apparent from a consideration of

fatty ester studies of the individual effects of branching and the position of unsaturation.

ACKNOWLEDGEMENT

The authors are indebted to the Colonial Sugar Refining Company Limited for a scholarship to one of us (I.D.A.) during the period that this investigation was undertaken.

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QUANTITATIVE GAS CHROMATOGRAPHIC DETERMINATION OF β-HYDROXYBUTYRIC ACID WITH APPLICATION TO EGGS*

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(Received June 5th, 1970)

SUMMARY

A quantitative method for the analysis of β-hydroxybutyric acid has been developed through the gas chromatography of its propyl ester. The ester was formed by reaction with BF_3-propanol and an internal standard, acetophenone, was added to the reaction mixture. The propyl ester and internal standard were extracted with chloroform and chromatographed isothermally on a diethylene glycol succinate column. Inasmuch as β-hydroxybutyrate may enter into polymerization reactions, calibration procedures were developed for the simultaneous determination of lactic, succinic, and β-hydroxybutyric acids as well as for β-hydroxybutyric acid alone. A discussion of the problems which arise and of the techniques needed for reliable results is given. The retention time of propyl β-hydroxybutyrate differs from those of the propyl esters of the α- and γ-isomers and of the propyl esters of a number of other acids. When the method was applied to the analysis of eggs, recoveries were greater than 90 % with good precision.

INTRODUCTION

The need for a reliable quantitative method for the analysis of β-hydroxybutyric acid exists in the fields of biochemistry and medicine. The available methods generally lack specificity and have been described as cumbersome and unsatisfactory[1].

Several papers have been published on the gas chromatographic (GLC) determination of β-hydroxybutyric acid as its methyl ester. However, none of them reported quantitative data on the recovery of the acid from natural materials[2–4].

Interest in developing a quantitative method arose from the identification in this laboratory of β-hydroxybutyric acid in incubator-reject eggs[5]. The acid was observed as a prominent component when such eggs were analyzed for lactic and succinic acids by a GLC procedure[6]. β-Hydroxybutyric acid may also have been responsible for one of the unidentified GLC peaks observed earlier by BETHEA AND WONG in incubator-reject eggs[7]. The GLC method for lactic and succinic acids[6] has

* This paper was presented at the 83rd Annual Meeting of the Association of Official Analytical Chemists, October 13–16, 1969, at Washington, D.C. (U.S.A.).

now been modified to include β-hydroxybutyric acid. Acetophenone is used as an internal standard and the acids are chromatographed as their propyl esters under isothermal conditions. Inasmuch as β-hydroxybutyrate may enter into polymerization reactions, calibration procedures were developed for β-hydroxybutyric acid alone and also in combinations with lactic and succinic acids. The procedure for the analysis of eggs is described but it should be readily applicable to other foods and biological materials through the use of appropriate sample extraction procedures.

EXPERIMENTAL

Apparatus

Gas chromatograph. A Barber-Colman Series 5000 gas chromatograph with flame ionization detector was used for measurements. The operating temperatures were: detector bath, 200°; injector block, 200°; and column temperature, 115 to 150°, depending upon the liquid phase. Gas flow rates were adjusted for optimum detector response with the carrier gas (nitrogen or helium) set at 75 ml/min. An 8 ft. column containing 10 % diethylene glycol succinate was operated at 130° and an electrometer setting of 9×10^{-10} A full scale deflection on a 5 mV recorder. Up to 15 μg (equivalent to 25 mg/100 g sample) of β-hydroxybutyric acid was chromatographed according to the procedure, without altering the electrometer controls.

GLC columns. Various columns were used with the choice dependent on the purposes intended. For the routine analysis of acids in eggs, an 8 ft. by 4 mm I.D. glass U-column of 100–120 mesh Gas Chrom Z (Applied Science Laboratories, Inc.), coated with 10 % DEGS (diethylene glycol succinate, stabilized, Analabs, Inc.) and conditioned 24 h at 200°, has proven very reliable. The propyl lactate peak separates from the solvent front and there is baseline resolution of acetophenone and propyl β-hydroxybutyrate. A typical chromatogram is shown in Fig. 1. The previously described slurry method was used to prepare the packing[6]. Reoplex 400 (Supelco, Inc.), 15 % on 60–80 mesh Gas Chrom Z, was also used with equivalent results.

FFAP (Supelco, Inc.), 5–10 % on 100–110 mesh Anakrom ABS (Analabs, Inc.),

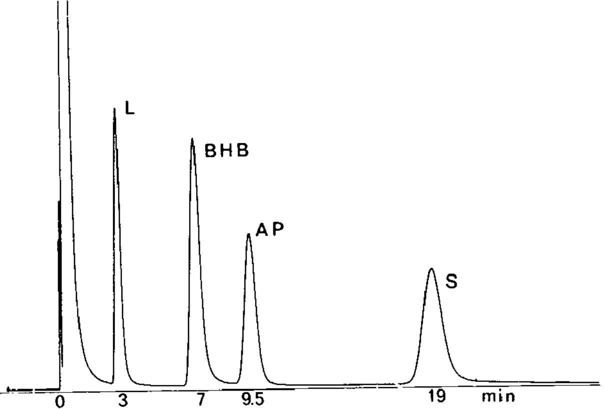

Fig. 1. Chromatogram of acids on DEGS column at 130°. Key: L = propyl lactate; BHB = propyl β-hydroxybutyrate; AP = acetophenone internal standard; S = dipropyl succinate.

was useful for special applications such as the high-temperature analyses described in this paper.

Reagents

Solvents. All were reagent grade quality. Anhydrous diethyl ether containing less than 0.05 % ethanol was used to avoid the formation of ethyl esters.

Calcium lactate standard. 0.171 g of N.F. grade calcium lactate pentahydrate was dissolved in distilled water and diluted to 100 ml (1 mg/ml as lactic acid).

Lactic acid standard. 0.100 g of crystalline L(+) lactic acid (Sigma Chemical Co.) was dissolved in anhydrous ether and diluted to 100 ml (1 mg/ml).

Succinic acid standard. 0.100 g of reagent grade succinic acid was dissolved in water and diluted to 100 ml (1 mg/ml). For an ether solution (1 mg/ml), 0.100 g of the acid was dissolved in anhydrous ether by gentle heating and shaking and diluted to 100 ml.

Sodium β-hydroxybutyrate standard. 0.121 g of the salt was dissolved in distilled water and diluted to 100 ml (1 mg/ml as β-hydroxybutyric acid).

Acetophenone standard. 0.800 g of acetophenone, 99 mole %, Chromatoquality Reagent (Matheson, Coleman and Bell), was dissolved in 1-propanol and diluted to 100 ml.

Diluting solution. 20.0 ml of 1-propanol, 10.0 ml of acetophenone standard solution and 20.0 ml of chloroform were pipetted into a 125 ml separatory funnel. 40 ml of a saturated ammonium sulfate solution was added and the funnel was stoppered and shaken for 1 min. The aqueous layer was discarded and the organic layer was dried over about 5 g of anhydrous sodium sulfate. The solution was made fresh daily.

Boron trifluoride–propanol reagent. A laboratory preparation of 10 % (w/w) boron trifluoride in 1-propanol[6] or the commercial 14 % (w/v) reagent (Applied Science Laboratories, Inc.) can be used. The latter can be used undiluted or diluted 1:0.6, v/v, with 1-propanol.

Calibration procedures

While the isolation of β-hydroxybutyric acid from eggs presents no special problem, precautions are required in preparing standard solutions of esters for calibrating the gas chromatographic column. The procedure varies, depending upon the kind and numbers of acids being esterified. The two calibration procedures described in this report were designed for application to the analysis of eggs containing β-hydroxybutyric, lactic and succinic acids. The procedures may require modification if additional acids are encountered in other applications.

Procedure A permits the simultaneous calibration for lactic, succinic and β-hydroxybutyric acids. In procedure B, the calibration for β-hydroxybutyric acid is performed separately.

Calibration procedure A. The number of calibration solutions used depends upon the precision required and the linearity of the system. See Table I for typical amounts of acids. Aliquots of the standard solution of sodium β-hydroxybutyrate were pipetted into 250 ml round-bottom ₸ 24/40 flasks and taken to dryness in a rotary evaporator at 50° and a pressure of 30 mm Hg or less. Aliquots of standard ether solutions of lactic and succinic acids were added to the flasks and the ether was removed in a

rotary evaporator at 30°. Two milliliters of the BF_3-propanol reagent was added to the dry residues and the flask was heated on a steam bath for 30 min with an air condenser attached. The flask was clamped on concentric rings so that only the lower half was immersed in steam. The flask was swirled after the reaction was initiated to insure contact between the solid residue and the reagent. When the esterification was completed, 4 ml of a saturated ammonium sulfate solution was added and the contents were cooled to room temperature. One milliliter of the acetophenone standard solution was pipetted into the flask, followed by 2 ml of chloroform. The flask was swirled to mix the contents and they were transferred to a 30 ml separatory funnel and shaken for 1 min. The lower layer (aqueous phase) was discarded and the organic phase was dried over 3 g of anhydrous sodium sulfate in a 4 dram screw-cap vial. A piece of aluminum foil was placed over the top of the vial before affixing the cap. When sampling the solution the syringe needle was inserted through the foil into the liquid. Duplicate injections of 3 μl each were made into the gas chromatograph and each peak height was measured to the nearest 0.5 mm. Peak height ratios (r) were calculated according to r = height of ester peak: height of acetophenone peak. For duplicate injections of the same solution of esters r values agreed within 5 % of their mean. A calibration graph was obtained by plotting r *versus* mg acid esterified (Fig. 2). The solutions of propyl esters should be stored at 4° if they are retained beyond the day of preparation.

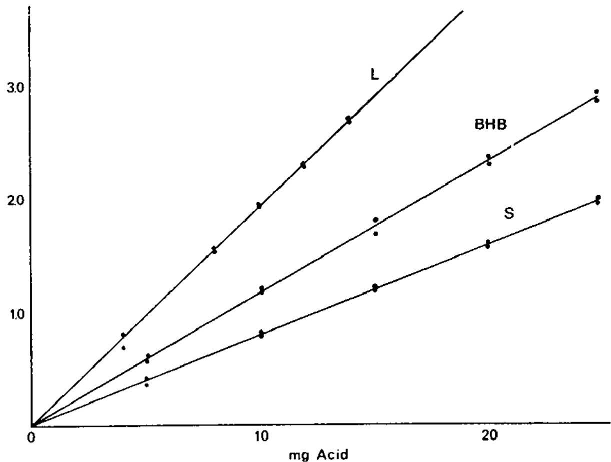

Fig. 2. Calibration data on DEGS column at 130° from solutions prepared in duplicate by Procedure A. Key: L = lactic acid; BHB = β-hydroxybutyric acid; S = succinic acid.

Calibration procedure B. Aliquots of the standard solution of sodium β-hydroxybutyrate were pipetted into 250 ml round-bottom $\mathfrak{T}$ 24/40 flasks and taken to dryness in a rotary evaporator as in procedure A. The residues were esterified for 10 min on a steam bath; the esters were isolated and chromatographed as in procedure A. Calibration solutions of propyl lactate and dipropyl succinate were prepared by combining aliquots of the calcium lactate and aqueous succinic acid solutions, evaporating to dryness and esterifying for 10 min. The esters were isolated and chromatographed as in procedure A.

Precautions in calibrations. The aliquots of sodium β-hydroxybutyrate were dried under vacuum to a white residue. In procedure A, when the ether was evaporated at 30° after the addition of lactic and succinic acids, the final residue no longer had a "dry salt" appearance but the esterification still proceeded quantitatively. The dried residues could not be stored and the BF_3–propanol was added immediately in order to convert them to their more stable propyl esters. The reaction mixtures were vigorously heated for the required length of time. The concentric rings of the steam bath served to keep the neck of the flask relatively cool by deflecting the steam.

Application to the analysis of egg. In the analysis of egg the organic acids were extracted according to the AOAC official method[8]. The ether extraction apparatus was checked for its efficiency by extracting a weighed amount of calcium lactate and quantitating the acid by titration. Recovery was greater than 98 %. When the acids were extracted from samples, reagent grade anhydrous ether containing less than 0.05 % ethanol was used to prevent the formation of ethyl esters in the subsequent BF_3-catalyzed esterification. The esters were prepared for chromatography as in calibration procedure A after the ether extract (with the omission of the 5 ml of water specified in AOAC 16.040) was evaporated to near dryness in a rotary evaporator at 30°. Two milliliters of BF_3–propanol were added, a 35 mm funnel or an air condenser was inserted in the neck of the flask and the reaction mixture was heated on a steam bath for 10 min. The BF_3–propanol reagent was added to the residue from the ether evaporation shortly after removing the flask from the rotary evaporator. It has been found that storage of the concentrated extract can result in polymerization between the organic acids and consequently in low recoveries.

When the ester concentration in a sample gave a recorder deflection greater than full scale for a 3 μl injection, 1.0 ml of the sample solution and 1.0 ml of the diluting solution were mixed in a screw cap vial, about 0.2 g of anhydrous sodium sulfate was added and the mixture was chromatographed. Additional diluting solution was added, when necessary, until the ester peak was on-scale. The amount of acid was calculated by multiplying the final result (obtained from calibration graph) by the dilution factor (F) where F is the volume after dilution: volume before dilution.

RESULTS AND DISCUSSION

Accuracy and precision

Fig. 2 shows the linearity of calibration procedure A over the ranges 0–14 mg lactic acid, 0–25 mg β-hydroxybutyric acid and 0–25 mg succinic acid. Similar results have been obtained on wider ranges (0–20 mg lactic acid, 0–40 mg β-hydroxybutyric acid and 0–40 mg succinic acid). Table I shows the close agreement in the calibration data obtained by procedures A and B and the reproducibility of each procedure on duplicate solutions. Table II is a further comparison of the precision of procedures A and B for β-hydroxybutyric acid at the 20 mg level. The S.D.'s of the mean r values were, respectively, 0.06 and 0.02.

Stability of calibration solutions

The solutions of propyl esters can be stored at 4° for periods of at least four weeks (Table III). The changes in the r values also reflect any change in the chromatographic system over the period of time. The aluminum foil lining and screw cap of the sample

TABLE I

r VALUES FOR CALIBRATION SOLUTIONS PREPARED IN DUPLICATE BY PROCEDURES A AND B
DEGS column at 130°; conditions as described in text.

Acid[a] *(mg)*	*r values*[b]					
	Lactic acid		*β-Hydroxybutyric acid*		*Succinic acid*	
	A	*B*	*A*	*B*	*A*	*B*
4:5:5	0.76	0.78	0.58	0.56	0.41	0.40
4:5:5	0.65	0.76	0.52	0.58	0.34	0.41
8:10:10	1.50		1.13		0.79	
8:10:10	1.53		1.16		0.78	
10:15:15	1.90	1.92	1.60	1.67	1.16	1.14
10:15:15	1.92	1.88	1.74	1.64	1.20	1.17
12:20:20	2.22		2.28		1.56	
12:20:20	2.25		2.20		1.52	
14:25:25	2.61	2.66	2.88	2.78	1.94	1.86
14:25:25	2.65	2.62	2.82	2.78	1.89	1.95

[a] Lactic acid–β-hydroxybutyric acid–succinic acid.
[b] r = height of ester peak: height of internal standard peak.

TABLE II

REPRODUCIBILITY OF r VALUES FOR β-HYDROXYBUTYRIC ACID ON SIX SOLUTIONS AT THE 20 mg LEVEL
DEGS column at 130°; conditions as described in text.

	r values	
	Procedure A	*Procedure B*
	2.28	2.26
	2.27	2.31
	2.20	2.28
	2.12	2.31
	2.30	2.28
	2.25	2.27
Mean	2.24	2.28
S.D.	0.06	0.02

vial are loose enough to permit some loss of chloroform during storage, but the presence of acetophenone as an internal standard avoids reliance upon solvent volumes.

Aqueous systems

When calibrations were attempted on aqueous systems containing sodium β-hydroxybutyrate, calcium lactate and succinic acid, recoveries of the propyl esters were low because of polymerization reactions during removal of water (Table IV). When one of the esterified mixtures was chromatographed at high temperature, the presence of several polymeric esters was observed (Fig. 3). Although the structures have not been determined, it has been established that peak A results from the reaction of lactate with β-hydroxybutyrate, peak B is the dimer ester shown below and peak C results from the reaction of succinate with β-hydroxybutyrate. In addition, much of the lactate-β-hydroxybutyrate polymer is insoluble in the reaction

TABLE III

STABILITY AT 4° OF CALIBRATION SOLUTIONS OF PROPYL ESTERS PREPARED BY PROCEDURE A

Acid[a] *(mg)*	*r values*					
	Lactic acid		*B-Hydroxybutyric acid*		*Succinic acid*	
	1[b]	*2*[b]	*1*	*2*	*1*	*2*
4:5:5	0.76	0.75	0.58	0.58	0.41	0.40
4:5:5	0.65	0.64	0.52	0.52	0.34	0.34
8:10:10	1.50	1.44	1.13	1.11	0.79	0.78
8:10:10	1.53	1.44	1.16	1.16	0.78	0.78
10:15:15	1.90	1.83	1.60	1.55	1.16	1.16
10:15:15	1.92	1.79	1.74	1.71	1.20	1.17
12:20:20	2.22	2.16	2.28	2.25	1.56	1.53
12:20:20	2.25	2.16	2.20	2.24	1.52	1.53
14:25:25	2.61	2.54	2.88	2.82	1.94	1.86
14:25:25	2.65	2.52	2.82	2.76	1.89	1.86

[a] Lactic acid–β-hydroxybutyric acid–succinic acid.
[b] 1 is freshly prepared; 2 is four weeks after preparation.

TABLE IV

RECOVERIES FROM AQUEOUS STANDARDS OF CALCIUM LACTATE, SODIUM β-HYDROXYBUTYRATE AND SUCCINIC ACID

Acid[a] *(mg)*	*Recovery*[b] (%)		
	Lactic acid	*β-Hydroxy-butyric acid*	*Succinic acid*
Mixture of three standards			
20:30:30	98	83	65
35:50:50	87	87	74
35:50:50	51	42	26
Mixture of three standards plus H_2SO_4			
20:30:30	99	99	96
35:50:50	99	100	99
35:50:50	97	100	94

[a] Lactic acid–β-hydroxybutyric acid–succinic acid.
[b] By comparison with procedure B.

mixture and thus remains in the flask. The existence of dimers of β-hydroxybutyric acid is known[9].

```
        H H C
        | | ||
   H3C-C-C-C-O-C3H7
        | |
        | H
        O
        | H H
        | | |
    O=C-C-C-CH3
          | |
          H OH
```

The interactions among the acids in aqueous solution could be minimized by the addition of a small amount of sulfuric acid (Table IV). However, the improvement

in results was not as dependable as that obtained with calibration procedure A in which the aqueous solution of sodium β-hydroxybutyrate is evaporated under vacuum to a dry residue before adding the other acids in ether solution.

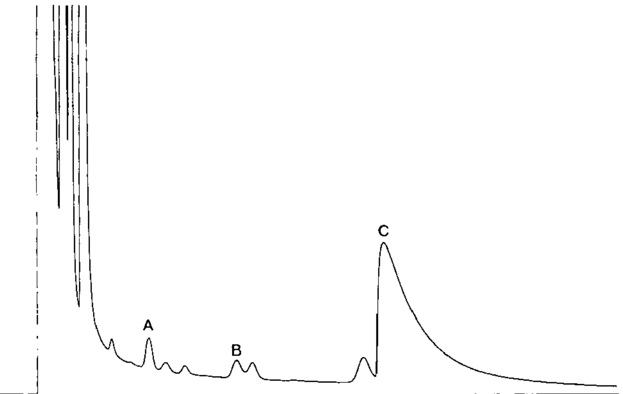

Fig. 3. Chromatogram of polymers from esterification of mixture of calcium lactate, sodium β-hydroxybutyrate and succinic acid on FFAP column at 225°. Key: A = β-hydroxybutyrate-lactate; B = β-hydroxybutyric acid dimer ester; C = β-hydroxybutyrate-succinate.

Aqueous systems could be avoided by using an ether solution of β-hydroxybutyric acid. However, the acid could not be completely esterified until the reaction time was extended to 90–120 min. At shorter times, recoveries of β-hydroxybutyric acid were low even when lactic and succinic acids were absent. Neutralization of the free acid, which is a syrup, by direct titration and by saponification indicated that it existed largely in polymer form. NMR spectroscopy also indicated the presence of polymeric forms. GC examination of the syrup after partial esterification with BF_3–propanol disclosed the presence of a compound which eluted at high temperature (BHB dimer, Fig. 4). The mass spectrum of the compound collected from the gas

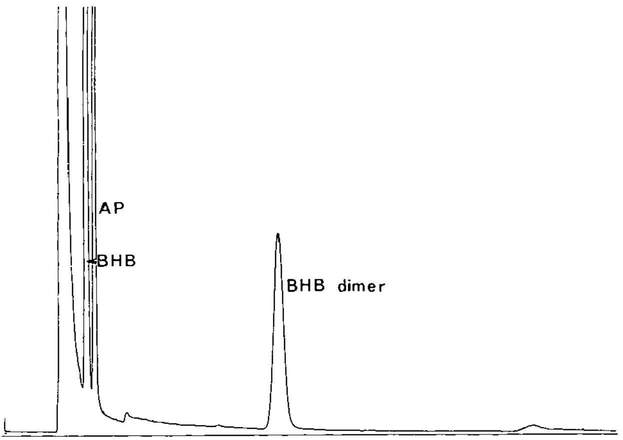

Fig. 4. Chromatogram on FFAP column at 185° of propyl esters of monomer and dimer forms of β-hydroxybutyric acid. Key: BHB = propyl β-hydroxybutyrate; AP = acetophenone internal standard; BHB dimer = propyl ester of dimer of β-hydroxybutyric acid.

chromatographic column was consistent with the dimer structure above. Peak B in Fig. 3 represents the same dimer. The esterification probably proceeds through a two-step process in which the dimer ester is first formed followed by cleavage and formation of the monomer ester.

Time of esterification

The esterification time is 30 min in calibration procedure A and 10 min in all other applications. The shorter time is adequate for converting combined residues of lactic acid (or its calcium salt) and succinic acid to their propyl esters[6]. Dry sodium β-hydroxybutyrate, in the absence of the other acids, can also be esterified in 10 min (procedure B). In like manner, a mixture of the three acids extracted from eggs can be esterified in 10 min. In that case, the ether solution of the free acids is evaporated with a minimum of heat, and interactions among the acids are not encountered. On the other hand, the stipulated conditions regarding the preparation of dry residues is more critical with the standard solutions. The reaction time was therefore extended to 30 min in procedure A in order to insure complete esterification despite occasional interactions among the acids.

Interference by other esters

The retention time of acetophenone is such that it does not interfere with the esters of a number of other low molecular weight acids. Table V lists some acids that were chromatographed as their propyl esters. The acids were used as received from commercial sources without further purification. Although β-hydroxypropionic acid could not be distinguished from acetophenone on a DEGS column it was separated on a 10 ft. by 4 mm I.D. column coated with 10 % FFAP at 150°. Under those conditions, the retention time relative to acetophenone was 1.40.

TABLE V

RELATIVE RETENTION DATA OF PROPYL ESTERS OF SOME LOW MOLECULAR WEIGHT ACIDS
DEGS column at 130°; conditions as described in text.

Acid	*Retention relative to acetophenone*
Crotonic	0.14
Pyruvic	0.27, 0.48
Lactic	0.34
Acetoacetic	0.64
β-Hydroxybuytric	0.77
α-Hydroxybutyric	0.39, 0.60
γ-Hydroxybutyric	0.48, 1.28, 2.55
β-Hydroxypropionic	1.04
Succinic	1.85
Fumaric	2.00
Maleic	2.91

Recovery of β-hydroxybutyric acid from eggs

Apparently, β-hydroxybutyric acid is extracted from incubated eggs as the monomer because, with an esterification time of 10 min, no significant amount of the dimer ester (BHB dimer, Fig. 4) was ever observed on the gas chromatogram. Furthermore, there was no increase in the amount of monomer ester when the esterification

time was increased. Thus, a 10 min esterification is adequate for esterifying the β-hydroxybutyric acid extracted from eggs. On the other hand, when the acid in syrup form was added to eggs it was recovered in part as the monomer ester and in part as the dimer ester. With an esterification time of 90 min, the acid was recovered entirely as the monomer ester.

Table VI shows recovery data for the sodium salt of β-hydroxybutyric acid added to passable eggs and to incubator-reject eggs. Quantitative recovery was obtained with the prescribed esterification time of 10 min. The recoveries of lactic and succinic acids were also quantitative. Extensive collaboration has already been accomplished on the application of the method to the determination of lactic and succinic acids in eggs[10,11]. It is now possible to quantitate the three acids from a single sample.

TABLE VI

RECOVERY OF β-HYDROXYBUTYRIC ACID FROM EGG

mg β-Hydroxybutyric acid/100 g egg		
Added as sodium salt	*Found*	*Recovery (%)*
Passable egg		
0	0	—
10.0	10.8	108
10.0	10.3	103
15.0	15.2	101
25.0	23.5	94
50.0	49.6	99
50.0	51.3	103
Incubator-reject egg		
0	13.0	—
10.0	23.2	102
10.0	24.0	110
15.0	28.0	100

ACKNOWLEDGEMENTS

The contributions of the following are gratefully acknowledged: ELIZABETH A. HANSEN for NMR analysis of β-hydroxybutyric acid and JAMES A. SPHON for mass spectral analysis of the propyl ester of the dimer form of β-hydroxybutyric acid.

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CHROM. 4844

GAS CHROMATOGRAPHY OF SOME NITROGEN AND SULFUR HETEROCYCLES BY MEANS OF SILICONE AND BENTONE–SILICONE PHASES

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(Received April 28th, 1970)

SUMMARY

Gas chromatographic retention ratios are reported for 49 compounds in the thiophene, benzo(*b*)thiophene, pyridine, quinoline, isoquinoline, and thienopyridine ring systems, variously substituted with alkyl and halo groups. Stationary phases of Silicone DC 710 and of Bentone 34–Silicone DC 710 on Chromosorb were used at 128° and 180°. Retention ratio data for silicone are interpreted in terms of volatility of the substrate. Changes in retention ratios on going to Bentone–silicone are ascribed to adsorption to Lewis acidic sites (on the Bentone aluminosilicate sheets) by coordination of the heterocyclic nitrogen atom of the substrate molecule, particularly in the pyridine, quinoline, and thieno(2,3-*b*)pyridine systems (studied most extensively).

INTRODUCTION

Recent synthetic studies in these laboratories have been concerned with thienopyridines and their derivatives[1–5]. Interest in examination of their physical properties and means of separation led us to compare the chromatographic retentivities of these compounds with those of analogous heterocyclic amines in thin-layer systems[6]. We have now extended these studies to comparison of gas chromatographic (GC) retention ratios, *R*, for parent, alkyl-substituted, and halo-substituted compounds in the thiophene, benzo(*b*)thiophene, pyridine, quinoline, isoquinoline, and thienopyridine systems. Selected as stationary phases were Silicone DC 710 on Chromosorb and Bentone 34-Silicone DC 710 on Chromosorb[7]. Modified Bentone columns have been found to be useful in effecting separations of isomeric aromatic hydrocarbons[7–13]. However, it seems that no previous use of such columns for gas chromatography of amines has been reported. In order to elucidate the nature of interactions between the substrate molecules and Bentone 34, retention ratios *versus* the same standard of reference,

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benzo(*b*)thiophene, were compared for each substrate on the two columns (under otherwise closely similar conditions). Compounds investigated are listed in Tables I–III.

EXPERIMENTAL

Starting materials

Unless otherwise stated, all compounds used were commercially available samples. 4,8-Dimethylquinoline[14] and thieno(3,2-*c*)pyridine[15] were synthesized by reported methods. Other thienopyridine compounds were available from research conducted in our laboratory[1–5].

Chromatographic procedure

The apparatus used was an F and M Model 810 dual column analytical gas chromatograph with a thermal conductivity detecting system and a Leeds and Northrup 10-mV electronic recorder. Stationary phase *S* was 10 % Silicone Fluid DC 710 on 60–80 mesh Chromosorb G, packed in copper tubing 3/8 in. (O.D.) × 6.26 ft. Stationary phase *BS* was 5 % Bentone 34 plus 5 % Silicone Fluid DC 710 on 60–80 mesh Chromosorbs G and W (4:5, by wt.), packed in copper tubing 3/8 in. × 5.26 ft.

TABLE I

VPC RETENTION RATIOS (R) OF THIOPHENE AND PYRIDINE COMPOUNDS AT 128°

No.	*Compound*	*B.p.*[a] (°C)	R_S[b]	R_{BS}[c]	R_{BS}/R_S
1	Benzo(*b*)thiophene[d]	221.5	1.00[e]	1.00[f]	1.0
2	2-Me-thiophene	113	0.10	0.09	0.9
3	3-Me-thiophene	115.4[g]	0.10	0.08	0.8
4	2-Cl-thiophene	128.3	0.14	0.23	1.6
5	Pyridine	115.3	0.08	0.07	0.9
6	2-Me-pyridine	129.4	0.15	0.07	0.5
7	3-Me-pyridine	144.1	0.14	0.11	0.8
8	4-Me-pyridine	145.4	0.16	0.13	0.8
9	2,4-DiMe-pyridine	158.4	0.21	0.16	0.8
10	3,4-DiMe-pyridine	178.8[h,i]	0.34	0.27	0.8
11	3,5-DiMe-pyridine	171.9	0.30	0.24	0.8
12	2-Et-pyridine	148.5	0.19	0.11	0.6
13	4-Et-pyridine	169.8[j]	0.27	0.21	0.8
14	2,4,6-TriMe-pyridine	176.5	0.30	0.19	0.6
15	2-Cl-pyridine	170	0.24	0.53	2.2
16	2,5-DiCl-pyridine	190.5[h]	0.46	0.70	1.5
17	2,6-DiCl-pyridine	211.5[h]	0.60	1.37	2.3
18	3,5-DiCl-pyridine	178.5[h]	0.34	0.42	1.2

[a] Unless noted otherwise, data are taken from ref. 17.
[b] $R_S = R$ for 10% silicone column, phase *S*.
[c] $R_{BS} = R$ for 5% Bentone-5% silicone column, phase *BS*.
[d] Internal standard in all runs.
[e] Corresponds to a retention time of 10–15 min.
[f] Corresponds to a retention time of 20–30 min.
[g] From ref. 18.
[h] From ref. 19.
[i] Ref. 17 gives a low value of 164°.
[j] At 750 mm.

TABLE II

VPC RETENTION RATIOS (R) OF QUINOLINES AND ISOQUINOLINES AT 180°

No.	*Compound*	*B.p.*[a] (°C)	R_S[b]	R_{BS}[c]	R_{BS}/R_S
1	Benzo(*b*)thiophene[d]	221.5	1.00[e]	1.00[f]	1.0
19	Quinoline	238	1.19	1.93	1.6
20	Isoquinoline	242	1.32	1.75	1.3
21	2-Me-quinoline	247.6	1.62	1.95	1.2
22	4-Me-quinoline	262	2.17	3.77	1.7
23	5-Me-quinoline	254[g]	1.86	3.52	1.9
24	7-Me-quinoline	252	1.86	2.97	1.6
25	8-Me-quinoline	247.8[h]	1.66	2.14	1.3
26	3-Br-quinoline	275	3.31	4.19	1.3
27	1-Me-isoquinoline	248	1.89	2.50	1.3
28	2,4-DiMe-quinoline	264.5	2.72	4.75	1.7
29	2,6-DiMe-quinoline	266.5	2.44	3.47	1.4
30	2,8-DiMe-quinoline	252	2.02	2.13	1.1
31	4,6-DiMe-quinoline	273.5	3.35	7.33	2.2
32	4,8-DiMe-quinoline	258.5	2.77	3.36	1.2
33	2-Cl-4-Me-quinoline	296	4.45	11.5	2.6

[a–d] See corresponding footnote in Table I.
[e] Corresponds to a retention time of 3–4 min.
[f] Corresponds to a retention time of *ca.* 5 min.
[g] At 735 mm.
[h] At 751 mm.

Before use, columns were conditioned at 200° for 24 h with a gentle flow of helium gas. Experiments were conducted at column temperatures of 128° (helium flow rate, 170 ml/min) for monocyclic compounds (Table I) and at 180° (helium flow rate, 225 ml/min) for bicyclic compounds (Tables II and III). Samples (10–20 μl) of solutions containing 25 mg of substrate plus 25 mg of benzo(*b*)thiophene (internal reference standard) per ml of solvent (benzene or acetone) were injected into the chromatograph. Adjusted retention times were measured in linear units from the air peak. In each run the adjusted retention time for the substrate was divided by that for the standard to give the relative adjusted retention ratio[16], designated here simply as "retention ratio". Average retention ratios (R_S for phase S, R_{BS} for phase BS) for duplicative runs (maximum variation in R, $\pm 5\%$) are given in Tables I–III.

RESULTS AND DISCUSSION

Observation of Tables I and II shows that there is a general increase in retention ratio with increasing normal boiling point (*i.e.* with decreasing volatility) in these simple derivatives of pyridine, thiophene, and their benzo analogs with silicone oil as the stationary phase. This approximate relationship is presented in Figs. 1 and 2, where log R_S is plotted *vs.* the temperature of the normal boiling point[20].

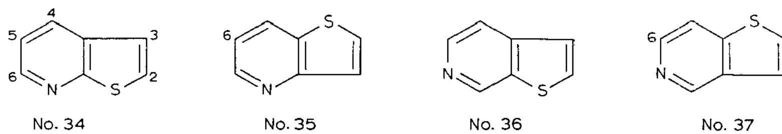
Boiling points of the thienopyridines in Table III have not been determined. However, one finds very close values of R_S for the thienopyridine isosteres[21,22] (*cf.* Nos. 34, 35; 36, 37) in Table III. Moreover, these values are 8–10 % higher than for their respective isosteres, quinoline and isoquinoline (Nos. 19; 20), in Table II. Also, derivatives of thieno(2,3-*b*)pyridine have R_S values *ca.* 5 % higher than do their iso-

TABLE III

VPC RETENTION RATIOS (R) OF THIENOPYRIDINES AT 180°

No.	*Compound*	R_S[a]	R_{BS}[b]	R_{BS}/R_S
1	Benzo(*b*)thiophene[c]	1.00[d]	1.00[e]	1.0
34	Thieno(2,3-*b*)pyridine[f]	1.30	2.86	2.2
35	Thieno(3,2-*b*)pyridine[f]	1.29	1.84	1.4
36	Thieno(2,3-*c*)pyridine[f]	1.45	2.73	1.9
37	Thieno(3,2-*c*)pyridine[f]	1.43	2.12	1.5
38[g]	4-Me-thieno(2,3-*b*)pyridine	2.26	5.23	2.3
39	6-Me-thieno(2,3-*b*)pyridine	1.70	2.97	1.7
40	4-Et-thieno(2,3-*b*)pyridine[h]	3.24	7.70	2.4
41	6-Et-thieno(2,3-*b*)pyridine[h]	2.48	3.57	1.4
42	4,6-DiMe-thieno(2,3-*b*)pyridine	2.86	6.70	2.3
43	4,5,6-TriMe-thieno(2,3-*b*)pyridine	6.05	11.0	1.8
44	3-Br-thieno(2,3-*b*)pyridine[h]	3.31	4.48	1.4
45	5-Cl-thieno(2,3-*b*)pyridine	2.46	4.68	1.9
46	5-Br-thieno(2,3-*b*)pyridine	3.49	5.37	1.5
47	2,3-DiCl-thieno(2,3-*b*)-pyridine[h]	3.64	5.08	1.4
48	6-Et-thieno(3,2-*b*)pyridine[i]	3.09	3.55	1.2
49	6-Me-thieno(3,2-*c*)pyridine[j]	1.81	2.24	1.2

[a–c] See footnotes b–d, respectively, in Table I.
[d,e] See footnotes e and f, respectively, in Table II.
[f] Structural formulas:



[g] For Nos. 38 to 47, see formula 34.
[h] Data on the syntheses and structures of these compounds will be reported elsewhere.
[i] See formula 35.
[j] See formula 37.

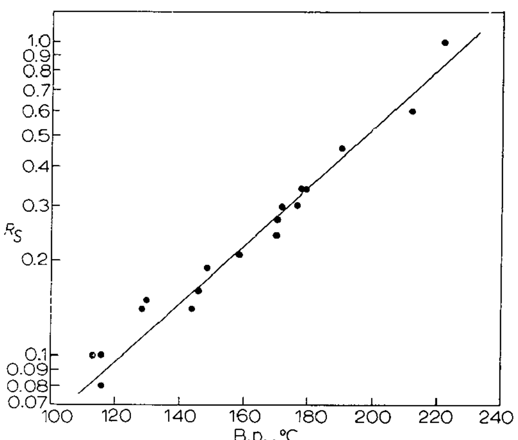


Fig. 1. Plot of log R_S *vs.* temperature of normal boiling point for compounds in Table I.

steric substituted quinolines (*cf.* 21, 39; 22, 38; 26, 46; 28, 42). It is, therefore, reasonable to assume that special polar or steric interactions do not occur between stationary phase S and the thienopyridine substrates studied. Hence, Fig. 2 and the R_S values in Table III might well serve as a means of estimating normal boiling points for compounds 34–49.

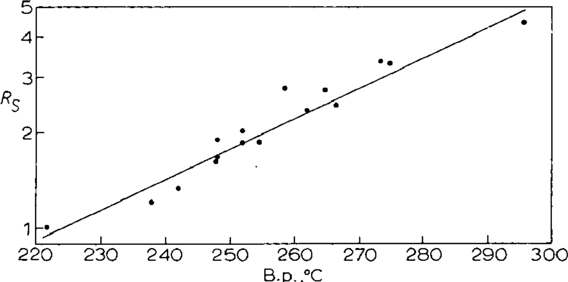


Fig. 2. Plot of log R_S *vs.* temperature of normal boiling point for compounds in Table II.

In general, retention times were larger for every compound with the BS phase than the S phase, despite the shorter column used in the former case. (See footnotes e and f in Tables I and II). The ratio $R_{BS}/R_S = r$ is taken as a simple criterion for evaluation of structural features pertinent to retention of substrates on Bentone *per se*. In this regard one notes that for all monocyclic compounds studied which have 0–3 alkyl groups (Nos. 2, 3, 5–14) $r < 1.0$. For all halogen-bearing monocyclic compounds and all bicyclic compounds, on the other hand, $r \geq 1.0$. For only eight substrates is $r > 2.0$. Three of these eight (Nos. 15, 17, 33) have at least one chlorine atom α to nitrogen and four (Nos. 34, 38, 40, 42) are thieno(2,3-*b*)pyridines (sulfur atom α to nitrogen). In fact for each of the 15 compounds studied which has either a chlorine or a sulfur atom α to nitrogen $r \geq 1.4$ and averages 1.9, as compared to an average of 1.5 for all other 21 substrates for which $r > 1.0$. For 2-chlorothiophene (chlorine atom α to sulfur) $r = 1.6$.

For interpretation of these r values it is instructive to consider briefly the known structure of the stationary BS phase. Bentone 34 (dimethyldi-*n*-octadecylammonium montmorillonite)[7] is a non-stoichiometric (but electrically neutral) compound containing aluminosilicate sheets intercalated with tetraalkylammonium ions, $(CH_3)_2$-N^+R_2, where $R = (CH_2)_{17}CH_3$[23–25]. Net negative electronic charge on the sheets results from isomorphous replacements of Al(III) and Si(IV) atoms with metallic elements of lower valence. The interfacial surfaces of the sheets bear incompletely coordinated metal atoms, M^{n+} (where M = Si or Al), which may serve as Lewis acidic sites. As determined by X-ray measurements, the interlayer distance is more than 18 Å. In accordance with proposals for monoalkylammonium montmorillonites[26], it is presumed that the positive centers of the intercalated ions lie close to the interfacial surfaces, while the long R groups from a relatively thick interlamellar non-polar region, also occupied by molecules of silicone in Bentone–silicone mixtures. Our substrate molecules are sufficiently small in size that they should readily penetrate into the non-polar region. The marked retention of polar molecules by montmorillonites[27] is ascribed to adsorption at the interfacial surfaces, where aspects of size, shape, and orientation of the substrate molecule become pertinent.

The high r values for the α-chloropyridines, the α-chloroquinoline (No. 33), and the thieno(2,3-*b*)pyridines (Nos. 34, 38–47) are consistent with retention of these molecules by Bentone 34 by means of simultaneous coordination (chelation) of both the ring nitrogen atom and its α-nucleophilic substituent atom (X) to a single Lewis acidic site on the aluminosilicate sheet (see Fig. 3). A similar chelate structure could

Fig. 3.

be formed with 2-chlorothiophene. The marked difference in R_{BS} values for thieno-(2,3-*b*)- and thieno(3,2-*b*)pyridines is especially notable. The general phenomenon of simultaneous anchoring by two favorably juxtaposed sites on the adsorbate to one site on the adsorbent is a familiar one in chromatography[6,28].

Steric hindrance by an alkyl group α to the heterocyclic nitrogen atom is readily apparent in the chelated thieno(2,3-*b*)pyridine system. Thus, contributions toward the r value fall in the orders γ-Me $>$ H $>$ α-Me (Nos. 34, 38, 39); γ-Et $>$ H $>$ α-Et (Nos. 34, 40, 41); α-Me $>$ α-Et; and α,γ-diMe $>$ α,β,γ-triMe (Nos. 42, 43 — buttressing effect). Steric hindrance to coordination at the nitrogen atom is also noted in compounds where chelation is impossible. One finds orders (in r) of γ-Me = β-Me $>$ α-Me (Nos. 6–8), γ-Et $>$ α-Et (Nos. 12, 13), and α, γ-diMe $>$ α,α′, γ-triMe (Nos. 9, 14) in the pyridine system as well as 5-Me, 7-Me, γ-Me $\geq$ H $>$ *peri*-Me $>$ α-Me (Nos. 19, 21–25) and γ,6-diMe $>$ α,γ-diMe $>$ H $>$ α,6-diMe $>$ γ,*peri*-diMe $>$ α,*peri*-diMe (Nos. 19, 28–32) in the quinoline system and H $>$ α-Me (Nos. 37, 49) in the thieno-(3,2-*c*)pyridine system.

For 3-bromoquinoline (No. 26) and the halo-substituted thieno(2,3-*b*)pyridines (Nos. 44–47) wherein the halogen atom occupies a position removed from the nitrogen atom, the halo derivative has an r value lower than that of its parent compound. This is consistent with electron withdrawal from the nitrogen atom by the halogen atom. The order 5-chlorothieno(2,3-*b*)pyridine $>$ 5-bromothieno(2,3-*b*)pyridine (*cf.* Nos. 45, 46) may reflect differences in bulkiness of the halogen atoms, in addition to differences in their electron withdrawing abilities. It might be noted, however, that 3,5-dichloropyridine (No. 18) does not fit this model well.

On the basis of the foregoing relationships it seems fairly clear that, in general, the pyridinoid ring in our compounds is adsorbed by coordination through the n-electrons of the nitrogen atom to a Lewis acidic site on the aluminosilicate sheet in the Bentone–silicone phase. Since only oxygen atoms are believed to occupy the outermost layer of each face of this sheet while the acidic sites occupy the second layer (partially exposed), it seems probable that the coordinated pyridinoid ring will assume a perpendicular or tilted geometry with respect to the plane of the sheet[29]. Effects of steric hindrance by alkyl groups α to the nitrogen atom would be most pronounced in such an orientation.

Insufficient data are presented for the thiophene, isoquinoline, and two thienopyridine systems (Nos. 35, 36) to permit meaningful interpretations to be made in these cases.

ACKNOWLEDGEMENTS

This work was supported by research grants No. CA-5969 from the National Cancer Institute and No. GM 12730 from the National Institute of General Medical Sciences, U.S. Public Health Service. In particular, funds for purchase of the gas chromatograph were derived from these sources.

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CHROM. 4866

THE UTILISATION OF 1-DIMETHYLAMINONAPHTHALENE-5-SULPHONYL CHLORIDE FOR QUANTITATIVE DETERMINATION OF FREE AMINO ACIDS AND PARTIAL ANALYSIS OF PRIMARY STRUCTURE OF PROTEINS*

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(Received May 15th, 1970)

SUMMARY

A dansylation method has been developed for the rapid partial determination of primary protein structures at the nanomole level. Modifications of published methods for the purification and chromatography of dansyl-amino acids and dansyl-peptides were necessary for quantitative analysis. The amino acid composition and N-terminal amino acids of proteins can be determined by this method. After finger-printing on a preparative scale, the amino acid composition and N-terminal amino acids of each dans-peptide can also be characterised.

INTRODUCTION

1-Dimethylaminonaphthalene-5-sulphonyl chloride (dans-Cl) covalently binds to free amino, phenol, imidazole and sulphhydryl groups[1]. Dansyl derivatives of amino acids (dans-AA) can be easily produced for all the amino acids. Since these derivatives are intensely fluorescent and are highly resistant to acid and alkaline hydrolysis, GRAY AND HARTLEY[2-4] have used them for the identification of the N-terminal amino acids of proteins. Other authors have used dansylation for revealing low quantities of amino acids and peptides separated by thin-layer chromatography[5-17]; but all these methods are qualitative, except for that of GROS AND LABOUESSE[14] in which the N-terminal amino acids are determined quantitatively.

After a preliminary study which established the optimal conditions for the dansylation of amino acids, and for the purification, chromatography and measurement of the fluorescence of dans-AA, we set up a quantitative method for the partial structural analysis of proteins on a microscale. The method has been used to determine

* Abbreviations used: dansyl or dans: 1-dimethylaminonaphthalene-5-sulphonyl. dans-Cl: 1-dimethylaminonaphthalene-5-sulphonyl chloride. dans-OH: 1-dimethylaminonaphthalene-5-sulphonic acid. dans-NH_2: 1-dimethylaminonaphthalene-5-sulphonamide. dans-AA: 1-dimethyl-aminonaphthalene-5-sulphonyl amino acid.

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the amino acid composition and N-terminal amino acids of a given protein and of the dans-peptides obtained from a peptide map after enzymatic hydrolysis of the protein on a preparative scale.

The method is rapid and inexpensive and requires only μg quantities of protein.

MATERIALS

Reagents

Dans-Cl (98 %), dimethylformamide (for UV spectroscopy) and amino acids (Chr., puriss.) were obtained from Fluka (Buchs, Switzerland), and dans-AA (A grade) from Calbiochem (Lucerne, Switzerland). Bovine serum albumin (Fraction V powder) and bovine pancreatic ribonuclease A (Type I A) were from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Trypsin (3 × crystallised) was from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.). Other chemical reagents were Analytical grade.

Chromatographic materials

Silica gel (Kieselgel G nach Stahl) was from Merck A.G. (Darmstadt, G.F.R.) and polyamide sheets were purchased from Chang Chin Trading Co. Ltd., (Taipei, Taiwan, Republic of China). Silica gel plates were coated by means of a Camag (Muttens, Switzerland) apparatus to a thickness of 0.3 mm.

Apparatus

The apparatus used was the following: a Cary 14 recording spectrophotometer (quartz cuvettes 1 cm path length); a Zeiss spectrofluorometer with two monochromators (Ref. PM QII + ZFM 4), (cuvettes Hellma QS, volume 1.5 ml).

METHODS

Dansylation of amino acids and purification of dans-AA

To avoid loss of fluorescence of dansylated proteins, peptides or amino acids, all operations from the dansylation to the last spectrofluorometric step, were carried out rapidly and as much as possible in the dark. Amino acid samples (between 1 and 50 nmole of each amino acid) were dissolved in 300 μl of 0.2 *M* sodium phosphate buffer (pH 8.85), and an equal volume of freshly prepared dans-Cl in acetone (10 mg/ml) was added, the tubes were tightly stoppered, shaken and left to stand at room temperature. After 120 min the acetone was evaporated under a light stream of air, then 2 ml of 0.1 *M* ammonium formate buffer (pH 3.5) was added to precipitate the excess dans-Cl. The precipitates were separated by low speed centrifugation, the supernatants decanted, and the pellet washed twice with 1 ml of buffer. The pooled supernatants were lyophilised and the dans-AA's were desalted by three successive extractions of the dans-AA's from the residue into 500 μl of acetone–1 *M* HCl (19:1). The pooled supernatants were rapidly dried in a stream of cold air while the tube was kept at 50°. The residues were taken up in 100 μl of acetone–1 *M* HCl (19:1) for chromatography.

Dansylation of peptides and purification of dans-peptides

The peptide mixture (between 1 and 20 nmole of each peptide) was dansylated

as described for the amino acids. The excess dans-Cl was converted to dans-OH by the addition of a few drops of 3 *M* KOH until the orange colour, typical of dans-Cl, had disappeared. The pH was then adjusted to approximately 3.5, by addition of glacial acetic acid.

Salts and dans-OH were then eliminated according to SCHMER AND KREIL[15] by adsorption onto columns of Dowex 50 X4 (2.0 × 0.5 cm) equilibrated with 0.01 *M* acetic acid. The blue-green fluorescence, typical of dans-OH, was washed from the column with 0.01 *M* acetic acid, then the dans-peptides were eluted with water–acetone–25 % ammonia (80:20:4) until all yellow fluorescence had disappeared from the column. This eluate was lyophilised and the residue dissolved in a small volume of acetone–water (1:1) for chromatography.

Dansylation and hydrolysis of proteins for N-terminal amino acid identification

From among several published methods for the determination by dansylation of N-terminal amino acids[2,13,14] we have chosen the technique of GROS AND LABOUESSE[14] with certain technical modifications, important for the quantitative estimations.

The protein (between 5 and 10 nmole) was dissolved in 200 μl of water. Then 250 mg urea, 150 μl of 0.2 *M* sodium phosphate buffer (pH 8.85), 250 μl of dimethyl formamide and 100 μl of dans-Cl in acetone (10 mg/ml) were added successively. The tubes were stoppered and left at room temperature. After 120 min, 5 ml of 10 % TCA was added, the tube was shaken and centrifuged (1000 g for 10 min). The precipitated dans-protein was washed twice with 1 ml of 1 *M* HCl, then twice with 1 ml of acetone.

Hydrolysis of the dansylated protein was performed with 250 μl of 6 *M* HCl at 115° in a sealed tube for 4 h. After the HCl had been removed *in vacuo*, the dry residue was taken up quantitatively in acetone–1 *M* HCl (19:1) for chromatography.

Bidimensional thin-layer silica gel chromatography of dans-AA

A homogeneous suspension of 54 g of silica gel in 125 ml of water was spread as a 300 μ layer over 20 × 20 cm glass plates. After standing until the plates became opaque, they were placed in an oven at 110° for at least 30 min, then kept at room temperature. The sample of dansylated amino acids was applied dropwise under a stream of warm air so that the spot diameter was kept less than 5 mm. Before development the plates were placed in an oven at 110° for 5 min, followed by 5 min at room temperature.

Chromatograms were developed in the first dimension with toluene–pyridine–acetic acid (150:50:3.5)[13] until the solvent front reached the edge of the plate (about 45 min). The plates were then dried under a stream of warm air until opaque, then at 110° for 5 min. The plates were then examined under UV light (365 mμ) to locate slightly fluorescent impurities eluted from the silica gel which migrated as a large band close to the solvent front. A horizontal groove was scratched on the silica gel to separate these impurities which interfere with the migration in the second solvent and the subsequent measurement of fluorescence of dans-leu, dans-ile, dans-val and dans-pro. Residual pyridine was eliminated by exposing the plates to an atmosphere of ammonia for 1 min. The chromatogram was then developed in toluene–chloro-2-ethanol–25 % ammonia (100:80:6.7) until the solvent front was 4 cm from the edge of the plate (about 45 min). The plates were then dried rapidly in warm air. The spots were marked under UV light and immediately eluted.

One-dimensional chromatography of dans-AA on polyamide sheets

Sheets of polyamide (5 × 5 cm) were divided by pencil marks, into a maximum of 10 lanes. To obtain acceptable resolutions each completely desalted sample must be applied as a spot less than 1 mm in diameter. The solvents used[8] were either benzene–acetic acid (9:1) (development 6 min) or formic acid–water (1.5:100) (development 4 min).

Bidimensional silica gel thin-layer chromatography of dans-peptides

The technique was identical to that used for the separation of dans-AA except for the solvents. After 45 min development in methyl acetate–isopropanol–25 % ammonia (9:6:4), the plates were dried in warm air, then placed in an oven at 110° for 5 min, then under a hood at room temperature for 10 min. It was necessary to observe these conditions strictly before proceeding to the second dimension, namely isobutanol–acetic acid–water (15:4:2) for 120 min.

Elution of dans-AA and dans-peptides and measurement of fluorescence

The spots of dans-AA were eluted from the silica gel by means of the technique of GROS with chloroform–methanol–acetic acid (7:2:2). Dans-peptide spots were eluted in the same manner with acetone–water (1:1). The completeness of elution was controlled by UV examination.

Five volumes of absolute ethanol were added to the eluate, and the fluorescence of this mixture was read at 340/510 mμ. A known quantity of a control dans-AA (dans-glu) was chromatographed and eluted in parallel to the sample. An area of silica gel equal to the spots was eluted to serve as a silica gel blank.

If the fluorescence measured for the standard is F^0 (the apparatus was adjusted so that $F^0 = 100$) the silica gel blank is F_b and that of the sample F_s, then:

$$F_s{}^0 = F_s - F_b \quad (1)$$

where $F_s{}^0$ is the "real" fluorescence of the sample. This value is then corrected for the fluorescence yield of each dans-AA, and for losses during separation, by the application of a "recovery factor relative to dans-glu" (α_s). The determination of this factor is discussed later. This correction allows calculation of the fluorescence equivalent of the sample F_s':

$$F_s' = \alpha_s \times F_s{}^0 \quad (2)$$

The percentage of each amino acid in a mixture can be calculated from the formula:

$$\text{amino acid } \% = \frac{F_s'}{\Sigma F_s'} \times 100 \quad (3)$$

The absolute quantity of each amino acid in nmole (n_s) is given by the equation:

$$n_s = n \times \frac{F_s'}{F^0 - F_b} \quad (4)$$

n being the number of nmole of standard dans-glu used.

Performic oxidation of proteins

This process was carried out according to the technique of HIRS[18]. It was necessary to observe the conditions specified strictly, in particular with regard to the temperature and the use of fresh hydrogen peroxide in order to prevent the rupture of certain peptide bonds.

RESULTS AND DISCUSSION

Determination of the composition of a mixture of amino acids by dansylation

The conditions for dansylation of amino acids reported in the literature[2–13] are extremely variable. GROS AND LABOUESSE[14] have studied this problem recently and we have confirmed that, in general, their conditions are satisfactory. However, we preferred to use a longer reaction time of 120 min, instead of 30 min because a kinetic study showed that the dicarboxylic amino acids, which react more slowly, were not completely dansylated within 30 min (Fig. 1). Complete dansylation of these amino acids occurs in 120 min without significant formation of dans-OH which interferes with later chromatographic steps. Significant formation of dans-OH occurs with the longer reaction times suggested by certain authors[2–4].

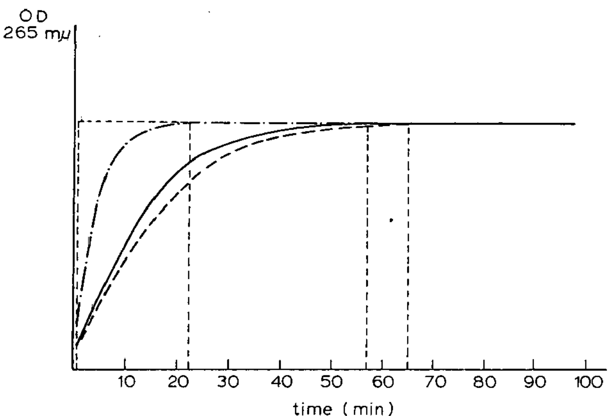

Fig. 1. Kinetics of the dansylation of some amino acids (———, glu; – – – –, asp; –·–·–, gly). 100 nmole of each amino acid in 300 μl of 0.2 *M* sodium phosphate buffer (pH 8.85) was mixed with 300 μl of dans-Cl in acetone (10 mg/ml) in a quartz cuvette. The absorption was followed at 265 mμ with a recording spectrophotometer against a blank containing the same mixture without amino acid.

The reaction medium must be kept as free as possible from salts other than disodium phosphate. The excess of dans-Cl (approximately 50 moles per mole of amino acid), the optimal amount for dansylation, strongly interferes with the chromatographic separation and therefore must be removed before chromatography. All other authors have suggested hydrolysis of the excess dans-Cl by alkali or strong acid[9,13,14], but both methods produce large amounts of dans-OH which then must be removed by chromatography on a Dowex 50 column[15]. This procedure is time-consuming, difficult to apply to a large number of samples simultaneously and causes a significant loss of fluorescence as a result of the UV irradiation necessary to follow the elution. An alternative technique[9,14] is to extract the dans-AA's into ether. However, dans-arg,

dans-his, ε-dans-lys, dans-$CySO_3H$ etc. remain in the aqueous phase with the dans-OH. The distribution of the dans-AA into two phases necessitates two chromatographic steps. Moreover, the dans-OH in the aqueous phase interferes with the chromatography.

We found that 0.1 *M* ammonium formate buffer (pH 3.5) will completely precipitate excess dans-Cl and this simple method can be used on several samples simultaneously. The formate precipitation produces very little dans-OH, provided that the operations are carried out rapidly. Desalting was then performed by extracting the dans-AA's from the residue, after lyophilisation, into acetone–1 *M* HCl (19:1). Under the conditions that we have described (1–50 nmole of each amino acid), all the dans-AA's were completely extracted except for dans-trp. An additional wash, necessary for complete extraction when dans-AA's were present in quantities larger than 50 nmole, will also quantitatively extract dans-trp (if present in quantities less than 20 nmole). Additional washes were required for larger amounts of dans-trp. The procedure described gives an over-all yield of amino acids, recovered as dans-AA's, of $92 \pm 2\%$ in a salt-free solution which contains very little dans-OH and no dans-Cl. The dans-AA's are now ready for quantitative chromatography on a single plate.

Bidimensional silica gel TLC of a standard mixture of amino acids treated by our procedure (Fig. 2) indicated that, with the solvents used, all dans-AA spots were

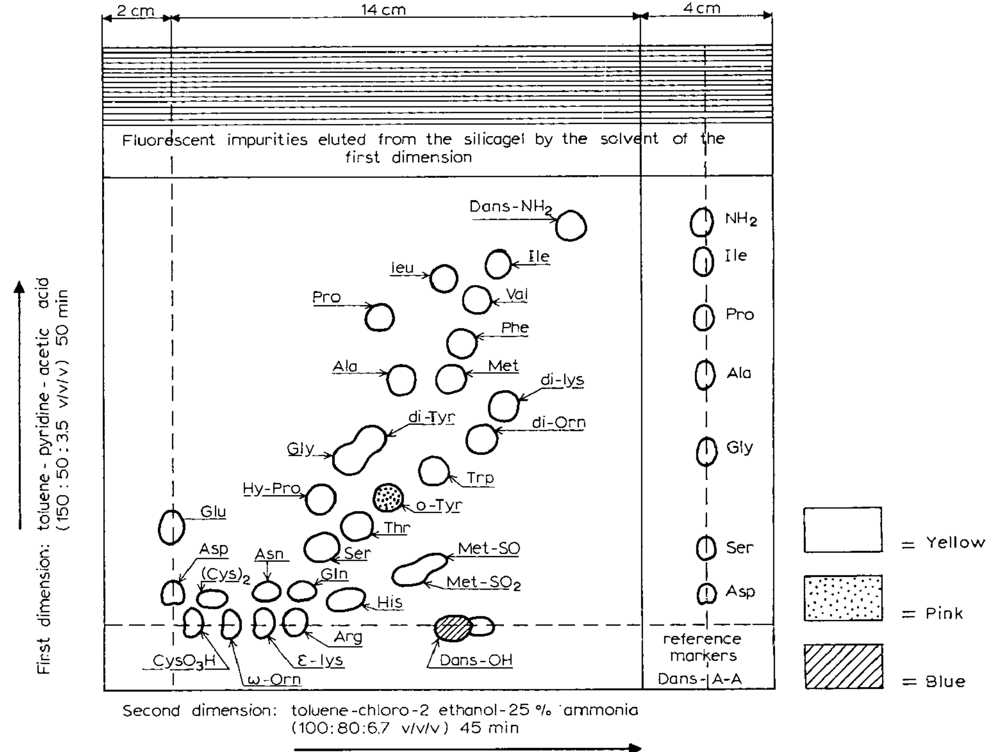

Fig. 2. Tracing of a two-dimensional separation of dans-AA's on a silica gel thin-layer plate. 5 nmoles of each dans-AA were applied.

well defined, with practically no trailing and, except for dans-gly and di-dans-tyr, completely separated.

The method reported has overcome the problem found by other[5–14] of overlapping of the dansyl derivatives of the branched chain amino acids with dans-pro, of dans-phe with dans-met, and among the dansyl derivatives of the basic amino acids.

TABLE I

$R_{DANS\text{-}NH_2}$ VALUES × 100 OF DANS-AMINO ACIDS, DANS-NH_2 AND DANS-OH ON SILICA GEL THIN LAYERS

Values are the mean ± S.D. of 20 determinations. Solvent I: toluene–pyridine–acetic acid (150:50:3.5); solvent II: toluene–chloro-2-ethanol–25% ammonia (100:80:6.7).

Dans-AA	*Solvent I*	*Solvent II*	*Dans-AA*	*Solvent I*	*Solvent II*
Dans-NH_2	100.0	100.0 ± 1	Dans-thr	25.0 ± 0.5	45.0 ± 1
Dans-ile	90.5 ± 1	83.5 ± 1	Dans-glu	24.0 ± 0.5	0.5 ± 0.1
Dans-leu	87.5 ± 1	67.5 ± 1	Dans-ser	19.0 ± 0.5	36.0 ± 0.5
Dans-val	82.5 ± 1	75.0 ± 1	Dans-met-SO	15.0 ± 0.5	58.0 ± 1
Dans-pro	78.5 ± 1	51.5 ± 1	Dans-met-SO_2	14.0 ± 0.5	57.0 ± 1
Dans-phe	73.0 ± 1	71.0 ± 1	Dans-asp	8.0 ± 0.5	0
Dans-met	66.0 ± 1	69.0 ± 1	Dans-asn	8.0 ± 0.5	20.0 ± 0.5
Dans-ala	62.0 ± 1	56.0 ± 1	Dans-gln	8.0 ± 0.5	29.0 ± 0.5
Di-dans-lys	55.0 ± 1	82.0 ± 1	Di-dans-$(Cys)_2$	5.0 ± 0.5	9.0 ± 0.2
Di-dans-orn	49.0 ± 1	79.0 ± 1	Di-dans-His	4.0 ± 0.5	42.0 ± 1
Di-dans-tyr	47.0 ± 1	49.0 ± 1	Dans-arg	0	29.0 ± 0.5
Dans-gly	44.0 ± 1	46.0 ± 1	ε-Dans-lys	0	20.0 ± 0.5
Dans-trp	41.0 ± 0.5	64.5 ± 1	Dans-orn	0	14.0 ± 0.5
O-dans-tyr	30.0 ± 0.5	50.0 ± 1	Dans-$CySO_3H$	0	4.0 ± 0.2
Dans-hyp	30.0 ± 0.5	36.0 ± 0.5	Dans-OH	0	67.0 ± 1

The method is reproducible (without any requirement for further purification of "Analytical grade" solvents), as shown by the migration characteristics of each dans-AA in the two solvent systems used (Table I). All amino acids can be unambiguously identified. Chromatography on polyamide (Fig. 3) is useful as a confirmation of the nature of certain dans-AA's eluted from the silica gel chromatogram. It is particularly useful for the unambiguous identification of dans-gly and di-dans-tyr, since their migrations on polyamide are quite different. Dans-AA's (1–50 nmole) are clearly separated on a single silica gel plate and can be quantitated reliably down to 0.5 nmole.

Ready-made commercially available silica gel thin-layer plates are not suitable for quantitative analysis since the material which strongly binds the silica powder to the support plates interferes with the fluorescence measurements, makes the recovery of dans-AA spots very difficult and limits the scale of operation to less than 10 nmoles of a given amino acid.

To demonstrate the reproducibility and the linearity of the method, four mixtures of amino acids were taken (Table II), mixture I contained equimolar amounts of each amino acid; in mixture II the concentrations of the amino acids were from 1–5 times greater and in mixture III from 1–10 times greater. Mixture IV contained amino acids in the proportions found in oxidised bovine RNAase[19]. Different quantities of each mixture were dansylated and purified and analysed by chromatography

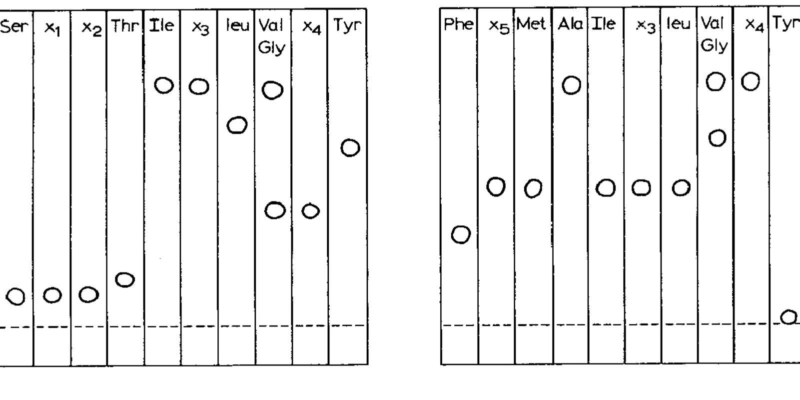

Fig. 3. Tracings of one-dimensional separation of dans-AA's on polyamide sheets. 0.01 nmole of each standard dans-AA was applied. The spots x_{1-5} were dans-AA's eluted from a silica gel thin-layer chromatogram to confirm their identity. A = benzene–acetic acid (9:1); B = formic acid–water (1.5:100).

TABLE II

COMPOSITION OF THE STANDARD MIXTURES OF AMINO ACIDS USED (nmole/100 μl)

The amino acids were dissolved in 0.2 *M* sodium phosphate buffer (pH 8.85).

Amino acid	*I*	*II*	*III*	*IV*
Ile	1	1	10	3
Leu	1	5	2	2
Val	1	2	10	9
Pro	1	2	5	4
Phe	1	2	2	3
Met	1	5	10	0
Ala	1	5	10	12
Lys	1	5	10	10
Orn	1	0	0	0
Tyr	1	0	10	6
Gly	1	5	0	3
Thr	1	2	10	10
Ser	1	5	5	15
Glu	1	5	10	12
Met-SO_2	1	2	0	4
Asp	1	5	10	15
Asn	0	2	0	0
Gln	0	1	0	0
$(Cys)_2$[a]	1	2	2	0
His	1	5	10	4
Arg	1	5	10	4
$CySO_3H$	1	5	0	8

[a] Expressed in nmole of cysteine/100 μl.

(Table III). It is clear that the method is satisfactory both with respect to the reproducibility and the linearity. However, dans-met was sometimes partially degraded during spotting, and also during migration, to dans-methionine sulphoxide and methionine sulphone. In practice, it is convenient to oxidise all the methionine to methionine sulphone before dansylation.

TABLE V

APPLICATION OF DANSYLATION TO THE DETERMINATION OF THE AMINO ACID COMPOSITION OF PROTEIN

These results are expressed in terms of the percentage of each amino acid and are the means of three analyses on 5 nmoles of protein.

Amino acid	*Bovine ribonuclease*			*Bovine serum albumin*		
	Native	*Oxidised*	*Literature data*[d]	*Native*	*Oxidised*	*Literature data*[e]
Ala	10.20	10.09	9.68	9.30	8.50	8.20
Arg	2.94	2.98	3.23	3.62	3.54	3.95
Asp	11.45	11.55	12.10	9.58	9.62	9.50
Cys	6.92	7.14[b]	6.45	6.94	6.80[b]	6.30
Gly } Tyr	6.96	6.88	2.42	5.50	5.50	2.84
Tyr			4.84			3.25
Glu	10.24	10.04	9.68	12.30	12.30	13.10
His	2.64	2.58	3.23	2.94	2.60	3.00
Ile	2.06	2.30	2.41	2.32	2.30	2.35
Leu	1.10	1.13	1.61	10.90	10.80	10.90
Lys[a]	8.71	8.71	8.06	10.85	10.60	10.70
Met	3.59	3.43[c]	3.23	0.90	0.50[c]	0.63
Phe	2.76	2.94	2.42	4.02	4.72	4.55
Pro	2.92	2.82	3.23	3.57	3.61	4.80
Ser	11.37	11.42	12.10	4.70	4.75	4.70
Thr	8.51	8.56	8.06	5.50	5.50	5.65
Val	7.63	7.43	7.25	5.80	5.80	5.80

[a] Recovered as di-dans-lys.
[b] Found as cysteic acid.
[c] Found as methionine sulphone.
[d] According to SMYTH *et al.*[19].
[e] According to WEBER AND YOUNG[21].

Certain authors recommend longer times for the hydrolysis of the protein when the N-terminal amino acid is Ile, Leu or Val[14]. Under our experimental conditions all the Ile N-terminal of trypsin (Table VI) was released after 4 h hydrolysis.

The effect of performic acid oxidation on the N-terminal amino acid determination of three known proteins was studied (Table VI). The results for native or oxidized RNAase and trypsin were similar. For serum albumin a preliminary performic oxidation was necessary in order to obtain a quantitative yield of the N-terminal amino acid. Since the presence of urea in the dansylation medium was insufficient for certain proteins to overcome steric hindrances, we recommend systematic rupture of disulphide bonds by performic acid oxidation prior to all quantitative determinations.

For the quantitative determination, the absolute amount (n_s') of each N-terminal amino acid, in nmole is given by:

$$n_s' = n_s \times l \tag{5}$$

where n_s is defined by eqn. 4 and l is the recovery coefficient after acid hydrolysis as measured by GROS AND LABOUESSE[14].

As a control for the quantitative recovery of the N-terminal amino acids, we have carried out the following calculations for the three proteins tested (Table VI).

TABLE VI

QUANTITATIVE DETERMINATION OF N-TERMINAL AMINO ACIDS AND OF LYS CONTENT OF SOME PROTEINS

Protein	*N terminal AA*	*Dansylation time (min)*	*Moles N-terminal amino acid/mole protein*		*Theoretical value*	*Moles lys/mole protein*		*Theoretical value*	*Mol. wt.*	
			Without performic oxidation	*With performic oxidation*		*Without performic oxidation*	*With performic oxidation*		*Calculated*	*Literature data*
Ribonuclease	Lys	30	0.92	0.93	1	8.62	9.43	10	15 000	14 500
		120	0.95	0.97[b]		8.65	9.77			
Trypsin	Ile	120	0.90	0.92[b]	1	12.60	12.90	14	24 400	22 500
Serum albumin[a]	Asp	30	0.08	0.65	1	24.00	38.50	55	76 800	66 000
		120	0.16	0.86[b]		51.20	46.00			

[a] Cohn fraction V powder.
[b] Values used to calculate mol. wt.

TABLE III

REPRODUCIBILITY AND LINEARITY OF THE METHOD FOR THE DETERMINATION OF THE COMPOSITION OF AMINO ACID MIXTURES

The fluorescence values (F_s^0, see eqn. 1) were expressed for 10 nmole of dans-AA. I, II, III and IV denote the standard amino acid mixtures whose composition is given in Table II.

<table>
<tr><th>Dans-AA</th><th>IA</th><th>IB1</th><th>IB2</th><th>IIA</th><th>IIB</th><th>IIIA</th><th>IIIB</th><th>IVA</th><th>IVB</th></tr>
<tr><td>Dans-ile</td><td>132</td><td>121</td><td>116</td><td>132</td><td>114</td><td>127</td><td>132</td><td>121</td><td>132</td></tr>
<tr><td>Dans-leu</td><td>150</td><td>150</td><td>140</td><td>150</td><td>140</td><td>140</td><td>140</td><td>140</td><td>140</td></tr>
<tr><td>Dans-val</td><td>170</td><td>162</td><td>173</td><td>170</td><td>165</td><td>172</td><td>170</td><td>165</td><td>161</td></tr>
<tr><td>Dans-pro</td><td>387</td><td>320</td><td>355</td><td>387</td><td>370</td><td>346</td><td>354</td><td>354</td><td>346</td></tr>
<tr><td>Dans-phe</td><td>183</td><td>180</td><td>168</td><td>173</td><td>168</td><td>175</td><td>168</td><td>178</td><td>171</td></tr>
<tr><td>Dans-met</td><td>traces</td><td>traces</td><td>295</td><td>275</td><td>245</td><td>255</td><td>325</td><td>0</td><td>0</td></tr>
<tr><td>Dans-ala</td><td>259</td><td>246</td><td>259</td><td>263</td><td>254</td><td>259</td><td>263</td><td>250</td><td>269</td></tr>
<tr><td>Di-dans-lys</td><td>300</td><td>272</td><td>286</td><td>272</td><td>300</td><td>272</td><td>293</td><td>293</td><td>286</td></tr>
<tr><td>Di-dans-orn</td><td>288</td><td>295</td><td>292</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>Di-dans-tyr</td><td rowspan="2">840</td><td rowspan="2">780</td><td rowspan="2">790</td><td></td><td></td><td>505</td><td>462</td><td rowspan="2">810</td><td rowspan="2">825</td></tr>
<tr><td>Dans-gly</td><td>302</td><td>318</td><td></td><td></td></tr>
<tr><td>Dans-thr</td><td>252</td><td>252</td><td>257</td><td>243</td><td>245</td><td>257</td><td>252</td><td>252</td><td>257</td></tr>
<tr><td>Dans-ser</td><td>288</td><td>288</td><td>288</td><td>288</td><td>288</td><td>288</td><td>288</td><td>288</td><td>288</td></tr>
<tr><td>Dans-glu</td><td>168</td><td>178</td><td>187</td><td>183</td><td>178</td><td>183</td><td>183</td><td>178</td><td>180</td></tr>
<tr><td>Dans-met-SO_2</td><td>236</td><td>193</td><td>220</td><td>325</td><td>330</td><td>traces</td><td>traces</td><td>236</td><td>225</td></tr>
<tr><td>Dans-asp</td><td>204</td><td>204</td><td>223</td><td>207</td><td>203</td><td>204</td><td>205</td><td>203</td><td>207</td></tr>
<tr><td>Dans-asn</td><td>0</td><td>0</td><td>0</td><td>190</td><td>195</td><td>0</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>Dans-gln</td><td>0</td><td>0</td><td>0</td><td>195</td><td>195</td><td>0</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>Di-dans$(Cys)_2$</td><td>295</td><td>272</td><td>295</td><td>295</td><td>300</td><td>276</td><td>295</td><td>0</td><td>0</td></tr>
<tr><td>Dans-his</td><td>420</td><td>378</td><td>397</td><td>403</td><td>407</td><td>427</td><td>412</td><td>412</td><td>425</td></tr>
<tr><td>Dans-arg</td><td>280</td><td>276</td><td>293</td><td>290</td><td>280</td><td>276</td><td>276</td><td>287</td><td>280</td></tr>
<tr><td>Dans-$CySO_3H$</td><td>276</td><td>287</td><td>300</td><td>285</td><td>295</td><td>0</td><td>0</td><td>280</td><td>275</td></tr>
</table>

Sample	*Volume dansylated (ml)*	*Aliquot chromatographed*
Ia	0.5	100 %
Ib	2	25 %
Ic	2	75 %
IIa	1	50 %
IIb	0.5	50 %
IIIa	0.5	100 %
IIIb	1	20 %
IVa	0.5	100 %
IVb	1	25 %

To determine the quantities of Gly and Tyr, when both were present in a mixture, the incompletely separated spots were eluted together after silica gel chromatography and the total fluorescence measured. After evaporation, the sample was re-chromatographed (with two migrations in each dimension). Dans-gly and di-dans-tyr, now clearly separated, were eluted and the relative amounts determined (see eqn. 3). Then the absolute quantity of each amino acid was calculated from the total fluorescence value after the first chromatography.

To prevent the rapid loss of fluorescence (50 % in 30 min) which occurs when the dans-AA's remain on dry thin layers, the elution of dans-AA's must be done rapidly. It is possible to elute twenty dans-AA's before the plaque is dry (about 3 min).

TABLE IV

RECOVERY FACTOR (α_s) RELATIVE TO DANS-GLU OF VARIOUS DANS-AMINO ACIDS

Fluorescence values (F_s^0, see eqn. 1) expressed for 10 nmoles of dans-AA, are the means ± S.D. of 9 determinations.

Dans-AA	*Fluorescence value*	α_s	*Dans-AA*	*Fluorescence value*	α_s
Dans-ile	125.2 ± 7.3	1.44 ± 0.08	Dans-thr	251.9 ± 4.0	0.71 ± 0.01
Dans-leu	143.3 ± 4.5	1.26 ± 0.04	Dans-ser	288.0 ± 0.5	0.63 ± 0.01
Dans-val	167.6 ± 1.5	1.08 ± 0.01	Dans-glu	179.8 ± 4.0	1.00 ± 0.02
Dans-pro	357.7 ± 15.4	0.50 ± 0.02	Dans-met-SO_2	230.0 ± 16.0	0.78 ± 0.04
Dans-phe	173.8 ± 4.5	1.03 ± 0.03	Dans-asp	206.6 ± 8.0	0.87 ± 0.03
Dans-met	290.0 ± 35.0	0.64 ± 0.08	Dans-asn	192.5 ± 2.5	0.94 ± 0.01
Dans-ala	258.0 ± 6.6	0.70 ± 0.02	Dans-gln	195.0 ± 0.5	0.92 ± 0.01
Di-dans-lys	286.0 ± 11.0	0.63 ± 0.03	Di-dans$(Cys)_2$	289.7 ± 10.5	0.62 ± 0.02
Di-dans-orn	291.7 ± 3.5	0.62 ± 0.01	Dans-his	409.0 ± 14.4	0.44 ± 0.02
Di-dans-tyr	483.5 ± 21.5	0.37 ± 0.02	Dans-arg	282.0 ± 6.0	0.64 ± 0.01
Dans-gly	310.0 ± 8.0	0.58 ± 0.02	Dans-$CySO_3H$	285.4 ± 9.6	0.63 ± 0.02

The dans-AA's in the elution mixture are stable in the dark and do not lose fluorescence after several hours.

Two factors must be taken into account when calculating the quantities of dans-AA's in these experiments; the different molar fluorescence of the dans-AA's[14] and fluorescence losses during chromatography. The "recovery coefficients" relative to dans-glu (α_S) of the amino acids, determined under our experimental conditions, are given in Table IV.

Application of the method to the determination of the amino acid composition of a protein

Two proteins of known composition (bovine serum albumin, bovine pancreatic RNAase) were chosen to illustrate the validity of the method. Five nmole of native or performic acid oxidised proteins were hydrolysed in sealed tubes for 24 h at 115° with 6 *M* HCl. The HCl was removed *in vacuo* and the dry residue treated as described for a mixture of free amino acids. The results are given in Table V and are compared to literature data obtained using the classical method of MOORE AND STEIN.

This method, without the use of an expensive instrument, is of a precision comparable to that of the classical method, with the advantages that it is possible to carry out several determinations in parallel, quickly, and requires 50–100 times less protein. On the other hand, contaminants reacting with dans-Cl, even in trace amounts, can be a significant source of error at this level of sensitivity; for this reason particular care must be taken in handling of the sample.

Determination of N-terminal amino acids by dansylation

The method of GROS AND LABOUESSE[14] was modified as follows: the time of dansylation was extended to 120 min, as indicated by the results for serum albumin (Table VI) and the excess dans-Cl was eliminated by washing the precipitate of dansylated protein with dilute HCl and with acetone. Therefore no dans-OH was formed during protein hydrolysis. This procedure allows direct chromatography of the dans-AA's, without the need for additional purification as previously described in the literature[9,14].

Since Lys is dansylated in the ε position, it is possible to determine the molar quantity of Lys in the sample[14] by using eqn. 5; the molar quantity of N-terminal amino acids relative to that of Lys can be easily calculated. By knowing the number of Lys residues per mole of protein it is easy to verify whether one mole of N-terminal amino acid corresponds to one mole of protein or less than one, thus giving an indication of the number and approximate size of the polypeptidic chains. The same argument is valid for the sum of the N-terminal amino acids if more than one is detected. On the other hand, the ratio between moles of N-terminal amino acid and moles of protein can indicate the presence of masked N-terminal amino acids. The principal advantage of this method is that quantitative determinations of unmasked N-terminal amino acid are possible with 1–10 nmole of protein.

Fingerprinting of proteins using dans-peptides

Elimination of excess dans-Cl was not performed by formate precipitation, for the purification of dans-peptides produced after enzymatic hydrolysis of a protein, since certain dans-peptides were also precipitated by this reagent. However, since the disadvantages of the alkaline hydrolysis method for elimination of dans-Cl do not apply in this case, given a limited number of hydrolysates to be handled and the fact that the hydrolyses are, themselves, not absolutely quantitative, this method was applied.

Several supports and solvent systems for chromatographic separation of dansylated peptides for the fingerprinting of proteins, have been described[15–17]. Peptides produced after tryptic digestion[20] of native and oxidised RNAase and serum albumin were dansylated as previously described and used to test the different methods.

Thin-layer silica gel chromatography was preferred to polyamide layers because of the low capacity and the difficulty of elution from the latter. Furthermore, the peptide maps are difficult to reproduce and trailing of spots is difficult to avoid on polyamide sheets.

All highly volatile solvents reported in the literature were rejected due to difficulties in getting reproducible maps. Solvent systems with low dielectric constants (around 10) like the chloroform–ethanol–acetic acid (38:4:3) mixture used by ATHERTON AND THOMSON[17] did not resolve the spots sufficiently, leaving a large number of dans-peptides at the origin. On the other hand, most of the peptides migrated toward the solvent front in chromatograms developed in solvents with a dielectric constant of the order of 50. We obtained the best results with solvents of dielectric constants of the order of 20–25.

Fig. 4 shows the map obtained for a tryptic hydrolysate of bovine pancreatic RNAase. The number of dans-peptides obtained corresponds to the number of Lys and Arg residues present in the molecule allowing for the fact that two of the "Lys-X" peptide bonds are not hydrolysed by trypsin[19]. Fig. 5 shows the map obtained for bovine serum albumin. 61 spots were clearly separated, whereas only 35 were observed in the system according to ATHERTON AND THOMSON[17]. The number of spots expected for bovine serum albumin would be around 70 assuming that all the "Lys-X" and "Arg-X" peptide bonds were hydrolysed.

Under our conditions the peptide maps obtained show good resolution, and thus the method can be used for proteins of high mol. wt. The dans-peptides can be detected at levels as low as 0.01 nmole; a reliable map can be obtained from 0.1–1

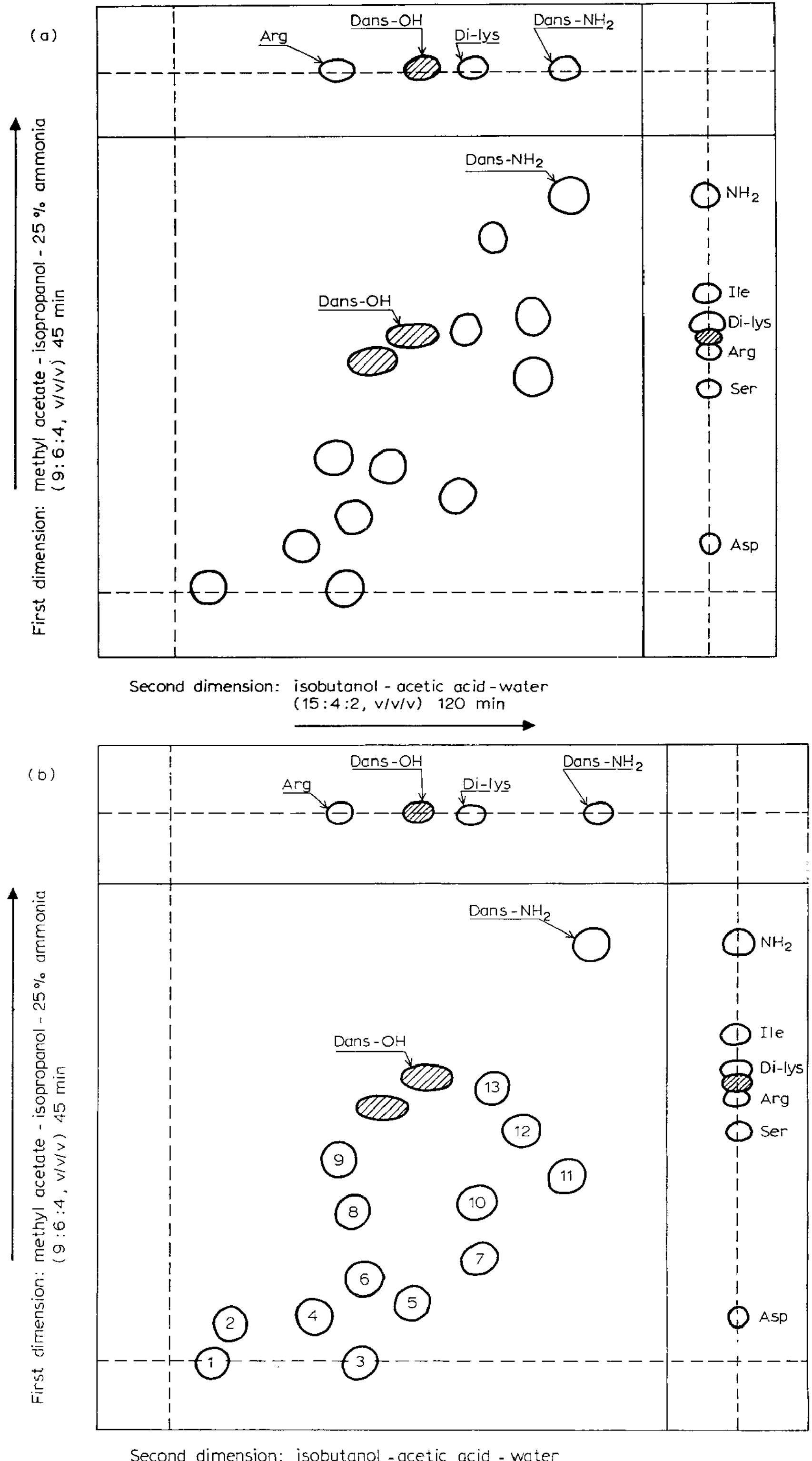

Fig. 4. Fingerprint of a dansylated tryptic digest (6 h, 37°, pH 7.8) of bovine pancreatic ribonuclease on a silica gel thin-layer. Spotted material corresponds to 1 nmole of protein. A = native ribonuclease; B = performic acid oxidised ribonuclease.

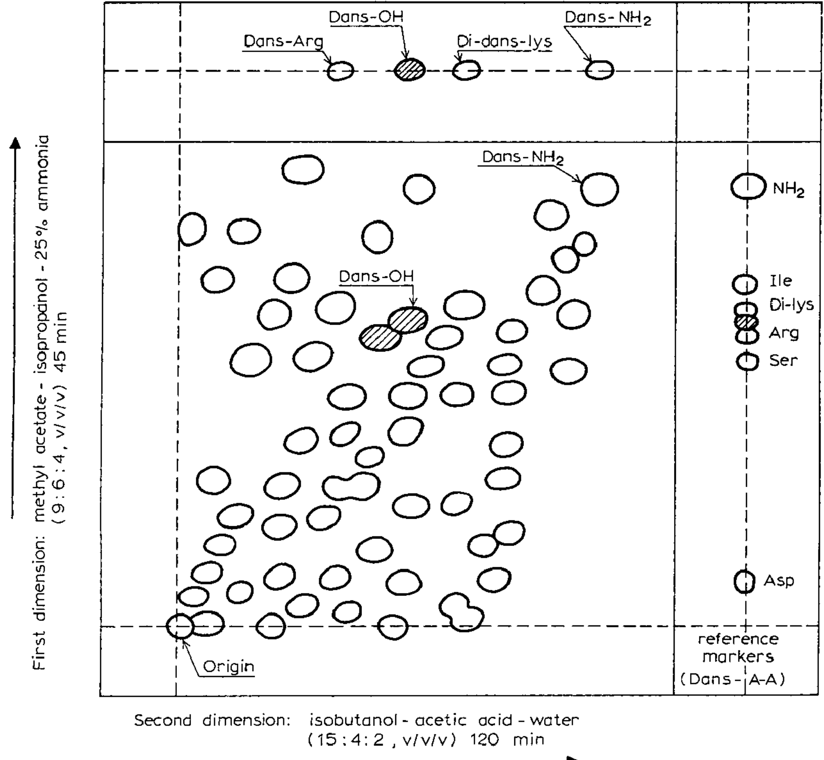

Fig. 5. Fingerprint of a dansylated tryptic digest (24 h, 37°, pH 7.8) of oxidised bovine serum albumin on a silica gel thin-layer. Spotted material corresponds to 1 nmole of protein.

nmole of protein. Peptides obtained from up to 20 nmole of protein can be separated on a single silica gel plate and higher quantities if necessary can be used for proteins of low mol. wt. Given the reproducibility of the chromatograms, corresponding spots eluted from several plates can be pooled in order to obtain sufficient quantities of dans-peptides for subsequent analysis. This technique which has been used to prepare dans-peptides is much quicker than the classical methods of fractionation of enzymatically hydrolysed proteins.

Partial determination of the primary structure of a protein

A combination of the various techniques described in this paper can be used to determine partially the primary structure of a protein on a microscale. Two aliquots were used for the quantitative determination of N-terminal amino acids and for the total amino acid composition of the protein. A third aliquot was used for the preparative fingerprinting.

The N-terminal amino acids and ε-dans-lys were determined on an aliquot of each eluted dans-peptide containing 2.5–7.5 nmole. After the lyophilisation of the acetone–water eluate, the residue was hydrolysed in sealed tubes in 250 μl of 6 *M* HCl for 4 h at 115°. HCl was removed *in vacuo* and the residue taken up in 50 μl of acetone–1 *M* HCl (19:1). 25 μl were then chromatographed on the silica gel thin layer

TABLE VII

DETERMINATION OF AMINO ACID COMPOSITION AND TERMINAL GROUPS OF DANSYLATED TRYPTIC PEPTIDES OF PERFORMIC ACID OXIDISED RIBONUCLEASE (20 nmole)

T_1–T_{13} denote the dans-peptides eluted from the peptide map (see Fig. 4).

AA	T_1[c]	T_2	T_3	T_4[f]	T_5	T_6	T_7	T_8	T_9[g]	T_{10}[g,h]	T_{11}	T_{12}	T_{13}
Ala	0.40	2.25		2.10				1.10	2.15	3.10	1.10		1.15
Arg	1.00[d]		1.00[d]		1.00[d]	1.00[d]							
Asp	2.90[e]	2.55		2.45		0.80[e]		0.95	1.90		0.95	0.85[c]	0.65[c]
Cys[a]	2.30	1.10		0.75				1.05	1.75[e]				1.10
Gly	0.95			0.75			1.05						
Tyr	2.40	1.20		1.05				1.65[e]					
Glu	2.40[e]	1.65[e]	0.95	1.00			0.65[c]		2.45	0.95	0.95		
His	0.20	1.25		2.25[e]					1.10				
Ile	0.95			1.75									
Leu									0.85			0.82	
Lys	0.20[d]	1.00[d]		0.25			1.00[d]	1.00[d]	2.00[d]	2.00[d,e]	1.00[d]	1.00[d]	1.00[d]
Met[b]	1.20	1.75											
Phe			0.78[c]	0.85					0.85				
Pro				1.55				1.15	1.15				
Ser	4.50	7.27		1.25	0.80[c]		2.15		1.75				
Thr	3.45	1.15		0.15			1.05		0.85	0.90	1.75[c]	0.95	
Val				3.85					3.65				0.90

[a] Found as cysteic acid.
[b] Found as methionine sulphone.
[c] T_1 was slightly contaminated with T_2 because of the high quantity of protein used.
[d] C-terminal amino acid.
[e] N-terminal amino acid.
[f] T_4 corresponds to the C-terminal peptide of the protein.
[g] Peptide containing a Lys-X peptide bond not cleaved by trypsin.
[h] T_{10} corresponds to the N-terminal peptide of the protein since it is the only peptide which has the same N-terminal amino acid as the protein.

and the 25 μl remaining was kept in case it proved necessary to confirm the nature of the N-terminal amino acids by polyamide chromatography. From the yield of N-terminal amino acids of a given spot it was possible to determine whether the peptide resulted from a specific enzymatic cleavage or from a non-specific lysis. A high molecular yield of N-terminal amino acid relative to protein used indicates a specific enzymatic hydrolysis. This is particularly helpful when two N-terminal amino acids are detected in the same spot. For tryptic fingerprinting, lysine containing peptides yield, upon acid hydrolysis, two major fluorescent spots: one due to the N-terminal amino acid of the peptide, the other to the C-terminal-lys. An N-terminal amino acid/ε-dans-lys molar ratio of 1 indicates that the peptide results from specific enzymatic cleavage. Since the ω-dansylation of arginine is very slow, it is not possible to carry out similar calculations for arginine containing peptides. The chromatograms of the acid hydrolysate of these peptide spots are characterised by absence of ε-dans-lys, and a weak spot of ω-dans-arg. The C-terminal peptide of the protein lacks both ε-dans-lys, and ω-dans-arg.

Another aliquot of 2.5–7.5 nmole of each dans-peptide was used to determine the amino acid composition of the peptide. The acetone–water eluate was lyophilised and the residue treated as described for the determination of the amino acid composition of a protein. Eqn. 4 gives the absolute quantity of amino acid in nmole (n_s). It was necessary however to introduce an additional correction for fluorescence losses due to partial destruction of ε-dans-lys during acid hydrolysis of the dans-peptide. The absolute amount of ε-dans-lys is given by:

$$n_{\text{lys}} = 1.6 \times n_s \qquad (6)$$

A similar correction is required for the N-terminal amino acid of the peptide.

The analysis of bovine pancreatic RNAase (Tables V–VII) shows that our results are in good agreement with the published data obtained by other methods[19].

The dansylation method allows the rapid partial determination of the primary structure on 15–35 nmoles of protein; *i.e.* 5–10 nmoles for N-terminal group determination, 5–10 nmoles for the determination of the amino acid composition and 5–15 nmoles for the tryptic fingerprinting and the subsequent analysis of the peptides. Using these quantities, all operations can be carried out with precision and give quantitative data. With smaller quantities (0.5–5 nmole), semiquantitative results can be obtained only if scrupulously clean glassware and reagents are used.

ACKNOWLEDGEMENTS

We thank Drs. N. Gros and B. Labouesse for their advice at the beginning of this work.

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CHROM. 4863

A SYSTEM FOR PREPARATIVE SEPARATION OF A WIDE RANGE OF SMALL MOLECULES BY GEL PERMEATION CHROMATOGRAPHY IN ORGANIC LIQUIDS

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(Received June 1st, 1970)

SUMMARY

This paper mainly deals with the description of the apparatus used; only a few examples of separations are given. More applications of the technique will be fully described in subsequent papers.

The procedures are applicable to high boiling compounds in the molecular weight range 100–1500.

For preparative purposes effective separations can be obtained with 30–100 mg amounts of mixture, using a single glass column, or at most a combination of 2–3 columns of 120 cm length and about 15 mm internal diameter, packed with gels of the Bio-Beads or Sephadex LH-20 type in organic liquids.

Low sensitivities of the $8–32 \cdot 10^{-5}$ R.I.U. full scale deflection of the detector, a differential refractometer, are normally used, the latter being thermostatically operated at 2° below room temperature.

Plate numbers of single columns are 3000–6000, HETP values being 0.4–0.2 mm at flow rates of 25–30 ml/h and *o*-dichlorobenzene as a test substance.

INTRODUCTION

Up to now only a relatively small amount of work has been reported in the literature on gel permeation chromatography (GPC) dealing with separations of low molecular weight substances in organic liquids. However, with the increasing interest during the last two years the literature on this matter has been growing steadily.

Apart from the basic literature on this subject[1–6], important studies about the elution behaviour of model compounds on polystyrene gels have been published by HENDRICKSON[6–8], EDWARDS AND NG[9], MOORE AND HENDRICKSON[10,11], CHANG[12,13], and by JOUSTRA *et al.*[14] and KRANZ[15] for alkylated dextran gels. Factors affecting the GPC of small molecules, mainly solvent–solute interactions caused by hydrogen bonding, are described by HENDRICKSON[16], CAZES AND GASKILL[17,18] and LARSEN[19]; adsorption effects of aromatic compounds on alkylated dextran gels are mentioned by WILK *et al.*[20], MAIR *et al.*[21], JANSON[22] and BROOK AND HOUSLEY[23].

Results obtained from separations on lipophilic dextran gels with different

methoxyl content are described by SJÖVAL AND NYSTRÖM[5]. These authors have also used complex solvent mixtures to obtain separations in the field of lipid analysis. ALTGELT[24] states that a small addition of a strong polar liquid to a less polar elution solvent decreases the adsorption effects. Using 2-propanol as mobile phase for GPC on Sephadex LH-20, OELERT[25] found that the elution order of aromatic compounds is given more by polarity than by the shape of the molecules. For applications of the technique to natural and commercial products, interesting information can also be obtained from the literature.

Separations of petroleum hydrocarbons on Sephadex LH-20 have been described by TALARICO *et al.*[26] and MAIR *et al.*[21]; separation of crude oil fractions on polystyrene gels is mentioned by COLEMAN *et al.*[27]. BOMBAUGH and coworkers[28–33] have described many separations in the field of hydrocarbons, alcohols, glycols, triglycerides, steroids, surfactants and ethylene oxide derivatives on polystyrene gels. SCHRÖDER AND MISCHE[33] give procedures for the separation of adipic plasticizers on lipophilic polydextran gels, while HAGEN AND SCHRÖDER[34] use polydextran as well as polystyrene gels for the fractionation of phenolic resins. The analysis of tall oil, using polydextran and polystyrene gels, is described by CHANG[35]. Other gel materials, *e.g.* isocyanate modified polydextran, cross-linked polymethyl- or polybutylmethacrylates and cross-linked polyvinylacetate have been used by HEITZ *et al.*[36] for the separation of oligomeric phenylenes and urethanes. The separation on a preparative scale of low molecular weight polystyrenes, polybutylmethacrylates, and polyethylene glycols on polystyrene gels is described by HEITZ AND ULLNER[37]; the optimal conditions and the limitations of GPC to the same oligomeric materials are given by HEITZ *et al.*[38].

Cyclo-oligomers from Nylon 6.6, 6.10 and 11 have been separated by ZAHN AND KUSCH[39] on a hydrophilic polydextran in acetic acid–water. These authors also separated oligomeric cyclo-glycolterephthalates on polystyrene and polydextran gels.

The papers cited above all deal with GPC in organic solvents; application of the technique in water or buffer solutions is mainly restricted to biological and biochemical analysis, using hydrophilic polydextran and polyacrylamide gels. Some examples of separation of low molecular weight commercial substances in aqueous solution are given by FEIST *et al.*[40], who separated polyethylene glycols on Sephadex G-50; the analysis of surfactants on Sephadex G-50 and G-75 is described by TOKIWA *et al.*[41–43].

Chromatography on Sephadex G-10, using two different eluants for the separation of an anionic detergent and an anionic non-detergent, combined with ion-exchange, is described by MUTTER[44].

In most cases cited above use is made of commercially available apparatus for liquid chromatography normally equipped with stainless steel columns, 4 ft. in length and 3/8 in. I.D., packed with polystyrene gels. For analytical purposes only a few mg of mixture can be separated with such an apparatus, using combinations of 2–5, sometimes as much as 10 and 40 columns, in series[28,29].

The use of glass columns is mainly coupled with the introduction of polydextran gels. Here, columns vary from 50–500 cm in length and from 16–50 mm in diameter; usually no exact description of the columns and other apparatus used is given. As far as we know, no commercial apparatus is used in combination with Sephadex gels. In addition the application of the technique using polydextran gels is mainly restricted to analytical separations. Preparative separations on a gram scale, using glass columns

of 200 cm length and 50 mm inside diameter, are described by HEITZ and coworkers[37,38].

Our purpose was to apply GPC to qualitative and quantitative analysis of mixtures from different origins. Generally, we only possess limited information about mixtures to be separated, based on an IR spectrum, a thin-layer chromatogram or/and the purpose for which such a mixture is or can be used.

For both qualitative and quantitative analysis, we need separation procedures, which have to be carried out on a larger scale than is possible with a commercial analytical GPC apparatus; furthermore, these apparatus are not flexible enough for our purpose.

As we are dependent on the solubility of the samples in organic liquids, and as in principle GPC is a simple technique[1,2], we thought an experimental apparatus, in which some types of different gels are used, each swollen in two or three solvents, to be the best suited to our analysis, *i.e.* separations on a 30–100 mg scale, which amount allows identification of the sample by means of IR and NMR, as well as quantitative gravimetric determination. Although this work has not yet been concluded, we thought it of interest to publish a description of the apparatus at this stage. In addition, some examples of separations are given.

CEL MATERIALS AND APPARATUS DETAILS

Gel materials

The choice of the gel is somewhat difficult, as the behaviour of polystyrene and polydextran gels is quite different. Association effects may occur in both cases. However, as the polydextran gel Sephadex LH-20 is not fully alkylated and possesses a certain number of hydroxyl groups, polarity of the substances to be separated plays an important role here. The effect may be diminished by the correct choice of the eluting solvent. An additional point is the adsorption of aromatics to polydextran gels. These phenomena may seem to be troublesome, but it is our experience that they can easily be overcome, and that it is sometimes even possible to exploit these properties and to obtain separation in cases where it was not expected.

A survey of commercially available gels, which may be used for the separation of small molecules, is given in Table I. For the sake of completeness some gels for use in aqueous solution are also mentioned here.

We have very good results with gels of the Bio-Beads and Sephadex LH-20 type. Comparative tests between the polystyrene gels of the Bio-Beads and Poragel type with approximately the same molecular weight exclusion limits showed that better results were obtained with the Bio-Beads gels; to obtain about the same separation we needed at least two Poragel columns, while with Bio-Beads only one column of the same dimensions was needed. It must, however, be mentioned, that the Poragels have a more rigid structure, which would probably allow the use of rather high pressures and facilitate the coupling of more columns in series.

Recently, Merckogel OR has been marketed. It is a gel based on cross-linked polyvinylacetate. According to the data from the manufacturer, the properties of this gel will probably lie between those of the polystyrene and the alkylated polydextran gels.

TABLE I

GEL MATERIALS FOR SEPARATION OF SMALL MOLECULES

Name	*Type*	*Swelling agent*[a]	*Mol. wt. exclusion limit*	*Manufacturer*
Sephadex LH-20	alk. polydextran	org.	5000	Pharmacia
Sephadex G-10	polydextran	H_2O	700	Pharmacia
Sephadex G-15	polydextran	H_2O	1500	Pharmacia
Sephadex G-25	polydextran	H_2O	5000	Pharmacia
Bio-Beads SX-1	polystyrene	org.	3500	Bio-Rad Laboratories
Bio-Beads SX-2	polystyrene	org.	2700	Bio-Rad Laboratories
Bio-Beads SX-3	polystyrene	org.	2000	Bio-Rad Laboratories
Bio-Beads SX-4	polystyrene	org.	1400	Bio-Rad Laboratories
Bio-Beads SX-8	polystyrene	org.	1000	Bio-Rad Laboratories
Bio-Gel P2	polyacrylamide	H_2O	1600	Bio-Rad Laboratories
Bio-Gel P4	polyacrylamide	H_2O	3600	Bio-Rad Laboratories
Bio-Gel P6	polyacrylamide	H_2O	4600	Bio-Rad Laboratories
Poragel A1	polystyrene	org.	1000	Waters Associates
Poragel A3	polystyrene	org.	3000	Wators Associates
Styragel 40 A	polystyrene	org.	1600	Waters Associates
Styragel 100 A	polystyrene	org.	4000	Waters Associates
Merckogel OR 750	polyvinylacetate	org.	750	Merck A.G.
Merckogel OR 1500	polyvinylacetate	org.	1500	Merck A.G.
Merckogel OR 5000	polyvinylacetate	org.	5000	Merck A.G.

[a] Swelling agents, according to the manufacturers, are: Sephadex LH20: DMF, water, alcohols, chloroform, *p*-dioxane, THF, acetone, ethyl acetate, toluene; polystyrene gels: benzene, DMF, chlorinated hydrocarbons, THF, *o*-dichlorobenzene; polyvinylacetate gels: THF, ethyl acetate, benzene, acetone, methanol.

The gels used in our experiments were the polystyrene gels Bio-Beads SX-1, SX-2 and SX-8 from Bio-Rad Laboratories, having exclusion limits of a mol. wt. of 3500, 2700 and 1000 respectively, and the alkylated polydextran gel Sephadex LH-20 from Pharmacia, having an exclusion limit of about 5000.

It should be mentioned that these limits, which are supplied by the manufacturers, have to be interpreted with caution, as it is our experience, that they are generally too high, and that the effective range for separation purposes starts considerably below these values. It should also be mentioned, that polystyrene as well as polydextran gels, having rather high exclusion limits of about 5000, may be successfully used for the separation of compounds in the mol. wt. range of 200–100 and lower.

The polystyrene gels were swollen in benzene and chloroform, the polydextran gel was swollen in methanol, acetone, or chloroform. In all cases, the gels were soaked for 24 h in an excess of solvent with occasional stirring. Before packing the columns, the suspension of gel is evaporated at room temperature for 1 h using a water jet pump.

Swelling agents

Liquids used for preparative GPC have to be absolutely free from nonvolatile matter; we therefore found it necessary to distil all solvents, even "pro analysi"

quality, until no residue could be detected by weighing after evaporation of 100 ml of solvent at 40° under N_2. The distilled solvents are stored in 30-l glass flasks, from which the liquids are pumped to the columns.

Solvents for preparative GPC should, on the one hand, be of low volatility but, on the other hand, it is desirable, that they are also good swelling agents; these two requirements considerably reduce the choice. In early experiments, tetrahydrofuran, the common solvent in GPC, was used. However, in spite of all the precautions to prepare and keep this solvent free from peroxides after removal of the stabilizer and distillation, so much peroxide was formed, where the solvent was exposed to air, *e.g.* in the fraction collector, that identification of the isolated substance was impossible. Therefore, this solvent was not used in further experiments.

Columns and plungers

Using stainless steel columns as in commercial apparatus, it is not possible to observe the gel bed. However, cracks and channels sometimes arise in the gel bed, even in columns which have been in use regularly for half a year and longer, so as we only intended to apply low pressures, it was decided to use glass columns, and so be able to observe the gel bed.

Experiences with commercially available columns were somewhat disappointing, except with the Sephadex SR column length 1 m and 1 in. I.D. which proved to be useful for separations on a 0.5–1 g scale under favourable circumstances. Resolution of such a column, however, is considerably lower than that of longer columns with smaller diameters.

We chose columns of 120 cm length and 14.5 mm I.D., as it appeared from our experiments, that good resolution could be obtained and that the capacity of these columns was amply sufficient for our purposes. As in the case of low-porosity polystyrene and polydextran gels, which are used for the GPC of small molecules, alterations in the packed gel bed are almost negligible. Columns, provided with only one small plunger, were made from Quickfit Visible Flow glass of the above-mentioned dimensions. Tolerances of the inside diameter of this type of glass tubing are narrow, so that the home-made, stainless steel plungers are interchangeable. Maximum pressure for these columns is 3.5 atm.

To avoid wall effects, the column walls are silylated with a 5 % (v/v) solution of dimethyldichlorosilane in chloroform for 1 h at room temperature; the columns are washed with chloroform, and then with synthetic detergent for complete removal of excess reagent and polysiloxanes formed. Column and plunger are shown schematically in Figs. 1a and 1b. Turning the nylon ring of the plunger pushes down the polyethylene ring over the oblique bottom part, and expands the ring firmly against the glass wall, so that it can easily withstand pressures up to 3 atm.

Coupling

For the connection of columns, plungers, and valves with flexible polyethylene tubing, stainless steel capillaries and hypodermic needles of exactly 1.3 mm O.D., about 0.8 mm I.D. and 4 cm length are used, over which a small piece of the said tubing, 2 mm O.D., 1 mm I.D. and 2 cm length is pushed. Capillary and tubing together are now pushed forcefully into the glass capillary at the bottom of the column (which has an O.D. of 9 mm and an I.D. of 1.9–2.0 mm); from this a piece of steel capillary

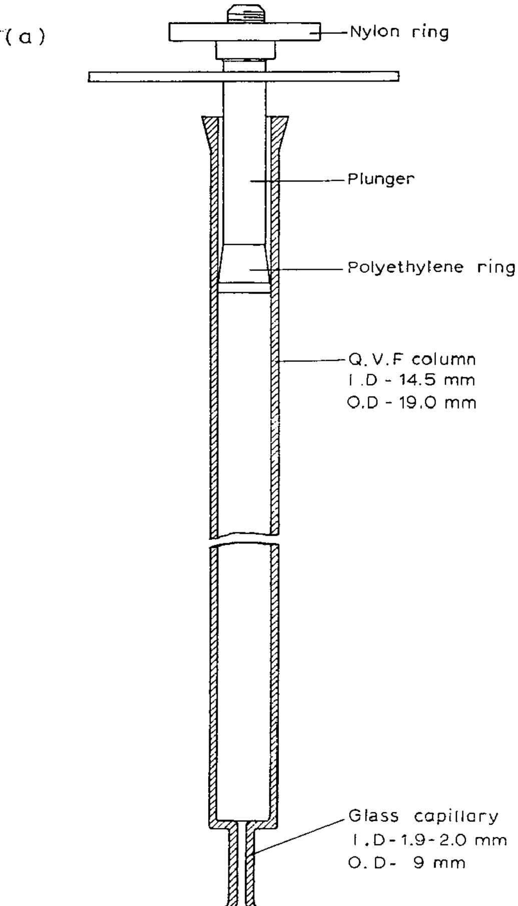


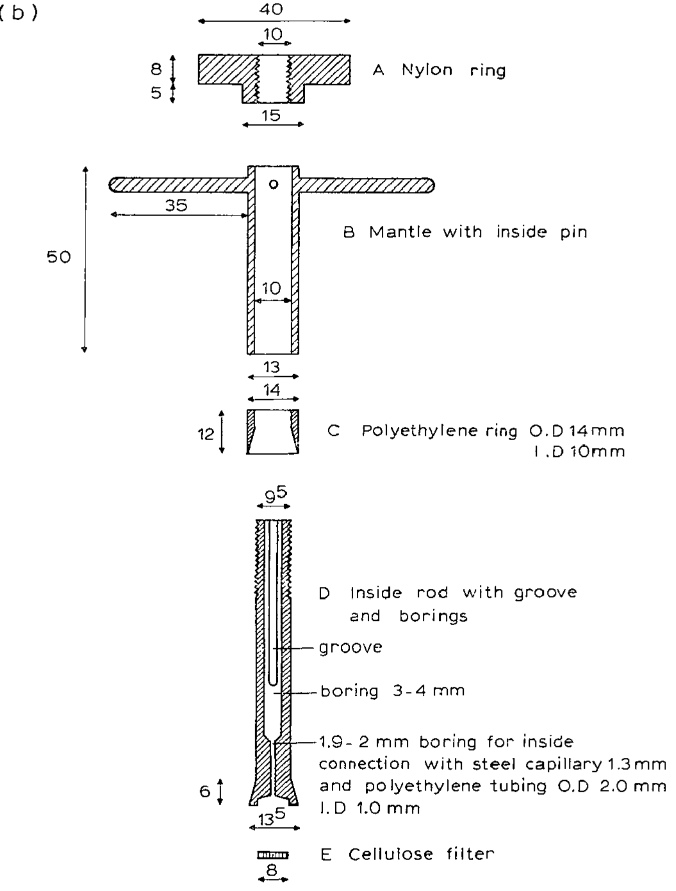
Fig. 1. Cross-section of (a) the chromatographic column and (b) the plunger for 14.5 mm I.D. chromatographic columns.

of about 2 cm, not covered with polyethylene tubing, extends. Over this free end the longer connecting tube is pushed over a length of 1.5–2 cm (Fig. 2a).

In the same way the plunger and its tubing are connected by means of a short piece of steel capillary in the 1.9–2.0 mm bore in the plunger (Fig. 2b). In our case, all columns are provided with Hamilton 3-way valves at the bottom; instead of steel capillaries, short hypodermic needles with Luer connections are used here, the valve being mounted on this (Fig. 2c). These connections, which we have used for years in column adsorption chromatography, have been found to be leak-proof. As with the plunger, pressures up to 3 atm. can easily be withstood here as well.

We prefer polyethylene tubing for flexible connections, as it is easier to handle than Teflon tubing; it is also equally stable against liquids such as benzene and chloroform at room temperature.

Before using the column, a very small wad of glass wool is pushed into the glass capillary above the stainless steel connecting piece, after which about 2 mm of sand



is placed in the sharply necked bottom of the column in order to obtain a real flat insert layer and thus to avoid irregularities in flow at the end of separation.

Column packing

Gel beds have to be carefully packed. Procedures have been described by DETERMANN[1], ALTGELT[24], HEITZ AND ULLNER[37,47], MOORE[45], FLODIN[46] and SIE AND VAN DEN HOED[48]. In our case no special procedures are used, however. In contrast to normal procedures, where a thin gel suspension is used, we used a rather thick slurry, which is poured into the column, in which 10–15 cm of solvent is already present. With the valve fully open at the bottom, the suspension above the settled particles is regularly kept moving by stirring. After the surface of the gel bed has reached the upper part of the column, the plunger is placed in position and liquid pumped down the column at a rate of about 60–80 ml/h until no decrease in volume of the gel bed can be observed. The plunger is now pushed down upon the gel bed, care being taken to avoid the entrance of air, until no dead volume between gel bed and plunger can be observed. Even with liquids such as chloroform and dichloromethane, in which the gels float, no difficulties arise.

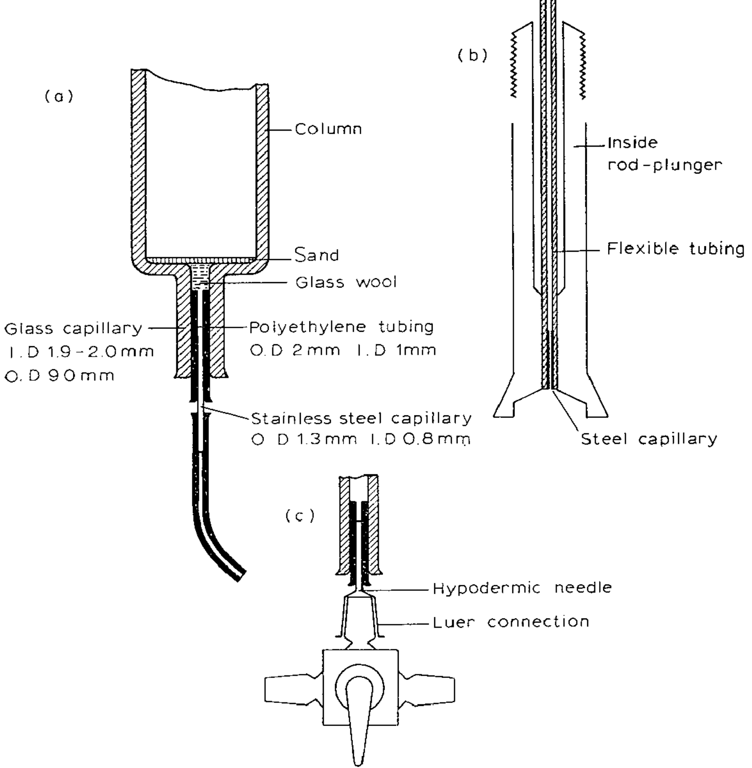


Fig. 2. Connection of (a) column–flexible tubing; (b) plunger–flexible tubing; and (c) column–Hamilton valve.

It appears that with this simple procedure good quality columns can be prepared; our polystyrene columns in benzene usually have plate numbers of 3000–6000, HETP values being 0.4–0.2 mm, obtained with *o*-dichlorobenzene as the test substance at flow rates of 25–30 ml/h. These values are comparable with those given in the literature[9,10,28,29].

The chromatographic system

Packed columns are inserted in the whole system, consisting of storage vessel, pump, pulsation damper, sample application system, thermostat, detectors, recorder, and fraction collector.

Liquid is pumped through the main liquid lines of heavy walled Teflon tubing (5 mm O.D. and 1 mm I.D.). Columns are placed between the main lines, making operation with one or two and three coupled columns easy. All connections between columns and main lines are as described above. A schematic diagram is given in Fig. 3.

Pumping; pulsation damping

The pumps are Hughes plunger type micro pumps with single short stroke mechanisms for each carrier solvent, operating at 20 strokes/min and having a maximum capacity of 185 ml/h.

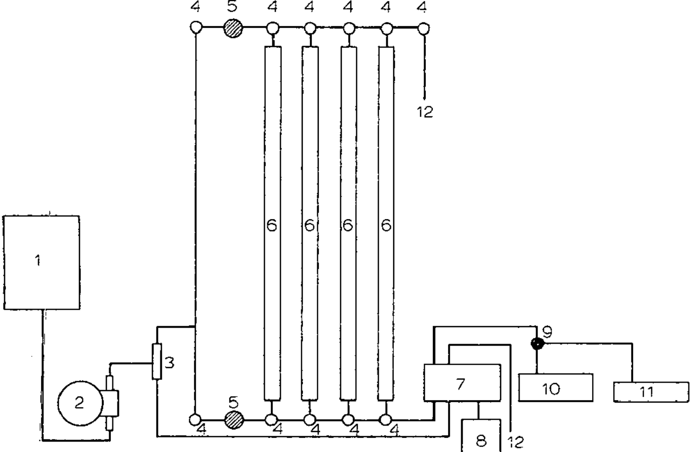


Fig. 3. Survey of apparatus. 1 = solvent reservoirs; 2 = micropump; 3 = pulsation damper; 4 = Hamilton 3-way valves; 5 = Chromatronix sample valves; 6 = columns; 7 = differential refractometer; 8 = recorder; 9 = stream splitter; 10 = fraction collector; 11 = TLC detector; 12 = waste.

It is necessary to damp the pulses of the pumps. This is possible by means of a shunt in the main line, provided with a manometer at the end. This construction is usually found in the commercial apparatus. However, it is also possible to damp pulses with the simple glass apparatus shown in Fig. 4a. It is essential, that the space between the air and liquid is separated by mercury, as in earlier experiments it appeared that, when the liquid was in direct contact with air, the latter was gradually dissolved, even in liquids such as benzene and chloroform, causing air bubbles at the exit of the column, where the pressure was reduced. With this apparatus it is also possible to measure pressures, as the longer and smaller tube acts as a manometer. A modification of this apparatus, now in use, is shown in Fig. 4b. In this case an air trap is also provided; a second improvement is a precision Teflon needle valve, which regulates the flow of the reference stream.

Sample application

Sample application systems in commercial apparatus are of two types. The first is derived from gas chromatographic injection systems, *i.e.* application of the sample with a hypodermic syringe *via* a septum; the second uses sample loops, which can simply be switched into the main liquid line. As the amounts of liquid we normally apply are 0.5–1.5 ml, we chose the latter system. In our apparatus we make use of several Chromatronix sample valves with stainless steel capillary sample loops. These valves have been in use for more than a year and we have never had any difficulties with them.

Formerly, we used a system of two Hamilton 4-way valves, provided with hypodermic needles and flexible Teflon tubing as a sample loop (Figs. 5a and 5b). It is true that a simple method of sample application is possible in this way, however, as Teflon tubing swells considerably in organic liquids, stainless steel capillary sample loops have to be used here. If columns are operated only by the gravity of the eluting

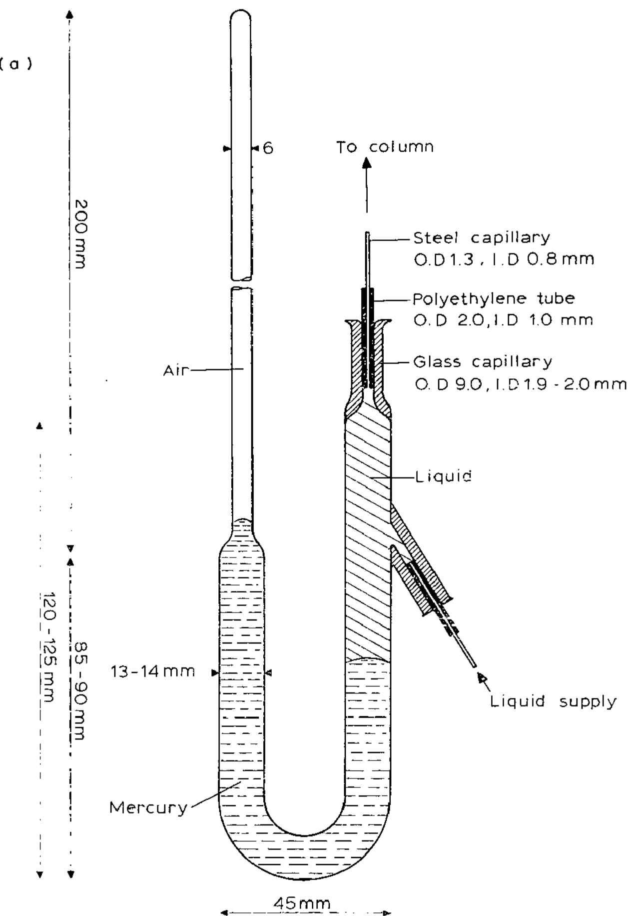


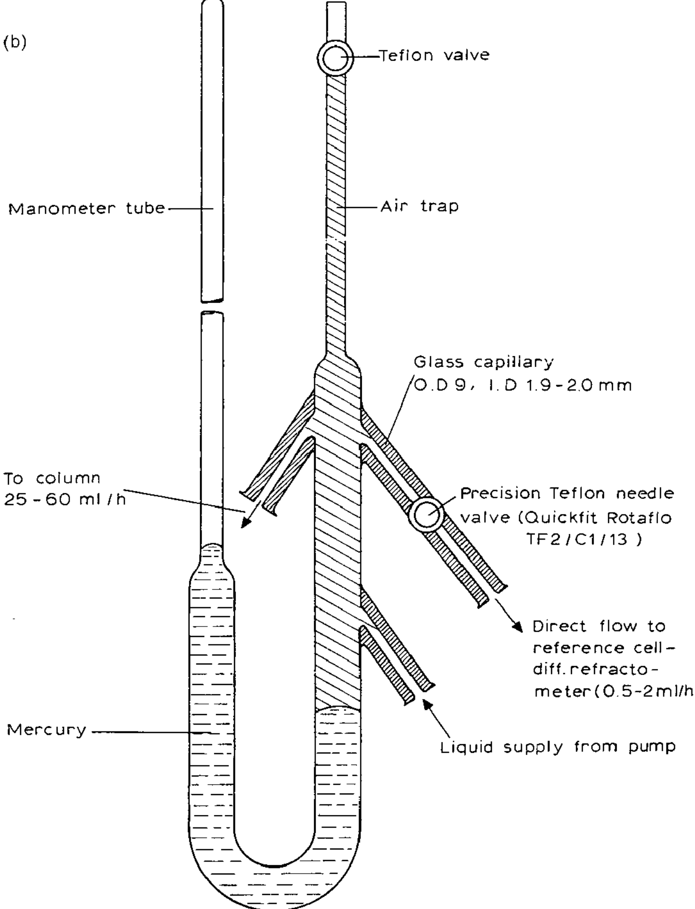
Fig. 4. Cross-section of the pulsation damper (a) and the modified pulsation damper (b).

solvent, a simple method of sample application is the use of a glass 3-way valve and a centrifuge tube, the latter containing the sample (Fig. 5c).

Detection

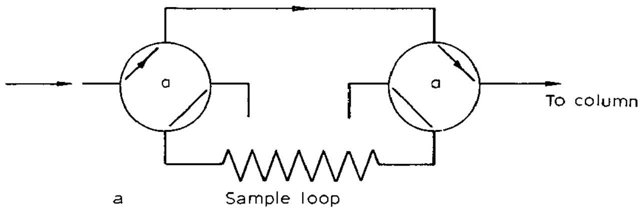
General methods for detection are flame ionization, heat adsorption, and differential refractometry. The last method was chosen, based upon the fact that this method seemed to be reliable, as it had been used for GPC of polymeric materials for years by several authors. However, flame ionization was also tried, but two types of apparatus, based on solvent transport by means of a chain, proved to be unreliable, and a third type, based on solvent transport upon a wire, had the disadvantage of inhomogeneous solvent application to the wire and also a somewhat irregular transport of the wire itself. We have not investigated heat adsorption detection.

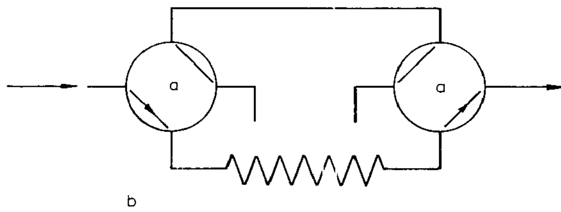
For our purposes we are using three differential refractometers of the type R4 from Waters Associates, equipped with 70-μl cells, which have been in operation almost continuously for one year. Two of them are used in combination with poly-



styrene gels in benzene and chloroform, the third is used for polydextran gels in methanol, chloroform, and acetone.

For the amounts of mixture (30–100 mg) we usually apply for separation, the detectors can be used at the low sensitivities of the $8–32 \cdot 10^{-5}$ R.I.U. full scale deflection of the recorder, whereby the whole effluent passes through the sample cell of the refractometer without splitting. The refractometers operate at $2° \pm 0.01°$ below room temperature with the aid of precision thermostats. It is necessary to keep the temperature of the cells between such close limits, as otherwise no stable base line can be obtained; in addition it is also advisable to work a few degrees below room temperature, as in this way the appearance of gas bubbles in the cells is prevented. Nevertheless, gas bubbles will sometimes arise with the use of such low volatile eluants as chloroform and dichloromethane. In this case it is advisable to place the outlets of the flexible tubing, connected to the outlets of the cells, about 10 cm above the differential refractometer. In this way the appearance of gas bubbles can be fully suppressed. After distillation of the carrier solvents, no further degassing of the liquids





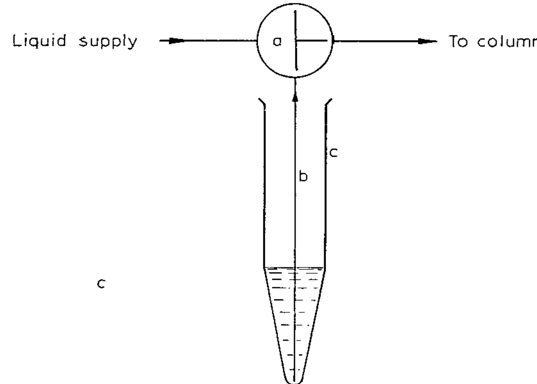


Fig. 5. Sample application using two Hamilton 4-way valves, hypodermic needles and flexible Teflon tubing (a + b); (a) flushing/loading, (b) sample application. (c) Sample application by gravity.

is carried out, the whole separation procedure being carried out at room temperature (21° ± 0.5°).

In contrast with GPC of polymeric materials, the eluant only passes through the columns which are used for separation, no reference column is used. Reference liquid is taken from the apparatus of Fig. 4b and directed to the reference cell at the low flow rates of 0.5–2 ml/h. The flow rate of the eluate itself amounts to 30–60 ml/h. As long as the ratio between the flow rates remains constant, no deflections of the recorder pen can be observed, and a stable baseline is obtained.

Normally, the whole effluent is guided to a time-operated fraction collector, but we split 2–3% from the effluent leaving the differential refractometer off to a new type of chromatographic detector, described by VAN DIJK[49]. The principle of this cheap method of detection is the continuous application of column effluent to a

series of TLC plates. This apparatus runs synchronously with the fraction collector. After development and visualization of the TLC plates, the spots are correlated with the corresponding tubes. Full details are given in the above-mentioned paper.

Flow rates

Flow rates formerly used were 15–25 ml/h. Under these conditions the separation procedures were carried out in 8–15 h, using the columns as described, having a total volume of about 200 ml. LITTLE and coworkers[50], however, have proved, that the elution volume of a chromatographic peak is less dependent upon the flow rate than expected, and it is demonstrated, that at very high flow rates peak widths are not really increased. Though the apparatus used was a modified analytical gel permeation chromatograph, operating at 2200 p.s.i. and 80°, we thought this finding interesting enough to be applied to our procedures. Though our working conditions are totally different, it appeared that our flow rates could be easily increased by at least 2–3 times without considerable increase in elution volume and loss in resolution. Normal procedures are now carried out at flow rates of about 60 ml/h, reducing the separation time to about 3 h. Further increases in flow rate are now being studied; the preliminary results are promising, as we have already been able to separate completely 75 mg of a test mixture containing 7 components in about 45 min, pumping at a rate of 120 ml/h. In this case a column of 120 × 0.9 cm, packed with Bio-Beads SX-2 in benzene, was used.

APPLICATIONS

Experiments with GPC of small molecules usually start with the investigation of test substances. Therefore, we thought it to be useful to give some examples in this field.

Fig. 6 shows the results of a separation of triglycerides and hydrocarbons, using a combination of 2 columns with Bio-Beads SX-1 and SX-2 in benzene, *i.e.* gels suitable for separations in the mol. wt. range of about 3000 and lower. Except in the

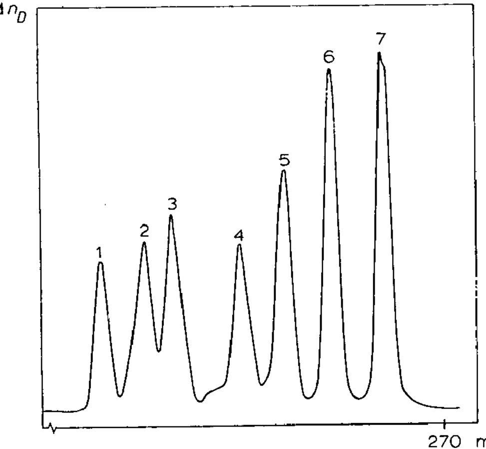


Fig. 6. Separation of test substances. Bio-Beads SX-1 + SX-2 in benzene; 2 columns. Sample application, 70 mg; attenuator, 32 ×; flow rate, 20.5 ml/h. 1 = tristearin; 2 = trimyristin; 3 = trilaurin; 4 = tricaprylin; 5 = tricaproin; 6 = hexadecane; 7 = undecane.

case of trimyristin (mol. wt. 723.2) and trilaurin (mol. wt. 639.0), a practically complete separation of all the components was obtained. The resolution of the substances in the higher molecular weight range seems to be somewhat disappointing, as has already been stated. On the other hand, however, separation of the lower molecular weight components is satisfactory beyond expectation.

It is interesting to compare here the results of BOMBAUGH and coworkers[28, 29]. In an analytical separation of a number of the same substances, combinations of 10 and 40 columns packed with Styragel are used, at flow rates of 66 and 24 ml/h, respectively. These authors, however, used THF as the carrier solvent. In spite of the fact that we used only two columns and applied 70 mg of mixture, our results can compete fully with those of the above authors.

A separation on a single column of Bio-Beads SX-8 in benzene (exclusion limit mol. wt. 1000) is given in Fig. 7. Here, too, the limit given is rather high, as tristearin is eluted with the void volume. Good separation is obtained with the lower molecular weight components, even *n*-amyl and isoamylbenzene tend to separate. Using more columns in series, one can obtain a complete separation in such a case.

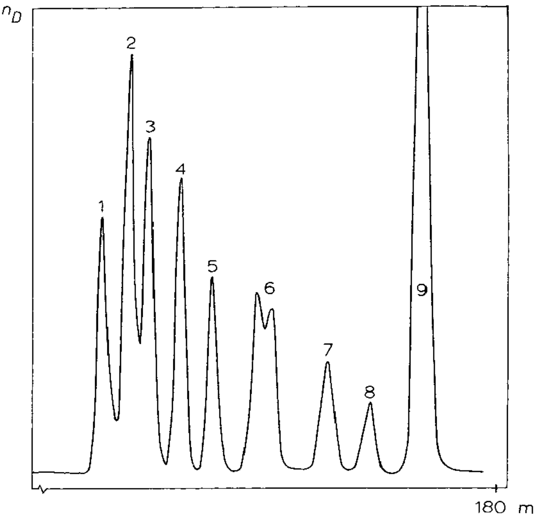


Fig. 7. Separation of test substances. Bio-Beads SX-8 in benzene; 1 column. Sample application, 41 mg; attenuator, 16 ×; flow rate, 24.5 ml/h. 1 = tristearin; 2 = tricaprylin; 3 = nonadecylbenzene; 4 = tridecylbenzene; 5 = nonylbenzene; 6 = *n*-amylbenzene + *iso*-; 7 = *n*-butylbenzene; 8 = toluene; 9 = methanol.

Sephadex LH-20 in methanol is used in the last example of separation with test substances (Fig. 8). The first tall peak originates from a propylene oxide–ethylene oxide copolymer, Pluronic F 68, having a molecular weight of about 4600. We have used this substance for the determination of the void volume of all Bio-Bead and Sephadex LH-20 columns, as it readily dissolves in all the solvents used.

The adsorptive properties of Sephadex for aromatics are clearly demonstrated in this case, as *p*-dichlorobenzene is eluted after water.

The following examples are all related to problems we met in practice. Investigating commercial mixtures of long chain acid alkylphosphates, neutralized with

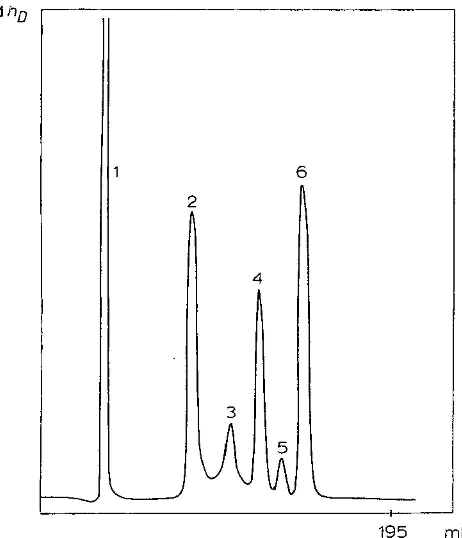


Fig. 8. Separation of test substances. Sephadex LH-20 in methanol; 1 column. Sample application, 15 mg; attenuator, 16 ×; flow rate, 24.5 ml/h. 1 = polypropylene oxide–polyethylene oxide; 2 = tristearin; 3 = stearic acid; 4 = ethylene glycol; 5 = water; 6 = *p*-dichlorobenzene.

bases such as morpholine or ethanolamines, we used ion exchange in an alcoholic solution for removal of the cationic part. The residue of the eluate, containing anionic and probably nonionic substances, was subjected to GPC in methanol on Sephadex LH-20 in methanol, but no complete separation was obtained, probably because of solute–solute interactions. However, after methylation of the residue, a complete separation was obtained on 3 columns of Sephadex LH-20 in methanol, and after isolation of the separated substances it appeared from NMR analysis that the main

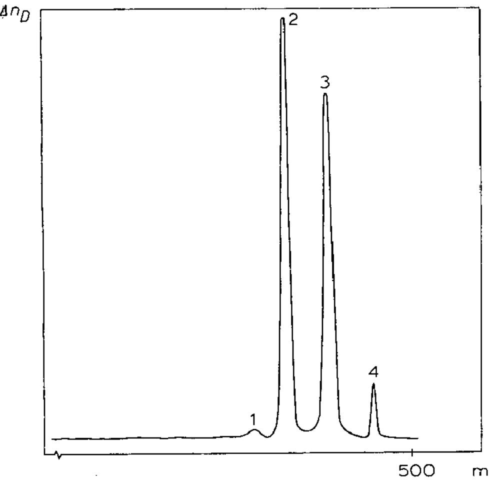


Fig. 9. Separation of methylated acid alkylphosphates. Sephadex LH-20 in methanol; 3 columns. Sample application, 60 mg; attenuator, 32 ×; flow rate, 40 ml/h. 1 = tridodecylphosphate; 2 = methyl-didodecylphosphate; 3 = dimethyl-dodecylphosphate; 4 = *n*-dodecylalcohol.

components in the mixture were mono- and didodecylphosphates. Also a small amount of the free alcohol was identified. Only one small peak, possibly from a triester, escaped identification (Fig. 9).

Dealing with oligomeric materials, we had the problem of separating cyclic oligomers, present in extracts from polyamide 6. In this case we were able to separate the oligomers up to the octamer on a simple combination of 2 columns Sephadex LH-20 in methanol (Fig. 10). By partial removal of the excess of lactam, the monomer, it is even possible to detect the nonamer. For quantitative analysis of the oligomer composition we use 3 columns in series, whereupon complete separation of all the oligomers is obtained. As GPC is only a part of a rather complex analysis procedure, further details of this procedure will be described in a subsequent paper.

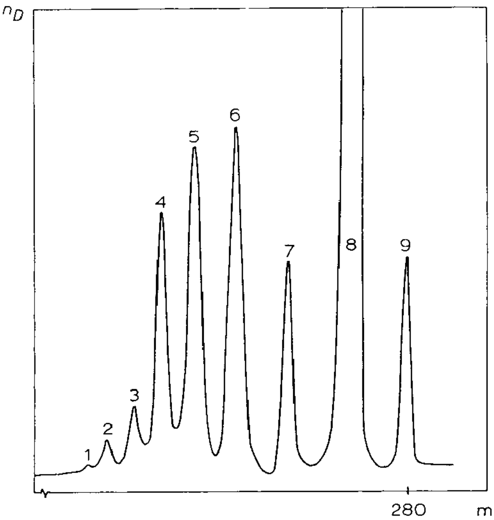


Fig. 10. Separation of cyclic caprolactam oligomers. Sephadex LH-20 in methanol; 2 columns. Sample application, 30 mg; attenuator, 32 ×; flow rate, 30 ml/h. 1–7 = octamer up to dimer; 8 = caprolactam; 9 = water.

In an investigation of the composition of deposits on machine parts, originating from an industrial yarn, a chloroform solution of the deposits was passed through a single column of Bio-Beads SX-2 in chloroform, and a usable separation was already obtained (Fig. 11). As in the case of the polyamide oligomers, an important improvement in resolution may here, again be expected by using more columns in series. However, with one column alone, several components in the mixture could be isolated.

Finally, two other examples are given in Figs. 12 and 13. In neither case was it our intention to identify the components separated. In Fig. 12 the separation is shown for components present in a polyester, prepared from a substituted diol and adipic acid. The tall peak at the end of the chromatogram, which comes from a small amount of chloroform added to the methanolic solution to dissolve a cloudy precipitate, is remarkable. Fig. 13 shows the chromatogram of a complex surfactant mixture, containing long chain acid salts of ethanolamines, free acid, and polyethyleneoxide derivatives.

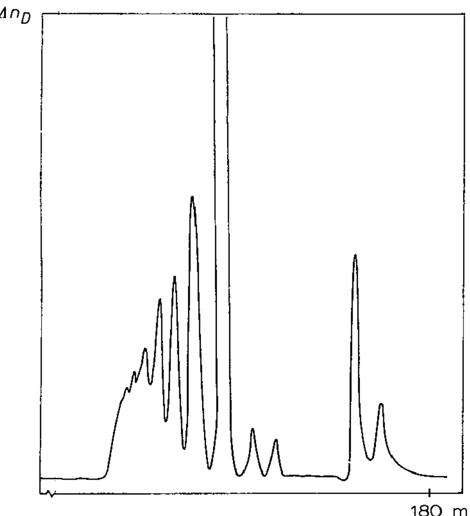


Fig. 11. Separation of components, present in deposits. Bio-Beads SX-2 in chloroform; 1 column. Sample attenuation, 30 mg; attenuator, 16 ×; flow rate, 47 ml/h.

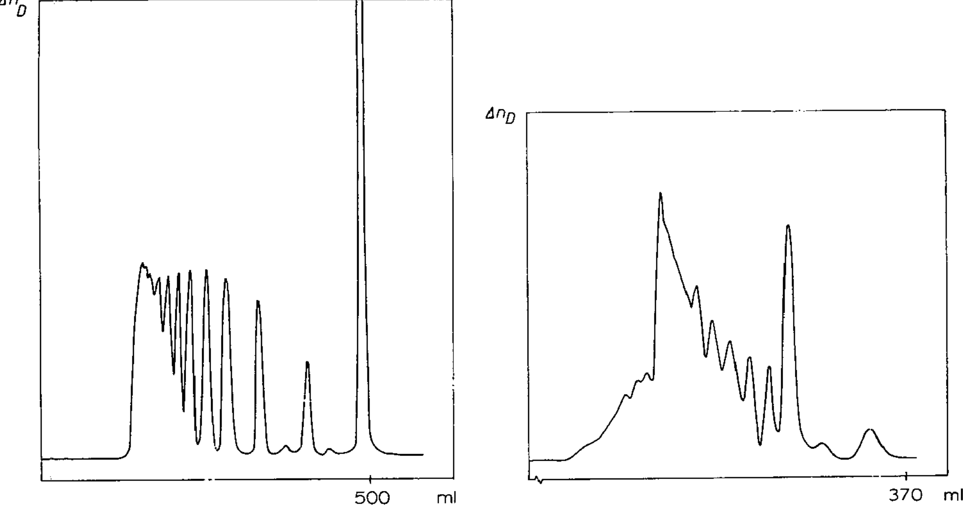


Fig. 12. Separation of low molecular weight polyester components. Sephadex LH-20 in methanol; 3 columns. Sample application, 30 mg; attenuator, 16 ×; flow rate, 53 ml/h.

Fig. 13. Separation of surfactant mixture. Bio-Beads SX-1 + SX-2 in benzene; 2 columns. Sample appication, 100 mg; attenuator, 32 ×; flow rate, 28 ml/h.

We believe we have demonstrated with these few examples, that GPC may be applied to many kinds of separations. Other applications in our laboratory are the analysis of glycols, plasticizers, sulphur compounds, resinous mixtures, competetive samples, and purifications.

It should be mentioned, that our apparatus seems to be rather complex, which is due to the many different kinds of samples we have to analyse. When, however, the field of analysis is limited, it is possible to obtain very satisfactory separations with one or two columns, which are operated by the gravity of the elution solvent only. In such cases sample application is carried out with the simple apparatus of Fig. 5c and detection by means of TLC can be carried out.

CONCLUSIONS

Gel permeation chromatography of small molecules in organic liquids is a versatile and efficient separation technique, which in principle may be carried out with simple apparatus.

As there is little or no irreversible adsorption of sample to the gel materials, repeated use of columns is possible.

Resolution is high and in some respects comparable with analytical GLC, even when using semi-preparative procedures. The technique is still more time-consuming than GLC and is in this respect more comparable with preparative GLC, but gel permeation chromatography is more versatile, as volatility of substances is of no account, and decomposition of sensitive substances is not to be feared.

In comparison with classic adsorption chromatography, sample loading capacity is high, polarity of substances plays a less important role, and separation time is short.

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CHROM. 4906

GROUP FRACTIONATION OF PLASMA PROTEINS ON DIPOLAR ION EXCHANGERS

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(Received July 3rd, 1970)

SUMMARY

The preparation of arginine- and sulphanilic acid-agarose is described. These dipolar ion exchangers have been characterised and applied to the fractionation of human serum by employing a modified displacement technique called pulse elution.

INTRODUCTION

A series of investigations dealing with the adsorption and desorption of proteins on group specific adsorbents[1] and dipolar ion exchangers[2] was initiated at this Institute. The programme was directed towards the development of improved fractionation methods for mixtures of proteins in general and plasma proteins in particular. In dipolar ion exchangers the distribution of ions rapidly reaches equilibrium as judged from the chromatographic behaviour of salts. Changes in bed volume due to alteration in ion concentration are usually very small or scarcely detectable under the appropriate conditions. These facts are of great importance for large-scale applications. There are also reasons to believe that suitable conditions for linear chromatography may be found.

Dipolar ion exchangers can be synthesised in many ways, for instance by reacting amino acids with an hydroxyl-containing polymer activated by cyanogen bromide[2,3]. In the present study we have used another simple 2-step procedure. Agarose was converted to an oxirane derivative by reaction with epichlorohydrin:

$$\text{(Polymer)-OH} + \underset{\text{O}}{CH_2\text{-}CH}\text{-}CH_2Cl \rightarrow \text{(Polymer)-O-}CH_2\text{-}\underset{\text{O}}{CH\text{-}CH_2} - HCl$$

After excess reagent had been removed, coupling was effected in alkaline solution:

$$\text{(Polymer)-O-}CH_2\text{-}\underset{\text{O}}{CH\text{-}CH_2} + RNH_2 \rightarrow \text{(Polymer)-O-}CH_2\text{-}\underset{\text{OH}}{CH}\text{-}CH_2\text{-NHR}$$

(where R contains at least one acidic group).

This method can be used not only to attach amino acids but also proteins and nucleotides containing an amino group, etc. to agarose, Sephadex, cellulose and other

hydroxyl-containing polymers. At this Institute we have produced biospecific adsorbents and matrix-linked enzymes. Oxirane groups can also be introduced in the polymer via bisepoxides or by other means.

EXPERIMENTAL

Material and methods

Chemicals. Analytical grade chemicals were used.

Arginine–agarose. 100 ml of sedimented Sepharose 6 B (obtained from Pharmacia Fine Chemicals, Uppsala, Sweden) was transferred to a glass filter funnel and thoroughly washed with distilled water and drained. After suspension in 80 ml 1 *M* NaOH, 10 ml epichlorohydrin was added and the mixture heated on a water bath at 60° for 2 h in a round-bottomed flask under vigorous stirring. Excess epichlorohydrin was removed by extensive washing with distilled water until no odour could be detected. The drained gel was suspended in 100 ml 0.01 *M* NaOH containing 10 g arginine. After being kept at 60° for 2 h, the mixture was allowed to cool overnight. The nitrogen content was determined and found to be 4.15 % (*ca.* 750 μmoles/g dry gel). The gel particles retained their spherical shape in aqueous solutions, but could not be freeze-dried without breakage.

Sulphanilic acid–agarose. 100 ml Sepharose sediment was treated as above and suspended in 80 ml 1 *M* NaOH. 10 ml epichlorohydrin was added and the suspension left for 24 h at room temperature under vigorous stirring. After washing with a large amount of water, the gel was suspended in 100 ml 2 *M* potassium carbonate buffer (pH 10) containing 3 g sulphanilic acid, and the pH was readjusted to 10. Coupling was allowed to take place without stirring for a period of 6 days. Nitrogen and sulphur contents were determined on dried samples of gel and found to be 0.93 and 1.78 %, respectively (*ca.* 600 μmoles/g dry gel). As in the case arginine–agarose, the gel particles retained their shape after the introduction of the dipolar substituents.

Titration. Potentiometric titrations were performed at room temperature with a pH-meter 26, Radiometer (Copenhagen) employing an alkali-resistant glass–calomel electrode (GK 2302 B). The titration vessel is schematically drawn in Fig. 1. It was provided with a lid with inlet for nitrogen, an ascarite trap to exclude carbon dioxide and an opening for the burette tip. A magnetic stirrer was introduced into the vessel to allow rapid mixing.

Human serum. The human serum was supplied by the Academic Hospital of Uppsala and kept frozen at —20° until used. Most experiments were performed with serum kept for less than two weeks.

Electrophoresis. The HJERTÉN apparatus was used[4] with a revolving tube of 36.5 cm length and 0.3 cm I.D. All analyses were performed with 4 μl samples in 0.1 *M* Tris–HCl (pH 8.5). 1200 V were applied across the electrodes which resulted in a current of 5.0 mA. By scanning the tube every 8th min, the progress of electrophoresis was conveniently followed. For identification the diagrams were compared with that of the serum obtained under identical conditions. (40-min run.)

Other equipment. Columns similar to that described by PORATH AND BENNICH were used[5]. The light transmission (254 nm) of the effluent was recorded on LKB-Uvicord (LKB-produkter AB, Bromma, Stockholm) and samples were collected in a time-based fraction collector (AB Stålprodukter, Uppsala). Absorption in the ultra-

violet or the visual range was measured by Hitachi spectrophotometer Model 101 and the conductivity was determined with a conductometer of type Philips PR 9501. The samples were concentrated with the Aminco Diaflo membranes.

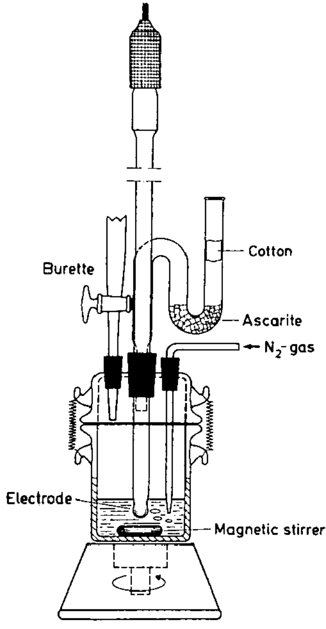

Fig. 1. Titration vessel (see text).

RESULTS

Characterisation of the ion exchangers

Titration. Gel was allowed to swell in excess water and then to settle in a cylinder. 20 ml of 0.5 *M* sodium chloride were added to 10 ml of the sediment, and the slurry was transferred to the titration vessel. The vessel was closed and nitrogen introduced in a slow current, and the stirring was started. Hydrochloric acid (1 *M*) was added to a pH below 2.0. Titration was effected by 0.05-ml portions of 0.1 *M* sodium hydroxide. The titration curves for arginine–agarose and sulphanilic acid–agarose are shown in Figs. 2 and 3, respectively.

From the curve in Fig. 2 for arginine–Sepharose, p*K* values of 2.95 and 8.10 were calculated for the carboxylic and amino groups, respectively. Due to the low concentration of guanidino groups in the gel, it was not possible to obtain a good estimate of the p*K* of this group. In a separate experiment in 0.5 *M* sodium chloride, the following p*K* values for the titratable groups of free arginine were obtained: 2.80, 9.07 and 12.65.

The p*K* values for sulphonic and amino groups were found to be 2.95 and 7.25, respectively, from the titration curve for sulphanilic acid–agarose (Fig. 3).

Adsorption capacity. The adsorption capacity was measured by frontal analysis. On a column of arginine–agarose (2.0 × 14.8 cm; V_0 = 21 ml; V_t = 47 ml) in 0.05 *M*

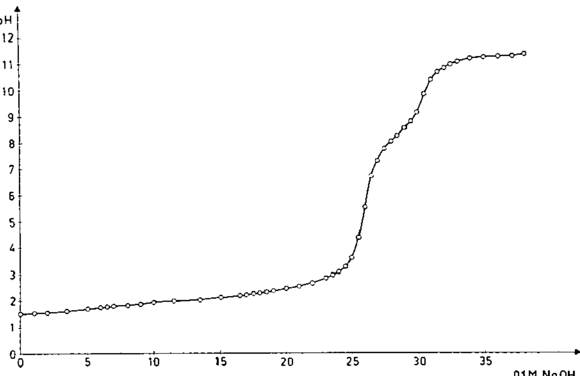

Fig. 2. Titration curve for arginine–Sepharose 6 B.

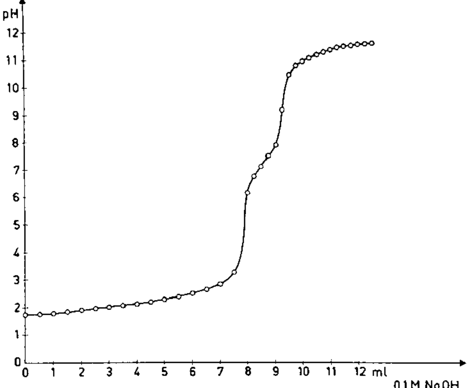

Fig. 3. Titration curve for sulphanilic acid–Sepharose 6 B.

Tris–HCl, pH 7.5, 0.5 % solution of serum albumin was continuously introduced at a speed of 24 ml per h. The absorption at 254 nm was recorded and the adsorption of albumin was calculated from the retention on the column. $V_e - V_0$ was found to be 218 ml which corresponds to 1.09 g of albumin. A similar experiment was made at pH 6.2 in 0.02 *M* imidazole–HCl, 1 *M* glycine. The column was then found to adsorb 2.31 g of albumin. After these two experiments the column was washed extensively with distilled water. The gel was transferred to a beaker and freeze-dried. The dry weight of the gel was found to be 1.717 g. The adsorption capacity of arginine–agarose for albumin was calculated to be 0.6 g and 1.3 g per g dry gel in the two buffers tested.

The adsorption capacity for γ-globulin on sulphanilic acid–agarose was measured in the same manner and found to be 0.02 g and 2.1 g in the Tris and imidazole buffers, respectively.

Flow properties. A column 2 × 15 cm of arginine–Sepharose 6 B was packed in 0.05 *M* Tris–HCl (pH 7.5) buffer by sedimentation[6].

The column was connected to a buffer reservoir. The pressure was adjusted by elevating or lowering the position of the reservoir. A series of flow-rate determinations were made at various pressure heads. Fig. 4 shows the flow rate as a function of the pressure.

Behaviour of salts and alkali. The chromatographic behaviour of buffer ions is of particular interest from a practical point of view. A "pulse" of salt, in high concentration, or of a different kind, migrates through the bed as a compact zone (Fig. 5).

Conversion to a new buffer medium can therefore be accomplished within a fraction of the total volume of the column. Since the buffer ions move at a somewhat different rate through the bed, a "pH wave" will be created (Fig. 5). The amplitude of this wave is small enough to be neglected in practice, at least in preparative chromatography. For routine fractionations of plasma and serum, it is of great value that a short concentration pulse effectively displaces the adsorbed proteins so that the bed can be rapidly regenerated. Dipolar ion exchangers are well-suited for cyclic operations. We will call this form of elution *pulse elution.*

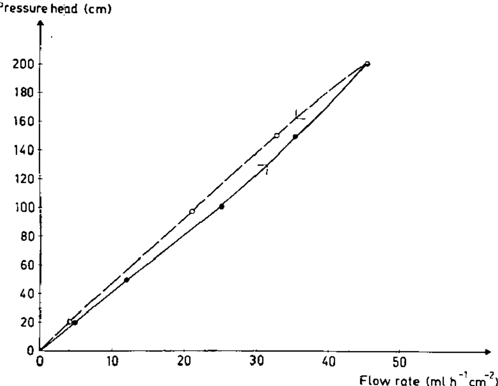

Fig. 4. Flow rate as a function of hydrostatic pressure. ———, increasing pressure; – – – –, decreasing pressure.

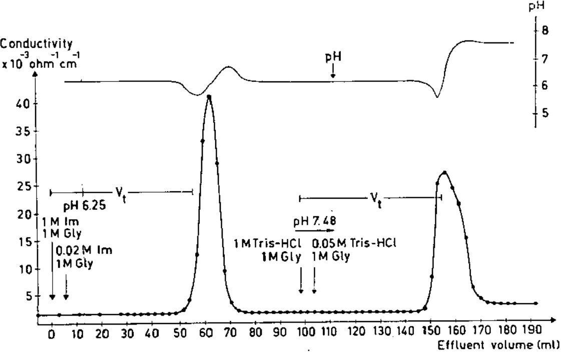

Fig. 5. Illustration of the rapid regeneration and buffer change on dipolar ion exchangers. Column: 2 × 36 cm, arginine–Sepharose 6 B. Flow rate: 27.6 ml/h.

Since buffer change can so easily be effected we considered the behaviour of alkali in the bed to be of great interest. The column from the previous experiment was loaded with 10 ml of 1 *M* sodium hydroxide followed by the original buffer, *i.e.* 0.05 *M* Tris–HCl in 1 *M* glycine, pH 7.6. The pH and conductivity of the effluent were measured and the results are shown in Fig. 6.

Fractionation of plasma proteins

In the experiments presented here, a simple strategy was chosen to achieve a group fractionation of the plasma proteins.

Step 1. 50 ml of serum, dialysed against 0.15 *M* Tris–HCl, pH 7.5, was introduced at a rate of 25 ml per h into a column, 2 × 39 cm, filled with arginine–agarose equilibrated with the same buffer. 8-ml fractions were collected. After 600 ml of buffer had passed, a pulse of 25 ml 1 *M* Tris–HCl, pH 7.5, was introduced into the bed followed by the original buffer. The distribution of material was determined spectrophotometrically and is shown in Fig. 7. The major part of the protein-containing material was collected in the elution zone: Fraction e_1. This fraction was red-coloured while the displaced material, Fraction A, was blue.

Step 2. Fraction e_1 was concentrated to 50 ml, dialysed against 0.10 *M* Tris–HCl, pH 7.5, and chromatographed on the 2 × 39 cm column, this time equilibrated

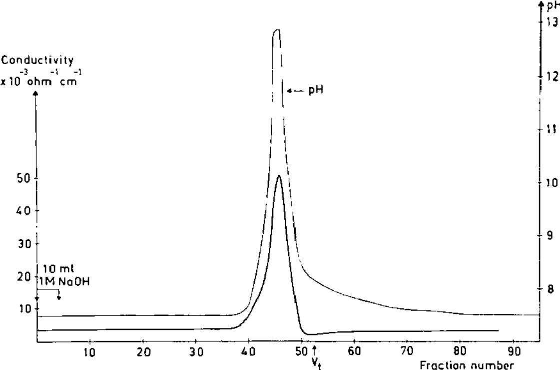

Fig. 6. Illustration of the rapid restoration of the original equilibration conditions after passage of a zone of 1 *M* NaOH (effective for displacement of residual proteins). Column: 2 × 36 cm, arginine–Sepharose 6 B. Buffer: 0.05 *M* Tris–HCl in 1 *M* glycine, pH 7.6. Flow rate: 26.5 ml/h. Fraction number: 12/h. ——, pH; ━━━, conductivity.

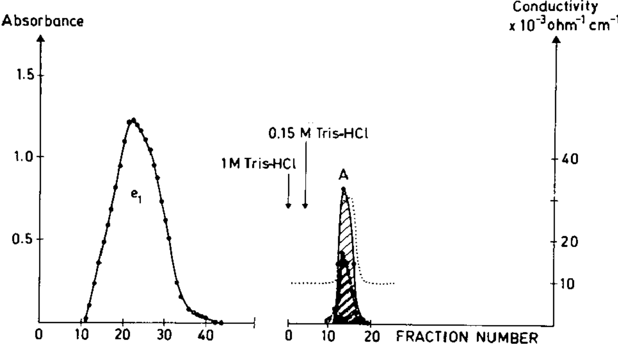

Fig. 7. Chromatogram of serum. Sample: 50 ml serum. Column: 2 × 39 cm, arginine–Sepharose 6 B. Buffer: 0.15 *M* Tris–HCl, pH 7.5. Flow rate: 28 ml/h. Fraction number: 3/h. ———, 280 nm; – – – –, 610 nm; · · · · · ·, conductivity.

with 0.10 *M* Tris-buffer. Displacement was again effected with a zone of 1 *M* buffer. Two fractions were obtained, e_2 and B (Fig. 8), the first being red, the second yellow-coloured.

Step 3. Fraction e_2 was concentrated to about 50 ml, dialysed against 0.05 *M* Tris–HCl, pH 7.5, and again chromatographed on the same column as before, but this time in 0.05 *M* Tris-buffer. As before, a broad peak appeared immediately after the passage of an effluent volume corresponding to the void volume of the column (Fraction F) (Fig. 9). This fraction was very weakly yellow-coloured and was found to consist of γ-globulins (Fig. 13). A slightly red-coloured zone passed the column at a slower rate and appeared in the eluate after passage of about four total bed volume equivalents of effluent ($V_e/V_t = 4.3$). Between these fractions D and F, less well-separated components were obtained (Fraction E).

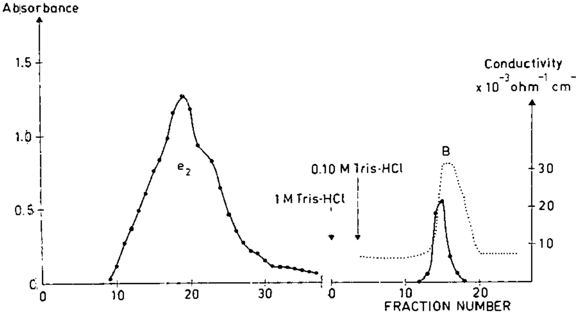

Fig. 8. Chromatogram of Fraction e_1 (Fig. 7). Sample: 50 ml concentrated Fraction e_1. Column: 2 × 39 cm, arginine–Sepharose 6 B. Buffer: 0.10 *M* Tris–HCl, pH 7.5. Flow rate: 25 ml/h. Fraction number: 3/h. ———, 280 nm; ······, conductivity.

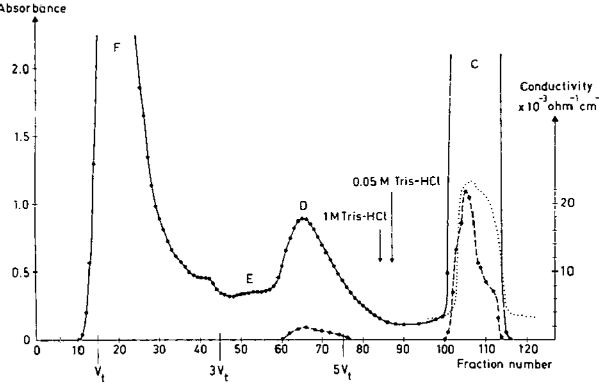

Fig. 9. Chromatogram of Fraction e_2. Sample: 50 ml concentrated Fraction e_2. Column: 2 × 39 cm, arginine–Sepharose 6 B. Buffer: 0.05 *M* Tris–HCl, pH 7.5. Flow rate: 25 ml/h. Fraction number: 3/h. —+—, 280 nm; – – – –, 410 nm; ······, conductivity.

It was noticed that the major part of the red material accumulated during the run in a concentrated zone, the speed of which was slowed down during the development with the buffer and finally stopped at a distance of 21–23 cm from the top. On displacement with 10 ml 1 *M* Tris–HCl the yellow material, distributed over most of the column, was pushed together with the red zone material and recovered in the

protein Fraction C. The column attained equilibrium against 0.05 *M* Tris–HCl immediately after the passage of the protein zone, as found by the decrease in the conductivity of the effluent.

Step 4. Fraction C was concentrated from 130 ml to 25 ml. After dialysis against 0.1 *M* imidazole–HCl in 1 *M* glycine, pH 6.3, 5 ml of the sample was transferred to a column (2 × 36 cm) of arginine–agarose equilibrated in the same buffer. As seen in Fig. 10, three components C_1, C_2, and C_3 were partly separated and a fourth component C_4 appeared just after an effluent volume corresponding to V_t. A 10-ml pulse of 1 *M* imidazole–HCl in 1 *M* glycine removed the remaining proteins (C_5). The material in Fraction C_4 was shown to be albumin by electrophoresis (Fig. 13).

Step 5. Fraction A was dialysed against 0.02 *M* imidazole–HCl in 1 *M* glycine, pH 6.3, and concentrated to 10 ml from 60 ml. 8.6 ml of the dialysate was fractionated on a 2 × 17.7 cm sulphanilic acid–agarose column in imidazole buffer. The chromatogram was developed at a rate of 26 ml per h and fractions were collected every 15 min.

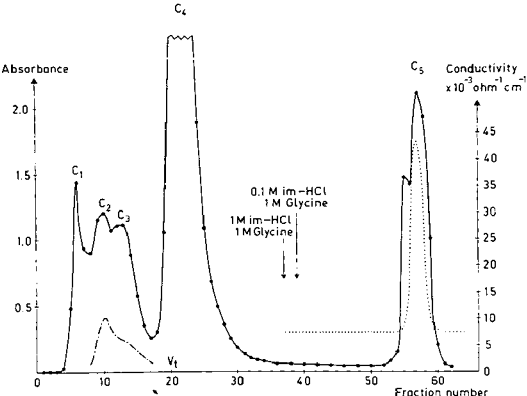

Fig. 10. Chromatogram of Fraction C. Sample: 5 ml concentrated Fraction C. Column: 2 × 36.3 cm, arginine–Sepharose 6 B. Buffer: 0.1 *M* imidazole–HCl, 1 *M* glycine, pH 6.3. Flow rate: 24 ml/h. Fraction number: 4/h. ———, 280 nm; –·–·–, 410 nm; ······, conductivity.

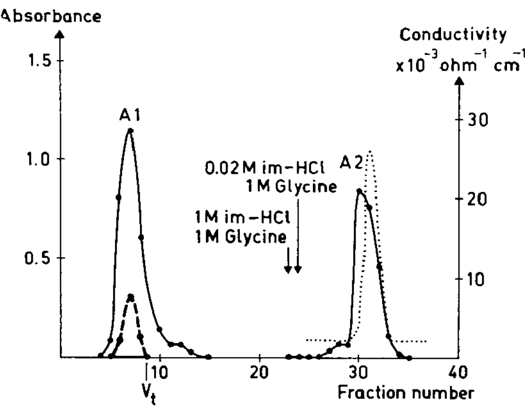

Fig. 11. Chromatogram of Fraction A. Sample: 8.6 ml concentrated Fraction A. Column: 2 × 17.7 cm, sulphonic acid-Sepharose 6 B. Buffer: 0.02 *M* imidazole–HCl, 1 *M* glycine, pH 6.3. Flow rate: 26 ml/h. Fraction number: 4/h. ———, 280 nm; – – – –, 610 nm; ······, conductivity.

When 150 ml of buffer had passed, a pulse of 10 ml 1 *M* imidazole–HCl in 1 *M* glycine was introduced followed by the original buffer. Absorbance was measured at 280 and 610 nm as well as the conductivity of the fractions around the displaced material peak. As seen in Fig. 11 two well-separated peaks (Fraction A_1 and A_2) were obtained.

The blue material of Fraction A, ceruloplasmin, moved without retention and was collected in Fraction A_1. The electropherograms of A_1 and A_2 are shown in Fig. 14.

Step 6. Fraction B was concentrated from 32 ml to 8 ml and dialysed against 0.02 *M* imidazole, 1 *M* glycine (pH 6.3). 3 ml of this solution was introduced into the column referred to in step 5 and the chromatogram was developed in the same way (Fig. 12). The electropherograms of the eluted and displaced fractions are shown in Fig. 15.

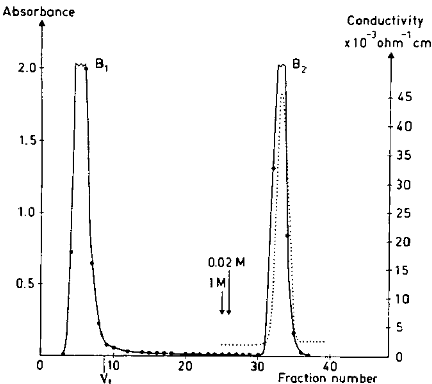

Fig. 12. Chromatogram of Fraction B. Sample: 3-ml concentrated Fraction B. Column: 2 × 17.7 cm, sulphonic acid–Sepharose 6 B. Buffer: 0.02 *M* imidazole–HCl; 1 *M* glycine, pH 6.3. Flow rate: 26 ml/h. Fraction number: 4/h. ———, 280 nm; · · · · · ·, conductivity.

DISCUSSION

The fractionation of plasma proteins with dipolar ion exchangers is based primarily on adsorption governed by electrostatic forces. In addition molecular sieving plays a major role, and sometimes ion exclusion may be a contributing factor. Of importance for the chromatographic behaviour are: (1) the structure and spatial distribution of the amphoteric groups responsible for the adsorption, (2) matrix density and (3) the nature of the medium. Extensive investigations are needed to clarify the interrelationships between these factors and thus provide the knowledge necessary for optimising the fractionation conditions for particular separation problems. From the limited experience so far, only crude judgements can be made.

Arginine–agarose acts as an anion exchanger and consequently the serum proteins will be eluted in the same or nearly the same order as on DEAE-cellulose or DEAE-Sephadex. At variance with the former, but like the latter, molecular sieving is superimposed upon ionic adsorption. The fact that the bed volume is not subjected to such large alterations as in the case of DEAE Sephadex is a significant advantage.

Alkali degrades native agarose. If agarose is sufficiently cross-linked, however, it is more stable and resists hot concentrated alkali (but not very strong acid)[7]. Dipolar ion exchangers based on cross-linked agarose, as those described in this paper, can therefore be generated or sterilised with alkali. Activation with the epoxy method simultaneously crosslinks the agarose such that if optimum conditions are employed the product will be alkali-stable yet not greatly altered with respect to its permeability. With arginine–agarose there is a risk of hydrolysis of the guanidino group upon prolonged contact with strong alkali. It is therefore important that the regeneration is accomplished by pulse elution with a rapidly moving zone of alkali (Fig. 6).

The adsorption capacity for plasma proteins is high for arginine–agarose at the degree of substitution, the pH values and the ionic strengths used here. In the experiments referred to in steps 1–3, 50 ml of serum was introduced into a column having a total volume of about 125 ml. With retained separation efficiency as in the experiment in steps 1, 2, 5, and 6, it should be possible to charge a bed with a sample volume of about 0.5 V_t and with good margin utilise only 1.5–2.0 V_t per fractionation cycle. Beds as large as 100–1000 l can be operated judging from experience with Sephadex. Large-scale plasma fractionation with agarose dipole ion exchangers employing pulse elution should therefore be possible.

A more effective separation can be obtained in the elution step at low ionic strength. The components of the elution Fraction e_1 (Fig. 7) emerge from the column in a fairly narrow zone, whereas the γ-globulins are separated from haptoglobin in step 3. In step 4 (Fig. 10) serum albumin is found in a retarded zone well separated from the other components of Fraction C. As the elution volume somewhat exceeds V_t, it is reasonable to assume that ionic adsorption is superimposed upon molecular sieving. (In a couple of experiments with another specimen of serum the albumin was divided in two peaks.)

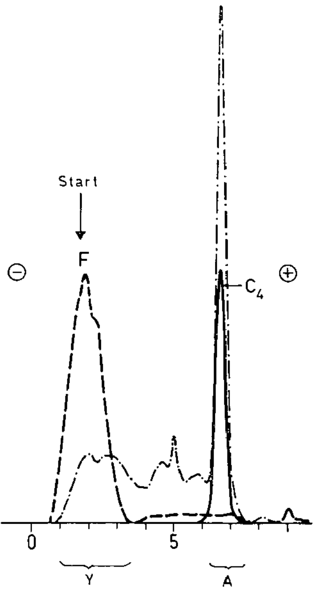

Fig. 13. Superimposed zone electropherograms. —·—·—, serum; – – – –, Fraction F; ———, Fraction C_4. Further details in the text.

The electrophoretic analyses reveal that sharp group separations were obtained. Fraction F consists exclusively of γ-globulins and Fraction C_4 contains only albumin (Fig. 13).

With the exception of Fractions F and C_4, all preparations are electrophoretically heterogeneous. Further purification should be possible by gradient elution or displacement chromatography on arginine–agarose. Fractions A and B were further purified on a dipolar ion exchanger containing more strongly acidic groups, *viz.* sulphanilic acid–agarose. On this adsorbent in 1 *M* glycine, 0.02 *M* imidazole–HCl, two subfractions were obtained in each case. The electrophoretic analysis showed that a sharp group separation had also occurred in these experiments (Figs. 14 and 15). For isolation of the various substances in the subfractions, further purification steps would be necessary. On sulphanilic acid–agarose, the salt concentration pulses move as compact protein-eluting zones.

The results so far obtained demonstrate that the dipolar ion exchangers offer an alternative to the adsorbents now in common use for protein chromatography. We feel, however, that the potentialities of these new types of adsorbents have as yet not been fully realised.

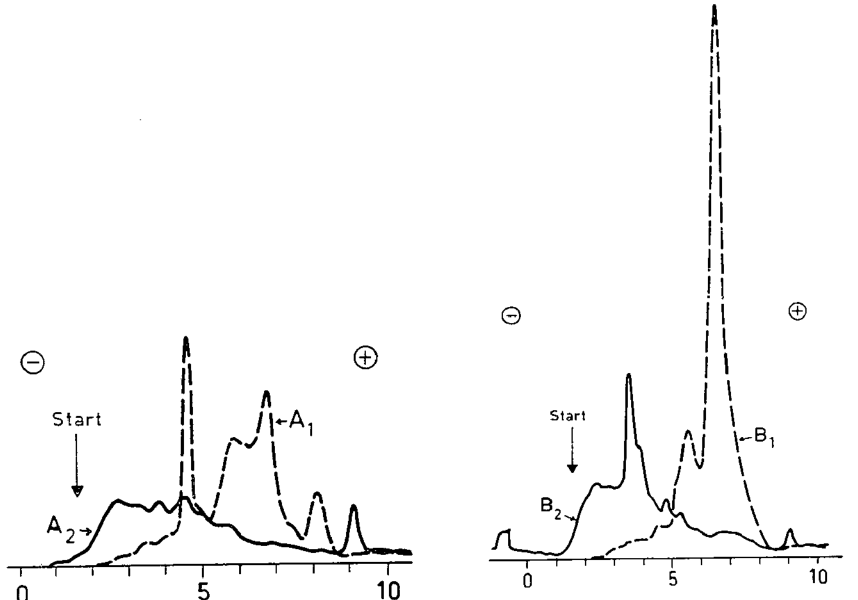

Fig. 14. Superimposed zone electropherograms. – – – –, Fraction A_1; ———, Fraction A_2.

Fig. 15. Superimposed zone electropherograms. – – – –, Fraction B_1; ———, Fraction B_2.

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CHROM. 4840

TRENNUNG VON PEPTIDEN UND AMINOSÄUREN IM URIN*

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(Eingegangen am 25. Mai 1970)

SUMMARY

Separation of peptides and amino acids in urine

A relatively simple method is described which allows the detection of differences in the peptide pattern in human urine. Urine is concentrated five times, shaken with $CuCO_3 \cdot Cu(OH)_2$, centrifugated, and the copper complexes are separated on a TEAE-cellulose column at pH 8.0 into a fraction A (amino acids, amines and neutral substances) and, by elution with 0.2 *M* acetic acid, into a fraction P (peptides and organic acids). After extraction of the copper by 8-hydroxy-quinoline in chloroform, fraction P is dinitrophenylated and the ether-soluble and acid-soluble DNP-peptides are separated by two-dimensional thin-layer chromatography on Silica Gel G. As little as about 0.5 nmole peptide may be detected.

EINLEITUNG

Während die Analytik der Aminosäuren in biologischen Flüssigkeiten bereits seit vielen Jahren gut ausgebaut und heute sogar vollautomatisiert ist, bleibt der Nachweis von Peptiden im Urin immer noch einigen spezialisierten Forschungsgruppen überlassen. Ein Screening-Verfahren zur Analyse der Peptide im Urin könnte aber unseres Erachtens für die Diagnose von Stoffwechselstörungen eine ähnliche Bedeutung erlangen, wie sie die Aminosäuren-Analyse[1] für die Diagnose von zahlreichen hereditären und erworbenen Krankheiten bereits besitzt. Der etwa 10–1000-fache Überschuss an Aminosäuren im Urin und deren physikalisch-chemische Ähnlichkeit mit den Peptiden haben bisher ein einfaches Screening-Verfahren zum Nachweis der Peptide verhindert und komplizierte Trennverfahren[2–5] erfordert. Weder die elegante Gelfiltration[6–8] noch die Säulenchromatographie an Ionenaustauschern[4,5,9,10], Aktivkohle[2], mit Kupferhydroxid präpariertem Sephadex[11] oder Chelex 100 (Lit. 12) sind für sich allein in der Lage, Urinpeptide generell von den in grossem Überschuss vorhandenen Aminosäuren zu trennen. Hingegen öffnet die Trennung als Kupferchelate bei pH 8 auf DEAE- oder TEAE-Cellulose nach dem Verfahren von TOMMEL *et al.*[13] einen prinzipiell neuen, vielversprechenden Weg. Wir haben dieses Verfahren

* Diese Arbeit wurde unterstützt vom Schweizerischen Nationalfonds zur Förderung der wissenschaftlichen Forschung, Projekt Nr. 5319.3. Teile dieser Arbeit wurden vorgetragen am VII. Internationalen Kongress für Klinische Chemie in Genf, 8.–13. Sept. 1969. Abstract Siehe Lit. 18.
** Direktor Prof. Dr. A. PRADER.

mit einigen Modifikationen übernommen und erreichen durch Kombination mit Dinitrophenylierung und zweidimensionaler Dünnschicht-Chromatographie einen nahezu generellen Nachweis von Peptiden bei erträglichem Arbeitsaufwand. Dem Nachweis unter unseren Bedingungen entgehen dabei lediglich Peptide, die keine freie Aminogruppe tragen, z.B. Acetylpeptide ohne Lysin oder Histidin. Neutrale oder basische Dipeptide verbleiben theoretisch in der Aminosäurenfraktion. Unser Verfahren ist in Fig. 1 zusammengefasst und wird im folgenden im Detail beschrieben.

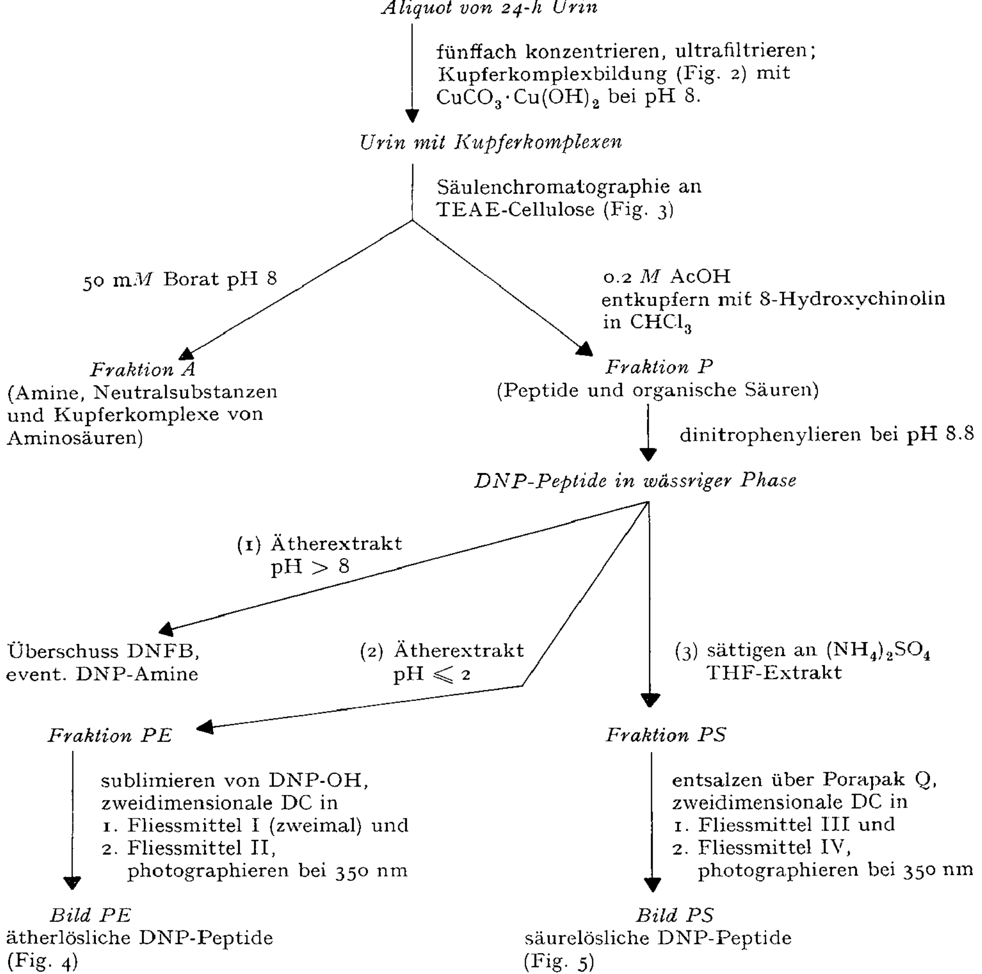

Fig. 1. Verfahren zum Nachweis von Peptiden im Urin als 2,4-Dinitrophenylderivate. DC = Dünnschicht-Chromatographie; DNFB = 2,4-Dinitrofluorbenzol; DNP-OH = Dinitrophenol; THF = Tetrahydrofuran.

Über erste Ergebnisse, die mit diesem Verfahren unter physiologischen und einigen pathologischen Verhältnissen erzielt wurden, werden wir in Kürze an anderer Stelle berichten.

EXPERIMENTELLES

Lösungen

0.05 M Boratpuffer pH 8.0. Borsäure, 30.9 g, in 41 ml 1 *N* NaOH lösen, portionsweise in etwa 6 l dest. Wasser einrühren, auf 10 l auffüllen, pH mit Glaselektrode prüfen und notfalls mit NaOH oder Eisessig korrigieren. CO_2-freies H_2O verwenden.

Fig. 2. Kupferkomplexe von Aminosäuren (oben) und Peptiden (unten). Bei pH 8 liegen neutrale Aminosäuren als positiv geladene 1:1 Komplexe oder als elektroneutrale 2:1 Komplexe vor. Peptide geben durch Ionisation von Amid-Protonen elektronegative Komplexe, dargestellt am Beispiel des Tetraglycins bei pH 8 (links) und bei pH > 8 (rechts), nach KIM UND MARTELL[14].

0.05 M Natriumacetat pH 5.0. 20.4 g Natriumacetat·$3H_2O$ in 3 l dest. Wasser lösen und pH mit etwa 4 ml Eisessig einstellen.

Carbonatpuffer pH 8.8. 8.4 g $NaHCO_3$ in 100 ml dest. Wasser lösen und mit 40 % NaOH und Glaselektrode pH auf 8.8 einstellen.

10 % g/v Dinitrofluorbenzol in abs. Äthanol. Im Kühlschrank aufbewahren.

Peroxidfreies Tetrahydrofuran (THF). 1 l THF (Fluka) mit 100 ml einer frisch zubereiteten Lösung von 5 % $Fe(II)SO_4$ in 0.5 *N* H_2SO_4 und mit 500 mg Hydrochinon versetzen, 30 min schütteln, und dann im Rotationsverdampfer bei etwa 100 Torr und 45° bis auf einen Rückstand von etwa 100 ml destillieren. Das Destillat mit 50 g Ammoniumsulfat schütteln, durch einen Wattebausch filtrieren, mit 50 g Natriumsulfat sicc. versetzen und sofort am Rotationsverdampfer bis fast zur Trockne destillieren. Destillat in brauner Flasche unter Stickstoff aufbewahren und bei Gebrauch durch Einleiten von Stickstoff auspressen. Vor Gebrauch auf Peroxide prüfen: 1 ml THF mischen mit 1 ml 4 % KJ-Lösung in Wasser; die Mischung muss farblos bleiben.

Fliessmittel

Die folgenden Fliessmittel wurden verwendet: (I) Toluol–2-Chloräthanol–Pyridin–25 % Ammoniak (50:35:15:7). Ammoniak genau abmessen; es darf sich keine Emulsion bilden! (II) Chloroform–Benzylalkohol–Eisessig (70:30:3). (III) *n*-Propanol–25 % Ammoniak (75:25). (IV) *n*-Butanol–Eisessig–Wasser (80:20:20).

Aufarbeitung der Probe

Probe. Es wird 24-h Urin verwendet. Während der Sammlung durch 5–10 ml

Toluol stabilisieren und im Kühlschrank aufbewahren. Danach Volumen messen, die Konzentrationen von Chlorid und Kreatinin bestimmen und bis zur Aufarbeitung tieffrieren.

Konzentrierung und Ultrafiltration. Im Rotationsverdampfer bei 45° 100 ml 24-h Urin unter Zusatz von etwa 10 ml *n*-Butanol auf etwa 10 ml einengen. Das Konzentrat mittels einer Pipette quantitativ in ein 20-ml Messkölbchen überführen und nach mehrmaligem Spülen mit H_2O dest. auffüllen (5-fache Konzentrierung des Urins). Nach guter Durchmischung das ausgefallene Salz 5 min bei 4000 r.p.m. abzentrifugieren. Die klare Lösung vorsichtig abhebern und bei 4° in ein 20 ml-Schliffrohr, das in die Zentrifuge passt, ultrafiltrieren. Wir verwenden zum Ultrafiltrieren ein Amicon Modell 52 mit einer Diaflo XM-50 Membran (80 % Retention von Pepsin, Molgew. 35000) bei 2 atm N_2. (Die Ultrafiltration kann unter Umständen weggelassen werden, vgl. DISKUSSION.)

Kupferkomplex-Bildung. Das Ultrafiltrat mit einigen Tropfen 40 % NaOH vorsichtig auf pH 8.0 bringen (Glaselektrode). Etwa 0.4 g Kupfer(II)hydroxid–Carbonat ($CuCO_3 \cdot Cu(OH)_2$) zugeben, verschliessen und 20 min bei 40° schütteln. Anschliessend den Überschuss abzentrifugieren (5 min, 4000 r.p.m.), und 10 ml des klaren, blaugrünen Überstandes auf TEAE-Cellulose chromatographieren (siehe unten).

Vorbereitung der TEAE-Cellulose. 250 g TEAE-Cellulose (Serva) in einem 5-l Becherglas in H_2O dest. aufschlämmen, 5-mal im randvollen Glas nach guter Durchmischung sedimentieren lassen und die feine Suspension jeweils nach 30 min absaugen. Den Rückstand in einer Glasfritte unter Umrühren und Absaugen nacheinander mit den folgenden Lösungen waschen (pH jeweils im Auslauf kontrollieren): 0.5 *N* NaOH, 0.1 *N* HCl, 0.5 *N* NaOH, 0.05 *M* Boratpuffer pH 8.0. Die TEAE-Cellulose daraufhin in einem Rundkolben in 0.05 *M* Boratpuffer pH 8.0 suspendieren, am Wasserstrahlvakuum entlüften und unter Rühren in ein 2.5 × 45 cm Chromatographierrohr mit Adapter (z.B. Pharmacia) einfüllen. Die Füllhöhe soll nach 16 h ständigen Äquilibrierens mit Boratpuffer (*ca.* 150 ml/h) etwa 30 cm betragen.

Säulenchromatographie an TEAE-Cellulose. 10 ml der klaren Kupferkomplex-Lösung durch einen Kapillaradapter mittels Pumpe in das 2.5 × 30 cm hohe TEAE-Cellulose-Bett einsaugen. Ohne Einschluss einer Luftblase anschliessend mit 0.05 *M*

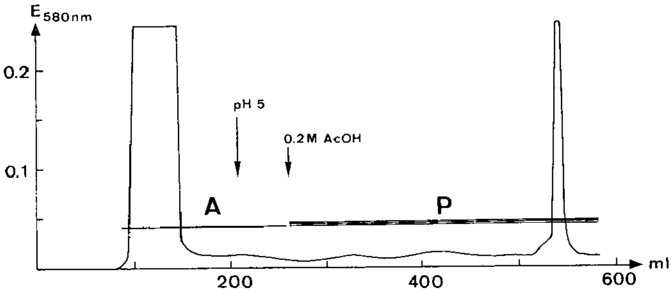

Fig. 3. Fraktionierung von Aminosäuren (A) und Peptiden (P) im Urin als Kupferkomplexe auf einer 2.5 × 30 cm Säule von TEAE-Cellulose, äquilibriert mit 50 m*M* Boratpuffer pH 8.0. Aminosäuren, Amine und Neutralsubstanzen gelangen in Fraktion A. Peptide und organische Säuren werden mit 0.2 *M* Essigsäure eluiert (Fraktion P.) Um die eventuell auf der Säule gebunene oder im Boratpuffer vorhandene Kohlensäure nicht plötzlich freizusetzen, wird vor der Essigsäure 30 min lang mit 50 m*M* Natriumacetat pH 5.0 eluiert. Durchflussgeschwindigkeit *ca.* 120 ml/h.

Boratpuffer pH 8.0 eluieren. Nach 105 min bei 120 ml/h auf 0.05 *M* Natriumacetat pH 5.0, nach weiteren 30 min bei gleichbleibender Pumpgeschwindigkeit auf 0.2 *M* Essigsäure umschalten. Das Eluat bei 580 nm im Durchlauf kolorimetrieren (wir verwenden dazu ein Beckman DB Spektralphotometer mit Schreiber) und in 5-min Fraktionen (*ca.* 10 ml) auffangen. Die Fraktionen gemäss Fig. 3 zu den Fraktionen A (Aminosäuren, Basen und Neutralsubstanzen) und P (Peptide und Säuren) vereinigen. Ihr Volumen soll etwa A $\approx$ 170 ml und P $\approx$ 370 ml betragen. Die Säule nach der Chromatographie 120 min mit 0.1 *N* HCl, 120 min mit 0.5 *N* NaOH waschen und über Nacht mit 0.05 *M* Boratpuffer pH 8.0 äquilibrieren.

Entkupfern und Dinitrophenylieren. Fraktion P auf etwa 30 ml einengen, mit NaOH auf pH 7.0 einstellen und 2-mal mit je 30 ml 0.6 % g/v 8-Hydroxychinolin in Chloroform und 2-mal mit je 30 ml Chloroform extrahieren. Die wässrige Phase am Rotationsverdampfer einengen, auf ein Volumen von 3–4 ml bringen, mit wenigen Tropfen 40 % NaOH vorsichtig auf pH 8.8 (Glaselektrode) einstellen, mit 1 ml Carbonatpuffer pH 8.8 und 1 ml 10 % Dinitrofluorbenzol-Lösung in abs. Äthanol versetzen und 90 min bei 40° im Dunkeln schütteln. Anschliessend das pH kontrollieren (muss grösser als 8 sein), die Probe quantitativ in einen 100-ml Scheidetrichter überführen (spülen mit jeweils zwei 10-ml Portionen H_2O dest. und Äther), 5-mal mit je *ca.* 30 ml Äther extrahieren und den Extrakt verwerfen. Die wässrige Phase mit 6 *N* HCl auf pH 2 einstellen und nochmals 5-mal mit jeweils 30 ml Äther extrahieren. Die vereinigten Extrakte mit Na_2SO_4 sicc. trocknen und (nach Spülen des Salzes mit frischem Äther) einengen, mit wenig Aceton quantitativ in ein Sublimations-Gefäss überführen und eindampfen (ätherlösliche Derivate; Fraktion PE). Die wässrige Phase mit festem Ammoniumsulfat sättigen und 2-mal mit 10 ml peroxidfreiem (Kontrolle!) Tetrahydrofuran extrahieren. Den Extrakt mit Na_2SO_4 sicc. trocknen, einengen, unter Zusatz von wenigen Tropfen Wasser quantitativ in ein 10-ml Schliffröhrchen überführen und im Stickstoffstrom bei 50° vorsichtig eindampfen (säurelösliche Derivate; Fraktion PS). Siehe ANMERKUNG BEI DER KORREKTUR

Sublimation (Fraktion PE). Dinitrophenol, das sich als Nebenprodukt bei der Dinitrophenylierung bildet, durch mehrmalige Sublimation bei $\leqslant$ 0.05 Torr und 50° entfernen. Um die Oberfläche zu erneuern, jeweils nach 15 min unterbrechen, den Rückstand in wenig Aceton und Äther lösen und beim Eindampfen auf eine möglichst grosse Fläche verteilen. Zur Kontrolle das Sublimat vom Kühlfinger mit Aceton in *ca.* 100 ml Wasser spülen und zum späteren Vergleich aufbewahren. Die Sublimation solange wiederholen (4–6-mal), bis die neue Spülflüssigkeit nur noch schwach gefärbt ist. Daraufhin den Rückstand vom Sublimiergefäss mit etwas Aceton quantitativ in ein 10-ml Schliffröhrchen überspülen und am Vakuum trocknen.

Entsalzen der Fraktion PS über Porapak Q. Den folgenden Vorgang ohne Unterbrechung durchführen, um einer teilweisen, irreversiblen Adsorption vorzubeugen! 1 g Porapak Q®, 150–200 mesh (ein poröses, mit Divinylbenzol quervernetztes Polystyrol der Firma Waters Associates, Inc., Framingham, Mass., U.S.A.) in 96 % Äthanol quellen lassen und in eine 1 × 10 cm Chrodatographiersäule mit Glasfritte einfüllen. Mit 96 % Äthanol, dann mit mindestens 20 ml entlüfteter 5 %-iger Essigsäure waschen. Die Fraktion PS in 2 ml und danach nochmals in 1 ml 5 %-iger Essigsäure aufnehmen und soweit möglich (es bleibt ein Rückstand) auf die Porapak Q Säule auftragen. Mit 12 ml entlüfteter 5 %-iger Essigsäure spülen und das Eluat (*ca.* 15 ml) verwerfen. Den im Schliffrohr verbliebenen Rückstand der PS-Fraktion in

3 ml Äthanol–Wasser (2:1) lösen und quantitativ auf die Säule überführen, mit 10 ml Äthanol–Wasser (2:1) und weiter mit 10 ml 96 % Äthanol langsam (!) eluieren. Eine 20-ml Fraktion auffangen, die praktisch alle gelben Farbstoffe enthält. Diese Fraktion einengen, quantitativ in ein 10-ml Schliffröhrchen überführen und im Stickstoffstrom bei 50° eindampfen. Die Säule zur Reinigung mit viel Äthanol nachwaschen.

Zweidimensionale Dünnschicht-Chromatographie

Allgemeines. Dünnschichten 20 × 20 cm aus Kieselgel G (Merck), selbstgestrichen, mindestens 16 h an der Luft getrocknet (rel. Luftfeuchtigkeit 50 %). Wie in Fig. 4 ersichtlich, auf der Dünnschichtplatte zwei 35 mm breite Randstreifen zur Aufnahme des Standards abtrennen und die Startpunkte jeweils 20 mm vom Plattenrand entfernt durch Einstich markieren. Alle Ränder 1–2 mm breit von Schicht befreien.

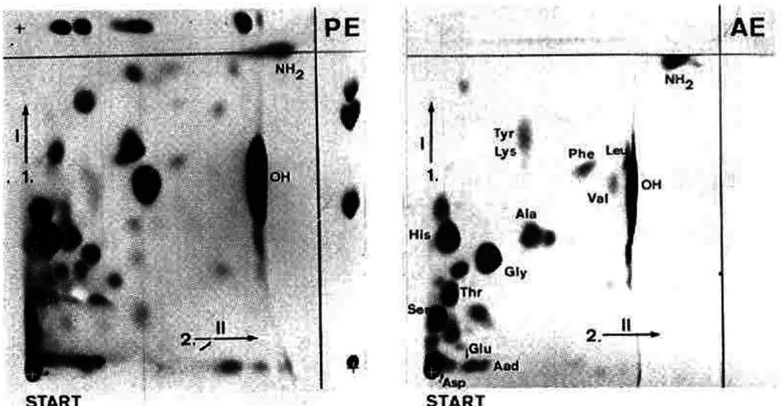

Fig. 4. Zweidimensionale Dünnschicht-Chromatogramme der ätherlöslichen DNP-Derivate der Peptidfraktion (PE, links) und der Aminosäurenfraktion (AE, rechts) eines Normalurins auf Kieselgel G. Die aufgetragenen Mengen entsprechen bei der Fraktion PE 2.5 ‰ und bei der Fraktion AE 0.1 ‰ des 24-h Urins. Die Fraktion A wurde analog zu P aufgearbeitet, doch unterblieb die Sublimation des Dinitrophenols. Fliessmittel I (zweimal in derselben Richtung) und II (einmal) nach WALZ *et al.*[15], siehe Text. Bei His, Lys und Tyr handelt es sich um die di-DNP-Derivate. Die am Rand aufgetragene Testmischung enthielt DNP-Alanin, DNP-Glycin, DNP-Glutaminsäure und di-DNP-Tyrosin. UV-Photographie. Aad = α-Aminoadipinsäure; NH_2 = 2,4-Dinitroanilin; OH = 2,4-Dinitrophenol.

Chromatographierkammern. Plattenkasetten der Firma Camag, Muttenz, Schweiz wurden verwendet. Die Schienen in den Kasetten unten 3 cm hoch abschleifen, um Störungen durch kapillares Hochklettern des Fliessmittels zu vermeiden. Die Ränder der zur Aufnahme des Fliessmittels dienenden Schalen beidseits etwas nach aussen biegen, um ein klemmfreies Einstellen und Herausheben der Kasetten zu gewährleisten. Zur Chromatographie die Schale mit 80 ml Fliessmittel füllen und sofort die aus Gründen der Reproduzierbarkeit immer vollständig mit sieben Dünnschichtplatten gefüllte Kasette (alle Dünnschichten zur selben Seite gewandt) hineinstellen und verschliessen. Sobald das Fliessmittel auf einer der Platten die Grenze des Randstreifens (siehe oben) erreicht hat, die Chromatographie unterbrechen und das Fliessmittel verwerfen.

Ätherlösliche DNP-Derivate (PE-Fraktion). Den Rückstand der PE-Fraktion in Aceton lösen, und zwar in 200/*v* µl, wobei *v* das 24-h Urin-Volumen in Liter bedeutet

(z.B. bei einer Ausscheidung von 0.5 l lösen in 400 μl Aceton). Das Gefäss unter gelegentlichem Schütteln 30 min gut verschlossen stehen lassen und dann mit Hilfe einer Microcap Kapillare (Drummond Scientific Comp., Broomall, Pa., U.S.A.) 10 μl auf die Dünnschicht portionsweise auftragen. An beiden Randstreifen der Platte je 1 μl einer Standard-Mischung von DNP-Glycin, DNP-Alanin, DNP-Glutaminsäure und di-DNP-Tyrosin (je 2 mg/ml Aceton) applizieren. Gut trocknen lassen und anschliessend in der 1. Dimension zweimal mit Fliessmittel I chromatographieren[15] (Zwischentrocknung 10 min im Luftzug*), danach 30 min im Luftzug trocknen und in der 2. Dimension einmal in Fliessmittel II chromatographieren (Fig. 4).

Säurelösliche DNP-Derivate (PS-Fraktion). Den Rückstand der Fraktion PS in peroxidfreiem Tetrahydrofuran–Wasser (2:1) oder in Aceton–Wasser (2:1) lösen, und zwar in 200/v μl (siehe oben), und 10 μl auftragen. Die an den Rändern aufzutragende Standardmischung enthält DNP-Arginin, DNP-Citrullin, DNP-Cysteinsäure, di-DNP-Histidin und DNP-Taurin (je 2 mg/ml in Aceton–Wasser, 2:1). In der 1. Dimension einmal in Fliessmittel III chromatographieren, 30 min im Luftzug* trocknen lassen und dann in der 2. Dimension einmal in Fliessmittel IV chromatographieren (Fig. 5).

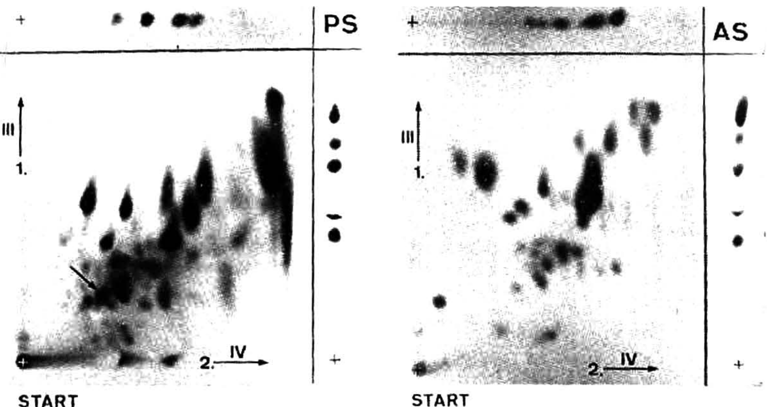

Fig. 5. Zweidimensionale Dünnschicht-Chromatogramme der säurelöslichen DNP-Derivate der Peptidfraktion (PS, links) und der Aminosäurenfraktion (AS, rechts) eines Normalurins auf Kieselgel G. Die aufgetragenen Mengen entsprechen 2.5‰ (Fraktion PS) und 0.1‰ (Fraktion AS) des 24-h Urins. Die Fraktion A wurde analog zu P aufgearbeitet. Fliessmittel III und IV, siehe Text. Die am Rand aufgetragene Testmischung enthielt DNP-Arginin, DNP-Citrullin, DNP-Cysteinsäure, di-DNP-Histidin und DNP-Taurin. UV-Photographie. Der Pfeil deutet auf die Stelle, an der von dem PS-Chromatogramm eines anderen Urins ein Fleck eluiert wurde; dieser Fleck ergab nach Weiterreinigung und Hydrolyse das in Fig. 8 gezeigte Aminosäuren-Chromatogramm.

Photographie im UV-Licht. Das im Luftzug* getrocknete Chromatogramm (Zeit standardisieren!) von zwei UV-Lampen (Camag Universal TL 900) bei 350 nm beidseitig im Winkel von 45° beleuchten. Der Abstand Lampe–Plattenmitte soll *ca.* 20 cm betragen. Im verdunkelten Raum 45 sec bei Blende 8 auf Agfa-Gevaert Copex Ortho 35 mm Film belichten (Abstand 65 cm) Die entwickelten Filme im Diaskop oder mittels eines Projektors betrachten. Falls erforderlich, Vergrösserungen anfertigen

* Platten unmittelbar hinter die Scheibe eines gut lüftenden Abzugs legen und die Scheibe bis auf einen etwa 3 cm hohen Spalt schliessen.

auf Agfa Brovira Papier BH1 (hart, extraweiss glänzend) oder auf Agfa-Gevaert Copyline P1m. Das letztere Material basiert auf Celluloseacetat, ist durchscheinend, matt und besonders gut geeignet zum Vergleich durch Übereinanderlegen der Bilder und zum Beschriften.

DISKUSSION

Die Methode von TOMMEL *et al.*[13] beruht auf der Eigenschaft vieler Peptide, bei pH 8 unter Abspaltung eines Protons vom Amid-Stickstoff negativ geladene Kupferkomplexe zu bilden, während die meisten Aminosäuren unter diesen Bedingungen positiv geladene oder elektroneutrale Komplexe bilden (vgl. Fig. 2) und daher z.B. durch Chromatographie an schwach basischem Ionenaustauscher, wie DEAE- oder TEAE-Cellulose, abgetrennt werden können. Saure Aminosäuren sollten ebenfalls negativ geladene Komplexe bilden und sich daher wie Peptide verhalten. Dies wurde von TOMMEL *et al.*[13] auch beobachtet. Um diese Schwierigkeit zu umgehen, haben die genannten Autoren vorgeschlagen, der zu trennenden Mischung Arginin als basische Aminosäure im Überschuss zuzugeben, um elektroneutrale Mischkomplexe zu erhalten. Wir können die Ergebnisse von TOMMEL *et al.* unter unseren Bedingungen nicht bestätigen und fanden keinen Einfluss von Arginin auf die Verteilung der sauren Aminosäuren. Da bei der Kupferkomplexbildung mit einem Überschuss an Kupfer gearbeitet wird, sollten 1:1 Kupfer Aminosäure-Komplexe bevorzugt und Mischkomplexe unterdrückt werden.

Wir haben quantitative Bestimmungen von Aminosäuren durchgeführt, die in einer Testlösung verkupfert, chromatographiert und entkupfert wurden. Ausser den Aminosäuren enthielt die Testlösung 5% NaCl und 20% Harnstoff, um den Bedingungen eines Urinkonzentrats besser zu entsprechen. Wie Tabelle I zeigt, gelangen nur relativ geringe Anteile von Asparaginsäure und Glutaminsäure in die "Peptidfraktion" P, aber ein hoher Anteil von Tyrosin, sowie Spuren von Serin, Glycin und Histidin. Offenbar wird der Kupferkomplex von Tyrosin durch teilweise Ionisation seiner phenolischen Hydroxylgruppe (pK-Wert = 10.07, Lit. 16) an TEAE-Cellulose gebunden. Phenylalanin, das diese Hydroxylgruppe bei sonst gleicher Struktur nicht besitzt, gelangt vollständig in die "Aminosäurenfraktion" A.

Bemerkenswert ist ferner, dass, entgegen der Theorie, der Kupferkomplex der basischen Aminosäure Lysin teilweise an den Ionenaustauscher gebunden wird und erst mit 0.1 N HCl eluiert werden kann.

Die Gesamtausbeute der meisten Aminosäuren liegt bei etwa 86%. Es wird vermutet, dass die grössten Verluste bei der Entkupferung auftreten. Die sauren und basischen Aminosäuren Asparaginsäure, Glutaminsäure, Lysin und Arginin werden nur zu etwa 76% wiedergefunden, und die schwefelhaltigen Aminosäuren Methionin und Cystin nur zu 73 bzw. 26%. Methioninsulfon und Cysteinsäure wurden nicht beobachtet. Eine Oxidation der schwefelhaltigen Aminosäuren durch Cu(II) und eine irreversible Bindung des dadurch entstandenen Methioninsulfons und der Cysteinsäure an den Ionenaustauscher kann aber nicht ganz ausgeschlossen werden, obwohl ein qualitativer Versuch zeigte, dass Cysteinsäure mit 0.1 N HCl von TEAE-Cellulose zumindest teilweise eluierbar ist. Frühere Versuche, die an einer anderen Charge von TEAE-Cellulose und ohne Zusatz von NaCl und Harnstoff zur Testlösung durchgeführt wurden, zeigten hohe Verluste von Asparaginsäure, Glutaminsäure und Serin,

TABELLE I

PROZENTUALE WIEDERGEWINNUNG VON AMINOSÄUREN NACH KUPFERKOMPLEXBILDUNG, CHROMATOGRAPHIE AN TEAE-CELLULOSE UND ENTKUPFERUNG

Eingesetzt wurden 10 ml einer wässrigen Lösung mit je 25 μMol Aminosäure, 5% NaCl und 20% Harnstoff. Angegeben ist das Mittel zweier Bestimmungen.

Aminosäure	*Fraktion A*	*Fraktion P*	*0.1 N HCl Waschflüssigkeit*	*Gesamtausbeute*
Asp	70.5	0.7	4.2	75.4
Thr	91.0			91.0
Ser	89.7	Spur		89.7
Pro	81.5			81.5
Glu	73.5	5.0		78.5
Gly	90.0	Spur		90.0
Ala	89.0			89.0
Val	85.2			85.2
$(Cys)_2$	26.4			26.4
Met	73.1			73.1
Ile	88.4			88.4
Leu	86.0			86.0
Tyr	55.9	30.2		86.1
Phe	84.4			84.4
Lys	70.0		3.1	73.1
His	81.2	Spur		81.2
Arg	79.3			79.3

sowie einen relativen Anteil von etwa 40 % Tyrosin, 4 % Lysin (!) und 5 % Arginin (!) in der "Peptidfraktion" P.

Der Einfluss der Salzkonzentration auf die Trennung von Aminosäuren und Peptiden wurde am Beispiel einer Mischung von Arginin, Glycin und Leucylglycylglycin untersucht. Fig. 6 zeigt eine deutliche Verschlechterung der Trennung bei NaCl-Konzentrationen über 6 %. Die störende Komponente ist dabei das Chlorid-Ion. Der zu verwendende Elutionspuffer darf daher kein Chlorid enthalten. Aus demselben Grund darf Urin im allgemeinen nur 5-fach konzentriert werden. Eine 10-fache Kon-

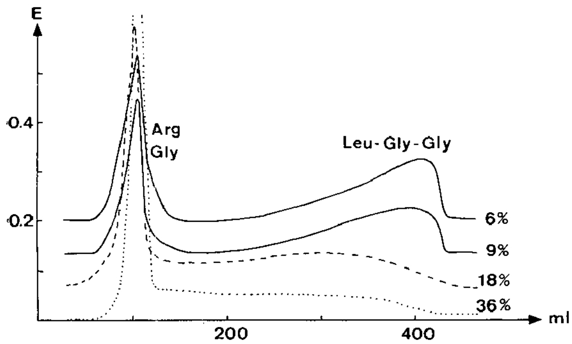

Fig. 6. Trennung von Arginin + Glycin und Leucylglycylglycin auf TEAE-Cellulose bei pH 8.0 in Abhängigkeit von der Chlorid-Konzentration. Aufgetragen wurden 12.5 mg Arginin, 25 mg Glycin und 50 mg Leucylglycylglycin in 1 ml 50 mM Natriumborat 6, 9, 18 bzw. 36%ig an NaCl, pH auf 8.0 eingestellt.

zentrierung ist nur bei einer Chloridkonzentration unter 110 mequiv./l zulässig.

Die "Peptidfraktion" P eines Normalurins ergab bei Ionenaustauschchromatographie nach Stein und Moore das in Fig. 7 gezeigte Bild, das auf die Anwesenheit zahlreicher saurer und neutraler Peptide schliessen lässt. Nach Hydrolyse der Peptidfraktion konnten alle Aminosäuren nachgewiesen werden, wobei Glycin und Glutaminsäure überwogen. Ein solches Totalhydrolysat sagt aber wenig aus, da Glycin bei salzsaurer Hydrolyse auch aus Nichtpeptiden freigesetzt wird, so z.B. aus der in der Fraktion P anwesenden Hippursäure. Hippursäure ist hingegen weder mit Ninhydrin (Fig. 7) noch mit Dinitrofluorbenzol (Fig. 4) nachweisbar.

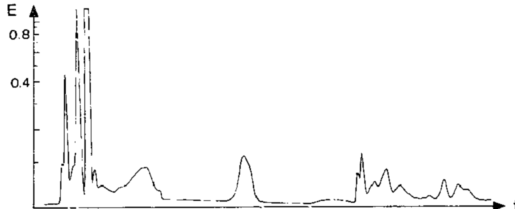

Fig. 7. Trennung der entkupferten Peptidfraktion P eines Normalurins durch Ionenaustausch-Chromatographie nach Stein und Moore. Saure und neutrale Verbindungen. Die breiten Peaks deuten auf unvollständige Trennung von Substanzgruppen mit sehr ähnlichen p*K*-Werten. Die zum Nachweis verwendete Ninhydrin-Reaktion (ohne vorherige Hydrolyse) ist bei grösseren Peptiden nur noch wenig empfindlich.

Bei den untersuchten Urinproben enthielt die 0.1 *N* HCl-Fraktion nur sehr wenig dinitrophenylierbares Material. Vereinigt man diese Fraktion mit der Fraktion P, so tritt häufig bei der Dünnschicht-Chromatographie der ätherlöslichen DNP-Derivative eine so schwere Störung auf (sehr langgezogene Flecken, Retention am Startpunkt), dass diese Chromatogramme nicht mehr auswertbar sind. Wir haben daher auf die Untersuchung der HCl-Fraktion verzichtet.

Die Ultrafiltration dient dazu, Zelldebris und grosse Proteine mit Sicherheit zu entfernen und auf diese Weise eine theoretisch mögliche Veränderung der Peptidzusammensetzung durch enzymatische Hydrolyse von Zellbestandteilen und Proteinen auszuschliessen. Da aber der Urin bis zur Aufarbeitung in gefrorenem Zustand gelagert wird und dann bis zur Verkupferung mit anschliessender Zentrifugation nur kurze Zeit verstreicht, dürfte eine nachträgliche Veränderung der Peptidzusammensetzung sehr unwahrscheinlich sein. Eventuell vorhandene Proteine stören in unserem Verfahren nicht, da diese denaturiert werden und bei der Chromatographie als DNP-Derivate auf Kieselgel am Startpunkt verbleiben. Im Hinblick auf eine Vereinfachung des Verfahrens kann daher auf die Ultrafiltration in der Regel verzichtet werden. Paralleluntersuchungen von Normalurin mit und ohne Ultrafiltration zeigten keinen Unterschied im Peptidmuster.

Die Extraktion der säurelöslichen DNP-Derivate mit Tetrahydrofuran aus der mit Ammoniumsulfat gesättigten Lösung (diese Arbeit) erfolgt leichter und schneller als dies bisher[15] möglich war. Die säurelöslichen DNP-Derivate lassen sich aus wässriger Lösung auf das Polystyrolharz Porapak Q adsorbieren und somit auf einfache Weise von anorganischem Material befreien[17]. Die zweidimensionale Dünnschicht-Chromatographie der säurelöslichen DNP-Peptide in den Fliessmitteln III und IV

ergibt von mehreren untersuchten Fliessmittelkombinationen die beste Trennung.

Bei der Photographie unter unseren Bedingungen werden nur Substanzen erfasst, die bei etwa 370–380 nm absorbieren. Die verwendete UV-Lampe emittiert maximal bei 350 nm, das verwendete Photoobjektiv (Asahi Pentax Takumar 1:1.8; f = 55 mm) ist aber, wie Kontrollmessungen ergaben, unterhalb von 370 nm lichtundurchlässig ($OD_{390\,nm} = 0.78$; $OD_{370\,nm} > 2$). Mit dieser Anordnung werden die intensiv gelben Dinitrophenylderivate von Peptiden und Aminosäuren mit grosser Empfindlichkeit erfasst (Absorptionsmaximum 366 nm). Die Nachweisgrenze dürfte bei dem beschriebenen Verfahren bei etwa 0.5 nMol Peptid liegen; dies entspricht einer Ausscheidung von etwa 200 nMol Peptid/24 h. Mono-O-DNP-Tyrosin, das ein Absorptionsmaximum von 310 nm aufweist — der Fuss des Absorptionspeaks endet bei etwa 390 nm — ist bereits nur noch sehr schwach sichtbar. Die Photographie der Chromatogramme im UV steigert daher die Selektivität unseres Verfahrens erheblich. Andere aromatische Verbindungen, die sich — wie z.B. die aromatischen Säuren — in der "Peptidfraktion" befinden, aber nicht dinitrophenyliert sind, werden nicht registriert. Andererseits werden dinitrophenylierbare Amine und Phenole, soweit sich diese überhaupt in der Fraktion P befinden, durch Extraktion mit Äther im alkalischen Milieu und durch die dünnschicht-chromatographischen Bedingungen weitgehend entfernt. Bei der Fülle der im Urin vorhandenen Substanzen ist es aber wahrscheinlich, dass trotzdem einige der auf den Chromatogrammen sichtbaren Substanzen keine Peptide oder Aminosäuren darstellen. Stichprobenartig untersuchte Flecken liessen nach weiterer dünnschicht-chromatographischer Reinigung, salzsaurer Hy-

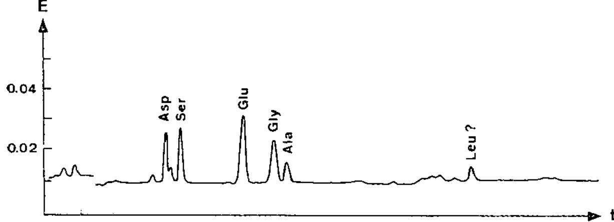

Fig. 8. Hydrolysat eines DNP-Peptids der PS-Fraktion. Das Peptid wurde nach zweidimensionaler Dünnschicht-Chromatographie (vgl. Fig. 5, Pfeil) mit Aceton–Wasser (2:1) eluiert, mehrmals, unter anderem mit Chloroform–Methanol–Eisessig–Wasser (60:40:1:10), auf Kieselgel H chromatographiert und mit 6 *N* HCl während 15 h unter N_2 hydrolysiert. Das Gesamtgewicht der nach dem Chromatogramm berechneten Aminosäuren betrug etwa 6 μg.

drolyse und zweidimensionaler Dünnschicht-Chromatographie mehrere Aminosäureflecken erkennen, u.a. Glycin, Glutaminsäure, Serin, Threonin, Alanin, Valin und Leucin. Man darf daher annehmen, dass es sich bei den dargestellten Flecken zumindest weitgehend um Dinitrophenylpeptide handelt. Sprühtests ergaben, dass zahlreiche gelbe Flecken, vor allem in der säurelöslichen Fraktion, Zucker enthalten und daher möglicherweise Glykopeptide darstellen. Die Untersuchung solch kleiner Mengen (μg bis ng-Bereich) stellt aber erhebliche Anforderungen. Fig. 8 zeigt als Beispiel das Hydrolysat eines Substanzfleckens (*ca.* 6 μg), der nach mehrmaliger dünnschicht-chromatographischer Reinigung hydrolysiert und nach Stein und Moore analysiert wurde. In der Regel reicht aber die Empfindlichkeit unserer Aminosäuren-Analysatoren nicht aus. Wir hoffen, in näherer Zukunft über ein besser geeignetes Analysierverfahren zu verfügen (Gaschromatographie). Die Dinitrophenylderivate besitzen die

Vorteile, dass sie unmittelbar sichtbar sind, leicht weitergereinigt werden können und nach Hydrolyse auch die endständige Aminosäure erkennen lassen.

ANMERKUNG BEI DER KORREKTUR

Vereinfachung: Die Extraktion der säurelöslichen DNP-Derivate (Fraktion PS, siehe Fig. 1) erfolgt nach der Dinitrophenylierung und den Extraktionen mit Äther aus der verbleibenden wässrigen Phase mittels Porapak Q (Lit. 17). Dabei entfallen Sättigung mit $(NH_4)_2SO_4$ und Extraktion mit dem wenig beständigen THF.

Man entfernt den in der wässrigen Phase gelösten Äther im Vakuum (5 min Rotieren bei *ca.* 20°) und lässt die Lösung zum Entsalzen (siehe dort) direkt durch eine Porapak-Q Säule laufen. Man spült mit *ca.* 12 ml entlüfteter 5 %iger Essigsäure und verwirft das Eluat. Wie unter *Entsalzen der Fraktion PS über Porapak Q* beschrieben, werden die DNP-Peptide langsam mit Äthanol–Wasser und Äthanol eluiert.

DANK

Herrn Dr. G. SCHOENENBERGER, Basel, danken wir für wertvolle Diskussionen und Frau L. GUIARD, Frau L. GUSTAFSON, Frl. H. KARPF und Herrn H.-R. BOSSHARDT für technische Hilfe.

ZUSAMMENFASSUNG

Es wird eine relativ einfache Methode zum Nachweis von Unterschieden im Peptidmuster im Urin beschrieben. Urin wird fünffach konzentriert, zur Komplexbildung mit $CuCO_3 \cdot Cu(OH)_2$ geschüttelt, zentrifugiert und der Überstand bei pH 8.0 auf TEAE-Cellulose in eine Fraktion A (Aminosäuren, Amine und Neutralsubstanzen) und, durch Elution mit 0.2 *M* Essigsäure, in eine Fraktion P (Peptide und organische Säuren) getrennt. Nach Entkupfern durch Extraktion mit 8-Hydroxychinolin in Chloroform wird Fraktion P dinitrophenyliert. Äther- und säurelösliche DNP-Peptide werden durch zweidimensionale Dünnschicht-Chromatographie auf Kieselgel G getrennt. Die Nachweisgrenze liegt bei etwa 0.5 nMol Peptid.

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SPURENANALYSE VON N-NITROSO-VERBINDUNGEN

III. QUANTITATIVE DÜNNSCHICHT-CHROMATOGRAPHIE VON NITROSAMINEN

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(Eingegangen am 14. Mai 1970)

SUMMARY

Trace analysis of N-nitroso compounds. III. Quantitative thin-layer chromatography of nitrosamines

Quantitative thin-layer chromatography of three typical nitrosamines has been investigated. To obtain reproducible and high recoveries the following conditions should be maintained: (1) Dry dichloromethane is a suitable solvent for spotting on the plate. (2) Evaporation of high solvent volumina should be done in a Kuderna–Danish evaporator to avoid losses of volatile nitrosamines. (3) Thin-layer chromatographic separation should be effected in the dark and at 4°. Layer thickness should be no less than 0.6 mm. (4) Separated nitrosamines from scratched-off sorbent can be quantitatively removed by steam-distillation. Under these conditions TLC can be used as part of a clean-up method in the trace analysis of carcinogenic nitrosamines.

EINLEITUNG

Viele organische N-Nitroso-Verbindungen (Nitrosamine und Nitrosamide) sind starke chemische Carcinogene[1,2]. Ihr Vorkommen als Naturprodukte und ihr mögliches Entstehen in der menschlichen Umwelt[3,4] machen selektive und empfindliche Nachweismethoden für diese Stoffklasse notwendig.

Im Rahmen unserer Arbeiten zur Spurenanalyse von Nitrosaminen prüfen wir qualitativ und quantitativ mögliche Einzelschritte eines Vorreinigungsverfahrens (clean-up)[5,6]. Hierbei erwies sich die Dünnschicht-Chromatographie (DC) von vorgereinigten Extrakten aus Lebensmitteln, die durch Wasserdampf-Destillation aus alkalischem und saurem Medium[6] gewonnen waren, als ein effektives Verfahren zur weiteren Abtrennung von störenden Begleitstoffen. Da jeder Einzelschritt des Anreicherungsverfahrens quantitativ gesichert werden musste, waren die Bedingungen für die DC von Nitrosaminen so zu standardisieren, dass reproduzierbar gute Ausbeuten erhalten werden konnten. Quantitative Untersuchungen hierzu liegen unseres Wissens bisher nicht vor, obwohl sich die DC als qualitative Nachweismethode für N-Nitroso-Verbindungen bewährt hat[7,8].

Als mögliche Verlustursachen bei der DC von Nitrosaminen kommen neben der Lichtempfindlichkeit besonders die hohe Flüchtigkeit der niederen Homologen von Dialkylnitrosaminen (z.B. Dimethyl-, Methyl-vinyl- oder Methyl-*n*-butyl-nitrosamin) in Betracht. Deshalb musste das Konzentrieren von Nitrosaminlösungen in verschiedenen Lösungsmitteln auf ein für das Auftragen auf die Platte geeignetes kleines Volumen ebenso untersucht werden wie Verluste durch Flüchtigkeit beim Auftragen und Entwickeln der Platte. Zur Isolierung getrennter Substanzen aus der abgelösten Sorbensschicht schliesslich war ein allgemein anwendbares Verfahren nötig, das die in ihrem physikalisch-chemischen Verhalten (z.B. Dampfdruck, Wasserlöslichkeit) sehr unterschiedliche Stoffklasse quantitativ erfasst. Hier erwies sich die Wasserdampf-Destillation der Sorbensschicht als geeignetes Verfahren.

Die in der vorliegenden Arbeit beschriebenen Untersuchungen zur dargelegten Problemstellung wurden an drei charakteristischen Vertretern der Stoffklasse durchgeführt, nämlich an Dimethyl-(DMNA), Diäthyl- (DENA) und Di-*n*-amyl-nitrosamin (DANA). Die ersten beiden Verbindungen sind relativ polar und gut wasserlöslich, DMNA hat einen hohen Dampfdruck und ist stark flüchtig. DANA wurde als Beispiel eines lipophilen Nitrosamins geringer Flüchtigkeit und Wasserlöslichkeit untersucht.

EXPERIMENTELLES

Materialien

DMNA, DENA und DANA wurden im eigenen Labor nach bekannten Methoden synthetisiert. Sie waren gaschromatographisch einheitlich. Alle verwendeten Lösungsmittel waren analysenrein. Zur Spektroskopie im UV wurde das registrierende Spektralphotometer Zeiss DMR 21, zur Polarographie das Metrohm-Gerät Polarecord E 261 R benutzt.

Herstellung der DC-Platten

Auf 20 × 20 cm Glasplatten wurden in üblicher Weise Schichten von 0.25 mm mit Kieselgel GF_{254} (Merck) bzw. von 0.6 mm mit Kieselgel PF_{254} (Merck) aufgebracht (Streichgerät Uniplan, Shandon). Nach 30 Min. Trocknen an der Luft wurde 2 Std. bei 120° aktiviert. Lagerung der aktivierten Platten erfolgte in einem Exsikkator über Blaugel.

METHODIK

Bestimmung der Abdampfverluste beim Konzentrieren von Lösungen

Lösungen von Dimethylnitrosamin (2.3–3.2 mg) in verschiedenen organischen Lösungsmitteln wurden nach zwei Methoden eingeengt: (1) Mittels Rotationsverdampfer im Vakuum ohne Heizung des Wasserbades unter Verwendung eines 250-ml Rundkolbens mit am Boden angeschmolzenem, graduierten 10-ml Ansatz. (2) Am Kuderna–Danish-Verdampfer mit aufgesetzter 3-Kugel-Synder-Kolonne bei Normaldruck. Bei beiden Verfahren wurden jeweils 200 ml auf ein Endvolumen von 2–3 ml eingeengt. Mit Wasser mischbare Lösungsmittel (Aceton, Acetonitril) wurden mit 0.2 *N* HCl auf 500 ml aufgefüllt und der Nitrosamin-Gehalt polarographisch[9] gemessen. Lipophile Lösungsmittel (z.B. Dichlormethan oder *n*-Pentan) wurden mit

n-Pentan auf 250 ml aufgefüllt und die Nitrosamin-Konzentration spektrophotometrisch gegen eine in gleicher Weise hergestellte Blindprobe bestimmt.

Bestimmung der Verluste während der DC

Mengen von 80 bis 170 µg des betreffenden Nitrosamins in 2 ml Dichlormethan p.A. wurden nach zwei Methoden strichförmig aufgetragen: (1) von Hand mittels einer Vollpipette (2 ml) bzw. (2) mit einem automatisch arbeitenden Auftraggerät (Autoliner der Fa. Desaga, Heidelberg). Das Auftragen erfolgte grundsätzlich ohne Benutzung eines Föhns. Die Auftragsstrecke war einheitlich 12 cm. Die Auftragezeit betrug mit Pipette etwa 5 Min., mit dem Autoliner etwa 15 Min.

Entwicklung und Detektion

Die Entwicklung erfolgte mit dem Fliessmittel *n*-Pentan–Diäthyläther–Dichlormethan (5:2:2) bei Kammersättigung und unter Lichtabschluss. Zur Chromatographie bei 4° wurde im Kühlschrank oder Kühlraum gearbeitet. Nach Entwicklung wurden die Substanzzonen durch kurzes Betrachten unter der Camag-Universal-UV-Lampe bei 254 nm markiert.

Isolierung

Die markierten R_F-Wert-Zonen (DMNA = 0.31; DENA = 0.55; DANA = 0.91) wurden als rechteckige Banden von 3–3.3 cm Breite mit einem Objektträger abgelöst. Für den Referenzwert wurde von jeder Platte ein Band genau gleicher Breite darüber bzw. darunter genommen. Die gewonnenen Sorbensschichten wurden anschliessend einer Wasserdampf-Destillation unterworfen. Hierbei hat sich folgende Methodik bewährt:

Platten mit 0.25 mm Schichtdicke. Das Kieselgel wurde in einem Rundkolben (50 ml) mit 6 ml Wasser, einigen Siedesteinchen und einem Magnetrührstab versetzt. Der Kolben wurde an eine kleine Schliff-Destillationsapparatur angeschlossen, und das Destillat in einem Spitzkölbchen (50 ml) unter Kühlung mit Eis-Kochsalz gesammelt. Beheizung erfolgte durch ein Ölbad, das von einem Magnetrührer mit Heizplatte auf einer Temperatur von 150° gehalten wurde; zur Vermeidung von Siedeverzügen musste stark gerührt werden. Die Destillation erfolgte bis zur Trockene. Unter Nachspülen der Apparatur wurden auf ein Volumen von 10 ml mit Wasser aufgefüllt.

Platten mit 0.6 mm Schichtdicke. Unter sonst identischen Bedingungen wurden anstelle von 6 ml hier 16 ml Wasser zugesetzt. Zur Isolierung schlecht wasserlöslicher Nitrosamine wie DANA wurde 16 ml 50 %iges wässriges Methanol anstelle von Wasser zugesetzt. Das Destillat wurde auf 20 ml mit Wasser bzw. Methanol–Wasser (50 %) aufgefüllt.

Konzentrationsbestimmung

Die Nitrosamin-Bestimmung in den Wasserdampf-Destillaten erfolgte UV-spektroskopisch durch Messung im Absorptionsmaximum der Nitrosamine bei 235 nm. Das Lambert–Beersche Gesetz ist über einen grossen Konzentrationsbereich erfüllt. Eichkurven für DMNA und DENA wurden in Wasser, für DANA in 50 %igem wässrigen Methanol aufgestellt. Es wurden jeweils die Destillate der Substanzzone gegen Destillate der Kontrollzone gemessen.

TABELLE I

ABDAMPFVERLUSTE BEIM EINENGEN VON DIMETHYLNITROSAMIN-LÖSUNGEN AUF EIN KLEINES END-VOLUMEN

Lösungsmittel	*Menge Nitros-amin* (μg)	*Verdampfer*	*Ausgangs-volumen* (*ml*)	*End-volumen* (*ml*)	*Rückge-winnung* (%)
CH_2Cl_2	3.2	Rotation	200	2	77.5
CH_2Cl_2	3.2	Kunderna–Danish	200	2.5	97
CH_2Cl_2, wassergesättigt, Trocknung über Na_2SO_4-säule (2 cm Durchmesser, 40 g)	3.2	Rotation	70	2	0
CH_2Cl_2, wassergesättigt, 4 Std. Trocknen mit 100 g Na_2SO_4 unter Rühren	2.3	Rotation	200	2	70
CH_2Cl_2, wassergesättigt, 4 Std. Trocknen mit 100 g Na_2SO_2 unter Rühren	2.3	Kuderna–Danish	200	2	96.5

TABELLE II

RÜCKGEWINNUNG VON NITROSAMINEN DURCH WASSERDAMPF-DESTILLATION VON ABGELÖSTEN KIE-SELGEL-SCHICHTEN OHNE ENTWICKLUNG

Lösungen der Nitrosamine in Dichlormethan (2 ml) wurden strichförmig mittels Pipette aufgetragen.

Nitrosamin	*Adsorbierte Menge* (μg)	*Zahl der Messungen*	*Rückgewinnung* (%)	
			Mittelwert	*Niedrigster und höchster Wert*
Dimethyl-	110–120	5	96.5	96–97
Diäthyl-	100–150	5	98	96–99
Di-*n*-amyl-	700	2	98	98

ERGEBNISSE UND DISKUSSION

In Modellversuchen wurden zunächst die Verluste beim Einengen von Lösungen des leicht flüchtigen Dimethylnitrosamins in verschiedenen organischen Lösungsmitteln auf ein kleines, zum Auftragen auf eine DC-Platte geeignetes Volumen (2–3 ml) untersucht. Es zeigte sich, dass beim Abziehen am Rotationsverdampfer im Vakuum ohne Badbeheizung teilweise beträchtliche Verluste eintreten. Sie betrugen bei Aceton als Lösungsmittel fast 50 %, bei Dichlormethan 22 % der eingesetzten DMNA-Menge. Bei der Verwendung unpolarer Lösungsmittel wie *n*-Pentan ergeben sich Verluste bis zu 100 % (vgl. Lit. 10). Abdampfen am Kuderna–Danish-Verdampfer reduzierte die Verluste signifikant. So wurden bei Verwendung von Aceton solche von 7 %, bei Verwendung von Dichlormethan Verluste von 3 % bestimmt.

Diese Vorversuche hatten ergeben, dass Dichlormethan das Lösungsmittel mit den günstigsten Eigenschaften ist: Es hat ein gutes Lösevermögen für Nitrosamine, hat einen niedrigen Siedepunkt und lässt sich leichter trocknen als andere Lösungsmittel. Wegen der starken Wasserdampf-Flüchtigkeit von Nitrosaminen[6] müssen

nämlich Lösungen vor dem Abdampfen gründlich getrocknet werden, um zusätzliche Verluste zu vermeiden. Die Ergebnisse mit Dichlormethan sind in Tabelle I zusammengefasst. Eine Schnelltrocknung wassergesättigter Dichlormethan-Lösungen über eine Säule mit Na_2SO_4 (40 g, 2 cm Durchmesser) erwies sich als völlig ungenügend: Es konnte kein Dimethylnitrosamin im Abdampfrückstand mehr gefunden werden. Gute Ergebnisse wurden dagegen erzielt mit einer 4-Std. Trocknung bei ständigem Rühren mittels eines Magnetrührers (Tabelle I).

Die Ausbeuten bei der destillativen Abtrennung der an Kieselgel adsorbierten Nitrosamine sind in Tabelle II aufgeführt. Es zeigt sich, dass die Methode der Wasserdampf-Destillation von Kieselgelfraktionen praktisch quantitative und gut reproduzierbare Ausbeuten liefert. Hierzu werden nur kleine Destillationsvolumina benötigt. Die Ergebnisse zeigen weiter, dass beim bandförmigen Auftragen der trockenen Nitrosaminlösungen in Dichlormethan praktisch keine Verluste entstehen. Verwendung von Acetonitril bzw. Aceton als Lösungsmittel führte im Gegensatz dazu zu Verlusten von rund 15 %.

Die Entwicklung der Dünnschichtplatten erfolgte prinzipiell im Dunklen, um mögliche Verluste durch Photolyse zu vermeiden. Das Fliessmittel *n*-Pentan–Diäthyläther–Dichlormethan (5:2:2) hat gegenüber dem früher vorgeschlagenen[7] Gemisch *n*-Hexan–Diäthyläther–Dichlormethan (4:3:2) den Vorteil, bei ähnlichen Trenneigenschaften leichter flüchtig zu sein und somit schneller Lösungsmittel-freie Sorbentien zu ergeben. In Tabelle III sind die Rückgewinnungsraten von DMNA und DENA nach Chromatographie auf einer 0.25 mm dicken Schicht von Kieselgel GF_{254} in Abhängigkeit von der Auftragmethode und der Temperatur während der Chromatographie dargestellt. Es zeigt sich, dass zwischen dem manuellen Auftragen mit Pipette und dem automatischen Auftragen (Autoliner) keine signifikanten Unterschiede bezüglich der Ausbeute bestehen. Unbefriedigend jedoch sind die Verluste von rund 30 % an Dimethylnitrosamin und vor allem die schlechte Reproduzierbarkeit der Ausbeuten unter diesen Bedingungen. Die Chromatographie bei 4° bringt keine

TABELLE III

RÜCKGEWINNUNG VON DIMETHYL- UND DIÄTHYLNITROSAMIN NACH DC AUF KIESELGEL GF_{254} IN ABHÄNGIGKEIT VON DER METHODE DES AUFTRAGENS UND DER TEMPERATUR BEI DER CHROMATOGRAPHIE

Schichtdicke: 0.25 mm.

Nitrosamin	*Menge* (*µg*)	*Auftrag-Methode*	*DC-Temp.*	*Zahl der Messungen*	*Rückgewinnung* (%)	
					Mittelwert	*Niedrigster und höchster Wert*
Dimethyl-	110–170	Pipette	22°	5	70.5	66–74
			4°	6	76	68–85
	110–120	Autoliner	22°	5	69.5	64–74
			4°	8	74.5	68–81
Diäthyl-	80–150	Pipette	22°	6	89.5	84–94
			4°	11	88.5	83–93
	90	Autoliner	22°	2	85.5	85–87
			4°	2	87	87

wesentlichen Verbesserungen (Tabelle III). Vor kurzem bekannt gewordene Versuche von SCHULLER[11] ergaben für Diäthylnitrosamin bei der DC an Kieselgel sogar Verluste bis zu 43 %.

Durch Erhöhung der Schichtdicke von 0.25 mm auf 0.6 mm gelang es uns jedoch, die DC von Nitrosaminen auch in quantitativer Hinsicht befriedigend durchzuführen. Wie in Tabelle IV dargestellt, ergeben sich mit dieser höheren Schichtdicke und bei Chromatographie bei 4° im Dunklen gute bis sehr gute Rückgewinnung aller drei untersuchten Nitrosamine bei guter Reproduzierbarkeit der Ergebnisse. Die Verluste betragen maximal *ca.* 10 %. Signifikante Unterschiede zwischen manueller und automatischer Auftragung bestehen nicht.

Die Ergebnisse beweisen, dass unter den angegebenen Bedingungen die Dünnschicht-Chromatographie von Nitrosaminen als wertvolle Reinigungsmethode in der Spurenanalyse dieser Carcinogene eingesetzt werden kann.

TABELLE IV

AUSBEUTEN DER DC VON NITROSAMINEN AN KIESELGEL PF_{254} BEI 4° UND DUNKELHEIT
Schichtdicke: 0.6 mm.

Nitrosamin	*Menge* (*µg*)	*Auftragmethode*	*Zahl der Messungen*	*Rückgewinnung* (%)	
				Mittelwert	*Niedrigster und höchster Wert*
Dimethyl-	110–130	Pipette	4	90	89–93
	110–130	Autoliner	4	89.5	88–92
Diäthyl-	95–160	Pipette	4	96	94–99
	95	Autoliner	3	94	93–95
Di-*n*-amyl-	735	Pipette	2	95	95
	735	Autoliner	2	94.5	94–95

DANK

Die Arbeiten wurden durchgeführt mit Unterstützung der International Agency for Research on Cancer, Lyon.

ZUSAMMENFASSUNG

Es wurde die quantitative Dünnschichtchromatographie dreier typischer Nitrosamine untersucht. Zur Erzielung reproduzierbarer und hoher Ausbeuten müssen folgende Bedingungen eingehalten werden: (1) Trockenes Dichlormethan ist als Lösungsmittel zum Auftragen auf die Platte geeignet. (2) Das Einengen grösserer Lösungsmittelvolumina sollte nach sorgfältigem Trocknen in einem Kuderna–Danish-Verdampfer vorgenommen werden, um Verluste flüchtiger Nitrosamine zu vermeiden. (3) Die dünnschichtchromatographische Trennung sollte unter Lichtschutz und bei 4° vorgenommen werden. Die Schichtdicke der Platte sollte nicht geringer als 0.6 mm sein. (4) Nach Entwicklung können die Nitrosamine quantitativ durch Wasserdampf-

destillation vom Trägermaterial abgetrennt werden. Unter diesen Bedingungen kann die Dünnschichtchromatographie als Teil eines clean-up-Verfahrens in der Spurenanalytik carcinogener Nitrosamine eingesetzt werden.

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CHROM. 4864

FLUORODENSITOMETRIC ASSAY OF PENICILLIC ACID

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(First received April 7th, 1970; revised manuscript received June 1st, 1970)

SUMMARY

A fluorodensitometric method was developed for the quantitative analysis of penicillic acid, a toxic secondary mold metabolite. The method depends on the conversion by ammonia fumes of penicillic acid after thin-layer chromatography to a fluorescent derivative. This technic was used successfully to analyze moldy corn and a liquid fermentation for this toxin.

INTRODUCTION

Penicillic acid, a secondary metabolite first isolated from *Penicillium puberulum*[1], has since been shown to be produced by a variety of fungi. The compound possesses antimicrobial activity[2,3], is toxic to mammals[4], and has proved carcinogenic to rats[5]. KURTZMAN AND CIEGLER[6] found that in blue-eye-diseased corn caused by *Penicillium martensii*, large quantities of penicillic acid are produced when high-moisture corn is stored at low temperatures (5 to 10°). Since blue-dye disease may be common in combine harvested corn, penicillic acid represents a potential mycotoxin hazard to humans and livestock. The colorimetric methods to detect this compound[7,8] lack the sensitivity required for a mycotoxin assay, the smallest detectable amount being 200 μg. We found that the product of the reaction between penicillic acid and ammonia fluoresced an intense blue and that this property could be used as the basis for a sensitive fluorodensitometric assay.

MATERIALS AND METHODS

Penicillic acid

The compound was produced fermentatively with *Penicillium cyclopium* NRRL 1888. Penicillic acid was recovered by the method of BENTLEY AND KEIL[8] except for two final recrystallizations from benzene to remove traces of a yellow pigment; the crystals were then sublimed under vacuum at 80–90°. The final product did not de-

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press the melting point of authentic penicillic acid (84.2 to 84.8° as determined with a Mettler FPI* melting point apparatus).

To produce the ammoniated derivative, an acetone solution of penicillic acid was streaked onto a preparative plate of Brinkmann Silica Gel GHR and developed in ether. The plate, after evaporation of solvent, was exposed for 5 min to concentrated ammonia fumes. The resulting bright blue fluorescing band was eluted with methanol and its spectral characteristics were determined in an Aminco-Bowman spectrophotofluorometer; excitation was at 350 mμ and emission at 440 mμ (Fig. 1).

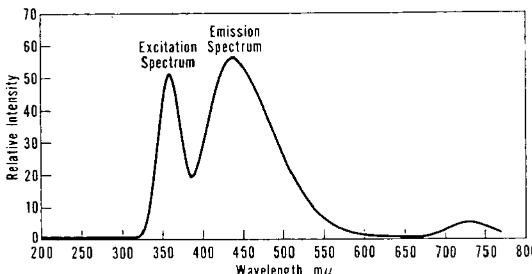

Fig. 1. Excitation and emission spectrum of the ammonia–penicillic acid reaction derivative in methanol.

TLC conditions

Standard 20 × 20 cm plates were coated with 250 μ of Silica Gel GHR. Various amounts of a standard penicillic acid solution in benzene (1 μg/μl) were spotted about 2 cm apart and 2.5 cm from the bottom of the plate. The plate was developed in chloroform–ethyl acetate–formic acid (60:40:1) in an unlined and unequilibrated Desaga–Brinkmann glass tank. After the solvent front had traveled about 16 cm, the plate was removed, dried under a gentle warm air stream from a hand dryer until formic acid could no longer be detected, and then exposed to concentrated ammonia fumes in a lined tank for 3 min. The R_F value of penicillic acid in the solvent system used was 0.45. Other solvent systems that can be used are as follows: chloroform–methanol (97:3), R_F value 0.5; chloroform–acetone (93:7), R_F value 0.1; chloroform–ethanol (90:10), R_F value 0.75; chloroform–ethyl acetate (50:50), R_F value 0.6.

Fluorodensitometric assay

About 1–2 cm of silica gel was removed from the bottom and top of the developed plate and small aluminum rails slipped along the cleared sides. The plate was placed on an automatic scanning stage of a Photovolt Model 530 densitometer. The stage contained a search unit (Model 52-C) equipped with a UV light source (320 to 390 mμ), a primary filter (365 mμ), a secondary filter (445 mμ), and a primary slit (0.1 by 15 mm). A Beckman 10-in. recorder with an automatic integrator was used to quantitate the assay. The distance between the TLC plate and the search unit was adjusted to 1 mm.

After the developed plate was placed face down on the stage, the densitometer was manually adjusted to the center of a standard zone containing 7 μg penicillic

* The mention of firm names or trade products does not imply endorsement or recommendation by the Department of Agriculture over other firms or similar products not mentioned.

acid. The response given to this amount was arbitrarily set at 70 % on the recorder with dilutions of unknowns being adjusted to read out between 10 and 90 %. The densitometer was also set to the lowest background at which no fluorescence occurred to obtain a base line. The fluorescent zones were manually centered inside the 15-mm slit of the search unit so that the path of development would be scanned. The stage was set so that the fluorescent zone was located near the origin of the plate, and the plate scanned automatically toward the solvent front. All analyses were carried out in a darkened room to prevent erratic readings of the densitometer.

A standard curve was prepared for each analysis by obtaining the area from the integration units from two different amounts of toxin, usually 3 and 7 μg, spotted in duplicate. The concentration of penicillic acid in unknowns was then determined by calculating the number of integration units from each of two samples analyzed in duplicate thus giving four readings per experimental condition; values were obtained by direct reading off the standard curve. The following formula was used to calculate the concentration of penicillic acid in an unknown where a solid substrate, such as corn, was analyzed: μg of penicillic acid per g of substrate $= (U_g/U_v)\ (V_D \cdot V_E)/W_x$, where U_g = weight of toxin (μg) determined from the standard curve; U_v = volume of solution spotted (μl); V_D = dilution of the solution spotted; V_E = volume of solvent (μl) used for the original extraction; W_x = weight of solids (g) extracted.

If an unknown solution was analyzed, the concentration of toxin in μg/ml was obtained readily from: $(U_g/U_v) \times 10^3$.

Extraction of solids

Generally, 50 g of molded grain, *e.g.* corn, was extracted with 250 ml of chloroform–methanol (90:10) for 3 min in a Waring Blendor. The extract was filtered through anhydrous soldium sulfate and the first 50 ml were collected for analysis.

RESULTS AND DISCUSSION

Standard assay

The densitometric assay using the procedure developed followed Beer's law between about 1 to 9 μg penicillic acid. A typical standard curve is shown in Fig. 2.

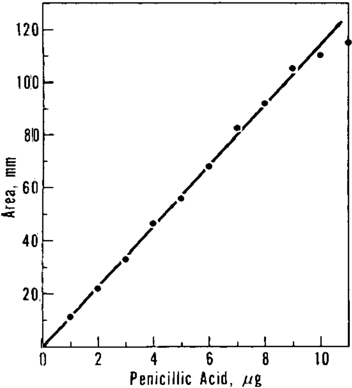

Fig. 2. Standard fluorodensitometric assay of penicillic acid TLC on Silica Gel GHR using chloroform–ethyl acetate–formic acid (60:40:1) for development; developed plate exposed to ammonia fumes for 3 min and fluorescence measured densitometrically.

At higher quantities some quenching occurred. Quenching could be partially avoided by eliminating formic acid from the TLC solvent system; a more diffuse spot results. However, the spot occasionally diffused too much so that it overlapped the slit of the search unit. The average deviation in values usually ranged from 3 to 7% with the largest deviation occurring with the smaller amounts spotted, *i.e.*, 1 to 3 μl, probably because of the difficulty in accurately dispensing small volumes of solution. This variation is similar to that noted for the fluorodensitometric analysis of aflatoxin[9]. Although the concentration of the unknown can be determined by visual comparison to a spotted series of standards, our experience with aflatoxin assays indicates a potential accuracy no greater than $\pm 17\%$ (ref. 10).

In the densitometric tracings, base-line variation caused by varying silica gel thickness across the plate was negligible. Even-thickness plates were more readily obtained by blending the Silica Gel GHR (30 g)–water (64 ml) mixture in a Waring Blendor for 30 sec before spreading. Commercial plates with a plastic backing were unsatisfactory because they passed too much light in areas lacking the fluorescent zones and because they were too flexible.

Ammonia exposure time

Increasing the time of plate exposure to ammonia from 3 to 5, 20, or 40 min only slightly increased fluorescence from the various amounts of toxin present as indicated by the increased slope of the dose-response curve, from 1.7 to 1.9 in one experiment. However, the final concentration of a given unknown was not affected since its fluorescence intensity also increased proportionately. After a 40-min exposure, the erratic data obtained indicated some degradation of the toxin.

Stability of fluorescence

No change in data was noted if the plates were stored 4 h after exposure to ammonia; this indicates relative compound stability. However, 24 h later, there was considerable loss of fluorescence and none could be detected at the lower amounts of acid (1–3 μg). Consequently, it would be advisable to assay the plates reasonably soon after preparative procedures are completed.

The identity of the fluorescent derivative is not known. An analogous reaction has not been reported in the literature. Reaction of penicillic acid with NaOH converts the toxin to its nonfluorescent tautomer, γ-keto-β-methoxy-δ-methylene-Δ^{α}-hexenoic acid. Hence, the reaction with ammonia to give a fluorescent derivative does not involve a simple alkaline hydrolysis of the lactone ring. In addition, no fluorescence results from ammoniation of the keto tautomer. Structural studies are now in progress.

Assay of moldy corn and a liquid fermentation

Whole corn that had been inoculated with *P. martensii* NRRL 3612 was incubated at 15° and analyzed periodically for penicillic acid content (Fig. 3). High concentrations of lipid did not interfere with the assay; hence, a cleanup procedure was not required.

Data in Table I indicate that only an average of 80 % of the toxin was removed on the first extraction. However, the concentration of toxin in the first solvent extract probably represents an accurate value for the amount of toxin in the entire sample

since an average of only 84% of the solvent is recovered after blending, the remainder being occluded in the residual gummy solids.

Penicillic acid was also produced by fermentation with *P. cyclopium* NRRL 1888 in static liquid culture[8]. The supernatant was sampled directly (2 to 10 μl spotted for TLC) and no difficulties were encountered in the analyses (Fig. 3).

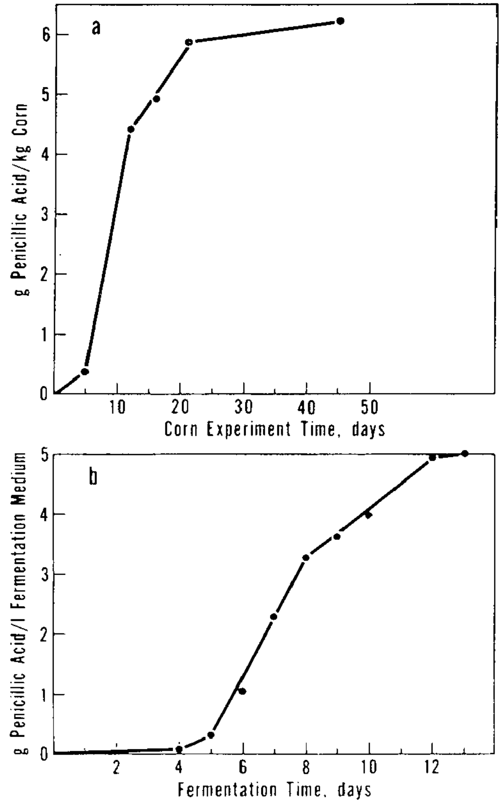

Fig. 3. Production of penicillic acid in corn and by liquid fermentation. Solid culture: 50 g corn inoculated with *Penicillium martensii*, incubated at 15°. Liquid culture: *Penicillium cyclopium* at 25°, static fermentation; supernatant analyzed.

TABLE I

ANALYSIS OF PENICILLIC ACID FROM MOLDY CORN

Sample[a]	*First extraction*[b] *(g toxin/kg corn)*	*Volume solvent recovered (%)*	*Second extraction*[b] *(g toxin/kg corn)*	*Total toxin (g)*	*First recovery (%)*
1	15.0	76	3.6	18.6	81
2	17.0	83	2.3	19.3	88
3	14.7	79	3.0	17.7	83
Av.	15.6	79.3	3.0	18.5	84

[a] Each sample contained 50 g corn molded with *Penicillium martensii* NRRL 3612 for 109 days at 5°.

[b] Each extraction used 250 ml of chloroform–methanol (90:10).

ACKNOWLEDGEMENTS

We are grateful to R. D. Stubblefield for the melting point analysis and to Dr. R. W. DETROY for the excitation–emission spectrum.

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CHROM. 4856

THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION OF ESTROGENS AND PROGESTOGENS IN ORAL CONTRACEPTIVES

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(Received May 4th, 1970)

SUMMARY

A two-dimensional thin-layer chromatographic procedure is described for the separation and identification of the active ingredients of oral contraceptive preparations currently available on the Canadian market. Short-wave ultraviolet light and concentrated sulfuric acid are used for the detection of spots. Observations after spraying with sulfuric acid are tabulated in a color chart which shows color development with respect to time for eleven compounds. The scheme also provides a method for the detection and tentative identification of certain impurities which are frequently found in such formulations.

INTRODUCTION

Because of the widespread and increasing use of oral contraceptive preparations in Canada, there is a need for a rapid method for the identification of such products and their components. In addition, procedures are required to determine the quality both of finished dosage forms and bulk drugs with respect to the presence of impurities, particularly foreign related steroids.

Although thin-layer chromatographic methods have been described for the analysis of synthetic gestogens[1,2] they do not include all of the eleven compounds presently on the Canadian market, and use of these procedures does not produce satisfactory separation of all compounds of interest. RÖDER's technique[1], employing two solvent systems for two-dimensional chromatography, was found to be quite adequate for the separation of the seven compounds which he examined, but three of our four additional progestogens were masked when incorporated with the original seven. KEAY's one-dimensional procedure[2] was not entirely satisfactory for the eight compounds with which he dealt: chromatography produced three poorly resolved areas corresponding to groups of three, two and three compounds; inclusion of the three additional progestogens resulted in a fourth unresolved area.

Analysis of a mixture of the eleven gestogens using the solvent systems recommended by the current *British Pharmacopoeia*[3] (for identification of five individual

gestogens) indicates that four of the components exhibit R_F values too similar to permit reliable identification or differentiation.

The work here reported describes the application of two solvent systems in a two-dimensional TLC sequence which is effective for the separation and identification of each of the eleven gestogens which may be present as a constituent of oral contraceptives.

EXPERIMENTAL

Solvent systems

(a) One-dimensional: (1) benzene–methanol (95:5)
(2) benzene–acetone (80:20)
(3) chloroform–methanol (90:10)
(4) methylene chloride–methanol–water (150:9:0.5)
(b) Two-dimensional: (5) solvent 3, followed by solvent 1
(6) solvent 2, followed by solvent 4

The solvents were all of analytical grade.

Preparation of TLC plates

Desaga equipment was used to prepare layers of 0.25 mm thickness, using a 1:2 w/v ratio of Silica Gel GF (Merck) and water. The plates were activated immediately before use by heating at 100° for 30 min and cooling in a desiccator.

Detection of spots

The plates were examined under short-wave UV light, then sprayed with concentrated sulfuric acid* and heated in an oven at 100° for 30 min.

Preparation of sample

An aliquot equivalent to one tablet was transferred to a 15-ml glass-stoppered graduated centrifuge tube, to which 5 ml of acetone was added. The active ingredients were extracted by shaking the tube on a vortex mixer for 2 min or manually for 10 min. The suspension was centrifuged to obtain a supernatant (A), from which 4 ml was transferred to another graduated centrifuge tube. The solvent was removed by evaporation on a sand bath at 100° with the aid of a stream of nitrogen. (The residue may be stored in a cool place without deterioration and then redissolved in 0.4 ml of acetone just before use (B).)

Standard solutions

(a) For development of the standard chromatograms, a mixture containing 10 mg of each of the progestogens and estrogens was made up to 25 ml in acetone. (b) For the preparation of the color chart and use as internal reference, individual solutions of standards in acetone were prepared. The concentration of the solutions varied from 0.1 mg/ml to 10 mg/ml. (c) For semi-quantitative determination of the active ingredients, a mixture of estrogen and progestogen of concentration equal to

* Fresh sulfuric acid should be used since excessive amounts of moisture adversely affect the color development.

that of the supernatant A was prepared (A_1) and treated in a manner identical to that used for the sample, to obtain a standard (B_1).

TLC procedure

A maximum width of 5 mm of silica gel was removed from two adjacent sides in order to prevent edge effect on the solvent during chromatography. The origin was marked with a dissecting needle. Solvent front lines were drawn 15 cm away from the origin and parallel to each scraped-off edge. The chromatographic chamber was lined with Whatman No. 1 filter paper. 100 ml of developing solvent were poured on to the paper and the system was allowed a minimum of 1 h for equilibration. (A fresh mixture of solvent was used for each plate.) The sample was spotted at the origin, the size of the spot being confined to a maximum diameter of 4 mm. (5 μl Drummond microcaps were found to be convenient.)

After chromatographing in the first solvent system to the 15-cm line, the plate was thoroughly dried and then was chromatographed, at right angles to the first run, in the second solvent system. The time required for development in each of the four solvent systems was approximately 30 min. The plate was examined under short-wave UV light, then sprayed with concentrated sulfuric acid and heated in an oven at 100° for 30 min.

Standard chromatograms

Amounts of 10 ml of standard solution (a), equivalent to 4 μg of each of the active ingredients, were spotted on two separate plates and were chromatographed in the two-dimensional solvent systems 5 and 6, respectively. An outline of the resulting patterns of spots was traced on paper and two corresponding templates* were prepared to assist in the identification of progestogen in formulations of unknown content.

Color chart.

To establish the color chart, 100 μg and 10 μg of each compound were spotted side by side on one plate. The plate was chromatographed in solvent system 1 and dried thoroughly. At time zero a light spray of sulfuric acid was applied and the immediate color (which changes rapidly in some cases) was observed. The plate was resprayed with sulfuric acid and placed in an oven at 100°. The color development of the 100-μg spots was followed closely for the first 30 min. The final colors were noted after 2 h of heating.

Identification of tablets containing a mixture of estrogen and progestogen

For unknown formulations, 20 μl of sample solution B, equivalent to 2–4 μg of estrogen and 10–500 μg of progestogen, were spotted. (The range of concentration of estrogens in anticonceptual formulations on the Canadian market varies from 0.05 mg to 0.1 mg per tablet and the amount of progestogen present is always greater than the amount of estrogen.) In order to select the proper internal reference standard for the two-dimensional procedure, the estrogen was identified first by one-dimensional chromatography in solvent system 1 by spotting sample solution B along with 2 μg of each of the two estrogens, namely mestranol and ethinyl estradiol. Then the pro-

* 209 Automatic Transparency 3M Company.

gestogen was identified by chromatography in solvent systems 5 and/or 6 by spotting sample solution B along with 2 μg of the appropriate internal reference standard. Ethinyl estradiol was added as internal reference standard when mestranol was present in the formulation and *vice versa*. The estrogen already identified was used as second internal reference standard. After the plate had been examined under short-wave UV light and sprayed with concentrated sulfuric acid, the mestranol and ethinyl estradiol marks on the template of the standard chromatogram were aligned with the corresponding internal reference standard spots on the chromatoplate. The unknown progestogen spot on the chromatoplate was readily identified from the corresponding progestogen mark on the template. The color chart served also as a guide for this preliminary identification work. To further substantiate the preceding identifi-

TABLE I

LIMITS OF DETECTABILITY UNDER SHORT-WAVE UV LIGHT

Compound	*Amount detected* (*μg*)
Chlormadinone acetate	0.5
Dimethisterone	0.1
Ethinyl estradiol	10
Ethynodiol diacetate	250
Lynestrenol	500
Megestrol acetate	0.5
Mestranol	10
Norethindrone	0.1
Norethindrone acetate	0.1
Norethynodrel	10
Norgestrel	0.1

CHLORMADINONE ACETATE l_Bu__ Ga__d-Bu__ ......Gr
DIMETHISTERONE O_Br_Bu(Ga)__ Ga __ Gr(Ga)__ Gr __ ......Gr
ETHINYL ESTRADIOL P__R(Br)__ ......P
ETHYNODIOL DIACETATE Br _V__Br(R)__ ......Br
LYNESTRENOL Y_O_Br__ ......Br
MEGESTROL ACETATE Y__Gr/Y __ ......Gr
MESTRANOL P__R(P)__ ......P
NORETHINDRONE Y_Gr_Bu_V__V(R) ......Br
NORETHINDRONE ACETATE Y_Gr_Bu_V__V(R)__V(Br).....Br
NORETHYNODREL V__V(R)__ ......Br
NORGESTREL Y_Gr_Bu(Ga)_V__ ......Br
0 5 10 15 20 25 30 2H
MINUTES

Fig. 1. Color development of 100 μg of each compound relative to time. Br = brown, Bu = blue, d = dark, Ga = gray, Gr = green, l = light, O = orange, P = pink, R = red, V = violet, Y = yellow.

cation a suitable aliquot of sample solution A or B containing about 4 μg of material plus 4 μg of the alleged progestogen as internal standard were chromatographed in solvent systems 5 and 6. The choice between sample solution A or B was made by visual evaluation from the size of the spot obtained from 20 μl of sample solution B during the identification of the estrogen. The volume of sample solution required was then determined on a separate plate by comparison of a series of spots of varied concentration of sample solution to the 4-μg spot of standard. For formulations of known label claim, the dilutions in the preparation of the sample may be adapted to the estrogen/progestogen ratio in the tablet so that 10 μl of A contains approximately 4 μg of progestogen and 10 μl of B contains approximately 2 μg of estrogen.

Semi-quantitative work

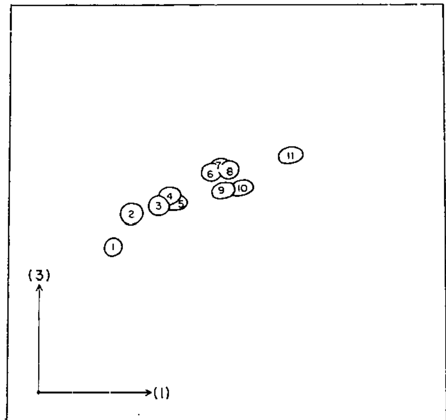
From the two standard chromatograms, the solvent giving the best separation of the progestogen–estrogen combination under study was selected for the semi-quantitative estimation of the active ingredients. For the estimation of the amount of progestogen present, the following solutions were spotted adjacently: (A) sample, (A_1) standard, progestogen standard (several spots containing amounts above and below the label claim concentration). The amount of drug in the sample was then estimated after chromatography by comparison of size and color intensity of the sample spot with the series of standard spots of varying concentration. Similarly, the amount of estrogen present was estimated using a sample solution B instead of A.

RESULTS AND DISCUSSION

Under short-wave UV light, the limits of detectability vary from 0.1–500 μg as indicated in Table I. Upon spraying with concentrated sulfuric acid and heating at 100° for 30 min all the compounds under study are detectable at levels of 1 μg or less.

The color development relative to time for progestogens and estrogens after spraying with concentrated sulfuric acid is illustrated in Fig. 1. (For example, code Gr/Y means green center with yellow edge and V(R) means reddish violet.) The color of the spots as well as their rate of development is affected by the following factors: (a) amount of H_2SO_4 sprayed, (b) amount of material in each spot, (c) heating time and temperature, (d) moisture content in H_2SO_4, and (e) residual solvent on the plate. Factors (a) and (b) were found to be the most critical in their effect on the rate of color development, but under standard conditions reproducible chromatograms could be readily obtained. The color development was recorded relative to time merely to illustrate the difference in the rate of color development from one compound to another at a fixed concentration. The time abscissa is valid only for a 100-μg spot of drug. 10 μg of drug were also spotted next to the 100-μg spot. The lower concentration spot aided the observation of the initial color changes while the higher concentration one was used to record the color development relative to time. For example, at the higher drug concentration the initial yellow color observed for norethindrone, norethindrone acetate and norgestrel changed so rapidly to green that it is easily overlooked.

Relatively short heating times (or low temperatures) caused non-reproducible results since although the same sequence of color developing reaction took place, the reactions had progressed to varying stages of completion. 30 min at 100° were found



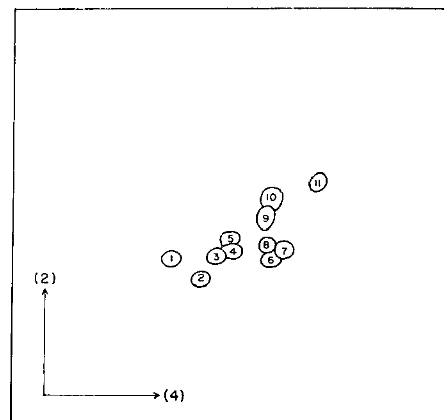


Fig. 2. Standard chromatogram in solvent system 5. 1 = Ethinyl estradiol, 2 = norethindrone, 3 = norgestrel, 4 = dimethisterone, 5 = norethynodrel, 6 = megestrol acetate, 7 = chlormadinone acetate, 8 = norethindrone acetate, 9 = mestranol, 10 = lynestrenol, 11 = ethynodiol diacetate.

Fig. 3. Standard chromatogram in solvent system 6. For numbering of spots, see legend to Fig. 2.

TABLE II

R_F VALUES FOR PROGESTOGENS AND ESTROGENS

Compound	*Solvent system*			
	1	*2*	*3*	*4*
Chlormadinone acetate	0.55	0.47	0.72	0.74
Dimethisterone	0.40	0.46	0.61	0.57
Ethinyl estradiol	0.23	0.43	0.46	0.39
Ethynodiol diacetate	0.77	0.68	0.76	0.84
Lynestrenol	0.61	0.63	0.65	0.69
Megestrol acetate	0.52	0.43	0.71	0.70
Mestranol	0.57	0.56	0.65	0.68
Norethindrone	0.29	0.37	0.57	0.48
Norethindrone acetate	0.58	0.48	0.71	0.69
Norethynodrel	0.42	0.51	0.63	0.57
Norgestrel	0.37	0.45	0.59	0.53

adequate in all cases to develop the color sequence but without causing charring, as is observed with a 2-h heating period.

Schematics of the two standard chromatograms are shown in Figs. 2 and 3, and the R_F values for each compound using the four solvent systems are listed in Table II (average of six determinations). Absolute R_F values are listed to show the amount of separation achieved with various solvent systems. However, the reproducibility of the relative R_F values (*i.e.* with respect to the internal reference standards) rather than absolute R_F values is important in the identification of tablets of unknown composition. The use of two internal reference standards provides a satisfactory means of checking the reliability of the template and thus eliminates the necessity of absolute R_F values as identification criteria. As long as the two estrogens in the chromatoplate

TABLE III

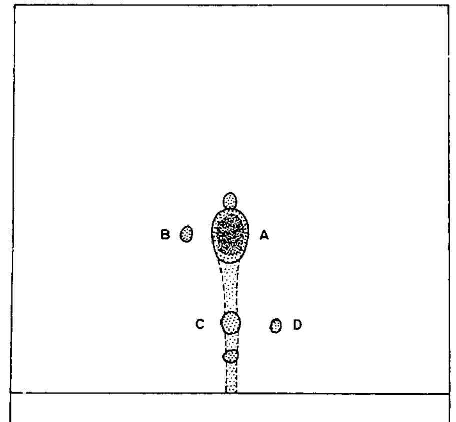
COMPOSITION OF ORAL CONTRACEPTIVE TABLETS EXAMINED

Progestogen	*Amount (mg)*		*Estrogen*	*Amount (mg)*
Dimethisterone	25.0	+	Ethinyl estradiol	0.1
Megestrol acetate	4.0	+	Ethinyl estradiol	0.05
Norethindrone acetate	2.5	+	Ethinyl estradiol	0.05
d-Norgestrel	0.25	+	Ethinyl estradiol	0.05
Chlormadinone acetate	2.0	+	Mestranol	0.08
Ethynodiol diacetate	1.0	+	Mestranol	0.1
Lynestrenol	2.5	+	Mestranol	0.075
Norethindrone	5.0	+	Mestranol	0.075
Norethynodrel	5.0	+	Mestranol	0.075

and the corresponding marks on the template are superimposable, the template is reliable for the identification of progestogens.

The method was assessed by examining a variety of tablets of unknown composition as well as a number of progestogen–estrogen formulations currently available on the Canadian market. The latter examples, listed in Table III, were selected because each one represents the largest progestogen/estrogen ratio in use for each particular combination. Employing solvent system 1, solvent system 5 and/or 6, along with the color chart and the template, satisfactory identifications and semi-quantitative estimations were effected in all cases. The results were duplicated by another analyst.

To facilitate the identification procedure, the sample preparation of the unknown and standards are carried out first. Then (on the same following day) the estrogen in all the samples is identified on one plate in solvent system 1. Finally the progestogen in each sample is identified individually in solvent system 5 and/or 6. If the unknown



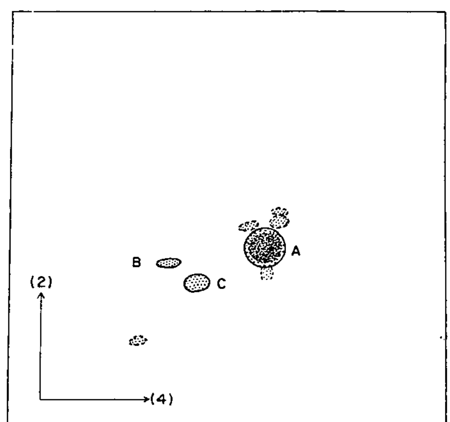


Fig. 4. Chromatogram of norethindrone acetate in solvent system 1. A = Norethindrone acetate (500 μg), B = norethindrone acetate (4 μg), C = norethindrone (impurity), D = norethindrone (4 μg).

Fig. 5. Chromatogram of norethindrone acetate in solvent system 6. A = Norethindrone acetate (500 μg), B = ethinyl estradiol as reference standard (2 μg), C = norethindrone (impurity).

is treated under experimental conditions identical to those used for the standard chromatograms, the corresponding template can be used repeatedly. Large variations in technique from the standardized procedure may make the template inapplicable. For instance, if a plate spotted with an unknown is run over the 15-cm line in one of the two solvent systems, the template cannot be used for its identification. But an error such as this is readily apparent since the template cannot be aligned with the two estrogen internal standards on the chromatoplate.

In addition to the direct identification of the compounds of oral contraceptive preparations, the two-dimensional scheme is also of value for:

(a) The detection and tentative identification of impurities such as: norethindrone in norethindrone acetate (Figs. 4 and 5) and in norethynodrel, and norethynodrel in norethindrone.

(b) The differentiation of an impurity from a "ghost" spot, especially when the spot suspected to be an impurity is partly masked by tailing of the main active ingredient.

(c) The separation of impurities that move together in a one-dimensional system.

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CHROM. 4858

THE NATURE OF SOME SOLVENT-DERIVED ARTIFACT SPOTS OBSERVED DURING THE CHROMATOGRAPHY OF ADRENALINE*

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(Received June 1st, 1970)

SUMMARY

Several spots are often detected on chromatograms obtained from solutions of a single pure β-(3,4-dihydroxyphenyl)ethanolamine derivative, such as adrenaline, particularly when the base is dissolved in strong acids prior to chromatography. The formation of some of these multiple spots can be explained by either the formation of complexes or salts, involving the catecholamine and the acids employed, or by the chemical modification of the catecholamine prior to chromatography. It has now been shown that substances responsible for certain artifact spots are formed by the interaction of one of the components of the chromatographic solvent system used with a reactive intermediate derived from the β-phenylethanolamine derivative being chromatographed. For example, adrenaline *n*-butyl ether is formed during the chromatography of adrenaline in *n*-butanol–hydrochloric acid solvent mixtures and β-(*p*-hydroxyphenyl)-β-(3′,4′-dihydroxyphenyl)ethylmethylamine is one of the artifact spots observed when adrenaline is chromatographed in phenol–hydrochloric acid systems.

INTRODUCTION

Several spots have often been detected on chromatograms of solutions of a single pure catecholamine, such as adrenaline (I). This phenomenon was first reported by CRAWFORD[1] in 1951 and a number of different explanations of this behaviour have subsequently been advanced. Some workers have suggested the formation of complexes or salts between the catecholamine and the acids employed, either in the extraction procedure or in the chromatographic solvent system used[2–7]. Other workers have proposed that distinct new chemical compounds are formed from the catecholamines by the action of acids prior to chromatography[1,8–12]. It has been suggested[11,12] that

* Issued as NRCC No. 11497.
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two of the new compounds detected when solutions of adrenaline in 10 N hydrochloric acid were chromatographed in a phenol–0.1 N hydrochloric acid system, were identical with the compounds described as "diadrenaline ether"[13,14] (2) and adnamine[15] (*i.e.*, 5-methylaminomethyl-2,3,7,8-tetrahydroxydibenzo[*a,e*]cycloheptatriene (3)). Both of these compounds can be obtained as crystalline solids by the action of hydrochloric acid on adrenaline[13–16]. However, it has recently been shown that the symmetrical ether structure proposed for the former is incorrect[17,18]. This compound is, in fact, 6-(3',4'-dihydroxymethylaminomethylbenzyl)adrenaline[17,18] (4) and the trivial name adrepine was proposed for this product[17,18].

Other workers have investigated the action of strong mineral acids on β-phenylethanolamines in general and report the formation of different types of product including: β-phenylnaphthalene derivatives[19,20], phenylacetaldehyde and phenylacetone derivatives[21–23]. It is also known that in the presence of acids, aliphatic alcohols form ethers with the secondary alcohol function in the β-phenylethanolamines[13,14,24–27].

When solutions of adrenaline in 10 N hydrochloric acid, which have been stored for varying periods of time, are examined chromatographically, several spots are observed on the chromatograms obtained. The number (which varies up to a maximum of 7 or 8) and nature of the spots observed depend on several factors, including acid strength and time and temperature of storage of the solution. In cases where the same

1

3

4

5 : R = H
8 : R = Cl

9

6 : R = CH_3
7 : R = $CH_2CH_2CH_2CH_3$
10 : R = C_6H_5

acid (*i.e.* hydrochloric acid) is used in the developing solvent it is unlikely that the new spots observed arise as a result of physical interactions of the type described previously by other workers[2-7].

In fact the new spots are due to chemical modification of the adrenaline molecule, and two of the substances which are eventually formed appear to be, as would be expected, adnamine and adrepine[11,28]. The nature of all the compounds formed when adrenaline is dissolved in 10 *N* hydrochloric acid and which may be at least partly responsible for the observed multispot phenomena is currently under investigation in these laboratories[28].

β-Phenylethylamine derivatives lacking a β-hydroxyl group have previously been shown not to produce spots analogous to adnamine and adrepine[9]. This has been confirmed by current investigations[28]. The most likely first step in the formation of adnamine and adrepine from adrenaline in the presence of strong mineral acids would be the elimination of the β-hydroxyl group with the formation of a highly reactive carbonium ion. This species would be expected to react readily with aliphatic hydroxyl groups to form the corresponding alkoxy derivatives and it is well known that reactions of this type do in fact occur[13,14,24-26]. In view of these facts it was decided to investigate the possibility that one or more of the extra spots observed when adrenaline was chromatographed using hydroxylic solvents, containing hydrochloric acid, were due to the formation of β-alkoxy or β-aryloxy derivatives. Two solvent systems that have been used extensively for the chromatography of adrenaline and related compounds are phenol containing 15 % v/v 0.1 *N* hydrochloric acid, and *n*-butanol saturated with 2 *N* hydrochloric acid. These systems have been used in this investigation.

EXPERIMENTAL

General

The melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. The IR and UV spectra were recorded on Perkin-Elmer Model 237 and Beckman DK-2 recording spectrophotometers, respectively. The NMR spectra were obtained on a Varian A-60-A instrument using tetramethylsilane as an external reference. The mass spectral data were obtained on a Bell and Howell/C.E.C. Model 21-110 instrument.

Paper chromatography

Radial development on Whatman No. 1 paper discs (diameter 32.0 cm) was used throughout these investigations.

Adrenaline, epinine (5) and adrenaline methyl ether (6)[29] (100 μg) were chromatographed from aqueous solution (as their hydrochlorides) or from solution in 10 *N* hydrochloric acid (10 mg/ml). The developing solvents used were: S_1, phenol containing 15 % v/v 0.1 *N* hydrochloric acid, and S_2, *n*-butanol saturated with 2 *N* hydrochloric acid.

The spots were located by viewing the developed chromatograms in UV light, before and after exposure of the papers to ammonia fumes and by the use of the following chromogenic reagents: (a) 4-aminoantipyrine followed by potassium ferri-

cyanide[30], (b) potassium ferricyanide, followed by exposure to ammonia fumes, and (c) ninhydrin (the papers were heated after spraying).

In some experiments phenol (10 mg/ml) or *n*-butanol (0.4 ml/ml) was added to the aqueous and 10 *N* hydrochloric acid solutions of the amines prior to chromatography.

Adrepine hydrochloride[14,17,18], adnamine hydrochloride[16], adrenaline *n*-butyl ether (7) hydrochloride and β-(*p*-hydroxyphenyl)epinine hydrochloride (β-(4-hydroxyphenyl)-β-(3',4'-dihydroxyphenyl)-N-methylethylamine (9)) hydrochloride (the last two compounds were prepared by the methods described below), were also chromatographed from solutions in distilled water and 10 *N* hydrochloric acid.

Solutions of methylaminomethyl-3,4-dihydroxyphenylchloromethane (8) hydrochloride[29] in 10 *N* hydrochloric acid and in the two chromatographic solvents mentioned above (*i.e.*, S_1 and S_2) were prepared and chromatographed in turn in these same two solvent systems.

Chemical

β-(p-Hydroxyphenyl)epinine hydrochloride (9)

A solution of L-adrenaline (10 g) and phenol (10 g) in 20 % aqueous hydrochloric acid (200 ml) was maintained at 40° for 48 h. The reaction mixture was then cooled to 4° and allowed to stand overnight at this temperature. A crystalline precipitate (13.75 g) was obtained, which afforded β-(*p*-hydroxyphenyl)epinine hydrochloride monohydrate in colourless prisms (m.p. 131°) on recrystallisation from 70 % aqueous alcohol.

Anal. Calcd. for $C_{15}H_{18}NO_3Cl \cdot H_2O$: C, 57.40; H, 6.42; N, 4.46; Cl, 11.29. Found: C, 57.85; H, 6.55; N, 4.61; Cl, 11.60 %. Anhydrous β-(*p*-hydroxyphenyl)-epinine hydrochloride (m.p. 198°) was obtained on drying the monohydrate at 120° *in vacuo* for 1 h. $\lambda_{max}^{EtOH} = 278$ mμ; $\varepsilon_{max}^{EtOH} = 5200$. Anal. Calcd. for $C_{15}H_{18}NO_3Cl$: C, 60.90; H, 6.13. Found: C, 60.71; H, 6.16 %. This compound has previously been obtained by KAPPE AND ARMSTRONG[31] from the interaction of β-(4-hydroxyphenyl)-N-methylethanolamine and catechol in 2 *N* hydrochloric acid at 100°. The m.p. reported[31] (126–128°) was presumably that of the monohydrate.

NMR. $\tau(D_2O)$: 2.62–3.24 (7H, m, aromatic, including 4H AA'BB' centered at 3.20, $J_{AB} = 9.0$ Hz); 5.70 (1H, t, methine H, $J = 8.5$ Hz); 6.35 (2H, d, $-CH_2-$); 7.16 (3H, s, $>N-CH_3$).

Mass spectral data. M = 259; 100 % peak 215; *m/e* (% relative height): 259 (34); 255 (10); 216 (31); 215 (100); 196 (10); 168 (11); 159 (19); 141 (12); 139 (18); 131 (11); 110(20); 102 (11); 92 (28); 91 (61); 89 (12); 77 (19); 76 (15); 64 (21); 63 (16); 58 (11); 55 (12); 52 (10); 51 (20); 45 (29); 44 (90).

Adrenaline n-butyl ether hydrochloride (7). A suspension of methylaminomethyl-3,4-dihydroxyphenylchloromethane hydrochloride (1.0 g, prepared by the action of pure thionyl chloride on adrenaline by the method of HUKKI AND SEPPÄLÄINEN[29]) in *n*-butanol (20 ml) was heated, under reflux, with stirring, at 85–95°. The clear solution which was obtained after 4–5 min heating was heated under reflux for a further 10 min. The reaction mixture was cooled and evaporated to dryness *in vacuo* (below 40°); a pale yellow oil was obtained which slowly formed a yellow solid (m.p. *ca.* 140°) on trituration with acetone. Adrenaline *n*-butyl ether hydrochloride was eventually obtained as a pale yellow crystalline solid (m.p. 140–142°) by repeated recrystal-

lisation from *n*-butanol–acetone mixtures. $\lambda_{max}^{EtOH} = 283$ mμ; $\varepsilon_{max}^{EtOH} = 4600$.

Anal. Calcd. for $C_{13}H_{22}NO_3Cl$: C, 56.60; H, 8.04; N, 5.09; Cl, 12.85. Found: C, 56.48; H, 7.96; N, 5.04; Cl, 12.89%.

NMR. $\tau(D_2O)$: 2.79–2.90 (3H, m, aromatic); the >CH–CH_2–N< protons occurred as an ABX system, with the signal due to the X proton centered at 5.17 (1H, dd, methine), the A proton of the AB at 6.30 (1H, d, J = 6.0 Hz) and the B proton at 6.5 (1H, d, J = 7.5 Hz); 6.45 (2H, dd, –O–CH_2–); 6.95 (3H, s, >N–CH_3); 8.00–9.00 (7H, m, –$CH_2CH_2CH_3$).

Mass spectral data. M = 239; 100 % peak 139; *m/e* (% relative height): 239 (12); 196 (26); 195 (62); 166 (11); 165 (10); 139 (100); 138 (11); 137 (18); 124 (12); 123 (10); 93 (11); 56 (14); 44 (92).

RESULTS AND DISCUSSION

The results of the chromatographic studies are summarised in Table I. The chromatography of adrenaline, epinine and adrenaline methyl ether hydrochlorides (100 μg) from solution in distilled water (10 mg/ml) resulted in the formation of dis-

TABLE I

R_F VALUES OF SOME AMINES AND THEIR SOLVENT-DERIVED ARTIFACTS

Amine[a]	*Added component (to amine solutions)*	*Developing solvent*[b]	*R_F values*[c] *from solutions*[a] *in distilled water*	*R_F values*[c,d] *of spots obtained from solutions in 10 N HCl*						
Adrenaline	—	S_1	0.49	0.00	0.56[e]	0.67[f]		0.80	0.91	
	phenol	S_1	0.49		0.59[e]	0.66[f]		0.80		
	n-butanol	S_1	0.50	0.00		0.66[f]		0.79		0.94
	—	S_2	0.31	0.00	0.19[e]	0.38[f]		0.83		
	phenol	S_2	0.32			0.38[f]	0.73			
	n-butanol	S_2	0.31	0.00		0.37[f]		0.85		
Epinine	—	S_1	0.65			0.81[f]				
	phenol	S_1	0.66			0.80[f]				
	n-butanol	S_1	0.65			0.80[f]				
	—	S_2	0.43			0.51[f]				
	phenol	S_2	0.44			0.52[f]				
	n-butanol	S_2	0.41			0.50[f]				
Adrenaline methyl ether	—	S_1	0.78	0.00	0.56[e]	0.68[f]		0.81	0.92	
	phenol	S_1	0.77		0.59[e]	0.68[f]		0.82		
	n-butanol	S_1	0.77	0.00		0.66[f]		0.79		0.94
	—	S_2	0.55	0.00	0.19[e]	0.38[f]		0.84		
	phenol	S_2	0.57			0.38[f]	0.73			
	n-butanol	S_2	0.55	0.00		0.36[f]		0.83		

[a] Solutions of the amine hydrochlorides in distilled water.
[b] S_1 = phenol containing 15% v/v 0.1 *N* HCl; S_2 = *n*-butanol saturated with 2 *N* HCl.
[c] Radial development.
[d] Single underlining = average intensity spot; no underlining = weak spot; broken underlining = intense spot.
[e] Adrepine.
[f] Parent amine.

crete spots in both solvent systems used in this investigation. The mean R_F values were 0.32, 0.43 and 0.55, respectively, in the *n*-butanol–hydrochloric acid solvent and 0.49, 0.65 and 0.78, respectively, in the phenol–hydrochloric acid solvent.

When chromatographed (100 μg) from solution in 10 *N* hydrochloric acid (10 mg/ml) epinine still only produced one spot, however, the mean observed R_F values were somewhat higher (0.51, *n*-butanol–hydrochloric acid; 0.80, phenol–hydrochloric acid) than those observed when the amine was chromatographed from aqueous solution. By contrast adrenaline and adrenaline methyl ether both produced several spots when chromatographed (100 μg) from solution in 10 *N* hydrochloric acid (10 mg/ml); it was impossible, however, to distinguish between the chromatographic behaviour of these two amines under such conditions and in each case the principal spot, due to adrenaline, had R_F values of 0.37 (*n*-butanol–hydrochloric acid) and 0.66 (phenol–hydrochloric acid) which were again higher than the R_F values for adrenaline obtained following chromatography of aqueous solutions. Three additional spots were obtained in the *n*-butanol–hydrochloric acid solvent system and four additional spots were observed with the phenol–hydrochloric acid solvent system. Retention of some products at the point of application (R_F 0.00) was common to both solvent systems. Colour reactions and comparisons with reference chromatograms allowed the spot having an R_F value of 0.19 in the *n*-butanol–hydrochloric acid system and 0.56 in the phenol–hydrochloric acid system to be identified as adrepine.

In the *n*-butanol–hydrochloric acid solvent system the unidentified spot (R_F 0.83) was probably a solvent-derived artifact. When *n*-butanol was added to the solution of adrenaline in hydrochloric acid prior to chromatography, the intensity of this spot was markedly increased. It therefore seemed most likely that the compound responsible for this spot was adrenaline *n*-butyl ether which has now been synthesised by an adaptation of the method used by HUKKI AND SEPPÄLÄINEN for the synthesis of other simple ethers of adrenaline[29]. The structure of this compound was confirmed by consideration of its mass and NMR spectra. The mass spectrum of adrenaline *n*-butyl ether showed the required molecular ion at *m/e* 239 as well as the expected fragment ions at *m/e* 195 [M — 44] and at *m/e* 139 [M — 44–56]. The NMR spectrum of adrenaline *n*-butyl ether showed the multiplet typical of an *n*-butyl ether grouping between τ 8.0 and τ 9.0 due to the $CH_3CH_2CH_2$– group and the triplet of the $-CH_2-O-$ group at τ 6.45 (J = 6.0 Hz). The other protons were observed at: (i) three aromatic protons as a multiplet between τ 2.79 and τ 2.90, (ii) the $>CH-CH_2-N<$ protons as an ABX system with the signal due to the X proton centred at τ 5.17 (J values: 6.0 and 7.5 Hz), the A proton at τ 6.30 (J = 6.0 Hz) and the B proton at τ 6.50 (J = 7.5 Hz), and (iii) the $>N-CH_3$ protons as a singlet at τ 6.95.

The chromatographic properties (*i.e.*, R_F values and colour reactions) of the synthetic adrenaline *n*-butyl ether were identical with those of the artifact spot with the same R_F value (0.83) observed when adrenaline solutions in 10 *N* hydrochloric acid are chromatographed in *n*-butanol–hydrochloric acid solvent systems. This assignment was verified by comparison of the IR spectra of a sample of the authentic material with a sample obtained by eluting the spot in question from a suitable chromatogram. In the phenol–hydrochloric acid solvent system the adrenaline *n*-butyl ether had an R_F value of 0.94, *i.e.* the same as that of the extra spot produced by addition of *n*-butanol to the adrenaline solution in hydrochloric acid prior to chromatography in the phenol–hydrochloric acid solvent system.

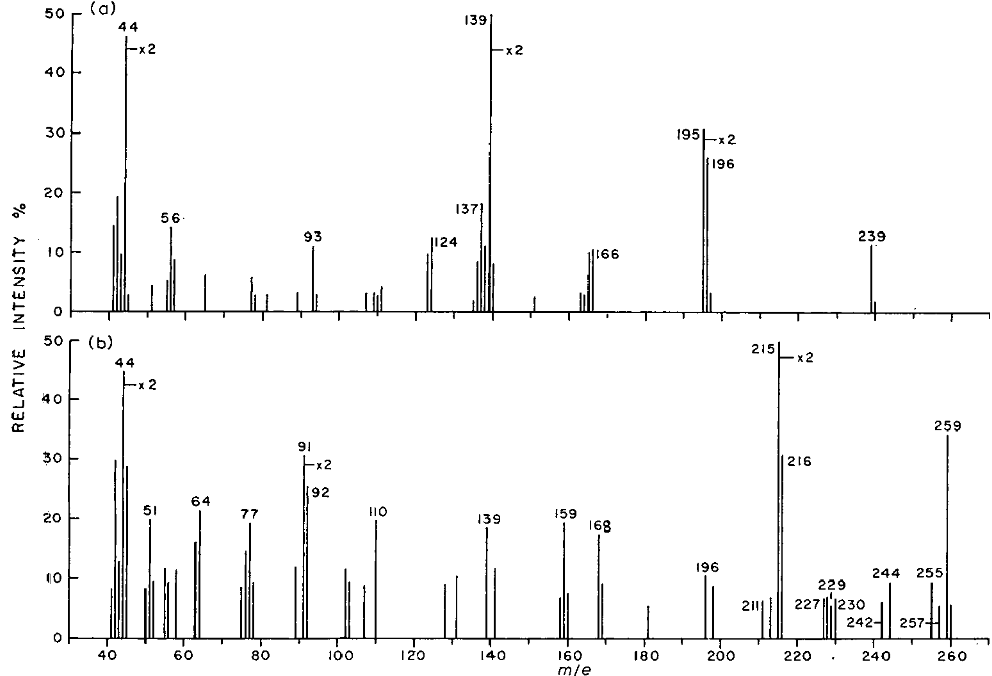

Fig. 1. Mass spectra of (a) adrenaline *n*-butyl ether (7) and (b) *β*-(*p*-hydroxyphenyl)epinine (9) hydrochlorides.

The two unidentified spots (R_F values *ca.* 0.80 and 0.90) observed on chromatograms of adrenaline or adrenaline methyl ether in the phenol–hydrochloric acid solvent system were possibly solvent-derived artifacts. Addition of phenol to the hydrochloric acid solution of adrenaline prior to chromatography resulted in the formation of an intense spot (R_F 0.80) as well as two weaker spots with R_F values of 0.59 (*i.e.* adrepine) and 0.66 (*i.e.* adrenaline). The spot at R_F 0.80 therefore appeared to be a compound derived from the reaction of adrenaline with phenol. In the *n*-butanol–hydrochloric acid solvent system an intense spot with R_F 0.73 and a weak spot with R_F 0.38 (adrenaline) were observed on chromatography of the adrenaline in hydrochloric acid solution which had been treated with phenol.

In some experiments involving the addition of phenol to the adrenaline–10 *N* hydrochloric acid systems a white crystalline product was eventually obtained after the reaction mixtures had stood at room temperature for several days. This product had the same R_F values in the two solvent systems as the artifact spot believed to be derived from the presence of phenol in the system. By employing higher concentrations of adrenaline and phenol and higher reaction temperatures the crystalline product could readily be obtained in good yield.

Microanalysis of the product obtained from phenol and adrenaline indicated that the compound had an empirical formula of $C_{15}H_{17}NO_3 \cdot HCl$, *i.e.* suggesting a 1:1 condensation had occurred between adrenaline and phenol with the loss of the elements of water. (The product was initially obtained as a monohydrate but the water of crystallisation could readily be removed *in vacuo*.) Whilst it was tempting

to assume that the product was a phenyl ether of adrenaline (10) the NMR spectrum of the compound (in D_2O) indicated that this could not be the case. The NMR spectrum showed the presence of: (i) seven aromatic protons in the region τ 2.5–3.0; the signals included those due to a definite AA'BB' system, centered at τ 2.75 ($J = 9.0$ Hz); (ii) a single $>CH-CH_2-$ grouping occurring as an AB_2 system with the B_2 protons at τ 6.35 and the A proton at τ 5.72 with a coupling constant of about 8.5 Hz; and (iii) one N-methyl group as a singlet at τ 7.2.

The presence of signals due to seven aromatic protons, including an AA'BB' system in the NMR spectrum, indicates that the phenol nucleus had been substituted in the *para* position and suggests that this product is not the phenyl ether 10, but is, in fact, β-(4-hydroxyphenyl)-β-(3',4'-dihydroxyphenyl-*N*-methylethylamine (9) produced by the electrophilic attack of the carbonium ion, obtained from the adrenaline by the loss of the side-chain hydroxyl group in strongly acid media, at the *para* position of the phenol molecule.

The mass spectrum of compound 9 confirmed that it has a basic β,β-diphenylethylamine structure. The molecular ion is observed as a strong peak at *m/e* 259. The 100% peak is observed at *m/e* 215 (*i.e.* [M — 44]) and the corresponding peak at *m/e* 44 is also present in the spectrum indicating that, similarly to other compounds of this type (*cf.* refs. 18 and 32–34), the expected fragmentation β to the nitrogen atom had occurred.

Methylaminomethyl-3,4-dihydroxyphenylchloromethane hydrochloride was a possible intermediate in the formation of the solvent-derived artifacts from adrenaline, and can be readily obtained by the action of HCl on adrenaline. Solutions of this compound (prepared by the method of HUKKI AND SEPPÄLÄINEN[29]) in 10 *N* hydrochloric acid and in the *n*-butanol–hydrochloric acid and phenol–hydrochloric acid solvent systems, were chromatographed in these solvents. The results obtained from these experiments indicated that the solvent-derived artifact spot obtained in the phenol-containing solvent having an R_F value of *ca.* 0.75–0.80 and that obtained in the case of the *n*-butanol–hydrochloric acid solvent having an R_F value of *ca.* 0.85 were the same as those derived from adrenaline or adrenaline methyl ether.

In contrast to the behaviour of adrenaline, the methyl ether and the chloro compound which reacted with both phenol and *n*-butanol in hydrochloric acid, the chromatographic behaviour of acid solutions of epinine was unaffected by the addition of phenol or *n*-butanol. As well, the chromatographic behaviour of aqueous solutions of adrenaline, epinine and adrenaline methyl ether hydrochlorides was unaffected by the presence of phenol or *n*-butanol in the distilled water used to prepare the solutions. It would appear from the results presented above that the presence of *n*-butanol or phenol in acid solutions of the catecholamines which have a substituent, such as halogen or oxygen on the β-carbon, decidedly modifies their composition as observed when they are chromatographed.

ACKNOWLEDGEMENTS

The authors wish to express their thanks to Mr. J. VAN INGEN for recording the NMR spectra, Mr. D. EMBREE and Dr. W. D. JAMIESON for the mass spectra, and Dr. R. BALTZLY of the Wellcome Research Laboratories (New York) for a generous gift of epinine hydrochloride.

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Notes

CHROM. 4865

Some problems in the purification of monoazo disperse dyes by column chromatography on alumina

During the course of a series of investigations concerned with the relationships between structure and properties of disperse dyes of all chromophoric types, the necessity of utilising dyestuffs of unquestionable purity arose. The value of correct microanalytical data was found in some cases to be a tenuous property when utilised as the sole criterion of dye purity.

In ranges of dyestuffs of the nitrodiphenylamine[1,2], anilinonitropyridine[3], nitroanilinopyridine[4] and benzothiazolylazo[5] type, the value of mass spectrometry as a control in ascertaining the presence and possible level of impurities of higher molecular weight than the parent dye was found to be particularly useful; in all these cases, the dyes were satisfactorily purified by conventional column chromatography on activated alumina (Type H, 100–200 mesh, Laporte Chemicals) using benzene or benzene–acetone mixtures as solvent and eluent, with, in some cases, small additions of ethanol. The basic principles behind this method of dyestuff purification have been reported recently[6], and some of the problems arising with certain anthraquinone dyestuffs discussed.

We report now some of the difficulties which we observed in the purification of some monoazo disperse dyes and the extent to which chromatographic techniques can be applicable in the context of both purifying and ascertaining the final purity of these dyes.

3-Methyl-4-N-β-cyanoethyl-N-β-hydroxyethylaminoazobenzenes

Diazotisation of a series of mono-substituted anilines and coupling to a range of N-substituted anilines afforded a range of monoazo disperse dyes of general formula I

X–C₆H₄–N=N–C₆H₃(R′)–N(R²)(R³)

(I)

The coupling components utilised were of a technical grade nature and contained minor amounts of impurities. Purification of the crude dyes was readily effected in most cases by column chromatography on activated alumina (Laporte Chemicals, Type H, 100–200 mesh). For dyes in which neither of R^2 or R^3 was β-hydroxyethyl, benzene was used as solvent and eluent; with dyes in which one of R^1 or R^2 was β-hydroxyethyl, addition of acetone was found necessary to obtain satisfactory elution

(benzene–acetone (9:1) being particularly useful); with dyes of very high absorptive power, *e.g.* where R^1 and R^2 both were β-hydroxyethyl, some impurities eluted readily with the above solvent mixtures, but the parent dyes required the use of acetone–ethanol (98:2) for satisfactory elution and separation.

In most cases, extraction of the principal zone with ethanol afforded dyestuffs of a high degree of purity, showing one spot only on TLC on Eastman chromagram sheets (Type K 301 R2, silica gel), giving excellent microanalytical data and showing no impurities above P^+ on the mass spectrum. The range of dyes in which $R^1 = Me$, $R^2 = C_2H_4OH$ and $R^3 = C_2H_4CN$, however, as extracted from an apparently homogeneous zone on alumina, whilst giving satisfactory microanalytical data, showed an impurity at $P \pm 44$ in the mass spectrum. TLC, as above, using ethyl acetate–toluene (1:1) as eluent, confirmed the presence of two components at R_F 0.23 and 0.39. Repeated column chromatography on these dyes on activated alumina as above resulted in no noticeable separation, irrespective of several variations in eluents used.

Use of a silica gel column packed with Kieselgel $PF_{254+366}$ (Merck), using ethyl acetate–toluene (1:1) as eluent, resulted in effective separation, permitting isolation in 5–8 % amount, and eventual identification of the impurity as I ($R^1 = CH_3$, $R^2 = C_2H_4CN$, $R^3 = C_2H_4OC_2H_4OH$), presumably arising during the preparation of the coupling component in the ethylene oxide condensation stage.

O-Acyl-N-β-hydroxyethylaminoazobenzenes

A range of monoazo dyes of general formula II and III were prepared by acetylation of the corresponding hydroxyethyl derivatives.

(II)

(III)

The course and extent of the acetylation can be readily followed by TLC (Eastman Chromatogram sheets, as above) using ethyl acetate–toluene (1:3) as eluent, the acyl derivatives having considerably higher R_F values. Acetylation was carried out so that only minor traces of unacetylated dyes remained. Attempted purification of the dyes and isolation of the pure acetyl derivatives was, however, found to be impractical using column chromatography on alumina.

In all cases, benzene was used as solvent and eluent; in the early stages of the chromatograph, the acyl derivative appeared to elute down the column as anticipated, for periods of 20–40 min, after which time separation and band movement ceased completely. No further development of the column occurred even on adding initially small, and then increasing, amounts of acetone to the benzene and only on use of an

eluent comprising acetone–ethanol (98:2) was any further band movement noted. The product, isolated by elution or extraction, was found in all cases to be the hydroxyalkyl derivative.

It is apparent that a fairly rapid de-acetylation occurs; it was found possible, using a very short column height, to elute some acetylated dye prior to deacetylation being complete.

Successful purification of the acetyl derivatives was, however, found to be satisfactory on a silica gel packed column (*loc. cit.*).

Conclusions

Whilst the use of activated alumina column chromatography for the purification of many azo disperse dyes is quite satisfactory, care must be taken in regarding the products from apparently homogeneous bands as being pure, even in conjunction with appropriate microanalytical data.

The use of additional TLC control tests, possibly in conjunction with mass spectrometry, is essential if problems such as these described above are to be overlooked. The conclusions reached[6] on the advantages of activated alumina columns for the purification of, *e.g.* anthraquinone dyes, must therefore not be taken to be generally applicable to all classes of dyes. Consideration should equally be given to preparative methods for the dyes involved and possible decomposition patterns to which it might be susceptible.

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Received May 11th, 1970

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CHROM. 4872

Gel filtration behaviour of sulphur and selenium-containing nicotinamide-analogues on Sephadex G-25

Most of the procedures described for the separation and purification of NAD(P)-analogues, *e.g.* ion-exchange chromatography, paper-chromatography and high voltage electrophoresis, possess certain inherent disadvantages, especially when working with labile nucleotides. Recently convenient procedures have been published utilising Sephadex or DEAE-Sephadex[1,2]. In the course of investigations with the relatively labile thio-$NADP^+$ and seleno-$NADP^+$ (refs. 3–5) we found that Sephadex was a suitable material for the purification of enzymatically synthesised $NADP^+$-analogues. A complete separation of small amounts of thionicotinamide and selenonicotinamide from the corresponding $NADP^+$-analogues was achieved by chromatography on Sephadex G-10 and G-25 using distilled water as eluant. During these experiments we observed an increase in the adsorption to the bed material if oxygen is replaced by sulphur or selenium in the carbamoyl-group of the nicotinamide.

Experimental

Materials. Nicotinamide was obtained from E. Merck, Darmstadt, G.F.R. Thionicotinamide was a product of Aldrich Chemical Co., Milwaukee/Wisc., U.S.A. Selenonicotinamide was prepared according to the method described previously[3]. Selenobenzamide was synthesised in a similar way from benzonitrile and hydrogen selenide. Thionicotinamide was recrystallised from water and the selenoamides, mentioned above, from *n*-propanol. Blue Dextran 2000 and Sephadex G-25 fine (particle size 20–80 μ) were purchased from Pharmacia, Uppsala, Sweden.

Methods. The dry Sephadex (20 g) was allowed to swell in distilled water overnight. The gel was sedimented in a 2 × 90 cm glass column to a height of 29.5 cm. After equilibration of the column with distilled water, the substances to be tested were put on the column in a volume of 2.0 ml. Elution was then started with distilled water with a hydrostatic pressure of 60 cm. This gave a flow rate of 2.6 ml per min. The effluent was collected in fractions of 5.0 ml in an automatic fraction collector. The column was alcohol jacketed and held at constant temperature (+4°) by means of a cryostat. The fractions were analysed by measurement of their visible or UV absorption with a Zeiss spectrophotometer, model PMQ II. Wavelengths used were: 625 nm for Blue Dextran, 260 nm for nicotinamide, 278.5 nm for thionicotinamide, 327 nm for selenonicotinamide and 323 nm for selenobenzamide. The substances tested were separately filtered through the column.

Results and discussion

A substance submitted to gel filtration is preferentially characterised by its K_D value, which is calculated from the expression

$$K_D = \frac{V_e - V_o}{V_i}$$

The elution volume, V_e, was determined by measuring the effluent volume from the time of addition of the test solution to the point where the concentration gradient

of the eluted substance is maximum. V_o, the outer volume was experimentally determined as the elution volume for Blue Dextran. The inner volume, V_i, was calculated from a reference value for Sephadex G-25, $V_i = 2.5$ ml per g dry weight[6].

TABLE I

NICOTINAMIDE ANALOGUES FILTERED THROUGH A COLUMN OF SEPHADEX G-25

Substance	*Molecular weight*	*Quantity (mg)*	*Eluant*	K_D
Nicotinamide	122.13	2	Distilled water	1.4
Thionicotinamide	138.20	2	Distilled water	2.0
Selenonicotinamide	185.08	3.5	Distilled water	2.2
Selenobenzamide	184.09	approx. 4–5	Distilled water	3.0

The nicotinamide analogues were eluted almost quantitatively, but selenobenzamide underwent partial decomposition on the top of the gel bed, producing red selenium. The K_D value of nicotinamide (Table I) is in good agreement with the one reported by GELOTTE[7]. This author demonstrated in detail that retention of heterocyclic and aromatic substances occurs when distilled water is used as eluant. As can be noticed in Table I there is a shift to stronger adsorption to the bed material on going from nicotinamide and thionicotinamide to selenonicotinamide. Furthermore, selenobenzamide which has practically the same molecular weight as selenonicotinamide is adsorbed more strongly than the latter. It is thought that perhaps there is a connection between the adsorption behaviour of nicotinamide analogues and the fact that thionicotinamide is in equilibrium with its tautomeric thiol structure $S{=}\overset{|}{C}{-}NH_2 \rightleftharpoons HS{-}\overset{|}{C}{=}NH$ thus increasing the tendency to interact with the gel matrix.

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Received June 9th, 1970

CHROM. 4877

Effects of ultrasonics on the resolution of DNA in gel filtration

Gel filtration on cross-linked polyacrylamide and Dextran, well established as a method for separating proteins on a molecular weight basis, has found some application in nucleic acid studies, *e.g.* for the removal of phenol and tRNA from DNA[1,2]. However, sphere-condensed agarose, with its relatively low porosity and incompressible structure, is becoming increasingly popular as a molecular sieve for DNA and deoxyribonucleoprotein[3,4].

In a previous communication[5] much of a specimen of native thymic DNA was delayed during elution on a column of Sephadex G-200 dextran, but in the presence of a trace of pancreatic DNAase eluted mainly in the exclusion volume. That this could be due to protein-mediated association of DNA molecules seemed unlikely at such low DNAase concentrations, and the effect could not be reproduced by the addition of similar amounts of bovine serum albumin.

As reported here, ultrasonication has been used to prepare a range of molecular size groups of DNA, in the absence of exogenous protein. The broad elution profile of native DNA, reproduced on columns of dextran, polyacrylamide and agarose, is shown to be sharpened by the briefest periods of sonication, and this is discussed in terms of the high viscosity of DNA solutions.

Calf thymus DNA was prepared as previously described[6]. ^{14}C-labelled DNA was prepared from *Bacillus subtilis* substantially by the phenol method of KIRBY as modified by KELLY AND PRITCHARD[7]. Sephadex G-200 was purchased from Pharmacia (G.B.) Ltd. Bio-Rad Laboratories supplied Bio-Gel P-300 polyacrylamide and Bio-Gel A-50m (100–200 mesh) 2% agarose gels.

Ultrasonication of DNA was performed in solutions containing thymic DNA at 950 μg/ml or *B. subtilis* DNA at 250 μg/ml[6]. The method of sucrose gradient centrifugation was as previously described[6], but with the following modifications. Using the 3×5 ml swing-out rotor, approximately 1 μg of bacterial DNA or 150 μg of thymic DNA, contained respectively in 0.1 ml and 0.25 ml of 10 mM potassium phosphate buffer (pH 7.2), were layered on 4.5 ml of sucrose gradient. Centrifugation was performed at 39,500 r.p.m. ($g_{max.}$ 171,300 $\times g$; $g_{min.}$ 92,700 $\times g$), for 144 min and 242 min, respectively. In the case of the bacterial DNA, gradient fractions were collected on circles of Whatman No. 1 filter paper and counted for carbon-14 in a liquid scintillation counter.

Gel filtration was performed routinely with 480 μg of thymic DNA or 30 μg of *B. subtilis* DNA contained in 1.2 ml of the column buffer, 20 mM potassium phosphate (pH 7.2), 0.5 M with respect to NaCl. The dimensions of the agarose column were 2.54×39 cm; those of the other gels were 2.54×34 cm. The columns were flowed upwards at 13.6 ml/h at 4°, effluent fractions of 3.25 ml being collected. $A_{260m\mu}$ values of fractions containing thymic DNA were read in a Unicam SP 800 recording spectrophotometer. ^{14}C levels were assayed after drying portions on filter paper. Gel filtrations were often repeated, and in all cases the degree of reproducibility was excellent.

Ultrasonication of thymic DNA for as little as 5 sec was sufficient to cause virtually complete exclusion by Sephadex G-200. Even after a 1 min pre-treatment, less

than 10 per cent of the DNA was small enough to be retarded during elution. Similar results were obtained using Bio-Gel polyacrylamide. In contrast, a much smaller load of *B. subtilis* [^{14}C]DNA, 30 μg, even in the native state was almost completely excluded by Sephadex G-200, and the effect of sonication for 5 sec or longer was simply to cause a gradually increasing degree of tailing during elution. It seemed consistent with the mean $s_{20,w}$ values of the native and sonicated specimens of these two DNA specimens, as shown in Table I, to ascribe the broad elution profile of the relatively high loads of native thymic DNA to the high viscosity of these solutions.

TABLE I

EFFECT OF SONICATION ON DNA SEDIMENTATION COEFFICIENTS

DNA	*Time of sonication*	*Mean* $s_{20,w}$	*90% of DNA recovered between*
Thymic	—	25.9 ± 0.38 S	4.5–52 S
	5 sec	14.5 ± 0.21 S	1.9–37.4 S
	15 sec	11.1 ± 0.14 S	2.0–23 S
	1 min	9.8 ± 0.13 S	1.5–20 S
	10 min	6.2 ± 0.10 S	0.4–13.2 S
B. subtilis	—	47.4 ± 0.29 S	6.3–62 S
	5 sec	13.5 ± 0.09 S	1.0–23.5 S
	15 sec	12.6 ± 0.09 S	1.0–21 S

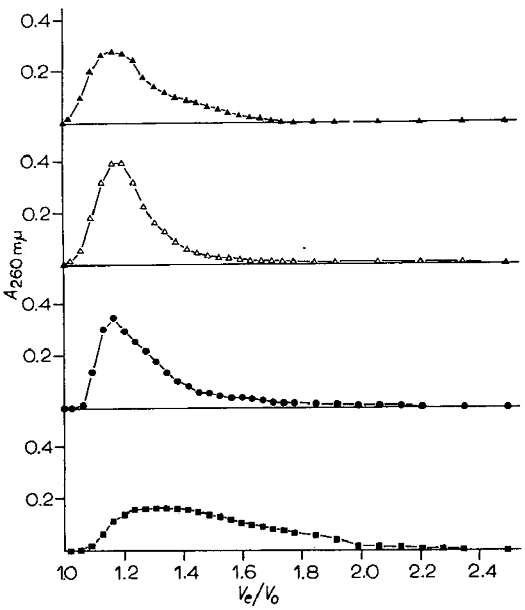

Fig. 1. Effect of sonication on elution of thymic DNA on Agarose A-50m. ▲—▲, native DNA; △—△, 5 sec sonicated DNA; ●—●, 15 sec sonicated DNA; ■—■, 1 min sonicated DNA.

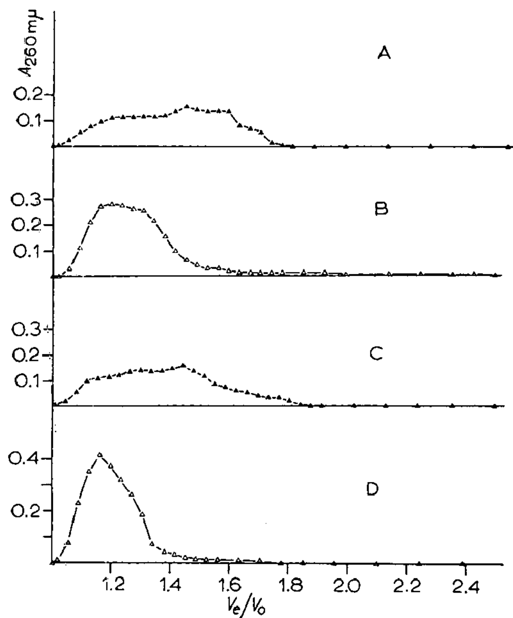

Fig. 2. Elution of thymic DNA on Agarose A-50m at extremes of ionic strength. A and B: elution in 20 mM potassium phosphate buffer (pH 7.2), 1 M with respect to NaCl. C and D: elution in 20 mM phosphate buffer alone. Symbols as in Fig. 1.

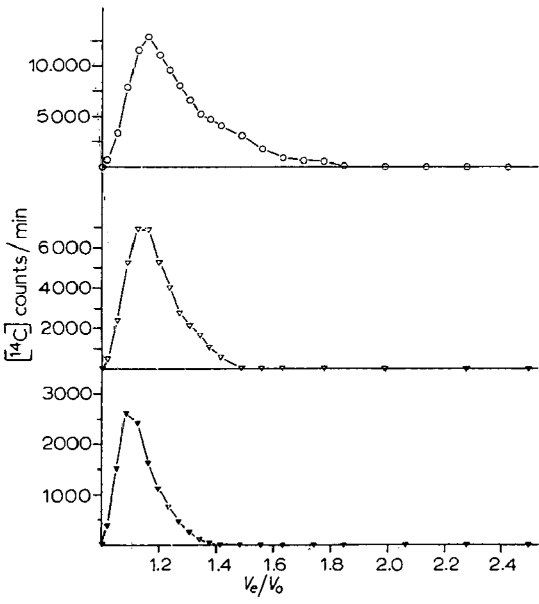

Fig. 3. Concentration dependence of elution of *B. subtilis* DNA on Agarose A-50m. DNA loads were: ○—○, 250 µg; ▽—▽, 100 µg; ▼—▼, 30 µg.

In extending the study to agarose, 480 μg of native and sonicated thymic DNA were run on an agarose column equilibrated in various buffers: the standard saline buffer; 20 m*M* potassium phosphate buffer (pH 7.2), 1 *M* with respect to NaCl; and saline-free 20 m*M* phosphate buffer. As may be seen in Figs. 1 and 2, at the two extremes of ionic strength the elution profile of the native specimen was greatly sharpened by limited sonication, yet in the standard saline buffer the profile of native DNA was little broader than that of the least sonicated specimen. The effect of ionic strength should find application in optimising the resolution of DNA gel filtration.

Because of the inferred effect of DNA load on the elution on Sephadex, several concentrations of bacterial DNA were run on the agarose column equilibrated in the standard buffer. As shown in Fig. 3, the proportion of DNA delayed by the gel increased with the DNA load. Even at 100 μg of [^{14}C]DNA, no peak sharpening was achieved by sonication.

These results may largely be explained by the high viscosity of solutions rich in native DNA. *Per se*, the concentration dependence of the elution of native *B. subtilis* DNA on agarose could implicate the involvement of DNA aggregates too large to pass through even the intersphere spaces, dissociating slowly as they are diluted. However, this hypothesis is difficult to reconcile with the sedimentation data, since the thymic DNA, when centrifuged at an overall concentration of the same order as that of the thymic DNA solutions routinely applied to the columns, sedimented rather more slowly than the 150 × lower concentration of bacterial DNA.

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Received June 15th, 1970

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CHROM. 4862

Effect of ionic strength on poly(D-Asp-L-Lys) in Sephadex gel filtration

Poly(D-Asp-L-Lys), a polypeptide, with a repeating sequence of trifunctional amino acids was found to behave anomalously on Sephadex G-50 fine with changes in ionic strength of the eluent. This kind of behavior of the protein model studied here is new and different from that reported in the literature for the other proteins[1]. In Sephadex gel filtration of proteins it has been observed[2] that the ionic strength of the eluent is independent of the elution volume of proteins. However, GELOTTE[3] and PORATH[4] have shown that phenylalanine, tyrosine and tryptophan are adsorbed to dextran gels. A protein with high contents of these amino acids might behave anomalously on Sephadex columns.

Poly(D-Asp-L-Lys), an unknown protein model[5] was synthesized for biological investigation. Polypeptides, with a repeating sequence of amino acids are usually prepared by the self-condensation of the active ester of the tri- or tetrapeptide. Normally this results in a mixture of higher and lower molecular weight polymers. The polymer obtained in this study showed an average molecular weight of 6600, as determined by ultracentrifugation.

For biological investigation we needed a polymer with the highest molecular weight. This was done by separating the fractions of polymer by using the Sephadex gel filtration technique. We wish to report here the anomalous results shown by poly(D-Asp-L-Lys) on Sephadex G-50 fine on changing the ionic strength of the eluent. The best separation was obtained with an aqueous buffer solution containing ammonium carbonate of an ionic strength of 0.03.

The column was standardized with proteins of accurately known molecular

TABLE I

ELUTION VOLUME (ml) OF POLYPEPTIDES

Conditions: 1.5 × 90 cm column at 25°–27° with a flow rate of 23 ml/h for all ionic strengths. Void volume (V_0) was determined by using Blue Dextran 2000 and was found to be 51.5 ml.

Protein	*Ionic strength*			*Mol. wt.*
	0.0	*0.03*	*0.3*	
Chymotrypsin[a] (beef pancreas)	58.5	57.0	56.0	22 500
Myoglobin[a] (horse heart)	67.0	68.0	69.5	17 800
Cytochrome[a] (horse heart)	78.0	78.5	78.7	12 400
Insulin[a] (bovine pancreas)	107.0	107.8	108.0	6 000
Adrenocorticotropic[a]	116.0	117.3	119.0	4 566
Poly(D-Asp-L-Lys)	58–89[b]	96.0	125–145[b]	6 900[c]
	—	112.0	—	5 400[c]

[a] These proteins were obtained from Mann Research Laboratories, New York.

[b] All the fractions showed the mean mol. wt. to be 6600 when determined by ultracentrifugation. But on the basis of standardization of the column (a graph of elution volume (V_1) / void volume (V_0) against the logarithm of molecular weight for all the standard proteins was drawn and was found to be a linear function), molecular weights of 24000–12000 were found when the ionic strength was 0.0 and 3000 to 1500 when the ionic strength was 0.3.

[c] These values, determined by ultracentrifugation, were in perfect agreement with standardization of the column when ionic strength was 0.03.

weights using glass distilled water (ionic strength thus zero) and aqueous ammonium carbonate buffer solutions with ionic strengths of 0.03 and 0.3, respectively. In all the cases the protein was eluted within the range of 3 to 5 ml.

Poly(D-Asp-L-Lys) was eluted from the column at all three ionic strengths. In each case thirty fractions of 3 ml each were collected and the presence of the polymer was detected by spectrophotometric analysis using a Cary-14 spectrophotometer. Only two major fractions showed the presence of polymer at an ionic strength of 0.03. Both the fractions showed the expected molecular weights on the basis of the standardization of the column and by ultracentrifugation. But, at ionic strength of zero, when glass distilled water was used as eluent the separation was found to be over a wide range. Twelve fractions which came immediately after the void volume (V_0), showed the presence of polymer. When the ionic strength was changed to 0.3 the polymer was eluted at a higher elution volume and over a wide range. In both the later cases each fraction was lyophilized and the molecular weight of each fraction was determined separately by ultracentrifugation. These values differ very much from the values obtained on the basis of the standardization of the column. The results are given in Table I. Currently we are investigating this problem further using protein models containing other amino acids, synthesized in this laboratory.

This work was supported by NIH Grants GM 06579 and GM 08795.

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Received June 1st, 1970

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CHROM. 4861

An improved one-dimensional thin-layer chromatographic separation of neutral lipid classes

A number of thin-layer chromatographic procedures were examined in an attempt to implement a rapid one-dimensional procedure suitable for densitometric quantitation[1-3]. None of the systems attempted gave satisfactory separations of neutral lipids from serum. One method reported required plates which were 34 cm long and required 2 to 3 h for development[4]. A system for separation of micro quantities of neutral lipids has been described by BIEZENSKI *et al.*[5]. The system described here was devised for routine separation of neutral lipid samples and quantitation by densitometry.

Materials and methods

Glass plates and spreader were obtained from Quickfit Reeve Angel, Clifton,

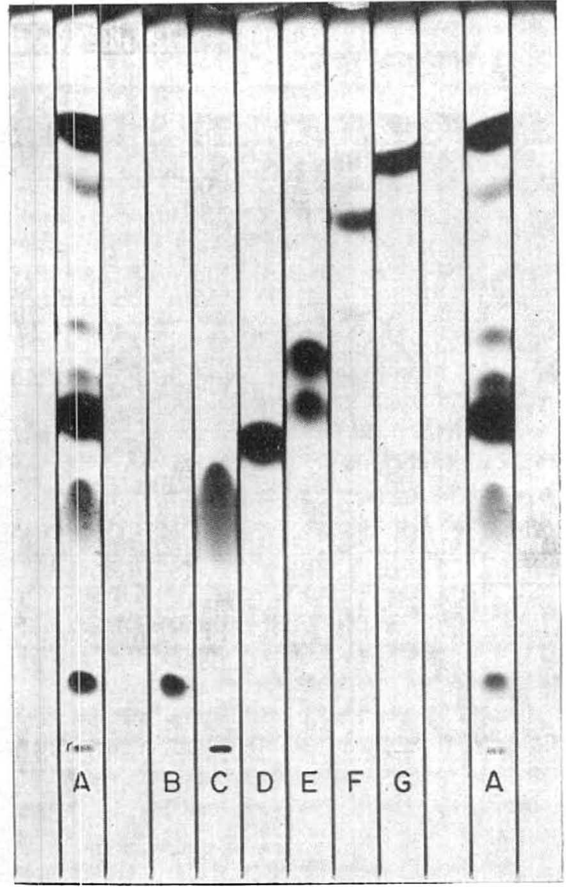

Fig. 1. Thin-layer chromatogram of standard neutral lipid classes. A = Mixtures of standard neutral lipids; B = monoglyceride; C = free fatty acid; D = sterols; E = 1,2- and 1,3-diglycerides; F = triglyceride; G = sterol esters.

N.J., U.S.A. Adsorbosil-5 silica gel was obtained from Applied Science Laboratories, State College, Pa., U.S.A. Spectroquality *n*-hexane and petroleum ether (boiling range 38.8–54.6) were obtained from Matheson, Coleman, and Bell, East Rutherford, N.J., U.S.A. Reagent grade methanol and anhydrous diethyl ether were obtained from Mallinckrodt, St. Louis, Mo., U.S.A.

Individual and mix standard neutral lipids were obtained from Supelco, Inc., Bellefonte, Pa., U.S.A. Spotting syringes and repeating dispenser were obtained from Hamilton Company, Whittier, Calif., U.S.A.

Glass plates, 20 × 20 cm, were coated with a 250 μ thick slurry of silica gel and air dried at ambient temperature for about 2 h. The air dried plates were scribed into 1.0 cm wide lanes which permitted multiple samples per plate. Samples containing 200 μg of lipid in 10 μl of chloroform–methanol (95:5) were spotted under nitrogen as a narrow band in the center third of a lane. The spotting solvent was allowed to evaporate and the plate transferred into a TLC tank lined with filter paper. The plate was developed to a height of 15 cm in a solvent of *n*-hexane–diethyl ether–methanol

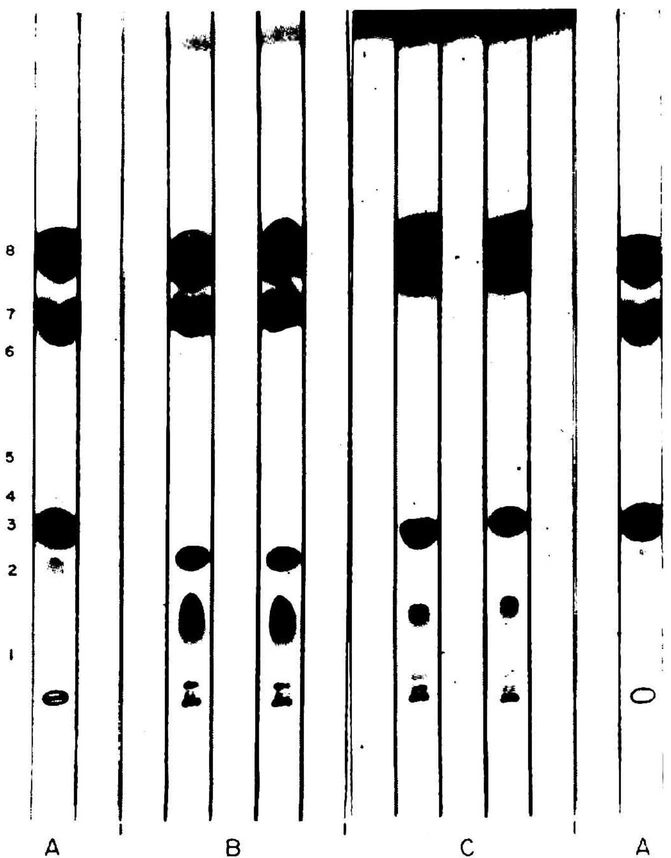

Fig. 2. Thin-layer chromatogram of serum neutral lipids and a standard neutral lipid mix. 1 = Monoglyceride; 2 = free fatty acid; 3 = sterols; 4 = 1,2-diglyceride; 5 = 1,3-diglyceride; 6 = unknown; 7 = triglyceride; 8 = sterol esters; A = mixture of standard neutral lipids; B = rat serum; C = baboon serum.

(90:30:5). Subsequent to development, the plate was placed in a dry nitrogen atmosphere for 5 min and then transferred into a second TLC tank lined with filter paper. The plate was developed in petroleum ether to the top and then left in the tank for an additional 25 min. Following development the plate was removed from the TLC tank, air dried at ambient temperature, sprayed with a 20 % aqueous solution of ammonium bisulfate[6], and charred at 170° for 90 min for lipid class detection. Total development including preparation of the plate for charring takes 1½–2 h.

Discussion

Fig. 1. illustrates a typical chromatogram obtained by the above method with neutral lipid standards. Fig. 2 illustrates a typical chromatogram, obtained by this method, with serum neutral lipids. Environmental and mechanical changes such as temperature, relative humidity, and lot variations in silica gel can affect the demonstrated separations. These changes can be alleviated by varying the height of development in the first solvent, the time the plate is left in the second solvent after full height development is attained, or by altering the amount of methanol in the first solvent.

Although methanol is incorporated as a solvent component in phospholipid chromatography it has been used very little in neutral lipid class separations. Solvents such as methanol have such a high polarity that triglycerides and sterol esters are not resolved and move on or very near the solvent front. The use of methanol, in this system, increased the polarity sufficiently to move the monoglyceride spot above the origin. This cannot be accomplished with most neutral lipid solvents. Subsequent development of the plate in a solvent such as petroleum ether, with a very low polarity, then moves the sterol esters above the triglycerides with little or no effect on the other neutral lipid classes. This second solvent also leaves the sterol esters below the solvent front which enhances quantitation. The use of these two solvent systems in one dimension accomplishes the desired separations with multiple samples per plate.

The excellent technical assistance of PHILIP J. FRAPPAOLO is gratefully acknowledged.

This investigation was supported by the Bureau of Medicine and Surgery, Navy Department, Research Task M4306.02.4010B. The opinions in this paper are those of the authors and do not necessarily reflect the views of the Navy Department or the naval service at large.

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First received April 16th, 1970; revised manuscript received May 29th, 1970

CHROM. 4868

The influence of pH in buffered reversed-phase thin-layer chromatography of penicillins and cephalosporins

The R_M values of penicillins and cephalosporins, as determined by a previously described reversed-phase TLC method[1,2], were shown to correlate well with the antibacterial activity[3]. This was in agreement with findings that the lipid-solubility of unionized molecules is one of the important factors in determining the passage of drugs through biological membranes[4,5]. A decrease in the permeability of a membrane to weak acids or bases can result from a change in the pH of the medium, which ionizes

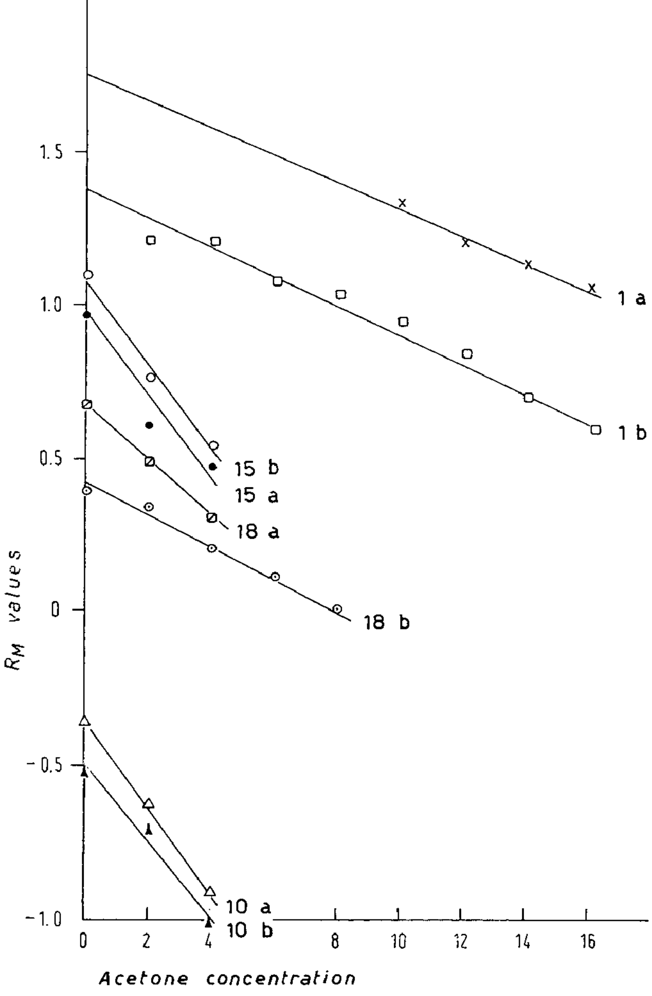

Fig. 1. The R_M values of some compounds plotted against the composition of the mobile phase. The straight lines were calculated from the R_M values in the range of linearity. Each point represents the mean of 8 determinations. The compounds are numbered as in Tables I and II. The pH 2.6 and 9.4 of the mobile phase correspond respectively to (a) and (b).

TABLE I

LIST OF THE PENICILLINS ACCORDING TO THE DECREASING LIPOPHILIC CHARACTER OF THEIR MOLECULES AS EXPRESSED BY THEIR EXTRAPOLATED R_M VALUES BOTH AT pH 2.6 AND 9.4

Carbenecillin was previously indicated as carboxybenzylpenicillin[1].

R—C(=O)—NH—CH—CH(—S—)C$(CH_3)_2$ / CO—N——CHCOOH

No.	Compound	R	R_M value	
			pH 2.6	pH 9.4
1	Dicloxacillin	Cl, Cl, N, O, C—CH_3	1.76	1.43
2	Cloxacillin	Cl, N, O, C—CH_3	1.67	1.21
3	Nafcillin	OC_2H_5	1.43	1.20
4	Oxacillin	N, O, C—CH_3	1.39	0.96
5	Phenethicillin	—O—CH(CH_3)—	1.35	0.91
6	Phenoxymethylpenicillin	—O—CH_2—	1.17	0.89
7	Benzylpenicillin	—CH_2—	0.84	0.45
8	Methicillin	OCH_3, OCH_3	0.78	0.41
9	Ampicillin	—CH(NH_2)—	0.11	0.05
10	Carbenecillin	—CH(COOH)—	−0.37	−0.50

TABLE II

LIST OF THE CEPHALOSPORINS ACCORDING TO THE DECREASING LIPOPHILIC CHARACTER OF THEIR MOLECULES AS EXPRESSED BY THEIR EXTRAPOLATED R_M VALUES BOTH AT pH 2.6 AND 9.4

Cephaloridine was not tested in the previous work on cephalosporins[2].

No.	Compounds	R_1	R_2	R_M value	
				pH 2.6	pH 9.4
11	(Acid)	O—CO—CH_3	NH—CO—$(CH_2)_6$—CH_3	1.94	1.72
12	(Acid)	O—CO—CH_2	NH—CO—CH_2-(naphthyl)	1.59	1.40
13	(Sodium salt)	O—CO—CH_3	NH—CO—CH_2S—CH_2-(phenyl)	1.48	1.20
14	(Sodium salt)	N_3	NH—CO—CH_2S—CH_2-(phenyl)	1.37	1.17
15	(Cephaloridine)	(pyridinium, N^+)	NH—CO—CH_2-(2-thienyl)	0.99	1.08
16	(Sodium Cephaloram)	O—CO—CH_3	NH—CO—CH_2-(phenyl)	0.86	0.63
17	(Sodium Cephalotin)	O—CO—CH_3	NH—CO—CH_2-(2-thienyl)	0.69	0.43
18	(Acid)	N_3	NH—CO—CH_2-(2-thienyl)	0.67	0.42
19	(Acid)	O—CO—CH_3	NH—CO—CH_2-(2-furyl)	0.38	0.22
20	(7-Amino-cephalosporanic acid)	O—CO—CH_3	NH_3^+	−0.17	−0.26
21	(Potassium cephalosporin C)	O—CO—CH_3	NH—CO$(CH_2)_3$—CH(NH_2)COOH	−0.49	−0.57

the molecules[4]. The present work shows that such a reversed-phase TLC method can demonstrate the influence of the pH of the mobile phase on the partitioning of penicillins and cephalosporins between the polar mobile and the non-polar stationary phases.

The R_M values of the penicillins and cephalosporins are reported in Tables I and II. The non-polar stationary phase consisted of silicone oil. The aqueous mobile phase was sodium acetate veronal buffer at pH 2.6 or 9.4, alone or in various proportions with acetone. The pH of the mobile phase was measured after each chromatographic run and found to be practically unchanged. The details of the TLC method have already been published[1].

Results and discussion

As previously pointed out[1,2], there is a range of linear relationship between R_M values and acetone concentration in the mobile phase (Fig. 1). The plots in Fig. 1 also show that a lipophilic compound such as dicloxacillin at pH 2.6 did not move from the starting line until a 10 % acetone concentration was used as mobile phase. On the other hand, at pH 9.4 a 2 % acetone concentration was sufficient to move the compound. More hydrophilic compounds such as compound No. 18 and carbenecillin migrated with a 0 % acetone concentration in the mobile phase both at pH 2.6 and 9.4. However the R_M values obtained at pH 2.6 were higher than those at pH 9.4. The only exception was cephaloridine, the R_M values of which were higher at pH 9.4 than at pH 2.6 (Fig. 1). By means of the equations of the straight lines it was possible to calculate an R_M value for each compound at 0 % acetone in the mobile phase at pH 2.6 and 9.4 (Tables I and II). In practice, the compounds migrated as round spots both at acid and basic pH's.

Higher R_M values indicate a more lipophilic nature; this means that at an acid pH more molecules are unionized and therefore are more lipophilic than at a basic pH. This is in agreement with the acidic character of penicillins and cephalosporins[6]. The behavior of cephaloridine could be considered a consequence of the basic character of the pyridine ring in its molecule which is ionized at an acid pH.

The influence of pH on the chromatographic behavior of acids and bases has also been pointed out by several other investigators[7–10]. HOWE[8] in particular, showed that, for several series of organic acids, an acid or basic mobile phase, where the acids were unionized or ionized, respectively, caused different migrations of each compound. BUSH[10] suggested the use of a formula derived by SOCZEWIŃSKI[11] for the calculation of the pK of an acid or base from paper chromatographic data. The classification of the compounds is practically the same as that previously found with a mobile phase of pH 7.4[1,2].

$$R_M\ (\text{acid}) = 0.190 + 1.096\ R_M\ (\text{basic})$$

In Fig. 2, the R_M values for each compound at pH 2.6 are plotted against those at pH 9.4. It can be seen that there is a linear relationship between the two series of data, expressed by the equation:

This means that all the compounds tested experimentally show the same increase in their R_M value when one changes the pH of the mobile phase from 2.6 to 9.4. Cephaloridine, which was not used in calculating the equation of the straight line, shows the greatest deviation from the regression line. In fact, its R_M values

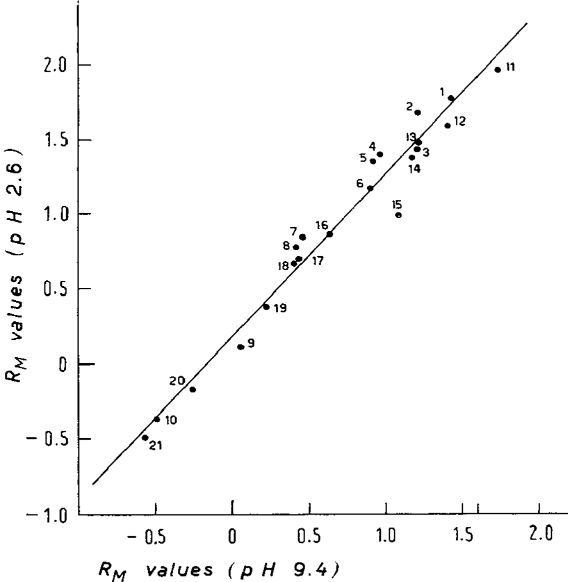

Fig. 2. The R_M values at pH 2.6 are plotted against those at pH 9.4. The compounds are numbered as in Tables I and II.

decrease by changing from a basic to an acidic pH in the mobile phase. The present data seem to suggest that buffered reversed-phase TLC can be used as a system, which permits one to study changes in the lipophilic character of a compound according to variations in the pH of the medium. The TLC technique described here could possibly provide a model system for studying the penetration of molecules through biological membranes.

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Received June 2nd, 1970

CHROM. 4874

Chromatography of the lignans of *Thuja plicata* Donn

The ten lignans in the heartwood of western red cedar (*Thuja plicata* Donn) have been isolated and characterised in these laboratories[1-4]. With the elucidation of the structure of the last lignan[4], we now present the data on the chromatographic behaviour of all of them. These data cover paper (PC), column (CC), thin-layer (TLC), and gas-liquid chromatography (GLC) systems of these compounds or some of their derivatives.

Experimental

PC was on Whatman No. 1 paper, TLC was on Merck Silica Gel G. Solvents and detecting reagents are listed in Table I. Gas chromatography was with a 5 ft. × 1/8 in. column filled with 3 % OV-1 on Gas-Chrom Q (100–120 mesh); nitrogen carrier gas at 20 ml/min; column oven temperature programmed for 200° to 300° at 2°/min; flame ionisation detector oven at 300°; and injector block at 275°. TMS derivatives of thujaplicatins and its methyl ethers eluted from the column at 280°, plicatic acid at 290°, and plicatin at 295°. These derivatives were formed by reacting a few milligrams of the heartwood extract, etc., with bis(trimethylsilyl)acetamide (100 mg) in pyridine (1 ml). The acetates of the lignans were formed by treating them with acetic anhydride (1 ml)–pyridine (1 ml) warming to 40°, allowing the reactants to stand for 2 h, and removing the solvent under vacuum, taking care that the temperature did not exeed 40°.

Results and discussion

Isolation of the ten lignans in amounts sufficient for characterisation has been by CC on deactivated silica[2,3]. When larger yields or a preliminary separation was desired, the ethyl acetate solubles were dried and triturated several times with hot chloroform and the soluble and insoluble fractions chromatographed. This system has recently been adapted to quantitative CC[5] on a micro-scale.

This system has been supplanted[4] by CC on a polyamide (Woelm) eluting with benzene followed by a gradient elution with ethanol. This separates the above lignans in the same order as shown for the R_F values of Table I system D, and faster and cleaner than the silica gel CC.

Table I presents the chromatographic data. With the exception of the thujaplicatin methyl ethers, most of the compounds listed have the unusual monomethylated pyrogallol (3,4-dihydroxy-5-methoxyphenyl) pendant ring and would therefore be expected to complex with molybdic acid. This reaction has been incorporated[6] in a PC system in which dilute molybdic acid has been substituted for water in the usual BAW solvent (Table I, C). The system is self developing because the *o*-dihydroxy groups react to form the yellow molybdate complex. Also, the R_F values of the complexes are much smaller than in the system without molybdic acid. Two other common paper irrigation solvents (Table I, A and B) separate the lignans by classes. No system was found which separated all ten lignans by PC.

Separation by TLC on silica gel had limited success in one direction (D). By making the acetates and using a two-dimensional system (E and F), however, sepa-

TABLE I

R_F VALUES FOR THE LIGNANS OF *Thuja plicata*

Solvent systems: (A) acetic acid–water (2:98); (B) butanol–acetic acid–water (60:15:25); (C) butanol–dilute molybdic acid–acetic acid[6]; (D) benzene–ethanol (9:1); (E) toluene–ethanol (9:1); (F) chloroform–acetone (95:5).

	Paper[a] *chromatography*			*Thin-layer*[b] *(silica gel) chromatography*			
	A	*B*	*C*	*D*[c]	*E*[d]	*F*[d]	
Thujaplicatin (4,3′,4′-trihydroxy-3,5′-dimethoxy-lignanolid-9,9′)[f]	0.62	0.76	0.61	0.21	0.29	0.31	brown
Dihydroxy thujaplicatin (4,3′,4′,8′,9′-pentahydroxy-3,5′-dimethoxy-lignanolid-9,9′)[f]	0.62	0.76	0.61	0.17	0.10	0.05	brown
Thujaplicatin methyl ether (4,4′-dihydroxy-3,3′,5′-trimethoxy-lignanolid-9,9′)[f]	0.64	0.88	0.75	0.30	0.29	0.31	rose
Hydroxy thujaplicatin methyl ether (4,4′,8′-trihydroxy-3,3′,5-trimethoxy-lignanolid-9,9′)[f]	0.64	0.88	0.75	0.25	0.28	0.23	rose
Dihydroxy thujaplicatin methyl ether (4,8,4′,8′-tetrahydroxy-3,3′,5′-trimethoxy-lignanolid-9,9′)[f]	0.64	0.88	0.75	0.17	0.15	0.11	rose
γ-Thujaplicatene (2-(3″,4″-dihydroxy-5″-methoxybenzylidene)-3-(3′-methoxy-4′-hydroxybenzyl)-butyrolactone)	0.25[c]	0.80	0.61	0.23	0.35	0.41	green
Plicatic acid (3,4,8,4′,8′,9′-hexahydroxy-5,3′-dimethoxy-9-cyclolignanoic acid)[f]	0.85	0.45	0.22	0.00	0.29, 0.27	0.31, 0.14	red-brown
Plicatin (4,3′,4′,8′,9′-pentahydroxy-3,5′-dimethoxy-cyclolignanolid-9,9′)[f]	0.81	0.68	0.48	0.05	0.16	0.06	red-brown
Plicatinaphthalene (6-hydroxy-2-(hydroxymethyl)-7-methoxy-4-(3′,4′-dihydroxy-5′-methoxyphenyl)-3-naphthoic acid lactone)	0.14[e]	0.74	0.63	0.16	0.25	0.28	UV-yellow
Plicatinaphthol (1,6-dihydroxy-2-(hydroxymethyl)-7-methoxy-4-(3′,4′-dihydroxy-5′-methoxyphenyl)-3-naphthoic acid lactone)	0.10[e]	0.74	0.62	0.10	0.25	0.30	UV-orange

[a] Whatman No. 1 paper was used and detection was carried out with Barton's reagent.
[b] Detection was carried out with a mixed sulphuric and nitric acid spray, followed by heating and 6 *N* NaOH.
[c] On Whatman No. 1 paper.
[d] Lignans were acetylated first (see text); colour reactions are given in the margin.
[e] Fluorescent before spraying.
[f] Nomenclature of FREUDENBERG AND WEINGES[8].

ration in most cases was achieved. Where separation was difficult due to similar R_F values, *e.g.* thujaplicatin, its methyl ether, and plicatic acid, differentiation was possible by running a standard added to the mixture on a duplicate plate. Acetylation conditions were chosen so that the primary and phenolic hydroxyl groups were esterified, but the tertiary hydroxyl groups were not. The exception was plicatic acid; it has been shown that, under mild acetylation conditions, plicatic acid gave two products — the major one a mixed anhydride, tetraacetate and a minor one a pentaacetate with one tertiary hydroxyl group reacted[7].

Some effort was expended in the search for detecting reagents giving specific colour reactions. Thus, the Maüle and Pauly reagents were examined, but the former gave colours which were too fugitive and the latter gave colours which were too similar. The usual reagents were those most commonly used here, *viz.* Barton's reagent for PC and mixed acids for TLC. Spraying the lignan acetates with nitric acid gave the nitro-derivatives, and the overspray with alkali gave the sodium salts of the nitrophenols which were detectable in very small amounts, especially under UV light. Six lignans could then be identified immediately because of characteristic colour reactions.

Attempts at separating the trimethylsilyl derivatives of the lignans by GLC were unsuccessful except for plicatic acid and its lactone. No doubt a GLC method could be evolved using longer columns, etc., but the high operating temperatures necessary for the separation (295°), even with light coatings of the latest substrate, make it probable that no gas chromatograph could operate for very long.

In conclusion, the chromatographic separations were complicated by the presence of other extractives from western red cedar, such as the thujaplicins, thujic acid, etc. However, the identification of any of the western red cedar lignans can be performed using a combination of the above PC and TLC systems. The co-chromatography of specific lignans is, of course, very helpful for identification.

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Received June 10th, 1970

CHROM. 4896

Ion-exchange paper chromatography of inorganic ions

XXVII. The adsorption of chlorauric acid on various exchangers from organic solvents

A new method for separating gold from platinum has recently been proposed by DYBCZYNSKI AND MALESZEWSKA[1] which is based on the adsorption of bromoauric acid on Dowex 50 and its subsequent elution with acetylacetone. These authors

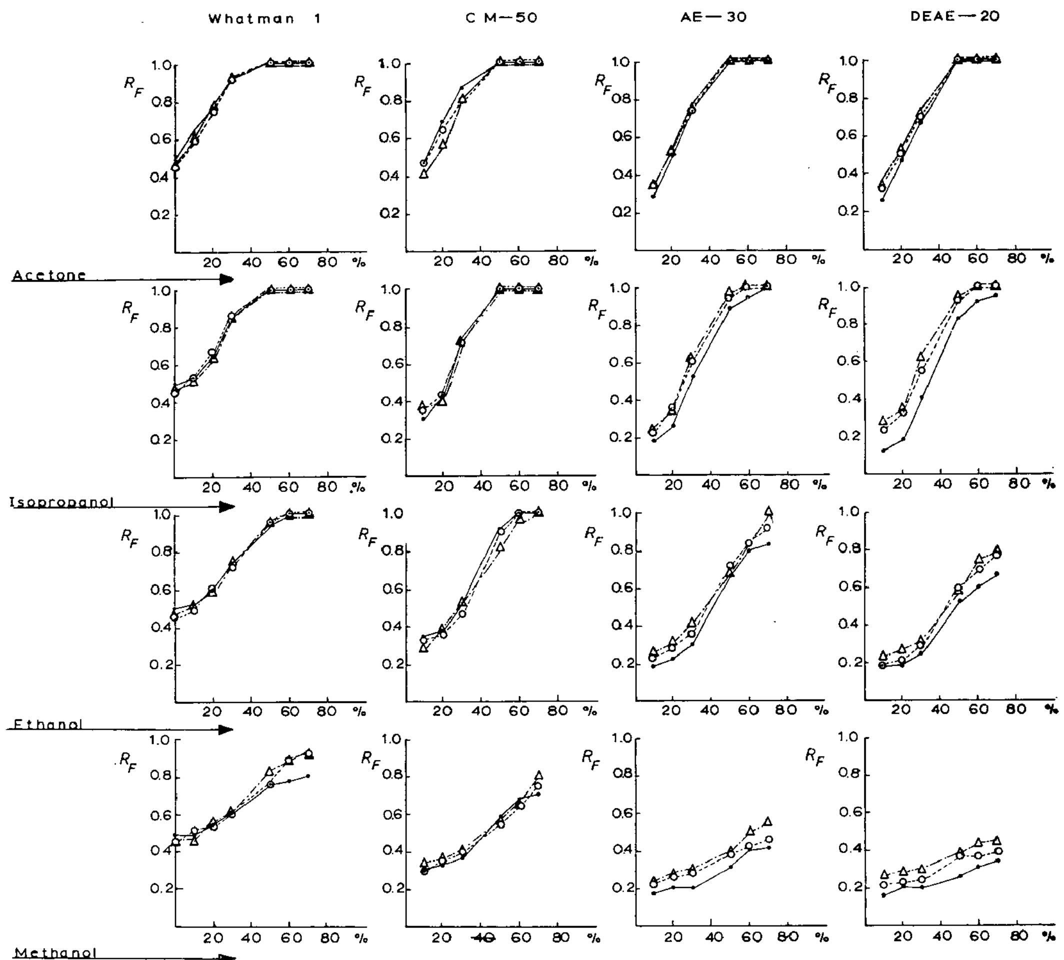
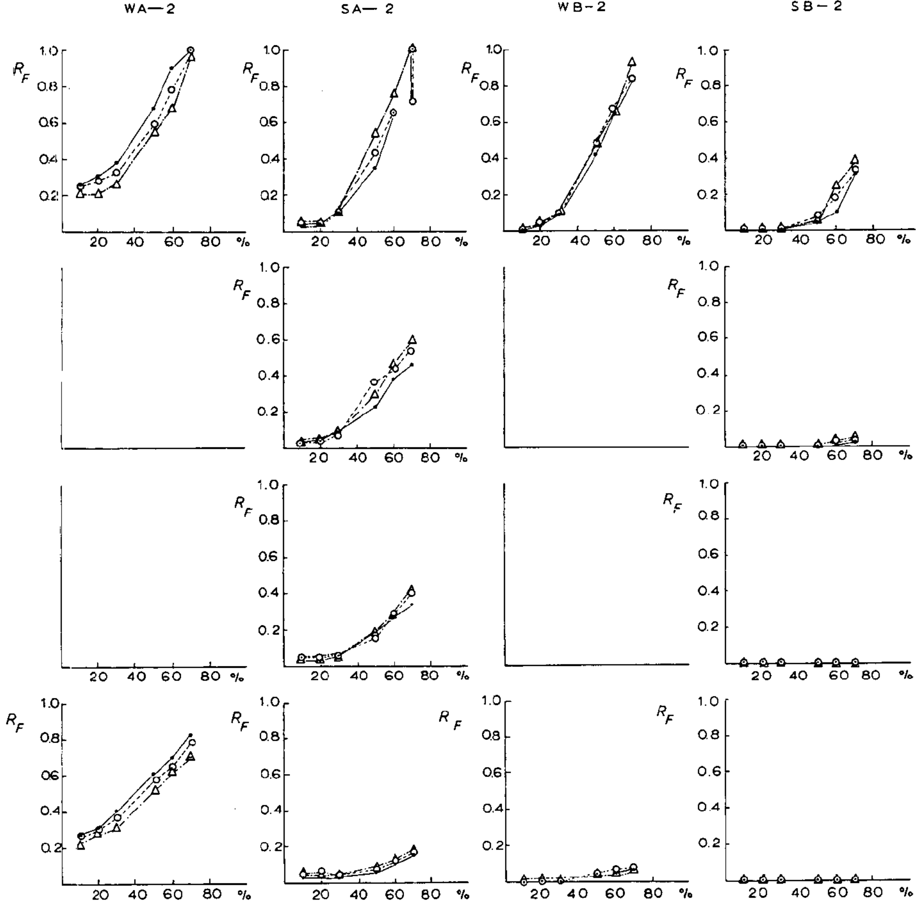


Fig. 1. R_F values of gold chloride plotted against the concentration of organic solvent in aqueous HCl (1 N, 2 N and 3 N). Solvents (from top to bottom): acetone, isopropanol, ethanol and methanol. Papers (from left to right): Whatman No. 1, Whatman CM-50 (carboxymethylcellulose), Whatman AE-30 (aminoethylcellulose), Whatman DEAE-20 (diethylaminoethylcellulose), Amberlite WA-2 (carboxylic resin paper), Amberlite SA-2 (sulphonic acid resin paper), Amberlite WB-2 (weak base anion-exchange resin paper) and Amberlite SB-2 (strong base anion-exchange resin paper). (●—●) 1 N HCl; (○---○) 2 N HCl; (△-·-·-△) 3 N HCl.

discuss the mechanism of the adsorption process and suggest adsorption of undissociated $HAuBr_4$ with the formation of a molecular (charge transfer) complex between the aromatic rings of the resin network and the complex acid.

The paper prompted us to go over our previous results[2] and we noted that the behaviour of Au(III) is still more interesting as it can be eluted from both cation and anion exchangers by organic solvents. Similar results have also been obtained in column and equilibrium experiments[3]. MARCUS[4] in a discussion of the ion-exchange behaviour of metal complexes concludes that gold chloride should exist mainly as an ion pair $H^+AuCl_4^-$ in more than 2 N HCl but that the species on the anion exchanger should be $AuCl_4^-$. However he states that spectra of $HAuCl_4$ in HCl contradict



this and tend to indicate that $AuCl_4^-$ exists even at high concentrations of HCl.

From aqueous solutions of HCl, gold chloride adsorbs very strongly not only on anion exchangers but also on cation exchangers and neutral surfaces. If it were present as $AuCl_4^-$ it should not really be desorbed from an anion exchanger by organic solvents, as a lowering of the dipole moment of the solvent should decrease the hydration of $AuCl_4^-$ and of the quarternary ammonium groups and thus produce rather the contrary effect.

We therefore felt that it would be interesting to study the behaviour of $AuCl_4^-$ further and adopted an approach used previously[2], namely to obtain data for various exchangers and adsorbants, which then give a clearer picture of the factors involved.

Experimental

The hydrogen or chloride form of the various ion-exchange papers were prepared by equilibrating twice with 2 N HCl (30 min) and then washing exhaustively with distilled water.

Chromatograms of chlorauric acid dissolved in 1 N HCl were developed by the ascending method in small jars at $20 \pm 1°$. The spots were usually visible as yellow spots and when sprayed with stannous chloride in HCl yielded purple spots.

The results are given in Fig. 1.

Discussion

Fig. 1 shows that the differences between cellulose, cellulose exchangers and resins (cationic and anionic) is rather one of degree and not of different types of mechanism, also that there is a regular decrease in effect with the decrease in the polarity of the solvent.

Spectrochemical evidence (see ref. 4) seems to indicate that $AuCl_4^-$ is the predominant species in aqueous HCl solution. Supporting evidence is also obtained by the R_F–HCl curve of $HAuCl_4$ on cellulose paper, which is practically linear from 1 N to about 10 N HCl ($R_F \sim 0.45$), together with the fact that $HAuCl_4$ is strongly anionic in paper electrophoresis in 0.5–2 N HCl (the practical upper limit); thus there is evidence of little change in the type of species present from 1 N to 10 N HCl as well as evidence that this species moves anionically.

We thus have to account for the adsorption of an anion (presumably $AuCl_4^-$) on surfaces less polar than aqueous HCl and its desorption by organic solvents. This can probably be done by numerous hypotheses, one that seems plausible to us is that $AuCl_4^-$ favours ion pair formation on non-polar solids and in non-polar solutions. When there are anion-exchanging groups on the solid, obviously three mechanisms are possibile: electrostatic attraction towards the groups; adsorption of $H^+AuCl_4^-$; and ion pair formation between $AuCl_4^-$ and the exchanging groups (unless this is identical with the first).

Elution with a non-polar solvent then either helps the ion-pair $[H^-AuCl_4^-]$ to re-enter the solution or destroys the electrostatic bond $-NR_3^+ AuCl_4^-$ to form preferentially $[H^+AuCl_4^-]$. Incidentally the latter does not seem to occur to a large extent with methanol.

On the anion-exchange celluloses a high HCl concentration gives a high R_F–solvent concentration curve while the contrary is observed on carboxylic cellulose, carboxylic resin and acetylated cellulose (Fig. 2). In the first case there seems to be

an anion-exchange (law of mass action) effect operating, while in the second the somewhat higher polarity of the mobile phase (in higher HCl concentration) will favour adsorption.

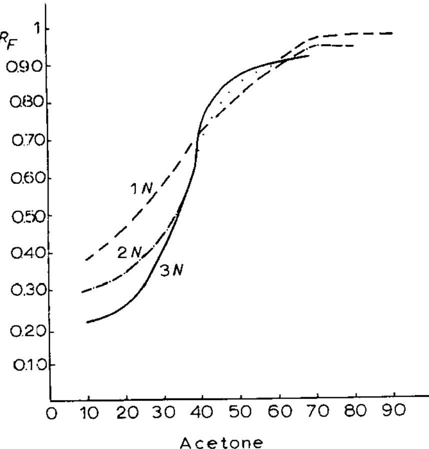


Fig. 2. R_F values of gold chloride plotted against the concentration of acetone in 1 *N*, 2 *N* and 3 *N* HCl on Polygram CEL 300 AC-10 (a ready-made thin layer consisting of acetylated cellulose).

To sum up, comparing the adsorption of $HAuCl_4$ on various types of cellulose and resin papers all effects can be explained by assuming $AuCl_4^-$ to be the preferred species in aqueous solution and an ion pair $[H^+AuCl_4^-]$ the preferred species in organic solids and solutions. There is no need to assume the formation of a charge transfer complex on the resin network.

Thanks are due to L. OSSICINI and R. MARINI-BETTÒLO for carrying out some of the experimental work described here.

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Received June 10th, 1970

Book Review

Electrophoresis of Proteins in Polyacrylamide and Starch Gels, by A. H. GORDON, North-Holland Publishing Co., Amsterdam, 1969, 149 pp., price 35/—.

Electrophoresis of proteins in polyacrylamide and starch gels by A. H. GORDON is a part of the series "Laboratory Techniques in Biochemistry and Molecular Biology". This small book will be valuable to those workers who intend to use gel electrophoresis as an analytical or semi-preparative tool in their investigations.

After an introductory chapter in which the applicability of starch and polyacrylamide electrophoresis is described in detail the following chapters are written under the headings:

(1) Applicability of starch and acrylamide zone electrophoresis; (2) acrylamide gel; (3) apparatus and techniques; (4) methods allowing recovery of separated materials (preparative methods); (5) starch gel.

Adequate subdivisions of the different chapters together with helpful tables on buffers and detection methods and about 130 references give sufficient information to the interested reader who may want to use this book as a practical guide.

It seems a pity that, as the book appeared in 1969, the very promising method of iso-electrofocussing in polyacrylamide gels has not been included.

The University of Nijmegen, Nijmegen (The Netherlands) H. BLOEMENDAL

News

Manufacturers' Literature

An issue of *Analytical Advances* from Hewlett-Packard, Avondale, Pa., U.S.A., contains two extremely interesting articles. One article discusses the use of the HP 7600A gas chromatographic system for the development of a method called "simulated distillation" which is of direct interest to petroleum and petrochemical industries, the other a new gas chromatographic detector for phosphorus containing pesticides based on the salt-modified flame ionization detector principle.

For further information apply to the publisher under reference No. Chrom. N-283.

Number 2 of Volume 20 (1969) of *Instrument News* (Perkin-Elmer Corporation) contains three articles of direct interest to the chromatographer. Amongst the application articles is one discussing gas chromatography–mass spectrometer analysis of flavours and fragrances, using the Perkin Elmer Model 270 instrument. New products described include the high performance, moderately priced Model 990 gas chromatograph, and a thin-layer chromatography accessory for the MPF-2A fluorescence spectrophotometer.

For further information apply to the publisher under reference No. Chrom. N-278.

A set of notes from Becker Delft NV, The Netherlands, has been received describing the gas–liquid chromatographic analysis of amino acids. The notes are quite detailed giving procedures for derivitization, examples of separations and general references, including those published by GEHRKE.

For further information apply to the publisher under reference No. Chrom. N-277.

An Atlas of diagrams and analysis conditions referring to the amino-acid separations carried out by the Carlo Erba Automatic Amino Acid analyzer 3A27 following the Spackmann accelerated method or the Spackman-Stein-Moore methods is now available from Carlo Erba, Milan, Italy.

For further information apply to the publisher under reference No. Chrom. N-287.

New Materials

An economic introduction to the complete range of Corning Controlled-Pore Glass granules for chromatography is being offered by Corning Glass Works in the form of a Corning Chromatography Test Kit.

Housed in a sturdy paperboard briefcase package, the kit contains five 50-cc jars of Corning Controlled-Pore Glass granules — one in each available pore diameter size — and a 50-cc bed volume analytical column. Also included are instructions for properly packing a chromatography column and a selection of application notes and product data sheets.

Corning Controlled-Pore Glass granules are available with pore diameters of 240, 370, 700, 1,250 or 2,000 Å. Pore diameters are accurate to within ± 10 per cent.

Advantages of controlled-pore glass packing material for permeation chromatography are listed in a two-page brochure (Data Sheet 103) published by the Chromatography Products Department of Corning Glass Works, Corning, N.Y., U.S.A.

For further information apply to the publisher under reference No. Chrom. N-284.

New Reagents

Pierce Chemical Company (Rochford, Ill., U.S.A.) announces a new reagent "MSTFA" for silylating polar materials to produce volatile and thermally stable derivatives for gas chromatography and mass spectrometry. MSTFA (N-methyl-N-trimethylsilyl-2,2,2-trifluoroacetamide) is the most volatile TMS-amide yet available; more volatile than BSTFA or BSA and is described as having similar donor strength (M. DONIKE, *J. Chromatog.*, 42 (1969) 103). Its reaction by-product, N-methyltrifluoroacetamide, has a lower GLC retention time than the MSTFA. This will be of considerable value in GLC determinations where the reagent or by-product peaks interfere with the derivative peaks. It will be of particular value in the trace analysis of materials where the interpretation of the small peak area on the chromatogram may be confused or lost in the tailing of other silylating reagents and by-products.

MSTFA boils at 132° and is a clear to light yellow liquid. It is quite sensitive to moisture and is therefore packaged under nitrogen in 1 ml-glass ampules.

For further information apply to the publisher under reference No. Chrom. N-279.

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Errata

J. Chromatog., 48 (1970) 406–411

Page 408, line 22: "10–15 kg/cm^2" should read "1.0–1.5 kg/cm^2".
line 36: "2–5 kg/cm^2" should read "0.2–0.5 kg/cm^2".

J. Chromatog., 49 (1970) 503–510

Page 507, line 6: "a depth of approximately 1.5 cm" should read "a depth of approximately 1.5 mm".

J. Chromatog., 49 (1970) 520–526

Page 525, line 7: "Komplexion $[Cr(SCN)_5(CH)]^{3-}$ wandert" should read "Komplexion $[Cr(SCN)_5(CN)]^{3-}$ wandert".

J. Chromatog., 50 (1970) 260–273

Page 263, 21st and 22nd line up: "In this way the overlap will be reduced and, since the separation will be improved, there will be a gain in sensitivity also." should read "In this way, since the separation will be improved, the overlap will be reduced, and a gain in sensitivity will also be obtained."